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Original Article

Biological and genetic characteristics of mesenchymal stem cells in vitro derived from human adipose, umbilical cord and placenta

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Abstract: Mesenchymal stem cells (MSCs) can be isolated from different tissues like bone marrow, adipose tissue, umbilical cord, placenta and milk tooth, and as a result may cause some differences in them. In this study, we separated MSCs from adipose tissue (ASCs), Wharton's Jelly of umbilical cord (WJ-MSCs) and chorionic plate of full-term placenta (CP-MSCs) using serum-free culture system with explant method, and compared the biological and genetic characteristics. The results indicated that these three types of MSCs, in low passages (less than 10 passages) are spindle-shaped cells, and the karyotype are normal within 15 passages. The surface of CD markers for these MSCs were negative for CD11b, CD19, CD34, CD45 and HLA-DR (I<1%), and positive for CD29, CD44, CD73, CD90 and CD105 (>95%), except that CD90 was 88.82±11.83% in CP-MSCs. The ASCs expressed higher level of P16, whereas CP-MSCs and CP-MSCs had more expression of Nanog. The growth factor of HGF (4~6 ng/mL) was secreted by CP-MSCs at a higher level than ASCs and WJ-MSCs. In conclusion, MSCs in low passages were suitable for clinical application, and various MSCs related products could to be exploited for different diseases.

Keywords: Adipose tissue, chorionic plate, mesenchymal stem cells, Wharton's Jelly

Introduction

After their first discovery in bone marrow and periosteum, mesenchymal stem cells (MSCs) had been proven to exist widely in perinatal and postnatal organisms from various species including mouse, rat, rabbit, canine, pig and human [1, 2]. Successful isolation of MSCs from many human tissues such as adipose tissue [3, 4], tooth pulp [5], placenta [6, 7], amnion [8], and amniotic fluid [9] have led to the increasing pursuit of researchers from all over the world. The enthusiasm surrounding these investigations are bored out of the opinion that MSCs would be the most promising adult stem cells suitable for regenerative medical application.

The results of comprehensive study have demonstrated that MSCs had the multipotent differentiation capacity to generate a variety of other cell types including osteocytes, adipocytes and chondrocytes, with additional ability of self-renew, the capability to secret many growth factors and to repair injured tissues [10]. Another attractive property is their immunoregulatory activity that provided the possibility of the allograft of MSCs without rejection [11]. In addition, MSCs have been proved to be safe when auto- or allo-transplantation was carried out [12].

There was a controversy on defining biological characteristics of MSCs among investigators, which could be caused by differences in human species, sources of tissues, isolation and expansion methods, as well as culture systems. To address this problem, the International Society for Cellular Therapy (ISCT) proposed three criteria, including adherence to plastic, specific surface antigen expression, and multipotent differentiation potential, to define MSCs [13]. It should be noted that manufacturing process systems would largely influence the characteristics of MSCs. Due to considerable therapeutic potential, manufacturing of MSCs should be under specific conditions which comply with

requirements of clinical application and current good manufacture practice (cGMP). However, the gap between experimental study and clinical application of MSCs was slowly highlighted, especially from bench to beside. To provide a guide for application of MSCs to regenerative medicine and cellular therapy, we chose MSCs derived from adipose tissue (ASCs), Wharton's Jelly of umbilical cord (WJ-MSCs) and chorionic plate of full-term placenta (CP-MSCs) to compare their biological and genetic characteristics under serum-free medium conditions in accordance with the requirements of clinical and cGMP guidelines.

Materials and methods

Isolation and expansion of ASCs, WJ-MSCs and CP-MSCs

All protocols were reviewed and approved by the Institutional Research Board of Pharmacy Department, Jiangsu University, and Sichuan Wuyan Biotechnology Co., Ltd., prior to the study.

ASCs

Lipoaspirates were collected from female inner thighs (N = 10) with aseptic technique after the informed written consents were sought from them. The collected adipose tissues were stored in a sterile bottle containing 100 microliter (mL) saline injection at 4~8°C. The adipose tissues were minced with surgical scissors. A 10 mL of the minced adipose tissues were transferred into a sterile 50 mL centrifuge tube (Corning) and 30 mL saline injection containing 0.9% sodium chloride was added. The adipose tissues were then centrifuged at 2000×g for 10 min at 20°C after which the lipid above the adipose was aspirated out. After washing the adipose twice, 4 mL adipose tissue was inoculated into a T-175 culture flask (Corning) and 10 mL StemPro MSC serum-free medium (SFM) (Life Technology) supplemented with 1% GlutaMAX™-I CTS (Gibco) was added. The flasks were placed in an incubator at the condition of 37°C, 5% CO_2 after mixing evenly. The medium was refreshed every 4 to 5 days. When the confluency was at 80 to 90 percent, the cell culture was dispersed with TrypLE™ Select (Gibco). And ASCs were passaged at a density of 1×10^5 cells/cm^2 feeding with 25 mL medium, which was replaced every 3 days.

CP-MSCs and WJ-MSCs

Fresh human placentas with umbilical cord (N = 10) were collected from full term births after written informed consent was obtained from their parents. Placenta and umbilical cord were stored in a sterile container and processed within 24 hours from the partum to obtain Chorionic plate and Wharton's Jelly tissues, respectively. Specifically, placenta and umbilical cord were washed with saline injection three times. The chorionic plate and Wharton's Jelly were separated with aseptic technique, followed by mincing into 3~5 mm^2 explants with surgical scissors. After washing three times, 2 g explants of chorionic plate and Wharton's Jelly, respectively, were inoculated separately into T-175 culture flask containing 10 mL StemPro MSC SFM supplemented with 1% GlutaMAX™-I CTS, respectively. Fresh medium was replaced every 4 to 5 days. When the confluency reached 80 to 90 percent, the cell culture was dispersed with TrypLE™ Select. The CP-MSCs and WJ-MSCs were then passaged at a density of 1×10^5 cells/cm^2 feeding with 25 mL medium, which was refreshed every 3 days.

Cell morphology and flow cytometry

The cell morphology was observed and pictured under inverted microscope (Olympus, CKX41). The MSCs were detached when the cells reached about 80% confluence. After digestion with a 0.25% trypsin/EDTA solution (Hyclone), the cells were washed with phosphate buffer saline (PBS) twice followed by centrifuging at 300×g for 5 min. The cells were resuspended in cold PBS containing 2% fetal bovine serum (FBS), and the concentration of cells adjusted to 1×10^6/mL prior to addition of monoclonal antibodies as follows: CD11b-FITC, CD19-ECD, CD34-PE, CD45-PC7, CD73-PE, CD90-PC5, CD105-PE, CD29-FITC and histocompatible locus antigen-DR (HLA-DR)-PC7 purchased from Beckman Coulter. The unmarked cells were used as negative control. Finally, the stained cells were analyzed using a Beckman Coulter flow cytometry system (Beckman Coulter, FC500).

Karyotyping

When the MSCs were at logarithmic growth phase, colchicine (Sigma) was added to the cell culture at a final concentration of 0.04 mg/L. The cell culture was incubated for another 3.5

Table 1. Primers for quantitative real-time PCR (qRT-PCR)

Primers Name	Primers Sequence	Product Length
P16-F	5'-GCA GCA TGG AGC CTT CGG-3'	197bp
P16-R	5'-CCG TAA CTA TTC GGT GCG T-3'	
P21-F	5'-TGT CCG TCA GAA CCC ATG-3'	219bp
P21-R	5'-GTG GGA AGG TAG AGC TTG G-3'	
P53-F	5'-AGG GAT GTT TGG GAG ATG TAA G-3'	179bp
P53-R	5'-TGT GAG GTA GGT GCA AAT GC-3'	
TERT-F	5'-CTA CGG CGA CAT GGA GAA CAA G-3'	150bp
TERT-R	5'-CCA TAC TCA GGG ACA CCT CG-3'	
K-ras-F	5'-TTG ATT TGT CAG CAG GAC CA-3'	149bp
K-ras-R	5'-GAG AGT TTC ACA GCA TGG ACT G-3'	
Nanog-F	5'-ATG CCT GGT GAA CCC GAC-3'	292bp
Nanog-R	5'-AGG ACT GGA TGT TCT GGG T-3'	
CCNE-F	5'-AAG GTT TCA GGG TAT CAG TGG TG-3'	185bp
CCNE-R	5'-TTT GCT CGG GCT TTG TCC-3'	
β-actin-F	5'-CAC GAA ACT ACC TTC AAC TCC-3'	265bp
β-actin-R	5'-CAT ACT CCT GCT TGC TGA TC-3'	

Expression of the β-actin was assessed as internal control.

h, after which, the cells were digested with 0.25% trypsin-EDTA, and collected into a tube before washing with PBS 3 times. The cells were treated with 5 mL KCl hypotonic solution at a concentration of 0.075 mol/L, and kept in a water bath at 37°C for 15 min. The supernatant was discarded after cell suspension was centrifuged at 300×g for 8 min. The cell pellet was fixed with 2 mL fixative, incubated at 37°C for 3 min, and then centrifuged. The supernatant was discarded and the fix step was repeated. At the second time, the cells were fixed with 8 mL Carnoy's fluid (Sigma) for 30 min. The re-suspended cells were dropped onto a slide and dried naturally followed by staining with Giemsa for 10 min. The cells were subsequently observed and analyzed under electron microscopy.

Tumor-related gene expression assay

The primers for P16, P21, P53, TERT, K-ras, CCNE and Nanog genes (**Table 1**) were designed using Primer Premier 5.0 software and then was synthesized by Sangon Biotech (Shanghai) Co., Ltd. Cells were digested with 0.25% trypsin-EDTA when the confluency arrived at 80%. Cells were washed twice with cold PBS and total RNA was extracted with RNAiso Plus (Takara) according to the manufacture's protocol. The cDNA synthesis was carried out using ThermoScript™ RT-PCR System (Invitrogen). Quantitative real-time polymerase chain reactions (qRT-PCRs) were carried out with SYBR Green master mix and ABI real-time thermocycler (ABI7500). The total volume (20 μL) of the PCR system comprised of the Fast SYBR Green Master mix (10 μL), forward and reverse primers of each gene (1 μL), cDNA template (2 μL) and sterile distilled water (7 μL). The reaction conditions were initial denaturation at 95°C for 5 min, followed by denaturation at 95°C for 15 s, extension at 60°C for 60 s of 40 cycles and final extension at 72°C for 1 min. The expression levels of the individual genes were normalized against β-Actin and the results were analyzed afterwards.

Detection of growth factors

The spent medium was replaced with 10 mL fresh Dulbecco's Modified Eagle Medium (DMEM) when cell confluency was up to 70%. Then the DMEM was collected and centrifuged at 5000 rpm for 5 min afterwards the cells were incubated for another 10 h at 37°C, 5% CO_2. The supernatant was then used to determine the expression level of growth factors secreted by MSCs with ELISA test kits for basic markers such as FGF, NGF, EGF, HGF, LIF and VEGF (R&D systems). The operation procedure kept pace with the manufacture's protocols.

Statistical analysis

All data were analyzed with GraphPad Prism version 5 (GraphPad software, San Diego California, USA; http://www.graphpad.com) and presented as the mean standard error of the mean. The ANOVA was used to compare numeric data among different experimental groups. Statistical analysis was performed with Statistical package for social sciences (SPSS) Software (SPSS Inc, Chicago, USA; http://www.spss.com) with differences between groups assessed by the paired Student's t-test and *p* values less than 0.05 were accepted as statistically significant.

Results

Comparison of biological characteristics of three types of MSCs

By 5 to 6 days post-inoculation, ASCs began to outgrow from explants of the adipose tissues

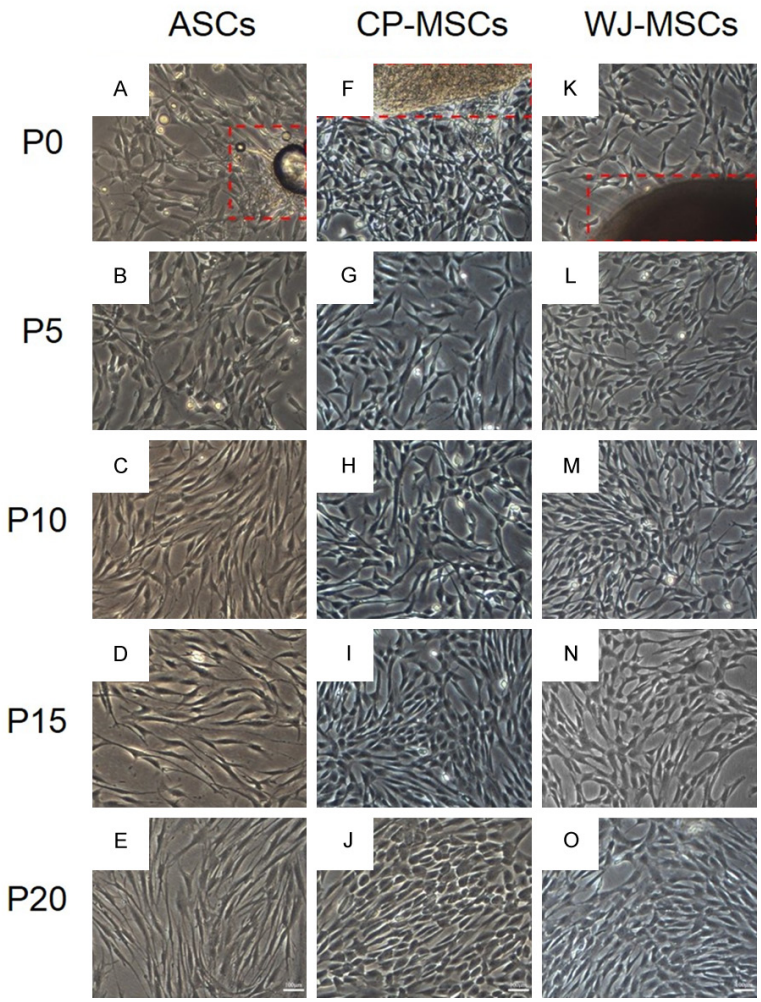

Figure 1. Comparison of cell morphology of MSCs. MSCs were isolated with explants methods, and the primary cells (P0) of ASCs (A), CP-MSCs (F) and WJ-MSCs (K) outgrew from explants (red dashed box) of adipose tissue, chorionic plate of placenta and matrix of umbilical cord. These cells in low passages (P0~P10) were typically spindle-shaped (A, B, C for ASCs; F, G, H for CP-MSCs; K, L, M for WJ-MSCs), and in high passages (P15~P20) changed to be abnormal with morphology of elongated ASCs (D, E), and polygonal CP-MSCs (I, J) and WJ-MSCs (N, O) with an extensive cytoplasmic volume. P: passage; scale bar: 100 μm.

Table 2. CD marker profile of MSCs (Mean ± SD)

CD marker	ASCs (N = 10)	CP-MSCs (N = 10)	WJ-MSCs (N = 10)
CD11b	0.08%±0.06%	0.04%±0.01%	0.23%±0.18%
CD19	0.16%±0.19%	0.12%±0.05%	0.14%±0.07%
CD34	0.22%±0.13%	0.20%±0.17%	0.26%±0.24%
CD45	0.37%±0.27%	0.40%±0.26%	0.56%±0.21%
HLA-DR	0.54%±0.29%	0.46%±0.34%	0.35%±0.27%
CD29	99.99%±0.01%	99.50%±0.50%	99.37%±1.16%
CD44	99.95%±0.04%	99.23%±1.07%	99.88%±0.17%
CD73	99.89%±0.32%	99.35%±0.84%	99.25%±0.92%
CD90	99.38%±0.52%	88.82%±11.83%	99.51%±0.99%
CD105	99.37%±1.71%	99.13%±0.91%	98.95%±1.15%

All cells were tested at passage 5.

with few explants attaching to the surface of flasks. In contrast, the outgrowth of CP-MSCs and WJ-MSCs were been observed around day 7 or day 8, with most of the explants adhering to the bottom of the flask. Marked alternations to cell morphology were discovered in low and high passages. The morphology of ASCs, CP-MSCs and WJ-MSCs were typically spindle-shaped when cells were passaged less than 10 times. At passage 15, ASCs were elongated in shape, and the morphology of CP-MSCs and WJ-MSCs were polygonal with an extensive cytoplasmic volume, which was observed with Ti-E (Nikon) (**Figure 1**). However, within the same period, the proliferation rate of the three types of MSCs slowed down (data not shown).

To compare the immunophenotyping of the three types of MSCs, the CD marker profile was examined at passage 5. The ASCs, CP-MSCs and WJ-MSCs expressed CD29, CD44, CD73, CD90 and CD105, which were considered as CD markers for MSCs [14]. In contrast, no expression of the endothelial cells or hematopoietic lineage markers including CD11b, CD19, CD34 and CD45 were observed. In addition, all MSCs were negative for HLA-DR, which was a major histocompatibility complex (MHC) class II cell surface receptor originally considered to mediate graft-versus-host disease (GvHD). The expression of CD90 in CP-MSCs was unstable and the expression level was 88.82±11.83% (**Table 2**).

Comparison of karyotype

Karyotyping of MSCs, which was performed at passage 1,

Figure 2. Karyotyping of MSCs. The fifth subculture of ASCs (A, female), CP-MSCs (B, male) and WJ-MSCs (C, male) were measured with a result of normal diploid karyotype.

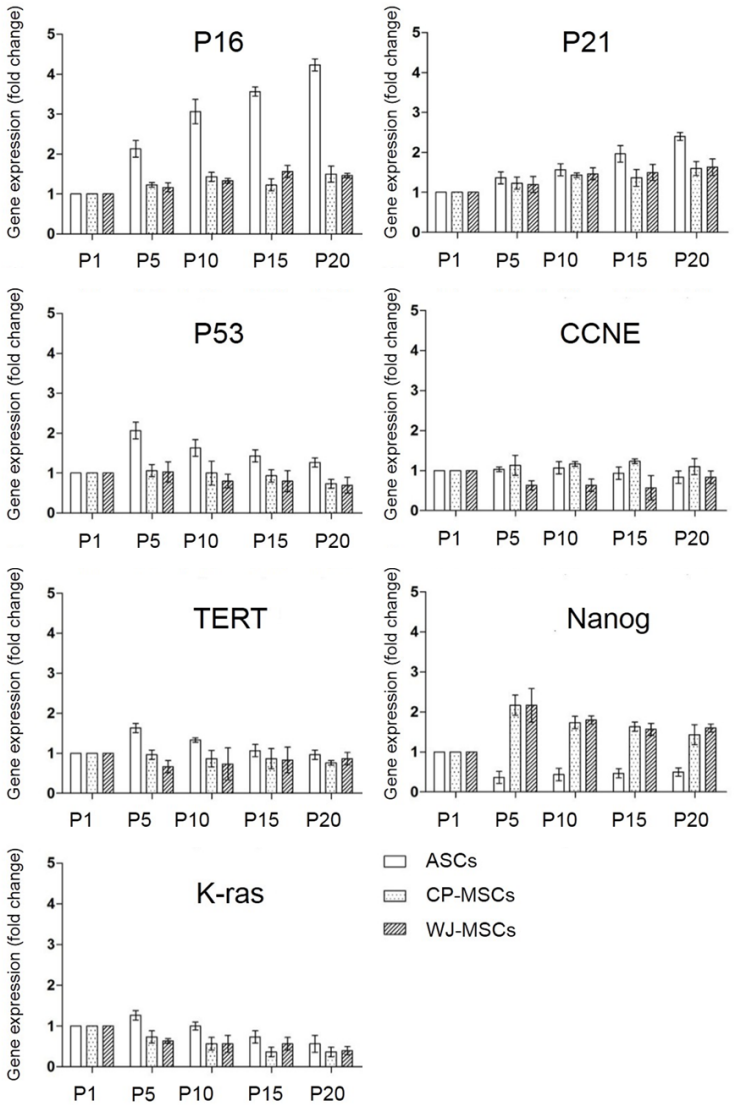

Figure 3. Expression level of tumor-related genes among three types of MSCs. Quantitative real-time PCR (RT-PCR) was carried out to measure the expression of tumor-related genes. P16, P21, P53 and CCNE were key regulators of cell cycle, and also related to formation of various tumors. The other three genes (TERT, Nanog and K-ras) were considered to regulate the pluripotency of cells, which were also relevant to tumorigenesis. The cells at passage one (P1) were used as control group.

5, 10, 15 and 20, demonstrated a normal diploid karyotype with no deletions, translocations and inversions. **Figure 2** shows the karyotype analysis results of 5th subculture of MSCs derived from adipose tissue, placenta and umbilical cord.

Expression of tumor-related genes

The expression levels of tumor-related genes including P16, P21, P53, CCNE, Nanog, TERT and K-ras were measured at 1st, 5th, 10th, 15th and 20th passages of ASCs, CP-MSCs and WJ-MSCs respectively. **Figure 3** shows that tumor-related genes P21, P53, CCNE, TERT and K-ras were stably expressed in ASCs, CP-MSCs and WJ-MSCs, except that of P16, was highly observed in ASCs after passage 1. Nanog expression in CP-MSCs and WJ-MSCs were higher than ASCs.

Secretion of growth factors

Growth factors expression level of MSCs was shown in **Figure 4**. The secretion level of basic FGF in ASCs and CP-MSCs was higher than that of WJ-MSCs, with the content between 15 and 25 pg per milliliter. No significant difference was observed in the expression of VEGF and EGF among

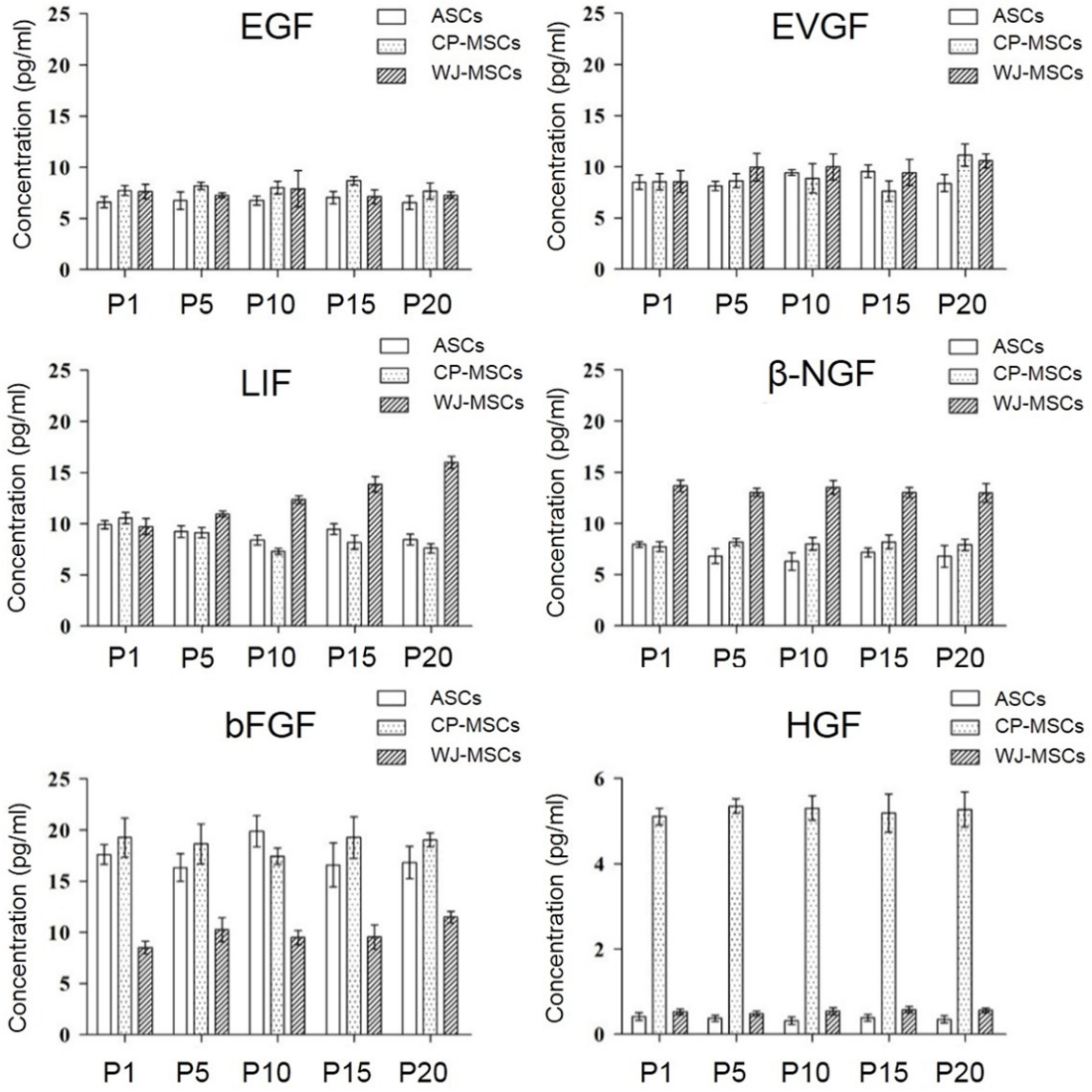

Figure 4. Determination of growth factors secreted by MSCs. The secretion level of growth factors of ASCs, CP-MSCs and WJ-MSCs was measured with the method of ELISA.

ASCs, CP-MSCs and WJ-MSCs cell cultures. The WJ-MSCs had a higher secretion level of β-NGF and LIF than the other two types of MSCs, which posed similar secretion level of these two growth factors. In particular, the expression level of HGF in CP-MSCs was much higher than that in ASCs and WJ-MSCs in cell culture with concentration of 4 to 6 ng/mL.

Discussion

In this study, we isolated the MSCs from adipose tissue (ASCs), placenta (CP-MSCs) and umbilical cord (WJ-MSCs) [2] via the explants method. They were expanded using commercial serum free medium (SFM), and the biological and genetic characteristics of different types of MSCs were compared. The results indicated that cell morphology of the MSCs at high passages was not typically spindle-shaped though these cells presented normal morphology at low passages under conditions of SFM. However, karyotyping assays did not show any chromosomal aberrations. It could be concluded that the change of cell morphology was not induced by alterations on chromosomes, but rather they were induced by culture conditions [14] or cellular senescence [15, 16] as reported by other investigators. This phenomenon also

indicated that it was at low passages, other than high passages and hence MSCs manufactured with SFM culture system according to cGMP guidelines were suitable for clinical application.

The immunophenotyping of ASCs, CP-MSCs and WJ-MSCs was similar to that of BM-MSCs [17, 18], which was positive for CD29, CD44, CD73, CD90 and CD105, and negative for CD11b, CD19, CD34, CD45. It should be noted that the expression level of CD90 from different CP-MSCs samples, even from same samples at different passages, were not always more than 95%, whereas some were even lower than 64.5% (data not shown). The CP-MSCs, however, expressed CD29 stably not only from different samples but also from same samples at different passages. These results showed that CD29, not CD90, might be a reliable reference of MSCs for CP-MSCs which were cultured with SFM systems, compared to the same result observed in a previous study [19]. No expression of HLA-DR among the three types of MSCs demonstrated that these cells had immunosuppressive properties without risk of graft-versus-host disease (GvHD) when cells carried out auto- or allo-transplantation [20]. This would accelerate the pace of MSCs as a good drug candidate for regenerative medicine and cell therapy [21].

As an important cell cycle regulator, P16 was reported to be correlated with cell senescence, especially when the cells were subcultured to high passages *in vitro* [22]. The ASCs were observed to express higher level of tumor-related genes including P16 than that of perinatal MSCs (CP-MSCs and WJ-MSCs) in this study, which indicated that perinatal MSCs posed more multipotent than ASCs. At the same time, the perinatal MSCs expressed high level of Nanog which was regarded as a gateway to the pluripotency of the stem cells [23]. Although being treated as a factor promoting the formation of diverse tumors [24], Nanog was found to reverse the senescence of cells [25]. To that point, the CP-MSCs or WJ-MSCs were, in theory, are more suitable as a cell therapy for Hutchinson-Gilford Progeria syndrome than ASCs which was collected from adult bodies. However, further clinical practice need to be carried out to validate that speculation.

As reported by many researchers, the therapeutic effects of MSCs might not only be due to the multipotency of MSCs differentiating into various tissues derived cells, but also owing to the paracrine mechanisms, which posit that MSCs promote tissue regeneration or wound healing [16, 26-28]. Six growth factors were measured and the results showed no obvious difference, except HGF expression in CP-MSCs among the three types of MSCs. The HGF was mainly secreted by hepatocytes from liver of human body. Previous studies declared that CP-MSCs could promote hepatic regeneration in CCl_4-injured rat liver model [29, 30]. Thus, our results could provide a theoretical proof for those findings.

In summary, using serum-free culture system with explants method, ASCs, WJ-MSCs and CP-MSCs displayed some common and slightly different biological and genetic characteristics. All these three types of MSCs were spindle-shaped cell in less than 10 passages and also the karyotypes were in normal status within 15 passages. The surface of CD markers for three types of MSCs were negative for CD11b, CD19, CD34, CD45 and HLA-DR, and positive for CD29, CD44, CD73, CD90 and CD105, but we suggest the use of CD29 for CP-MSCs identification instead of CD90, because CD90 expression was lower than 90% in CP-MSCs. ASCs expressed higher level of P16, whereas CP-MSCs and CP-MSCs had more expression of Nanog. The HGF secretion by CP-MSCs was higher than ASCs and WJ-MSCs, which means CP-MSCs are more suitable for hepatopathy therapy. In conclusion, MSCs in low passages are stable and show prospect for clinical application, while MSCs from different tissue needed to be exploited for more research when they are to be employed for the cure of different kinds of diseases.

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Disclosure of conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work. There is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled "Biological and Genetic Characteristics of Mesenchymal Stem Cells in Vitro Derived from Human Adipose, Umbilical Cord and Placenta".

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