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Comparative characterization of mesenchymal stem cells from different age groups of cynomolgus monkeys

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Bone marrow mesenchymal stem cells (BM-MSCs) are a potential tool for cell therapy and tissue engineering. In this study, we carried on a comparative study of the characteristics of MSCs from different age cynomolgus monkeys. A variety of factors, including donor age, must be considered before further applications, and various tests should be used to properly assess MSCs before the clinical application, especially when a prolonged culture and *ex vivo* expansion is necessary.

cynomolgus monkey, mesenchymal stem cells, differentiation, expansion, safety

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Bone marrow mesenchymal stem cells (BM-MSCs), a heterogeneous population of adult stem cells, have been extensively investigated for their various therapeutic approaches [1–3]. The reason is that MSCs have the ability to expand *ex vivo* and differentiate into osteocytes, adipocytes, and chondrocytes, which are potentially usable for cell therapy and tissue engineering [4–7], and provide support for primitive hemopoietic cells as hematopoiesis-supporting stroma [8]. MSCs are also easy to genetically modify to express therapeutic genes, using lentiviral, adenoviral, or adeno-associated viral vectors [9]. It has also been shown that MSCs have limited immunogenicity and display a unique immunosuppressive and immunoregulatory function [10]. Indeed, MSCs as a prospective cell source have already been experimentally used in preclinical and clinical trials.

In a genetic sense, NHPs and humans share about 95% of their respective DNA in common [11,12]. Experimental evidence suggests that bone marrow-derived MSCs isolated from NHPs are very similar when compared to MSCs from other species including humans [13]. Cynomolgus monkey MSCs (cMSCs) have been invaluable in developing and assessing new therapeutic transplant approaches for the treatment of human diseases [14]. However, the characteristics of cMSCs from different ages of NHPs are not yet clear. In this study, we accomplished a comparative study of cMSCs isolated from different age groups, and found that cMSCs from adolescents and young adult groups had significantly higher proliferation and differentiation potential than those from the middle-aged group. Due to long-term culture and different age groups, part of the cell surface markers significantly changed.

Safety is a great concern in MSCs application. Several studies reported that MSCs are highly resistant to transformation *in vitro* [15–18]. However, other studies showed that MSCs undergo spontaneous transformation at a high frequency in long-term culture [19–22]. In this study, we examined the telomerase activity and the karyotype of cMSCs in long-term culture, and found that the telomerase activity of cMSCs from different age groups is unchanged. However, with long-term culture, spontaneous transformation oc-

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curred in two cases of the YM group, and telomerase activity of TMCs significantly increased, the frequency of chromosome instability increased in cMSCs accordingly.

Our results showed the *ex vivo* expansion and the differentiation capacity of cMSCs declined with the age of the donors, and suggested that their therapeutic potential might be diminished as well. Therefore, it may be that cells with both proliferation and differentiation potency would be an optimal tool for future cell-based therapeutic applications. These findings suggest that various tests should be used to properly assess MSCs before clinical application, especially when a prolonged propagation period is required.

1 Materials and methods

1.1 The primary culture of cMSCs

According to the age standard of rhesus monkeys [23], the animals were grouped as adolescent monkeys (AM, 3–4 years old; *n*=6), young adult monkeys (YM, 8–10 years old; *n*=6) and middle-aged monkeys (MM, 14–16 years old; *n*=6). Bone marrow aspirates (5 mL) were collected from the iliac crest, as previously described [24]. Mononuclear cells (MNCs) were isolated using Ficoll-Paque TM Plus (StemCell Tech Inc., Vancouver, Canada). MNCs were washed, and plated at 4×10^5 cells cm^{-2} in an Alpha-MEM (Invitrogen, Carlsbad, CA, USA) medium containing 10% MSC Qualified Fetal Bovine Serum (Qualified FBS, GIBCO AUS), 1% GlutaMAX™-I Supplement (Invitrogen, Carlsbad, CA, USA), and 1% Penicillin-Streptomycin liquid (Invitrogen, Carlsbad, CA, USA). The medium was replaced every other day until the cells reached ~80% confluence. The cells were harvested with 0.25% Trysin-EDTA (Invitrogen, Carlsbad, CA, USA) for 3–5 min at 37°C, and replated at 1×10^4 cells cm^{-2} in the medium described above.

1.2 PCR assay of simian foamy viruses (SFV)

Genomic DNA was extracted from cMSCs using a DNA mini kit (Qiagen Ltd., Crawley, UK) according to the manufacturer's specifications. RNA was extracted using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). PCR on purified DNA and cDNA was performed on a PTC-100 thermal cycler (Bio-Rad Laboratories, Inc., Waltham, MA) using rTaq polymerase (Takara, Bio, Inc., Kyoto, Japan). The following primer pairs were used: round 1: forward 5′-AGGATGGTGGGGACCAGCTA-3′; reverse 5′-GCTGCC CCTTGGTCAGAGTG-3′ and round 2: forward 5′-CAGT GAATTCCAGAATCTCTTC-3′; reverse 5′-CACTTATCC CACTAGATGGTTC-3′ as previously described [25,26]. Amplification was performed in an ABI 7700 Sequence Detection System (Applied Biosystems).

1.3 Cell counting

After the primary culture, cMSCs were cultured in an Alpha-MEM medium containing 20 ng mL^{-1} bFGF (R&D System, Minneapolis, MN, USA) and 10 μmol L^{-1} Tenofovir (LGM Pharmaceuticals Inc., Boca Raton, FL, USA), as well as 10% MSC Qualified Fetal Bovine Serum, 1% GlutaMAX™-I Supplement and 1% Penicillin-Streptomycin liquid. cMSCs were seeded at a density of 2×10^4 cells cm^{-2}, and were digested and counted on a hemocytometer at each passage. From the primary passage of 1×10^6 cells derived from the different age groups, the total number of cells was calculated. Values were averaged from 3 independent experiments.

1.4 Cell surface marker expression

The cells were collected and washed with cold PBS twice, and re-suspended at 4°C in PBS containing 1% BSA, and stained with the fluorescent antibody at 4°C for 30 min (Supplemental Table 1). FITC-CD106 (clone 51-10C9), PE-CD73 (clone AD2), FITC-CD90 (clone 5E10), Percp-CD14 (clone MφP9), APC-CD34 (clone 8G12), Percp-CD45 (clone 2D1), FITC-CD19 (clone SJ25C1) and FITC-HLA-DR (clone L243 (G46-6) were purchased from PharMingen (San Diego, CA). FITC-CD105 (clone SN6) was purchased from Serotec (Oxford, UK). PE-STRO-1 was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Five thousand events were acquired using a FACS Calibur Flow Cytometry System (Becton Dickinson Cytometry Systems, San Jose, CA) and analyzed with Win MDI 2.9 software.

1.5 Differentiation of cMSCs

cMSCs were differentiated into osteocyte, adipocyte, and chondrocytes using a differentiation medium (Lonza Walkersville, Inc., Walkersville, Maryland, USA) following the manufacturer-provided protocols (http://www.lonza.com). As previously described [27], for adipogenic differentiation, cMSCs were plated at 2.1×10^4 cells cm^{-2}, when reaching a 100% confluence, cells were incubated in three cycles of the induction/maintenance medium. cMSCs were subsequently cultured for 7 d in a supplemented adipogenic maintenance medium. Cells were fixed with 10% buffered formalin, and stained with Oil Red O. Oil Red O stain was extracted from differentiated adipocytes using 100% isopropanol. The absorbance was determined at 510 nm using a spectrophotometer (Bio-Rad, Hercules, CA). For each chondrogenic differentiation, 2.5×10^5 cMSCs were pelleted at 150×*g* for each differentiation. The pellets were incubated in a complete chondrogenic induction medium containing 10 ng mL^{-1} transforming growth factor-β3 (TGF-β3) for 28 d. Paraffin-embedded pellets were sliced at 5 μm, and stained with Safranin O stain. Ten sections stained Safranin O were randomly selected to calculate the volumes of the chondrogenic fraction using the Cavalieri method as previously described [28]. For osteogenic differentiation, cMSCs were plated at 3.1×10^3 cells cm^{-2}, and incubated for 24 h with a

proliferation medium. The medium was replaced with an osteogenic induction medium, and the medium was replaced every 3 d for a total of 21 d. The cells were fixed and stained with Von Kossa stain to assess mineralization. Calcium production was also measured at 560 nm using a spectrophotometer (Bio-Rad) as previously described [27].

1.6 Senescence cells staining

The assay of senescence cells was based on a histochemical stain for β-galactosidase activity. β-galactosidase activity is easily detectable in senescent cells, but undetectable in quiescent, immortal, or tumor cells. The Senescence Cells Histochemical Staining Kit (Sigma-Aldrich, Saint-Louis, Missouri, USA) was performed according to the manufacturer's instructions.

1.7 Karyotype analysis

The adherent cells were treated with Colcemid (Sigma-Aldrich, Saint-Louis, Missouri, USA), and were detached utilizing 0.125% trypsin (Gibco BRL, Grand Island, NY, USA) and treated with 0.075 mol L^{-1} KCl in a 37°C water bath. Cells were fixed and spread onto slides. Air-dried slides were stained with freshly prepared 10% Giemsa stain (Invitrogen, Carlsbad, CA, USA) in a Gurr buffer (Invitrogen, Carlsbad, CA, USA) for 30 min. The slides were observed under a light microscope, before printing out each karyotype and counting each chromosome by writing a number on each sister chromatid pair.

1.8 Telomerase activity

To measure and compare the telomerase activities of cMSCs, the Telomerase ELISA Assay Kit TeloTAGGG (Roche Applied Science) was performed according to the manufacturer's instructions. For every single reaction, 2×10^5 cells were examined. The mean value of the telomerase was averaged from three independent experiments.

1.9 Statistical data analysis

The results were expressed as means±SEM for all groups. Multiple comparisons between groups were analyzed by ANOVA. Pairwise comparisons were made using Student's *t*-test. A value of $P<0.05$ was considered to be significant.

2 Results

2.1 The primary culture of cMSCs

Bone marrow mononuclear cells (MNCs) were plated at a density of 4×10^5 cells cm^{-2} in a 25 cm^2 flask. The medium was replaced after 2 d, and proliferated colonies emerged in 2–4 d. cMSCs of the AM group and the YM group are spindle-shaped cells with a whirlpool-like array, and showed a strong proliferative potential (Figure 1A), while MM group cMSCs showed greater heterogeneity, inconsistent morphology and a larger cell size (Figure 1B). The cell numbers of the primary culture derived from 1×10^7 MNCs exhibited no significant difference between the groups (Figure 1C),

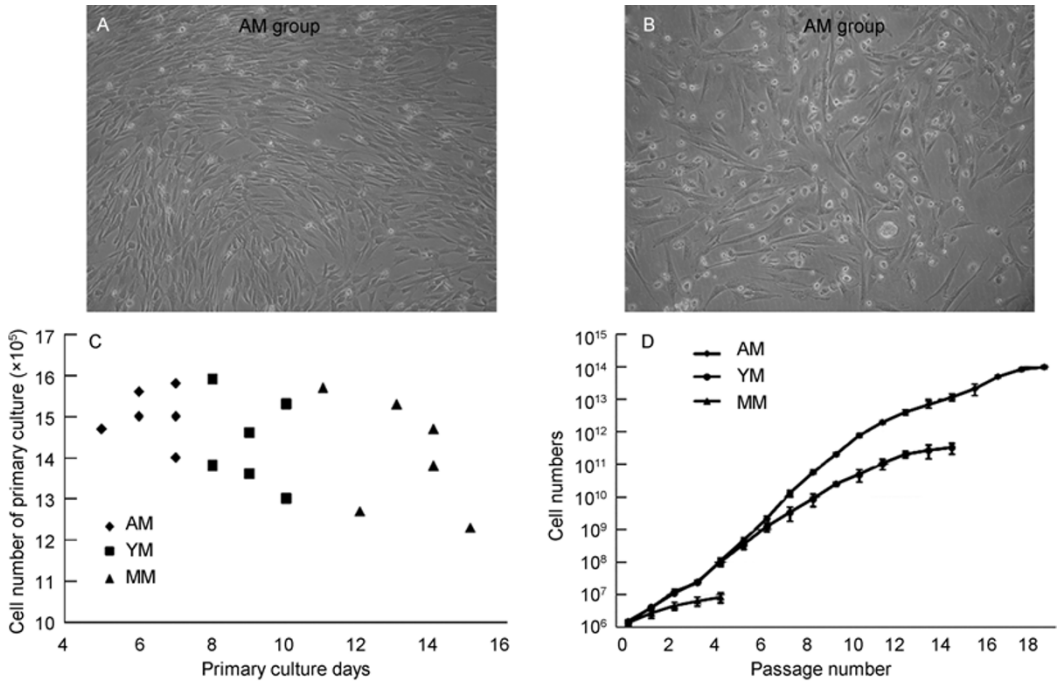

Figure 1 Primary culture of cMSCs from different age groups. A, AM group cMSCs in the primary culture; B, MM group cMSCs in the primary culture; C, the time required for primary culturing and the cell numbers harvested in the primary culture; D, passage number and cell number in different age groups. A and B, original magnification 100×.

respectively as (15.02±0.65), (14.37±1.10) and (14.08±1.39), $\times 10^5$ cells 25 cm^{-2} flask in the AM, YM and YM groups. However, there were significant differences in the culture time required for the primary culture to reach confluence between the groups ($P<0.05$) (Figure 1C), respectively, AM group, (6.33±0.82) d; YM group, (9.00±0.89) d; YM group, (13.17±1.17) d. In our cultures the cells were maintained for about 20 passages in the AM group and about 14 passages in the YM group, and no more than 4 passages in the MM group (Figure 1D). According to this protocol, started with about 1×10^6 cMSCs of the primary passage, we expanded the YM group cMSCs to about 10^{11}–10^{12} cells, and the AM group cMSCs to about 10^{13}–10^{14} cells before the cell cycle entered quiescence (Figure 1D). However, MM group cMSCs are unable to be expanded to a large number *in vitro* (Figure 1D).

2.2 Proportion of SFV infection in cMSCs

Studies from captive and free-ranging NHPs over 3 years old show that up to 100% are infected with SFV [29]. Therefore, consistent with a previous report [30], we used Tenofovir in our culture system to avoid SFV infection (Figure 2A). Fusion cells were observed in cultured cMSCs infected by SFV without Tenofovir (Figure 2B). In order to explore the proportion of SFV infection in cultured cMSCs, RT-PCR was applied to confirm the SFV infection in cultured cMSCs (Figure 2C). The report of rhesus monkey MSCs showed that cells cultured with Tenofovir maintained normal morphology and growth [30], and were therefore usable in other experiments. Consistent with previous studies [29], up to 100% of cultured cMSCs from the YM and the MM group were infected with SFV, except for the AM group, whose percentage was 66.67%.

2.3 Surface markers of cultured cMSCs

To further characterize the phenotype of cultured cMSCs, we examined the surface markers by flow cytometry. In line with previous reports [18], cMSCs expressed high levels of CD73 and CD105, moderate levels of CD90, CD106 and Stro-1, and did not express the following cell surface markers: CD14, CD19, CD34, CD45 or HLA-DR (Figure 3A). Compared with AM group cMSCs (p4), CD90 and Stro-1 of YM and MM group cMSCs (p4) were significantly increased (Figure 3B, Supplemental Table 2). With long-term culture, CD90 markedly increased in the AM group (Figure 3C, Supplemental Table 2).

2.4 Differentiation potential of cMSCs

To examine the differentiation potentials of different age groups cMSCs, the differentiation of cMSCs (p4) into osteocytes, adipocytes and chondrocytes was carried out. AM and YM group cMSCs (Figures 4A, B and C) showed greater differentiation potential than MM group cMSCs (Figures 4D, E and F). Quantitative comparison results also demonstrated that the cMSCs of the AM and the YM group

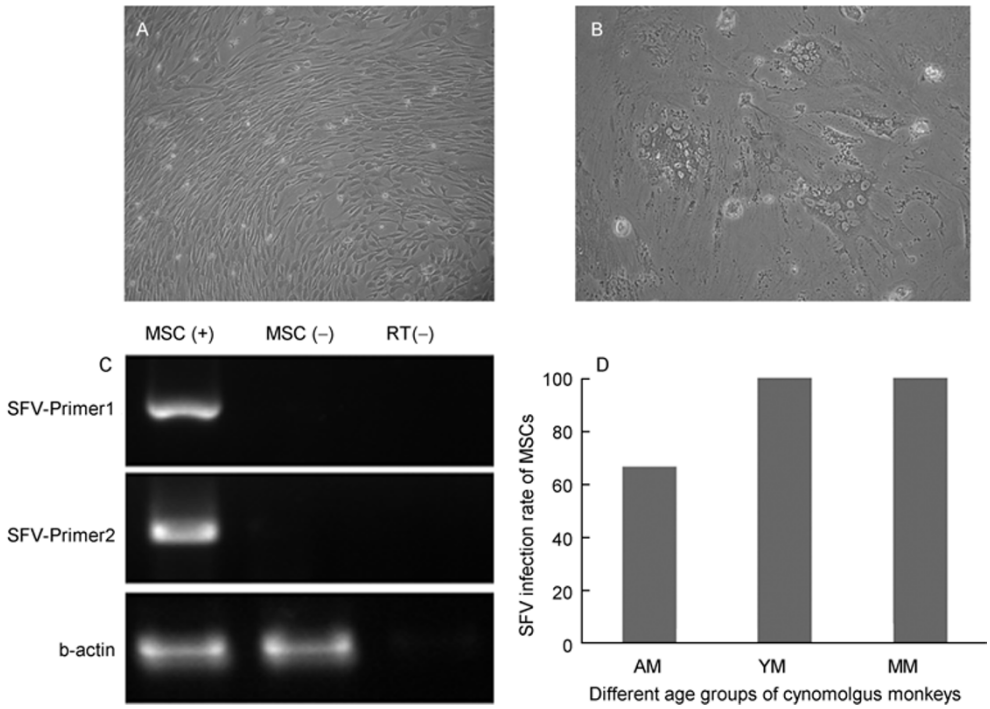

Figure 2 SFV infection of cMSCs from different age groups. A, the culture of cMSCs with the additive Tenofovir; B, the culture of cMSCs without Tenofovir showed the morphology of fusion cells; C, reconfirmation of cMSCs with SFV infection by RT-PCR; D, the proportion of SFV infection in cMSCs from different age groups. A, original magnification 100×; B, original magnification 200×.

had significantly higher differentiation potential than those of the MM Group (Figures 4G, H and I). Differentiation potential had no statistically significant difference between the AM group and the YM group cMSCs (p4). In order to detect the influence of long-term culture on the differentiation capacity of cMSCs *in vitro*, AM group cMSCs were differentiated into adipocytes at p4, 10 and 16. The results found that the differentiation potential began to decrease at p10, and strikingly decreased at p16 (data not shown).

2.5 Senescence and spontaneous transformation of cMSCs

At early passage cMSCs were spindle shaped in nature, similar to a fibroblast (Figure 2A). After a period of time culture, the changes of cMSCs were shown in both size and shape (Figures 5A, B and C). A corresponding increase in senescence-associated β-galactosidase activity was observed in the 18th passage of the AM group (Figure 5D), the 14th passage of the YM group (Figure 5E), and the 4th passage of the MM group (Figure 5F). Following senescence, many granular cell inclusions began to appear in the cytoplasm, and form debris in the medium (Figure 5G). During and after senescence, spontaneous transformation of cMSCs occurred in two cases of YM group cMSCs. Compared with the original cMSCs, TMCs were clearly morphologically changed (Figures 5H and I). The telomerase activity of TMCs significantly increased compared with that of the original cMSCs (Figure 5K) (P<0.001). There was no statistically significant difference in the telomerase activities between different age groups (Figure 5J).

2.6 Karyotype analysis of cultured cMSCs

Karyotype analysis of cultured cMSCs was performed by Giemsa staining. The results showed chromosome instability in long-term cultured cMSCs. Common chromosomal abnormalities included: extra chromosomes, 43, XY i(14) and 43, XX i(19) (Figure 6B), missing chromosomes: 41, XY del(13) and 39, XX del(16;20;20) (Figure 6C). With the prolongation of culture time, the proportion of cells with abnormal chromosomes significantly increased in the AM and the YM group (respectively 37.5% and 44.4%, Table 1). The karyotype of TMCs did not show any aberrations by Giemsa staining. The normal karyotypes of cMSCs, 44, XY and 44, XX are shown in Figure 6A.

3 Discussion

MSCs are an attractive adult stem cell source, possessing the capacity for self-renewal and differentiation into a variety of mesodermal cells under appropriate conditions, providing a prospective therapeutic approach for tissue regeneration [30–33].

Prior studies have described the characterization of

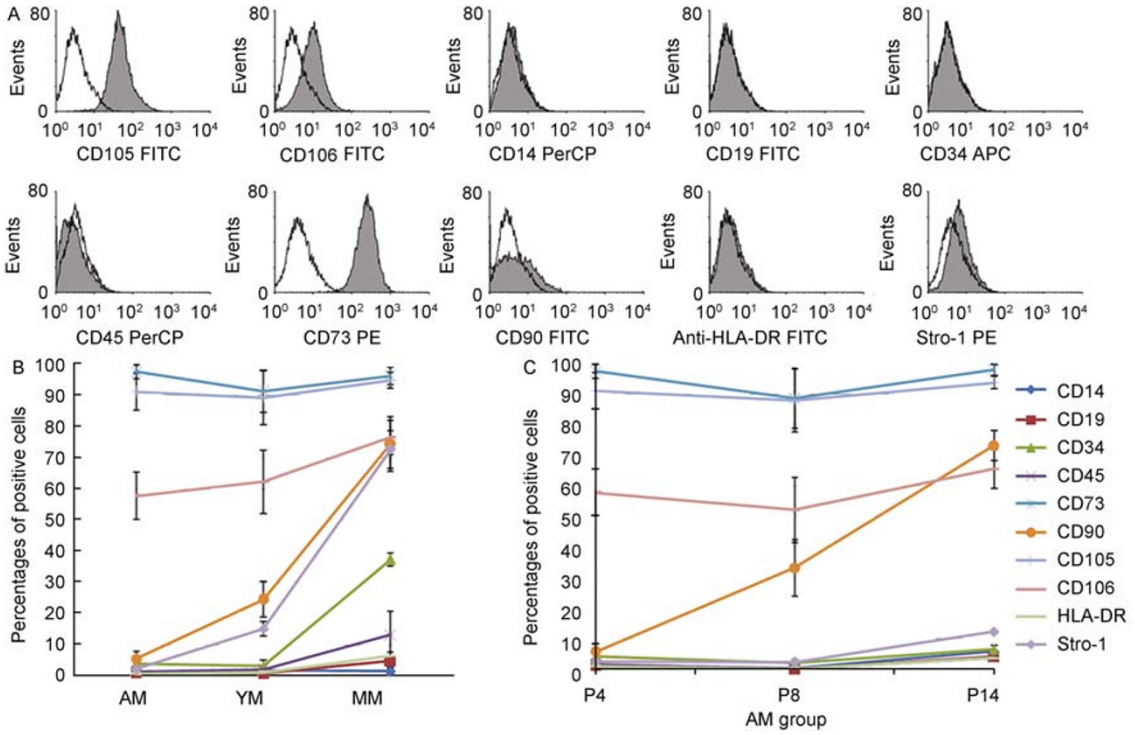

Figure 3 Cell surface markers of cMSCs from different age groups or at different passages. A, representative cell surface markers of cMSCs by a flow cytometer (AM group, p8); B, comparison of cell surface markers in different age groups (p4); C, comparison of cell surface markers at different passages.

Table 1 The proportion of an abnormal number of chromosomes

	Passages	Number of detected cells	Number of chromosomes					
			39	40	41	42	43	Proportion of abnormal
AM group	P10	9	0	0	1	8	0	11.1%
	P18	8	0	1	1	5	1	37.5%
YM group	P7	8	0	1	0	7	0	12.5%
	P14	9	1	0	2	5	1	44.4%
MM group	P4	4	0	0	0	4	0	0%

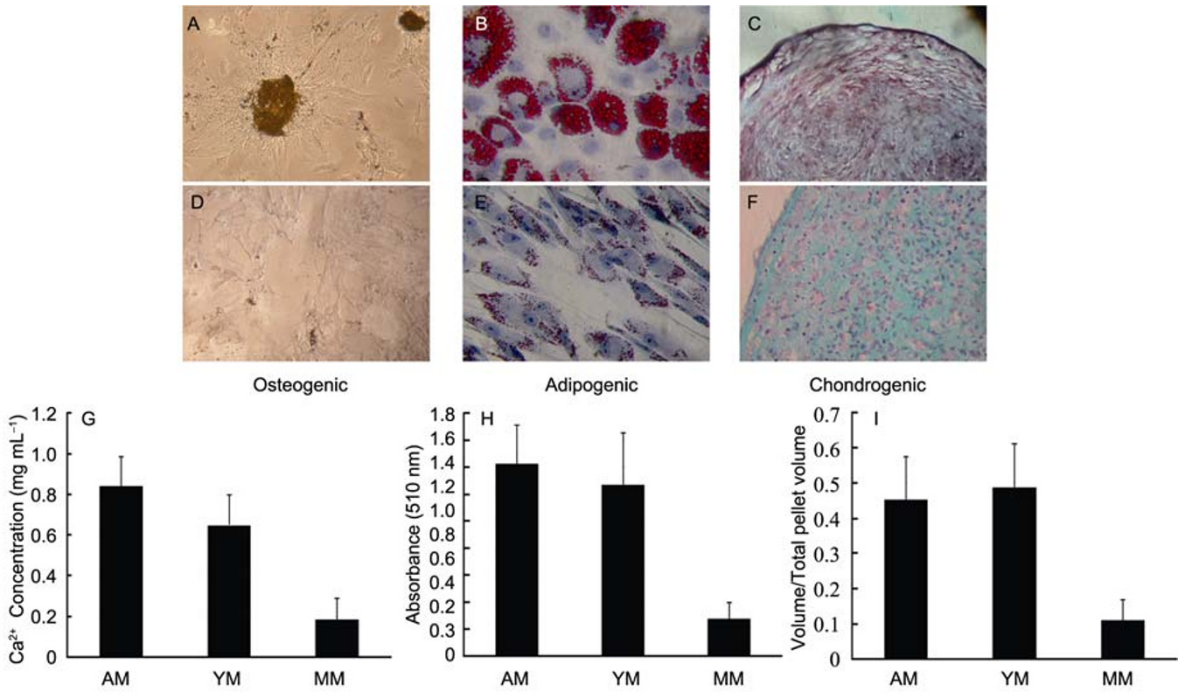

Figure 4 Differentiation potential of cMSCs from different age groups. cMSCs were differentiated toward osteocytes (A and D), adipocytes (B and E), and chondrocytes (C and F). AM group cMSCs (A–C) showed greater differentiation potential compared with that of MM group cMSCs (D–F). AM and YM groups cMSCs had significantly higher differentiation potential than that of the MM group by quantitative analysis (P<0.05, G–I). A–D, original magnification 200×.

rhesus monkey MSCs from different age groups [30,34]. The results suggested that rhesus monkey MSCs derived from older donors have a shorter life-span, diminished proliferative and differentiation potential [35]. The purpose of this study was to compare the biological characterization of bone marrow-derived MSCs from cynomolgus monkeys of different age groups. In this study, we found that cMSCs isolated from adolescent and young adult donors exhibited significantly shorter population doubling times, significantly more cumulative population doublings and markedly higher differentiation efficiency than cMSCs from middle-aged animals. This data suggests that adult MSCs obtained from younger sources have greater self-renewal capabilities and differentiation potential. The donor age is a factor which must be considered for further clinical and biomedical applications of MSCs.

Studies of rhesus monkeys from fetus to juvenile have addressed the negative correlation between growth potential of MSCs and the donor age [34,36]. However, there has not yet any report about the culture of adult monkey MSCs. In this study, we demonstrated the biological characterization of adult monkey MSCs, and suggested that MSCs from middle-aged donors were unable to be expanded to an adequate cell number *in vitro*, and significantly reduced in cell differentiation potential. These results suggested that MSCs from older donors may not be suitable for experimental and preclinical studies.

Generally, in successive passages, MSCs isolated from adults showed an increase in cell size and displayed a broadened polygonal morphology related to senescence [37].

Our results demonstrated that significant morphological changes of cMSCs were shown in the 18th passage of the adolescent group, the 14th passage of the young adult group, and in the primary culture of the middle-aged group.

Studies found that telomerase activity increased in immortal cell lines and malignant tumors, but did not in somatic cells [38,39]. Previous reports about human MSCs showed that telomerase activity was very low, and not significantly different compared to controls during long-term culture [22]. In this study, we showed that telomerase activity was not significantly different between different age groups. However, in line with previous reports [21], telomerase activity significantly increased in TMCs compared with its original cells. Unlike the previous reports [22], karyotypes of cultured cMSCs did not remain normal, and chromosomal instability occurred at a high frequency in long-term culture.

Our study showed that the total number of MSCs following *ex vivo* expansion and their differentiation capacity declined with age, and their therapeutic potential might also be diminished. Therefore, cMSCs from middle-aged or older donors might be unsuitable for experimental and preclinical studies. These findings suggest that a variety of factors, including donor age and the culture time *in vitro*, must be considered before further applications of MSCs, and various tests are needed to properly assess MSCs, particularly when a prolonged and extended propagation period is required.

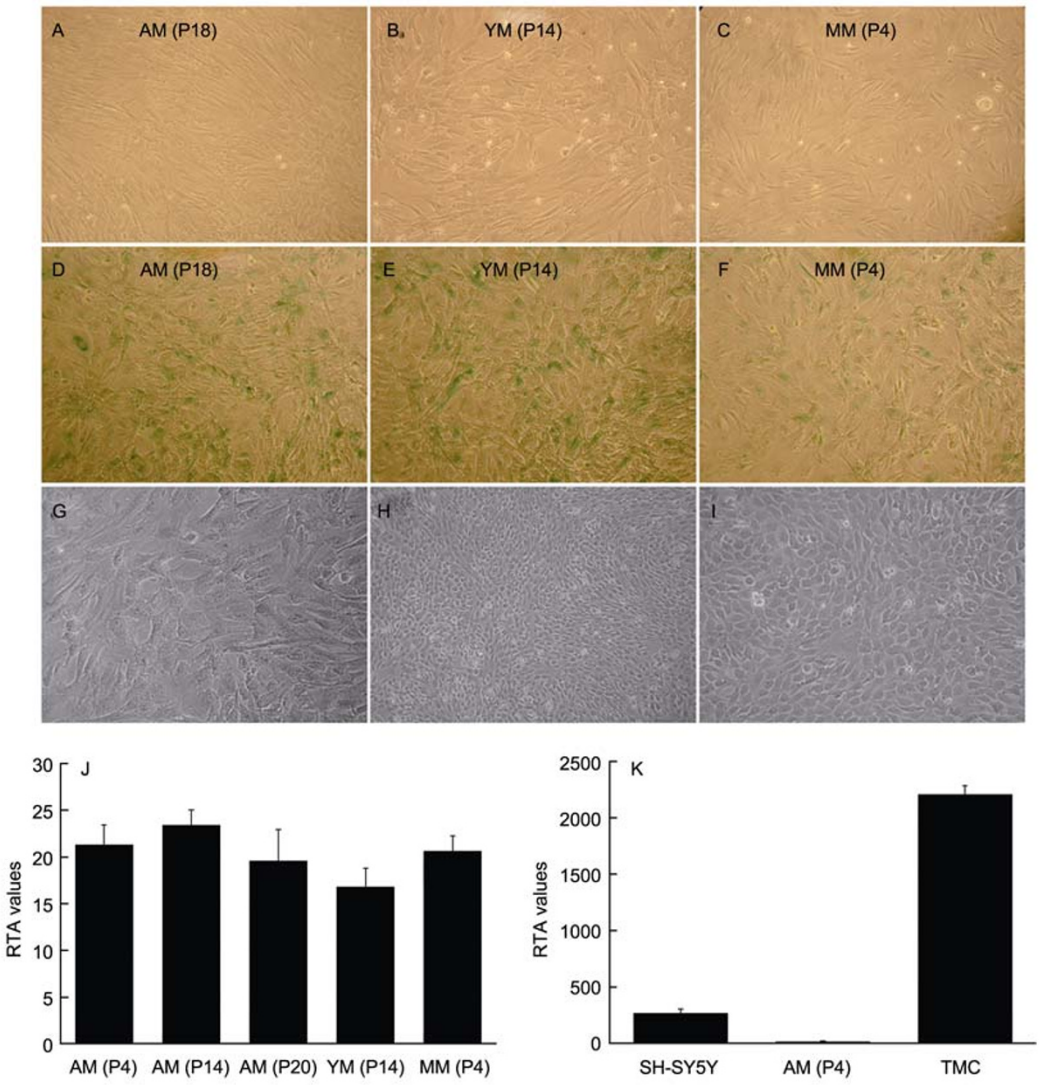

Figure 5 Senescence and spontaneous transformation of cMSCs. Associated with long-term culture, the cell growth decreased. The cell size and morphology significantly changed (A–C). Corresponding increased β-galactosidase activity was observed (E–F). Cell cytoplasm began to appear granular within many inclusions related to senescence (G). Markedly morphologic changes were observed in TMCs after spontaneous transformation (H–I). Telomerase activity significantly increased in TMCs compared with that of the original cMSCs (K) (P<0.001). Telomerase activities had no significant difference between different age groups (J). H, original magnification 100×; A–G and I, original magnification 200×.

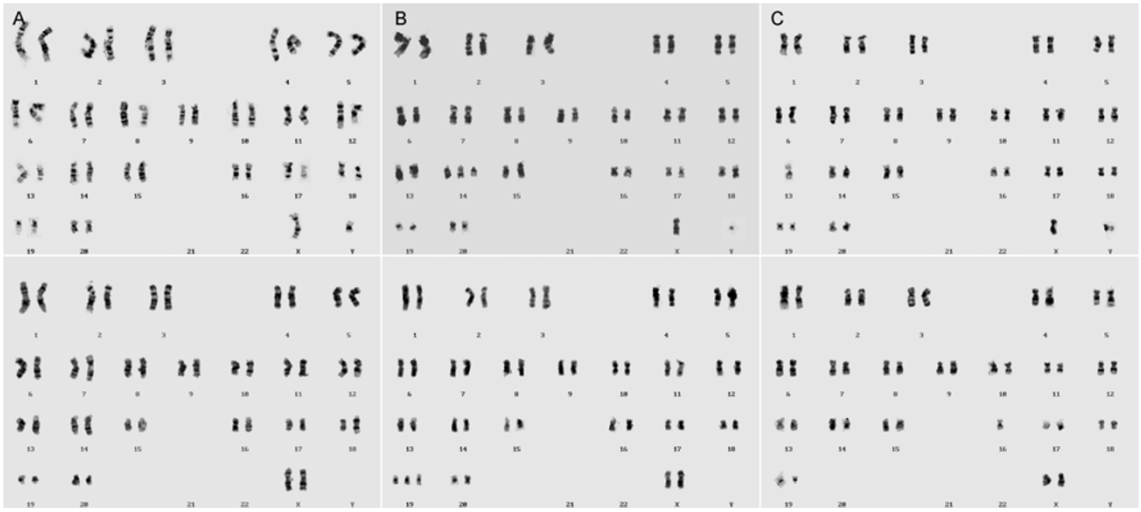

Figure 6 The karyotype of cMSCs. The G-banded karyotype of normal cynomolgus monkey MSCs, 44, XY and 44, XX (A). Common chromosomal abnormalities of cynomolgus monkey MSCs in long-term culture include extra chromosomes, 43, XY i(14) and 43, XX i(19) (B), missing chromosomes, 41, XY del(13) and 39, XX del(16;20;20) (C).

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Supplemental Table 1 Details of fluorescent antibody used in Flow Cytometry

fluorescent antibody	clone NO.	Fluorescent	Source	Website
CD106	51-10C9	FITC	PharMingen, San Diego, CA,	http://www. pharmingen. com
CD73	AD2	PE	PharMingen, San Diego, CA,	
CD90	5E10	FITC	PharMingen, San Diego, CA,	
CD14	MφP9	Percp	PharMingen, San Diego, CA,	
CD34	8G12	APC	PharMingen, San Diego, CA,	
CD45	2D1	Percp	PharMingen, San Diego, CA,	
CD19	SJ25C1	FITC	PharMingen, San Diego, CA,	
HLA-DR	L243 (G46-6)	FITC	PharMingen, San Diego, CA,	
CD105	clone SN6	FITC	Serotec, Oxford,	http://www.serotec.com
STRO-1	--	PE	santa cruz, Biotechnology Inc.	http://www.scbt.com

Supplemental Table 2 Surface antigen profile of cMSCs[a)]

Antigen		AM	YM	MM
P4	CD14	1.42±0.44	2.14±0.24	1.72±1.02
	CD19	1.15±0.94	1.06±0.56	5.01±1.68
	CD34	4.21±1.33	3.41±2.01	37.45±2.19
	CD45	1.68±0.73	2.34±0.65	13.39±7.41
	CD73	97.42±2.22	91.26±6.78	96.14±2.70
	CD90	5.74±2.47	24.75±5.67	74.35±8.63
	CD105	91.09±5.96	89.17±8.64	94.71±2.61
	CD106	57.79±7.57	62.32±10.11	76.44±5.57
	HLA-DR	0.94±0.75	1.46±0.78	6.63±1.22
	STRO-1	2.47±0.91	15.31±2.31	72.60±6.048
P8	CD14	0.46±0.52	1.53±0.33	–
	CD19	0.09±0.11	4.54±0.29	–
	CD34	2.11±1.08	2.42±1.13	–
	CD45	0.28±0.31	0.53±0.324	–
	CD73	88.60±9.79	96.16±10.34	–
	CD90	46.17±9.26	66.18±9.36	–
	CD105	87.97±10.27	94.56±11.64	–
	CD106	52.10±10.74	57.42±8.34	–
	HLA-DR	0.25±0.11	0.57±0.17	–
	STRO-1	2.39±0.87	36.24±6.52	–
P14	CD14	5.86±1.14	–	–
	CD19	4.17±1.52	–	–
	CD34	6.62±1.17	–	–
	CD45	3.78±1.15	–	–
	CD73	97.85±1.85	–	–
	CD90	73.17±4.93	–	–
	CD105	93.75±1.91	–	–
	CD106	65.56±6.31	–	–
	HLA-DR	3.72±0.64	–	–
	STRO-1	12.35±0.31	–	–

a) Data are presented as the mean of percentages of positive cells±SE.