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Adipose-derived stem cell: a better stem cell than BMSC

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To further study the proliferation and multi-differentiation potentials of adipose-derived stem cells (ADSCs), the cells were isolated with improved methods and their growth curves were achieved with cck-8. Surface protein expression was analyzed by flow cytometry to characterize the cell phenotype. The multi-lineage potential of ADSCs was testified by differentiating cells with adipogenic, chondrogenic, osteogenic, and myogenic inducers. The results showed that about 5×10^5 stem cells could be obtained from 400 to 600 mg adipose tissue. The ADSCs can be continuously cultured *in vitro* for up to 1 month without passage and they have several logarithmic growth phases during the culture period. Also, the flow cytometry analysis showed that ADSCs expressed high levels of stem cell-related antigens (CD13, CD29, CD44, CD105, and CD166), while did not express hematopoiesis-related antigens CD34 and CD45, and human leukocyte antigen HLA-DR was also negative. Moreover, stem cell-related transcription factors, Nanog, Oct-4, Sox-2, and Rex-1 were positively expressed in ADSCs. The expression of alkaline phosphatase (ALP) was detected in the early osteogenic induction and the calcified nodules were observed by von Kossa staining. Intracellular lipid droplets could be observed by Oil Red staining. Differentiated cardiomyocytes were observed by connexin43 fluorescent staining. In order to obtain more stem cells, we can subculture ADSCs every 14 days instead of the normal 5 days. ADSCs still keep strong proliferation ability, maintain their phenotypes, and have stronger multi-differentiation potential after 25 passages. Copyright © 2008 John Wiley & Sons, Ltd.

KEY WORDS — adipose tissue; mesenchymal stem cells; proliferation; doubling time; multipotential

INTRODUCTION

Adult stem cells hold great promise for use in tissue repair and regeneration. Research interests continuously exist in both the biology and potential therapeutic applications of adult stem cells from bone marrow, referred to as either mesenchymal stem cells or marrow stromal cells (BMSCs). The key characteristics of stromal stem cells include the ability to adhere to plastic to form fibroblastic-like colonies, extensive proliferative capacity, the ability to express several common cell surface antigens, and the ability to differentiate into several mesodermal lineages, including bone, muscle, cartilage, fat, epithelium, and neural progenitors.[1,2] Because of these characteristics, stromal stem cells may potentially be useful in the field of regenerative medicine. However, the stem cell incidence in bone marrow is estimated to be about 1 per 10^5 cells,[3] and other tissues contain even lower stem cell numbers.[4,5] In recent years, interest has rapidly grown in the developmental plasticity and therapeutic potential of stromal cells isolated from adipose tissue, called adipose-derived stem cells (ADSCs). The acquisition of adipose tissue is much less expensive than bone marrow, with less invasive operation and available in greater quantities. Clinically relevant stem cell numbers can be extracted from isolated adipose tissue since it possesses higher stem cell proliferation rate than BMSCs.[6] Therefore, adipose tissue represents an abundant, practical, and appealing source of donor tissue for autologous cell replacement.

The successful use of ADSC on regeneration therapy can be achieved only after we learn its extensive characteristics in details. However, the

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published data about ADSC primary culture and their growth characteristics are quite different. It was reported that $2–6 \times 10^8$ processed lipo-aspirate cells can be obtained from 300 ml adipose tissue.[7] The average frequency of ADSCs that Brian obtained from processed lipo-aspirate was 1–2% of nucleated cells, and the yield of ADSCs was approximately 5000 stem cells g^{-1} of adipose tissue.[8] While others obtained different numbers of ADSCs from adipose tissue with some different methods.[9,10] All these data indicate that with diverse isolation methods, different amount of ADSCs could be obtained from equal quantities of adipose tissue. If we want to obtain enough high quality cells for tissue engineering and regeneration therapy, a better and simple isolation method must be developed.

In addition, the majority of studies showed the growth cycle of stem cells including ADSCs was about 1 week before harvest and passage.[10] However, it has been demonstrated that protein synthesis in ADSCs continuously increased over 2 weeks.[11] Therefore, the detailed growth characteristics of ADSCs are not well understood to date.

In this study, we firstly established an improved method for isolating stromal stem cells from human adipose tissue. The proliferation ability and multi-differentiation potential of the human ADSCs were then further investigated to obtain high quantity and quality ADSCs for clinical cell-based therapy and tissue engineering.

MATERIALS AND METHODS

Isolation of human adipose-derived stem cells

Human subcutaneous adipose tissue was obtained from subjects undergoing surgery with informed patient consent. In brief, approximately 400–600 mg of human subcutaneous adipose tissue was washed several times in D-Hank's buffer. Since adipose tissue is highly vascularized, careful attempts were made to dissect away any visible blood vessels from the tissue. The adipose tissue was minced finely using surgical scissor and incubated in digestion buffer (collagenase and trypsin dissolved in Hank's buffer) at 37°C with constant agitation for about 20 min. Digestion buffer comprised 0.1% collagenase (type I, Sigma) and 0.25% trypsin (Sigma) which dissolved in Hank's buffer. Once digested, the liquid was separated into three layers: an upper layer is yellow oily lipocytes, an intercellular layer of adipose tissue, and a bottom layer of buffer including mononuclear cells. The buffer in the bottom layer was collected carefully and transferred to centrifuge tubes with medium (high glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, National Hyclone Bio-engineering)). The mixture was centrifuged at 1500 rpm for 10 min and trypsin-collagenase was re-added to the remnant adipose tissue. The above steps were repeated for two to three times, until adipose tissue was digested completely. Cell pellets were then suspended in medium (DMEM + 10%FBS) and plated in T flasks. To avoid the influence of red blood cell (RBC) lysis on the growth of ADSCs, KRB (Krebs-Ringer-Bicarbonate), or NH_4Cl was not used. Cells were cultured at 37°C and 5.0% CO_2 in humidified incubators, with 100% of the media replaced every 3 days. The primary cells were cultured for 3–4 days until they reached confluence completely, and these cells were defined as passage "0." At 100% confluence, the ADSCs were split. The ADSCs after the 4th passage were trypsinized with 0.25% trypsin and 0.04% EDTA (v/v 1:1) and centrifuged at 1000 rpm for 5 min. The cells were suspended with medium (DMEM + 15%FBS) and counted with a hemocytometer, then plated into flasks or plates at 5×10^4 cells ml^{-1} and cultured.

Morphology

ADSCs at each passage were examined under light microscopy.

Cytokinetics analysis by cck-8

ADSCs were plated in 96 well plates at 6.25×10^4, 1.25×10^5, 2.5×10^5, 5×10^5, 1×10^6, 2×10^6 cells ml^{-1}, respectively, to get a standard curve relating cell density and optical density values. ADSCs of passages 3, 7, and 15 at 5×10^3 cells ml^{-1} -1 were plated to develop growth curves. Ten microliters of sterile cell counting kit-8 were added to each well and incubated for 3 h at 37°C. The percentage of viable cells was determined everyday for 15 days. The optical density values were determined at least in triplicate against a reagent blank at a test wavelength of 450 nm and reference wavelength of 630 nm.

Additionally, after passage 20, we subcultured ADSCs with different subculture times. One population of ADSCs was subcultured every 5 days (99% confluence), and the other population of ADSCs was subcultured every 14 days (overlapping growth). Both of them grew until passage 25. Growth curves of two populations of ADSCs of passage 25 were drawn to

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compare the growth ability after subculture for different times. The percentage of viable cells was determined for 25 days.

Cumulative population doubling time

The doubling time was calculated during the logarithmic phase of the growth curve. The time of population doublings was calculated using the formula:

$$T_d = T \times \frac{\log 2}{\log\left(\frac{N_t}{N_0}\right)}$$

T is the time of the logarithmic phase of the growth curve, N_0 the number of cells after seeding, N_t the number of cells at the end of the logarithmic growth phase. Doubling time T_d was determined in cultures until passage 25. The mean cumulative population doubling time was expressed as a function of passage number.

Flow cytometric analysis

hADSCs were examined for surface protein molecule expression by flow cytometry. ADSCs of passages 4, 20, and two populations of passage 25 were stained using antibody CD13-PE, CD29-PE, CD34-FITC, CD44-FITC, CD45-FITC, CD105-FITC, CD166-PE, and HLA-DR-PE for 20 min. After staining, the cells were washed twice in PBS and analyzed using a standard Becton–Dickinson FACSAria instrument (BD, San Jose, CA, USA). The data were acquired and analyzed using the FACSDiVa software (BD, San Jose, CA, USA). All antibody and isotype controls were purchased from BD.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from 2×10^6 cells of passages 4, 20, 30, and two populations of passage 25, respectively, by using an RNA extraction kit. Oligo (dT)-primed reverse transcription was performed on aliquots (1 mg) of total RNA as a template and the resultant cDNA used for PCR amplification with the following primers:

Nanog primers 5′-GCCCTGATTCTTCCACCA-GT- 3′ and 5′-CAAAGCAGCCTCCAAGTCAC-3′ (approximately 200 bp).

Oct-4 primers 5′-CGCACC-ACT-GGC-ATT- GTC-AT-3′ and 5′-TTCTCC-TTG-ATG-TCA-CGC- AC-3′ (approximately 200 bp).

Sox2 primers 5′-GGC-AGC-TAC-AGCATG-ATG-CAG-GAG-C-3′ and 5′-CTG-GTCATG-GAG-TTG-TAC-TGC-AGG-3′ (approximately 200 bp).

Rex-1 primers 5′-TGA-AAG-CCCACA-TCC-TAA-CG-3′ and 5′-CAA-GCT-ATCCTC-CTG-CTT-TGG-3′ (approximately 200 bp).

RT-PCR reactions were performed for the GAPDH gene as a control for efficiency of the amplification in the reactions (5′-ATG-GGGAAG-GTG-AAG-GTC-GG-3′ and 5′-GGA-GTGGGT-GTC-GCT-GTT-GAA-GTT-GAA- 3′; approximately 500 bp).

The PCR products were visualized by 1.5% agarose gel electrophoresis. Gels were analyzed using the Scion Image System and band densities were expressed as percentage of control.

ADSC multi-differentiation potential assays

Adipogenic, osteogenic, chondrogenic, and cardiomyogenic differentiation were induced and assayed according to Zuk' protocols.[7] Passage 4 and two populations of passage 25 hADSCs were induced, respectively. The acquisition of the adipogenic phenotype was determined by staining the monolayers with 2% Oil Red-O solution. To assess the osteoblastic phenotype, alkaline phosphatase (ALP) activity was measured after 7 days of induction, Von Kossa staining was used to assess matrix mineralization after 21 days. For chondrogenic differentiation, the cells were stained with Toluidine Blue. For cardiomyogenic induction, ADSCs were induced with 5-azacytidine for 24 h. After cells had been cultured for 4 weeks, cardiac-specific connexin-43 with IgG-FITC was used to examine myogenesis. We chose 10 visual fields randomly, and analyzed the positive products of two populations of passage 25 ADSCs semiquantitatively.

RESULTS

Morphorlogy of hADSCs

After the cells from subcutaneous fat adhered to the tissue culture flask, nonadherent cells, such as RBCs, were removed by changing the culture medium after 3 days. The initially adherent cells grew into spindle- or stellate-shaped cells, which then developed into visible colonies 3 days after the initial plating (Figure 1A). The cells began to proliferate rapidly and were passaged by trypsinization every 3–4 days until they were completely confluent. After the second passage, hADSCs appeared to adopt a more uniform fibroblast-like shape with directionality and regularity

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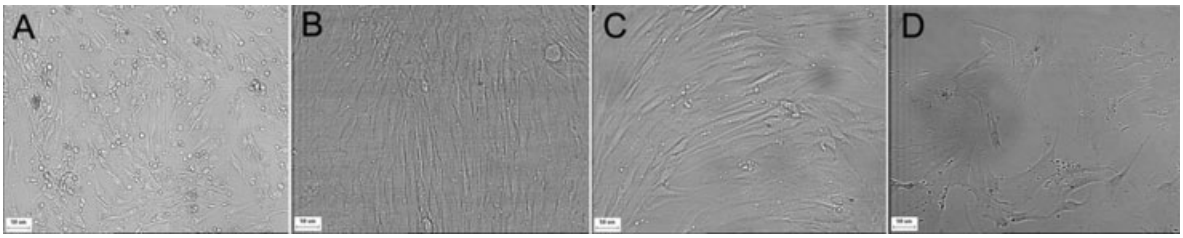

Figure 1. Morphology of hADSCs from subcutaneous fat tissue cultured *in vitro*. (A) After being plated in tissue culture flask for 3 days. The initially adherent cells grew into spindle-shaped cells; (B) clone formation of passage 3 cells at the 3rd day, the hADSCs had reached complete confluence; (C) hADSCs of passage 20 at the 3rd day and the cells were approx. 80% confluent; (D) hADSCs showed augmented volume at passage 30. Scale bars measure 50 μm

(Figure 1B), similar to bone marrow stromal cells. The cell morphology (size and shape) persisted with only minimal alterations after passage 20 (Figure 1C). ADSCs showed augmented volume once passage 30 was exceeded (Figure 1D).

Growth kinetics of ADSCs of different passage

Cell viability and growth kinetics were assessed by cck-8. Under our culture conditions, hADSCs were capable of proliferating beyond 30 passages. The growth curves in Figure 2B showed that ADSCs were initially in stationary phase during the first 3 days. From the 4th day to the 7th day, the cells underwent the first logarithmic growth period. Then, the cells stayed at a lag phase during the following 2 days. After this, however, the cells did not show any contact inhibition as normally seen in cell culture. In fact, they grew continuously until the 10th day to reach a growth peak. Although, the number of cells decreased significantly in the following day, the ADSCs underwent another logarithmic phase after the 12th day. Therefore, the growth characteristics of hADSCs include multi-logarithmic phases without complete contact inhibition. Also, from the growth curves it can be seen that the growth ability of the cells decreased gradually with the increase of passages.

Figure 2C displays the growth ability of two passages 25 ADSCs which had been passaged with different subculture times (every 5 days and every 14 days) from passage 20 to 25. Similar to those in Figure 2B, the growth curves of the two types of cell in Figure 2C all illustrated more than one logarithmic phase and growth peak. We also can see that the two types of cell have comparable strengths of proliferation ability but the cells passaged every 14 days had grown for a much longer time *in vitro*.

The hADSCs morphology change under the microscope (Figure 3) was consistent with the growth curves. At the 6th day, cells reached 90% confluence, and then localized overlapped growth appeared on the 10th day. On the 11th day, some twisted and detached cells were present on monolayers of ADSCs. In the following days, cell overlap increased and the cell slice became thicker. After 20 days, the cell slice rolled over from the edge of the well, some cells continually grew in the margin and even crossed over the rolled cell slice. When hADSC grew in 96 well plates for over 35 days, the cell slice nearly rolled up completely. At this time, we examined the cell viability by cck-8, and the absorbance had no significant difference from that of around 20 days.

Moreover, the cell doubling time was calculated according to the growth curves in Figures 2B and C, and the results are shown in Figure 4. It can be seen from Figure 4A that the time required for population doubling significantly increased with passages, from 36 h at passage 3 to 96 h at passage 25. Figure 4B indicated that there was no obvious increase in the doubling time of each passage cells during their two or three logarithmic growth phases. In fact, the doubling times of the cells during their second logarithmic phase were significantly less than those of the other phases for each passage of cells, and the doubling times during this phase also increased with passages.

Surface protein expression by flow cytometry analysis

The hADSCs surface markers were examined by flow cytometry. We examined the expression of CD13, CD29, CD44, CD34, CD45, CD105, CD166, and HLA-DR of hADSCs at passages 4 and 20, and the results are summarized in Table 1. CD13, CD29, CD44, CD105, and CD166 were positive in expression, while CD34, CD45, and HLA-DR were negative. The positive expression ratios of P4 cells were a little higher than those of P20 cells. Additionally, we

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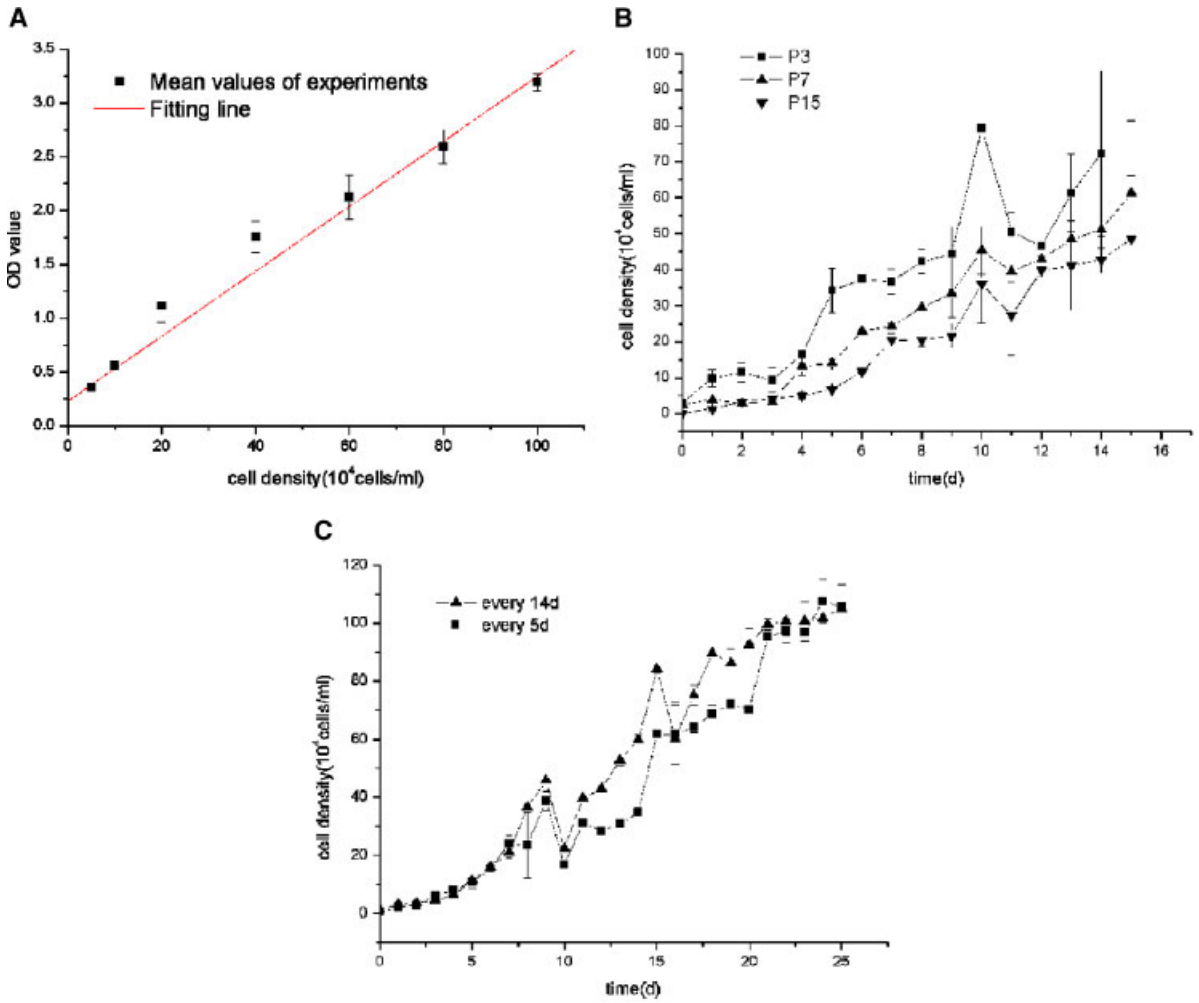

Figure 2. Growth curves of hADSC. (A) Standard curve; (B) growth curves of P3■, P7▲, P15▼; (C) growth curves of two P25 ADSCs after being passaged with different subculture times from passage 20 to 25, ▲subcultured every 14 days, ■subcultured every 5 days

compared the CD marker expression ratio of two P25 cells which had been passaged from P20 with different subculture times, every 5 and 14 days, respectively, as shown in Table 2. The results showed that the positive expression ratios of ADSCs with subculture time of 14 days were slightly higher than those of cells with subculture time of 5 days although, there was no statistical significance ($p > 0.05$).

hADSCs transcription factor expression

The expression of genes such as Nanog, Oct-4, Sox-2, and Rex-1 was examined for the cells of P4, P20, and P30 in culture. Nanog, Oct-4, Sox-2, and Rex-1 mRNA were expressed at consistent levels in P4 and P20, but the mRNA expression decreased significantly at passage 30 ($p < 0.05$) (Figures 5A and 6A). The mRNA expression ratio of P25 ADSCs after being subcultured every 14 days was a little higher than that of the other type of P25 ADSCs (Figures 5B and 6B).

Multi-differentiation potential

Adipogenesis. The efficiency of adipogenesis was different for the hADSCs of early and later passages (Figure 7A). ADSCs of passage 4 showed multiple intracellular lipid filled droplets in 80–90% of cells (Figure 7A2) in adipogenic media. The cells containing lipid vesicles exhibited an expanded morphology with the majority of intracellular space occupied by

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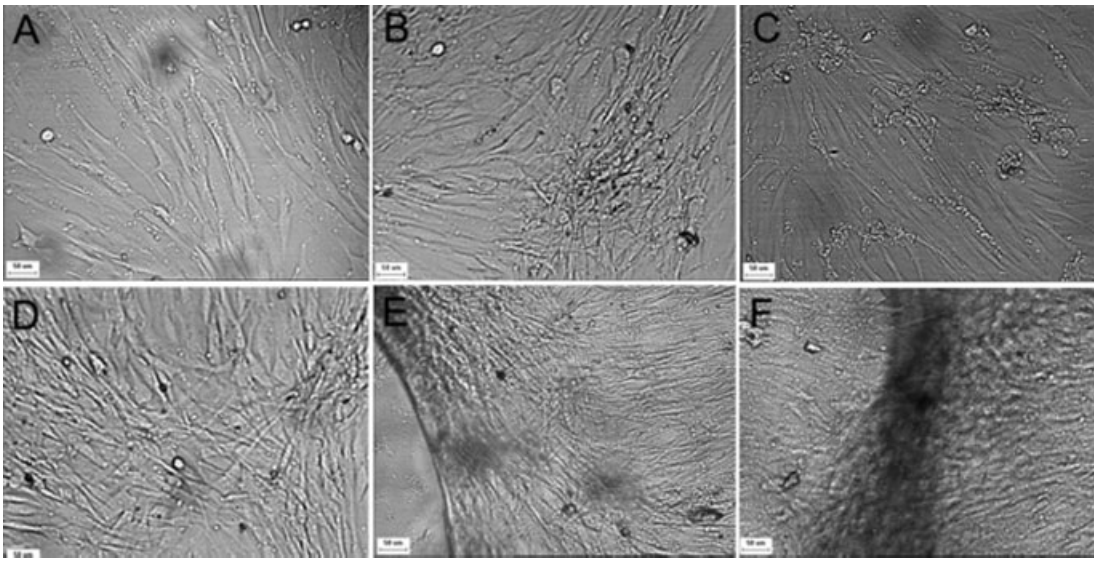

Figure 3. Morphological changes of hADSC in accordance with the growth curve. (A) hADSC reached 90% confluence at the 6th day; (B) overlapped growth of hADSCs appeared locally at the 10th day; (C) some twisted and detached cells presented on monolayers of hADSC at the 11th day; (D) hADSC in bulk of regions were overlapped at the 13th day; (E) after 20 days, the cell slice rolled up from the edge of well; (F) after 35 days, the cell slice was almost completely rolled. Scale bars measure 50 μm

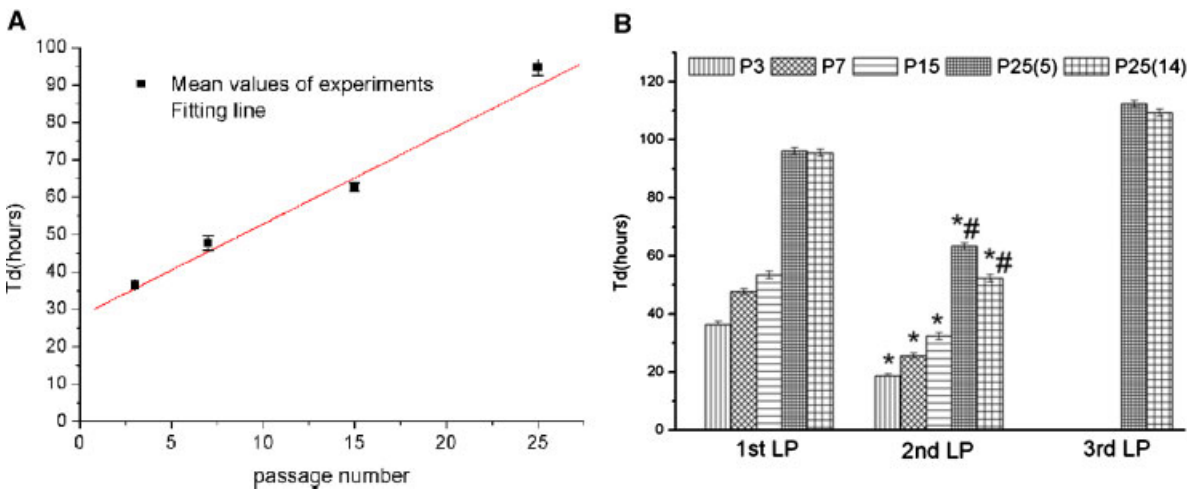

Figure 4. (A) Linear relationship of Td and passage number during the first logarithmic phase. (B) Doubling time of passages 3, 7, 15, and 25 ADSCs in each logarithmic phase. P25(5), P25 ADSCs after being subcultured every 5 days from P20 till P25; P25(14), P25 ADSCs after being subcultured every 14 days from P20 to passage 25. 1st LP, the first logarithmic phase; 2nd LP, the second logarithmic phase; 3rd LP, the third logarithmic phase. $^{*}p < 0.05$, compared with other phases at the same passage; $^{\#}p < 0.05$, compared with the other two P25 cells at the first and third logarithmic phases

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Table 1. Expression of cell surface CD markers of ADSCs of passages 4 and 20

Antibody	Conjugate	P4 cells	P20 cells
CD13	PE	99.44% (+)	91.16% (+)
CD29	PE	78.5% (+)	74.8% (+)
CD34	FITC	(−)	(−)
CD44	FITC	83.43% (+)	83.43% (+)
CD45	FITC	(−)	(−)
CD105	FITC	68.5% (+)	60.76% (+)
CD166	PE	52.91% (+)	48.98% (+)
HLA-DR	PE	(−)	(−)

droplets and lipid vesicles, which is consistent with the phenotype of mature adipocytes. No lipid droplets were observed in undifferentiated ADSCs. Such strong differentiation potential of the hADSCs after passage 25 declined, however, with only about 40–55% ADSCs differentiated into adipocytes. Additionally, the adipogenesis potential of two types of P25 ADSCs had no significant difference (Figures 7A3, A4 and 8).

Osteogenesis. ADSCs of passage 4 showed increased ALP activity after 7 days of culture in osteogenic medium (Figure 7B2). However, with the same induction time, ADSCs of passage 25 had lower ALP activity. There was no significant difference in ALP activity between the two P25 ADSCs (Figures 7B3, B4 and 8).

After the cultures had been incubated for over 3 weeks, multiple layers of cells often formed. In some cases, these colonies gave rise to dense nodules from which radiated highly elongated spindle-shaped cells with large nuclei. Calcium deposition was demonstrated by Von kossa staining. The percentage of ADSCs that underwent osteogenic differentiation was about 90% in all ADSCs at passage 4 (Figure 7C2), it was possible to see the mineralization nodule by naked eyes. However, this frequency decreased to 30–40% at passage 25. Calcium deposition between the two P25 ADSCs still had no significant difference (Figures 7C3, C4 and 8).

Table 2. Expression of cell surface CD markers of ADSC of passage 25 after being passaged from passage 20 with different subculture time

Antibody	Conjugate	Cells passaged every 5 d	Cells passaged every 14 d
CD13	PE	96.8% (+)	99.89% (+)
CD29	PE	70.8% (+)	85.7% (+)
CD34	FITC	(−)	(−)
CD44	FITC	41.03% (+)	53.37% (+)
CD45	FITC	(−)	(−)
CD105	FITC	32.61% (+)	48.1% (+)
CD166	PE	28.54% (+)	48.15% (+)
HLA-DR	PE	(−)	(−)

Chondrogenesis. ADSCs were differentiated into chondrocytes by using a hanging drop culture technique. After 3 weeks induction, the volume of cells became larger and cells were clear to see. The differentiated cells were stained positively by Toluidine Blue which is specific for the highly sulfated proteoglycans of cartilage matrices (Figure 7D2). The two P25 ADSCs with different subculture time all showed the potential to differentiate into chondrocytes, and the differentiation ratio between the two P25 cells had no obvious difference (Figure 7D3, D4). They were; however, lower than that of P4 cells (Figure 8).

Myogenesis. After induction by 5-AZA for 24 h, ADSCs were further cultured for 4 weeks and grown until they overlapped. We could not find obvious morphology changes up to this time, and spontaneous cellular beating was also not seen. After the fluorescent staining for connexin-43, several cells showed strong green fluorescence in the differentiated ADSCs of passage 4 (Figure 7E2). However, the induced ADSCs of passage 25 did not express green fluorescence after the staining (Figure 7E3, E4).

DISCUSSION

Like bone marrow, adipose tissue is derived from the mesenchyme and contains a supportive stroma that is easily isolated. ADSC may be an alternative and less-invasive source of mesenchymal stem cells existing within human adipose tissue.[12,13] Based on this, adipose tissue may represent a source of stem cells that could have far-reaching effects on many fields. However, because of the divergent nature of ADSC in the present studies,[11] additional studies are required to further assess the characteristics of these cells and to elucidate their true biological nature.

Isolation of a population of stem cells with similar biological potential from human adipose tissue has been described earlier.[14–18] We sought to study the biological properties of hADSCs with our improved techniques.

Firstly, when isolating ADSCs from adipose tissue, usually collagenase or trypsin has been applied. We

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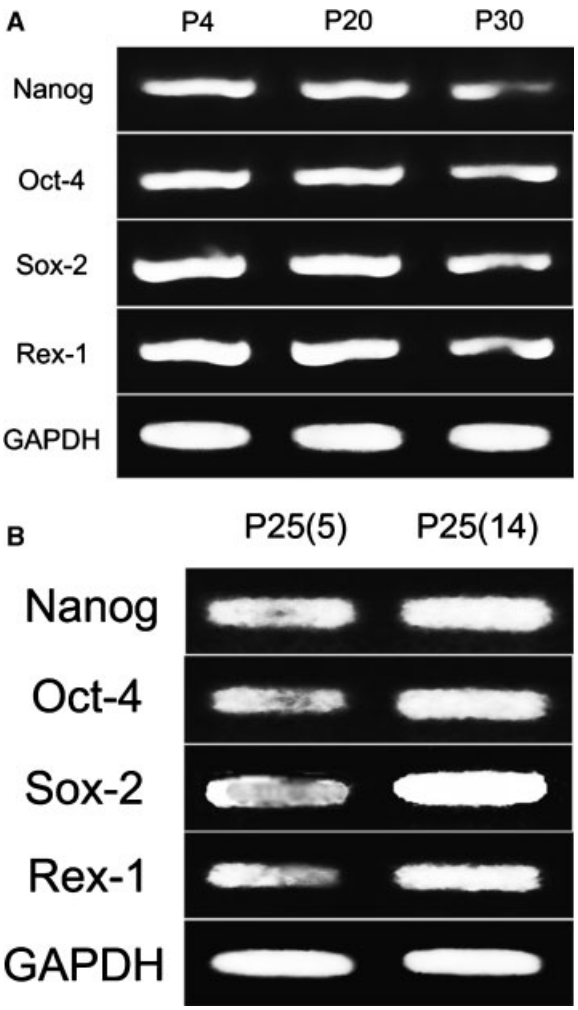

Figure 5. Transcription factors mRNA expression in hADSC of different passages. Total cellular RNA was analyzed by RT-PCR for Nanog, Oct-4, Sox-2, and Rex-1 mRNA expression. (A) mRNA expression of hADSC of passages 4, 20, and 30; (B) mRNA expression of hADSC of passage 25. Lane P25(5), ADSCs after being subcultured from P20 every 5 days; lane P25(14), ADSCs after being subcultured from P20 every 14 days

combined the use of collagenase and trypsin and digested adipose tissue several times. This can shorten the digestion time from 1–3 h to 20 min, make tissue completely digested and decrease the lesions caused by trypsin. Secondly, the current methods for removing RBCs during primary culture usually employ RBC lysis KRB or NH_4Cl. We removed RBC by exchanging the medium, thus the impairment caused by KRB or NH_4Cl to ADSCs was excluded. With the above improvements of isolation and culture, obvious improved results were obtained. The adipose specimens for our experiments from human subcutaneous tissue were between 400 and 600 mg (about 1.5 ml). The number of nucleated cells isolated per adipose tissue sample was about $1–2 \times 10^7$, and the number of stem cells was 5×10^5. While Aust gained $1–2 \times 10^8$ nucleated cells per 100 ml of lipo-aspirate, and 200 ml lipo-aspirate would typically yield in excess of 1×10^6 stem cells,[15] this was approximately 20 fold less than ours.

Under our culture condition, we plated all cells at a density of 10 000 cells ml^{-1}. The ADSCs grew to 90% confluence within 3 days only; and we could obtain 10^5 cloned cells from 10^7 nucleated cells. Others need longer times and gain fewer cloned cells.[10,19] Moreover, BMSCs took a markedly longer period of time to reach to 90% confluence, typically 1 week.[20]

Nearly all growth curves of stem cells including BMSC and ADSC appear to reach a platform phase at the 6th or 7th day.[21–23] However, the growth curves we obtained showed a much longer growth time. There were three logarithmic phases occurring within 25 days, although, there was a lag stage between each logarithmic phase, which indicated that a slight contact inhibition happened. We also examined the cell OD value of the 30th day which was close to that of the 25th day. This may be because the cells stop growing further once they reach a certain thickness. This result is different from others,[21–23] which indicates ADSCs have stronger growth ability than was known previously.

A linear relationship between population doubling time and passage number was observed in the first and second logarithmic growth periods, which means that the cell proliferation ability declines with the passage number. These are similar to the results reported by others previously.[10] Therefore, the less subculture, the less lesion to the cells since passage operation can impair cells.

Also, our results showed that there was no obvious increase of the doubling time of each passage cells corresponding to their multi-logarithmic growth phases. This implies that ADSCs can be continuously cultured *in vitro* for a long time without deterioration of their proliferation. Thus, we can obtain more progenitor cells with strong proliferation ability by adopting less passage operation and long time culture.

The hADSCs we cultured contain distinct cell populations which express stem cell markers CD13, CD29, CD44, CD105, and CD166, and these results are consistent with others.[10,24–27] CD34 and CD45, commonly used as a hematopoietic stem cell or endothelial progenitor cell marker, were negative. About CD34 expression, there are many different

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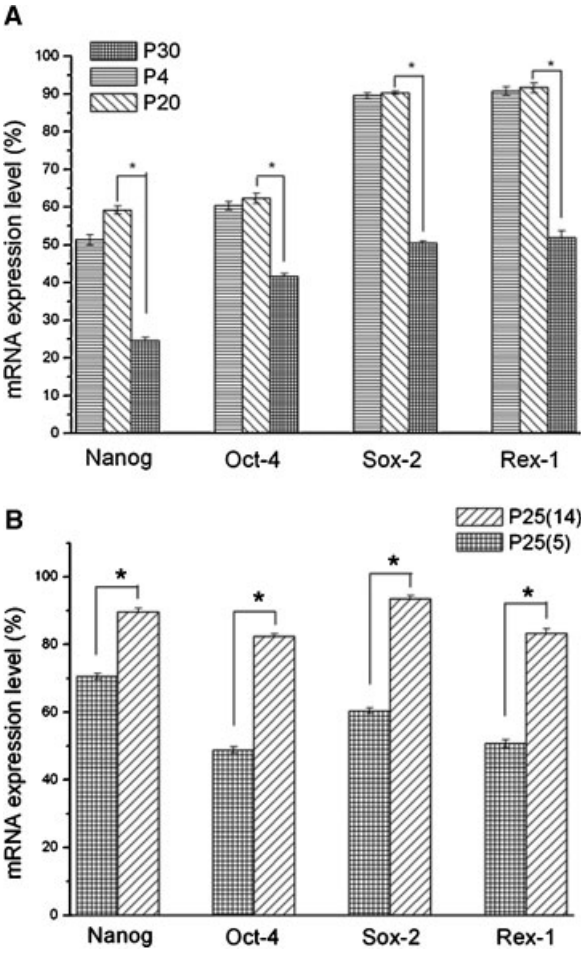

Figure 6. (A) Semiquantitative analysis of mRNA expression in hADSC of passages 4, 20, and 30. (B) Semiquantitative analysis of mRNA expression of hADSC of passage 25. P25(5), P25 ADSCs after being subcultured every 5 days from P20 till P25; P25(14), P25 ADSCs after being subcultured every 14 days from P20 till passage 25. Ratios of Nanog, Oct-4-, Sox-2-, and Rex-1-to-GAPDH in percentage for ADSCs of different passages with the same cell number. Data are represented as means $\pm$ SD of three different experiments. *$p < 0.05$, compared with other groups

reports.[10,18,28,29] Some researchers reported that ADSC can express CD34,[18,28] but this is different from our results and of some others.[10,29] It is possible that at the early time of culture, some hematopoietic cells may be mixed with the cultured cells. We examined ADSC after passage 4, and the purity was higher. Less than 1% of ADSCs express the HLA-DR protein, suggesting their potential for allogeneic transplantation. The surface protein expression of two P25 ADSCs after being subcultured for different times had no significant difference. Therefore, we could obtain more progenitor cells if we subcultured cells every 14 days, and these cells still maintain their stem cell characteristics.

Transcription factors are responsible for regulating the biology of stem cells. The co-localization of Nanog, Oct-4, and Sox-2 may play important roles in differentiation potentials of ADSCs.[30,31] The ADSCs we cultured still expressed transcription factor mRNAs even at passage 30, but the expression level was significantly lower than that of passage 4. These stem cell-related genes expression of ADSCs after subculture every 14 days was higher than the expression of ADSCs with normal subculture intervals. Since these factors have been suggested as candidates for the master regulator of initiation, maintenance, and differentiation of pluripotent cells,[32] our results indicate that if ADSCs are subcultured every 14 days instead of the normal 5 days, they may have stronger differentiation potential than normal subcultured ADSCs.

Due to the lack of a specific and universal molecular and gene marker to identify adult stem cells, functional assays are required to identify them. Our isolated ADSCs with strong proliferation ability also can differentiate *in vitro* toward the osteogenic, adipogenic, and chondrogenic lineage, and even endoderm cardiomyocyte when treated with established lineage specific factors, as also reported by others.[26,33] ADSCs of passage 25 after being subcultured every 14 days also have multipotential differentiation ability, that is, even stronger than passage 25 cells that were subcultured every 5 days, but the ability of P25 cells is weaker than that of P4 cells. This result is consistent with the result of gene expression. In other words, after subculturing ADSCs every 14 days, we can obtain more ADSCs with renewable ability and stronger multi-differentiation potential.

Sparse data exist on ADSCs differentiation toward tissues of nonmesodermal origin.[26,33,34] We tried to differentiate ADSCs into cardiomyocytes which are endodermally derived. ADSCs can differentiate into cardiomyocytes at lower passages by using 5-AZA. However, after passage 25, ADSCs lost the ability of differentiating into cardiomyocytes. So, if we want to transplant ADSCs into impaired heart or to develop engineered cardiac tissue, we must use the lower passage ADSCs.

The main benefits of our isolated ADSCs are that they can be easily harvested from patients by a simple, minimally invasive method and can be easily cultured (5×10^5 stem cells from 400 to 600 mg tissue).

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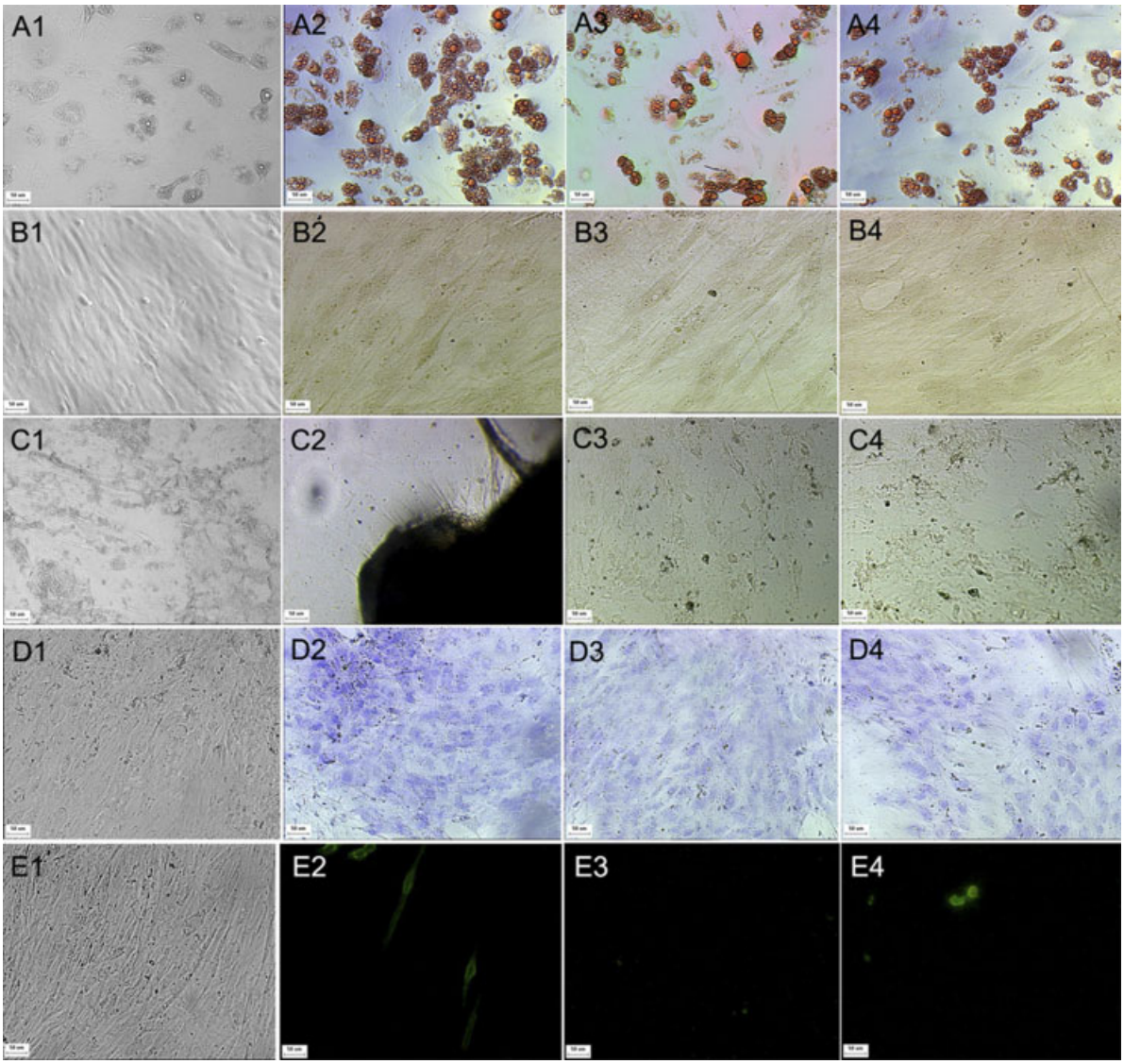

Figure 7. Multi-differentiation of hADSCs towards mesodermal and endodermal lineages. (A) hADSCs were differentiated toward adipogenesis and formed lipid vesicles stained with Oil Red-O; (B) Osteogenic-differentiated ADSCs were stained with ALP at the early stage; (C) After 3 weeks differentiation of ADSCs into osteocytes, mineralization was revealed using Von Kossa staining; (D) ADSCs under chondrogenesis condition promoted the formation of chondrocytes, differentiated cells were stained with Toluidine Blue; (E) ADSCs differentiated into cardiomyocytes by 5-AZA, cardiomyocyte were connexin-43 positive. (1) Morphology of differentiated ADSCs before staining; (2) stained pictures of differentiated ADSCs of passage 4; (3) stained pictures of differentiated ADSCs of passage 25 after being subcultured every 5 days from P20; (4) stained pictures of differentiated ADSCs of passage 25 after being subcultured every 14 days from P20. Scale bars measure 50 mm

Moreover, the cells can be expanded more rapidly and permanently (three logarithmic phase in 1 month), and higher passage ADSCs (over 25 passages) still retain stem cell phenotypes and mesenchymal pluripotency. In order to obtain more and higher quality cells, we can subculture ADSCs every 14 days instead of the normal 5 days. hADSCs as a source of stem cells are very appealing, and could also potentially be an alternative source to BMSCs for being used in allogeneic transplants and tissue engineering.

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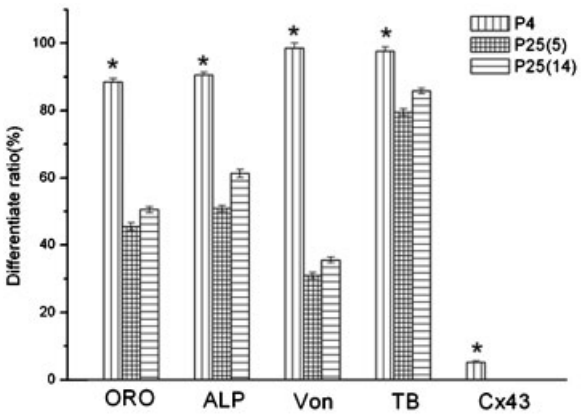

Figure 8. Semiquantitative analysis of differentiate ratio of ADSCs. ORO, adipogenesis, Oil Red-O staining; ALP, osteogenesis, ALP staining; Von, osteogenesis, Von Kossa staining; TB, chondrogenesis, Toluidine Blue staining; Cx-43, myogenesis, immunofluorenscence staining. $^{*}p < 0.05$, compared with other groups. P4, ADSCs of passage 4; P25(5), P25 ADSCs after being subcultured every 5 days from P20 till P25; P25(14), P25 ADSCs after being subcultured every 14 days from P20 till P25

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