hASCs acquisition and identification

Materials and Methods

Cell Culture

Recruitment of donors and acquisition of patients who accepted flap reconstruction was approved by the ethical committee of the Nanjing Medical University. Total 6 patients, 3 males and 3 females, aged from 33-55 year, the average age was 46 year. hASCs were obtained after digesting human subcutaneous adipose tissue by collagenase I (Sigma, USA) and were incubated in alpha-modified Eagle medium (α-MEM) (GIBCO BRL, USA) containing 10% fetal bovine serum (FBS) (GIBCO BRL, USA) at 37℃ in a 5% CO_2 incubator. Cells were plated into the plastic flask at the density of 1×10^8 cells/L; passage 3 cells were prepared for the following experiments.

Cell proliferation assay

Briefly, cells were plated into 96-well plates at the density of 3×10^3 cells per well and incubated in α-MEM containing 10% FBS at 37℃ in a 5% CO_2 incubator. Fifty-four wells were divided into 9 groups randomly. Each group had 6 duplicate wells. Cell proliferation rate was measured every day for 8 consecutive days (d) by adding 10 ml of CCK8 solution (Dojindo, Japan) to each well, which was followed by incubation at 37˚C for 2 hours (h). Absorbance was measured by spectrophotometry using SPECTRA MAX190 (Sunnyvale, USA) at 450 nm. The growth curve of cells was drawn.

Flow cytometry detected cell cycle changes and immunophenotypes

For analysis of cell cycle changes, cells were collected, and precooled 70% ethanol was added for fixation overnight. Then, the cells were harvested. Cell cycle changes were performed by staining cells with propidium iodide (PI) using the Annexin V Apoptosis Detection Kit I (BD, USA) for 30 minutes (min). The fluorescence intensity of DNA-PI was measured by flow cytometry. Flow cytometric immunophenotypes of CD29, CD34, CD45, CD90 and CD105 (BD, USA) were analyzed by staining cells with allophycocyanin (APC), phycoerythrin (PE), PE, Annexin V-fluorescein isothiocyanate (FITC) and PerCP cy5.5 respectively for 30 min on ice in the dark.

Untreated hASCsserved as negative control. The results were analyzed using the FlowJo software 7.6.1 (Leonard Herzenberg, USA).

Detection for the multi-lineage potential of cells

Adipogenic differentiation was induced by culturing cells for 14 days in adipogenic medium (10% FBS, 1 μM dexamethasone, 200 μM indomethacin, 10 mg/L insulin and 0.5 mM 3-isobutyl-1-methylxanthine in α-MEM) and examined by Oil Red O stain. The hASCs inducted by adipogenic medium for 14 d were fixed with 4% paraformaldehyde for 30 min, and then stained with 1% Oil Red O stain (Leagene, China) for 10 min at room temperature.

Osteogenic differentiation was induced by culturing cells for 21 days in osteogenic medium (10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerol phosphate, and 50 μM vitamin C in α-MEM) and examined via Alkaline phosphatase (ALP) and Alizarin Red stain. ALP stain was performed with the BCIP/NBT ALP Color Development Kit (Beyotime, China) according to the manufacturer's instructions after osteogenic induction for 5 d. The hASCs inducted by osteogenic medium for 21 d were fixed with 4% paraformaldehyde for 30 min, and then stained with 1% Alizarin Red S (Leagene, China) for 1 h at room temperature. For semi-quantitative assessment of ALP and Alizarin Red stain, stained colors were eluted by 10% (w/v) cetylpyridinium chloride (Sigma-Aldrich, USA) for 30 min and the spectrophotometric absorbance of extracted dye was measured at 562 nm.

Chondrogenic differentiation was induced by culturing hASCs for 14 days in chondrogenic medium (10% FBS, 10 ng/ml transforming growth factor-β1, 200 μMindomethacin, 6.25 μg/ml insulin and 50 nM vitamin C in α-MEM) and assessed by Alcian Blue stain. Alcian Blue stain was performed with the Alcian Blue stain Kit (Leagene, China) according to the manufacturer's instructions after chondrogenic induction for 14 d.

Results

Cell growth and cycle for hASCs

The acquired hASCs displayed typical fibroblast-like morphology (Fig S1. A) under a light microscope (magnification 100×; Leica Microsystems GmbH, Germany). CCK-8 was used to evaluate cell proliferation of hASCs. The growth curve was plotted as the overall shape of "S" (Fig S2. B). The slow growth phase was in the first 3 d. The exponential growth phase ranged from 3 to 6 d when the cell proliferation rate increased exponentially, and then the hASCs plateaued after 7 or 8 d in the number of cells.

The results of cell cycle changes showed that 86.91% ± 7.97% of the cells were in the G1 phase and 13.09% ± 2.64% of the cells were in the S/G2 phase (Fig S1. C). The results of cell proliferation and cycle changes revealed that good proliferation ability of hASCs in our study.

Immunophenotypes and differentiation of hASCs

Flow cytometry detected immunophenotypes of hASCs. The cells were positive for CD29 (99.58% ± 1.48%), CD90 (99.53% ± 1.94%) and CD105 (98.03% ± 2.07%) and were negative for molecular markers of the hematopoietic cell line CD34 (5.61% ± 0.95%) and CD45 (3.95% ± 0.87%), as examined in immunophenotypes (Fig S1. D). Thus, these results of immunophenotypes supported the identity and purity of the mesenchymal stem cells from adipose tissue.

Furthermore, the cells were stained with ALP and Alizarin Red for semi-quantitative assessment of mineralized matrix, respectively. The eluted stain assay showed that staining of ALP, compared to the hASCs without osteogenic induction, increased 3.55 ± 0.27-fold ($p < 0.001$) in 5 d after osteogenic induction (Fig S1. E); the staining of Alizarin Red, compared to the hASCs without osteogenic induction, increased 4.72 ± 0.45-fold ($p < 0.001$) in 14 d after osteogenic induction (Fig S1. E). When multi-differentiations were induced respectively, the formation of lipid droplets were detected by Oil Red O stain and exhibited adipogenic differentiation potential; the mineralization nodules were evidenced by Alizarin Red stain and showed osteogenic differentiation potential; the proteoglycans were examined by Alcian Blue stain and exhibited

chondrogenic differentiation potential. The staining of Adipogenic, osteogenic and chondrogenic differntiation was shown in Fig S1. F.

Figure Legends in Supplementary Materials

Fig.S1 Biological characteristics of hASCs. (A) hASCs of the third passage. (magnification 100×) (B) Cell proliferation detected by CCK-8. Slow growth phase was in the first 3 d. From 3 to 6 d, cells were in exponential growth phase; and achieved the platform growth phase at 7 or 8 d. (C) Cell cycle detected by flow cytometry. 86.91% ± 7.97% of the cells were in the G1 phase and 13.09% ± 2.64% of them were in the S/G2 phase. (D) Immunophenotypes of cells detected by flow cytometry. Cells were positive for CD29 (99.58% ± 1.48%), CD90 (99.53% ± 1.94%) and CD105 (98.03% ± 2.07%); and were negative for CD34 (5.61% ± 0.95%) and CD45 (3.95% ± 0.87%). (E) The multi-lineage potential of cells. Lipid droplets were stained by Oil Red O; the mineralization nodules were stained by Alizarin Red; the proteoglycans were stained by Alcian Blue. (magnification 100×)