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ORIGINAL ARTICLE

Effects of the donor age on proliferation, senescence and osteogenic capacity of human urine-derived stem cells

Peng Gao · Peilin Han · Dapeng Jiang · Shulong Yang · Qingbo Cui · Zhaozhu Li

Abstract To study the effects of the donor age on the application potential of human urine-derived stem cells (hUSCs) in bone tissue engineering, by comparing proliferation, senescence and osteogenic differentiation of hUSCs originated from volunteers with different ages. The urine samples were collected from 19 healthy volunteers (6 cases from children group aged from 5 to 14, 5 cases from middle-aged group aged from 30 to 40, and 8 cases from the elder group aged from 65 to 75), and hUSCs were isolated and cultured. The cell morphology was observed by microscope and the cell surface markers were identified by flow cytometry. Their abilities to undergo osteogenic, adipogenic and chondrogenic differentiation were determined in vitro, and cell proliferation analyses were performed using Cell Counting Kit-8 (CCK8) Assay. The senescence of hUSCs among three groups was assessed by senescence-associated β galactosidase staining. After osteogenic differentiation, the alkaline phosphatase (ALP) activity of hUSCs was measured and expression of osteogenic-related runt-related transcription factor 2 (RUNX2) and osteocalcin (OCN) was determined by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot. The hUSCs isolated from urine samples were adherent cells displayed "rice gain"-like and "spindle-shaped" morphology, expressing surface markers of mesenchymal stem cells (MSCs) (CD73, CD90, CD105) and the peripheral cell marker (CD146), but not hematopoietic stem cell markers (CD34, CD45) or the embryonic stem cell marker (OCT3/4). The obtained hUSCs could be induced into osteogenic, adipogenic or chondrogenic differentiation. The hUSCs from the children group showed higher proliferation and lower tendency to senescence than those from the middle-aged and elder groups. After osteogenic induction, the ALP activity and RUNX2 and OCN expression of hUSCs from the children group were higher than those from the elder group. While no significant differences were observed when comparing the middle-aged group with the children group or the elder group. Donor age could influence the potency of hUSCs on proliferation, senescence and capacity of osteogenic differentiation. hUSCs from children group have shown higher proliferation, lower tendency to senescence, and stronger osteogenic capacity, which means to be more suitable for basic research and have better clinical application. Furthermore, hUSCs from all groups suggest the application potential in bone tissue engineering as seed cells.

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Abbreviations

AgNPs	Silver nanoparticles
ALP	Alkaline phosphatase
BMPs	Bone morphogenic proteins
BMSCs	Bone marrow mesenchymal stem cells
β-Gal	Senecence-associated β galactosidase staining
β-TCP	B-Tricalcium phosphate
CCK-8	Cell counting kit-8
CS	Calcium silicate
ECL	Enhanced chemiluminescence assay
FBS	Fetal bovine serum
hUSCs	Human urine-derived stem cells
KSFM	Keratinocyte-serum free medium
MSCs	Mesenchymal stem cells
PBS	Phosphate buffered saline
PLGA	Poly lactic-co-glycolic acid
qPCR	Quantitiative real-time polymerase chain reaction

Introduction

Bone tissue engineering is based on the cultivation of seed cells (autologous osteoblasts, marrow stromal stem cells and cartilage cells, etc.) on natural or synthetic cell scaffolds to repair defects. As seed cells, stem cells, such as umbilical cord mesenchymal stem cells, bone marrow stromal cells (BMSCs), and placenta-derived mesenchymal stem cells, have been widely utilized in the field of bone tissue engineering research (Diao et al. 2009; Zhou et al. 2014; Siddappa et al. 2007; Zhong et al. 2012; Frese et al. 2016). Human urine-derived stem cells (hUSCs) firstly reported by Zhang et al. (2008) have several features, such as adhesive growth, cloning, expression of most mesenchymal stem cell (MSC) markers and peripheral cell markers, multiple differentiation potential, keeping stable karyotype of generations and non-tumorigenicity. They may be a new source of seed cells in tissue engineering because of noninvasive and convenient to be obtained, stable cultivation and multiple differentiation potential (Zhang et al. 2008, 2014).

Currently, hUSCs were studied as seed cells in bone tissue engineering (Guan et al. 2015a, b, c; Qin et al. 2014). However, the impact of donor age on biological characteristics of hUSCs has not been reported. In the present study, we explored the effects of donor age on application of hUSCs in bone tissue engineering by comparing proliferation, senescence and osteogenic capacity. Our work will answer whether hUSCs from all donor ages could be applied in bone tissue engineering, and the differences of hUSCs from different donor ages in bone tissue engineering.

Materials and methods

Ethics statements

Approved by the Harbin Medical University Review Board (IRB81572117), all adult volunteers signed informed consents according to the Helsinki Declaration (amended by 55th WMA General Assembly, Tokyo, Japan, 2004). Furthermore, by signing consents, we received the participation permission from the children volunteers' parents or their legal guardians in this study.

Isolation and culture of hUSCs in vitro

200–250 ml urine samples from 19 healthy volunteers, including six children (age from 5 to 14, with an average age of 8.2), five middle-aged (age from 30 to 40, with a mean age of 34.8) and eight elders (age from 65 to 75, with an average age of 68.4), were collected and transferred into 50 ml sterile tubes. After centrifuging at 1000 r/min for 10 min, the supernatant was carefully discarded. The pellet was resuspended and washed with steriled phosphate buffered saline (PBS) buffer twice. The obtained cells were resuspended in complete culture medium and seeded into 6-well culture plates. The cells were cultured at 37 °C in a 5% CO_2 humidified incubator (NU-5500E, Nuaire, Plymouth, MN, USA). The non-adherent cells were washed away before changing medium on day 7. The first passage was maintained when cells reached 80–90% confluence on day 15 or day 17. The cells from the fourth passage ($2–10 \times 10^6$/urine sample) were used in our experiment.

hUSCs were cultured in a 1:1 mixture of Keratinocyte-serum free medium (KSFM) and Dulbecco's modified Eagle's medium/nutrient mixture F12 (DMEM/F12) supplemented with 5% fetal bovine serum (FBS), 1% penicillin–streptomycin, 1% glutamine, 10 ng/ml human epidermal growth factor and 50 ng/ml bovine pituitary extract (Gibco, Carlsbad, CA, USA).

Analysis of cell surface markers by flow cytometry

The hUSCs suspension containing 5×10^5 cells was washed with PBS twice and then with sodium azide staining buffer. 5 µl of antibodies (CD34-FITC-A, CD45-FITC-A, CD73-FITC-A, CD90-FITC-A, CD105-FITC-A, CD146-PE-A, STRO-1-FITC-A, OCT3/4-PE-A) (BioLegend, San Diego, CA, USA) (Table 1) and 95 µl sodium azide staining buffer were added and mixed gently and incubated at room temperature in the dark for 20 min. After washing with PBS twice, 500 µl of 1% paraformaldehyde was added for fixation and the cell samples were subjected to fluorescence-activated cell sorting analysis (FAC-Saria, BD Biosciences, San Jose, CA, USA).

Osteogenic differentiation and alizarin red stain analysis

For osteogenic differentiation, human mesenchymal stem cell osteogenic differentiation basal medium (175 ml) and mesenchymal stem cell-qualified fetal bovine serum (20 ml) containing penicillin–streptomycin (2 ml), glutamine (2 ml), ascrobate (400 µl), β-glycerophosphate (2 ml) and dexamethasone (20 µl) were used. hUSCs were seeded at 3×10^5 cells/well

Table 1 Antibodies used for FACS analysis

Primary antibody	Source/Cat#	Dilution
CD34(FITC)	BioLegend/343604	1:20
CD45(FITC)	BioLegend/368512	1:20
CD73(FITC)	BioLegend/344015	1:20
CD90(FITC)	BioLegend/328108	1:20
CD105(FITC)	BioLegend/323208	1:10
CD146(PE)	BioLegend/361006	1:20
STRO-1(FITC)	BioLegend/340106	1:20
OCT3/4(PE)	BioLegend/653704	1:20

in 6-well tissue culture plates. When cells were 80–90% confluent, the growth medium was removed and 2 ml human mesenchymal stem cell osteogenic differentiation medium was added. Cells were refed every 3 days for 2 weeks with fresh osteogenic differentiation medium. The cell samples were washed with PBS twice and fixed with 2 ml of 4% paraformaldehyde for 30 min. The cells were rinsed twice and stained with 1 ml alizarin red working solution for 3 to 5 min. After rinsing with PBS twice, the cells were observed under light microscope (TS100, Nikon, Tokyo, Japan).

Adipogenic differentiation and oil red O stain analysis

For adipogenic differentiation, human mesenchymal stem cell adipogenic differentiation basal medium A (175 ml) and mesenchymal stem cell-qualified fetal bovine serum (20 ml) containing penicillin–streptomycin (2 ml), glutamine (2 ml), insulin (400 µl), IBMX (200 µl), rosiglitazone (200 µl), dexamethasone (20 µl) were used as "Adipogenic Differentiation Medium A", and containing penicillin–streptomycin (2 ml), glutamine (2 ml) and insulin (400 µl) were used as "Adipogenic Differentiation Medium B". The hUSCs were seeded at 2×10^5 cells/well in 6-well culture plates. When cells were 80–90% confluent, the growth medium was discarded and 2 ml of adipogenic differentiation medium A (induction solution) was added. Three days later, the medium A was changed to adipogenic differentiation medium B (maintenance solution). The medium B was changed back to medium A 24 h later. After repeating the cycles three times, the cells were cultured in medium B for 7 days, and then washed with PBS and fixed with 2 ml of 4% paraformaldehyde for 30 min. The cells were washed with PBS twice and stained with 1 ml of oil red O working solution for 30 min and rinsed three times with PBS, then observed under light microscope.

Chondrogenic differentiation and alcian blue stain analysis

For chondrogenic differentiation, human mesenchymal stem cell chondrogenic differentiation basal medium (97 ml) containing dexamethasone (10 µl), ascrobate (300 µl), ITS + supplement (1 ml), sodium pyruvate (100 µl), proline (100 µl) and TGF-β3 (1 ml)

were used. 10×10^5 cells were transferred into 15 ml sterile tube, washed with incomplete chondrogenic medium once, centrifuged at 1000 r/min for 5 min at room temperature. 2 ml of incomplete chondrogenic medium was used to resuspend the cells and the cell samples were divided into four tubes. After centrifuging at 1000 r/min for 5 min at the room temperature, the samples were incubated for 24 h. Feeding the cell pellets every 2–3 days by completely replacing medium in each tube. Flicking the bottom of each tube to keep the pellet free-floating. Chondrogenic pellets were harvested after 28 days of culture and fixed with 4% paraformaldehyde.

The slides were deparaffinized, hydrated and stained with alcian blue solution for 30 min and then washed in running tap water for 2 min. The samples were observed under light microscope after rinsing in distilled water.

Cell proliferation assay

The hUSCs from all groups (n = 9) were seeded at 2×10^3 cells/well in 96-well plates and cultured with growth medium in humidified incubator for 24 h. The culture medium was changed every 3 days. The medium was changed to 100 μl of fresh growth medium containing 10 μl of CCK8 solution (Dojindo, Kumamoto, Japan) and kept in humidified incubator for 1 h. The absorbance (OD value) at wavelength of 450 nm was measured by microplate reader (MRX type-II, DYNEX, Chantilly, VA, USA). Every well measure was performed in triplicate.

Senescence-associated β galactosidase staining (β-Gal)

The hUSCs from all groups (n = 5) in 6-well culture plates were washed with PBS once when reaching to 60–70% confluence. 1 ml of β-Gal stationary solution (Beyotime, Shanghai, China) was added to the wells and incubated at room temperature for 15 min. After washing with PBS for three times, 1 ml of β-Gal working solution was added for staining at 37 °C for 12 h and then washed with PBS for observation under light microscope. Five fields of view were randomly chosen in each well. The number of senescent cells and total number of cells in the field of view were counted.

Alkaline phosphatase (ALP) activity assay

After osteogenic differentiation for 14 days, the hUSCs from all groups (n = 5) were washed with PBS for three times, then 0.1% Triton X-100 were added and transferred to 1.5 ml tubes for centrifuging. The ALP activity of cells was measured according to the manufacture instructions of ALP Activity Assay kit (Jiancheng, Nanjing, China) and the total protein content was measured with Pierce BCA Protein Assay Kit (Pierce Biotechnology, Waltham, MA, USA). The ALP activity was expressed as the absorbance (OD value) at 520 nm/μg total proteins.

Quantitiative real-time polymerase chain reaction (qRT-PCR)

The total RNA of hUSCs osteogenic differentiated for 14 days from all groups (n = 5) was exacted with RNeasy Mini Kit (Qiagen, Valencia, USA). Complementary DNA was prepared from 2 μg of total RNA with superscript reverse transcriptase (Promega, Madison, WI, USA) and oligo (dT) primers. The mRNA levels of *RUNX2*, *OCN* and *β-actin* were determined by quantitative real-time PCR (qRT-PCR) using C1000 instrument (Bio-Rad, Hercules, CA, USA). The used primers are listed in Table 2. The expression of *RUNX2* and *OCN* was normalized to *β-actin*. Each sample was assayed in triplicate.

Western blot analysis

The hUSCs after osteogenic differentiation were lysed with RIPA lysis buffer and centrifuged at 4 °C at 12,000 r/min for 15 min. Cell lysates were boiled for 5 min and then loaded onto 8–12% sodium dodecyl sulfate–polyacrylamide gels and transferred onto nitrocellulose (NC) filter membrane for 1 h (constant current, 180 mA). The NC membranes were blocked in 5% skim milk in TBST at room temperature for 1 h and incubated overnight at 4 °C with anti-RUNX2 (1:500), anti-OCN (1:1000) and β-actin (1:1000) (Thermo Fisher, Waltham, MA, USA). After washing with TBST for three times, the NC membrane was incubated with appropriate diluted HRP-conjugated secondary antibody at room temperature for 1 h. After washing with TBST for three times, the NC membrane was developed with ECL Chemiluminescence Reagent.

Table 2 Primers for qRT-PCR

Gene	Forward primer sequence	Reverse primer sequence	Annealing. Temp (°C)	Amplicon length (bp)
RUNX2	5′-AACCCACGAATGCACTATCCA-3′	5′-GTAGTGAGTGGTGGCGGACAT-3′	60	90
OCN	5′-AAGAGACCCAGGCGCTACCT-3′	5′-AACTCGTCACAGTCCGGATTG-3′	60	110
β-actin	5′-TCGTGCGTGACATTAAGGAGA-3′	5′-GTCAGGCAGCTCGTAGCTCTT-3′	60	110
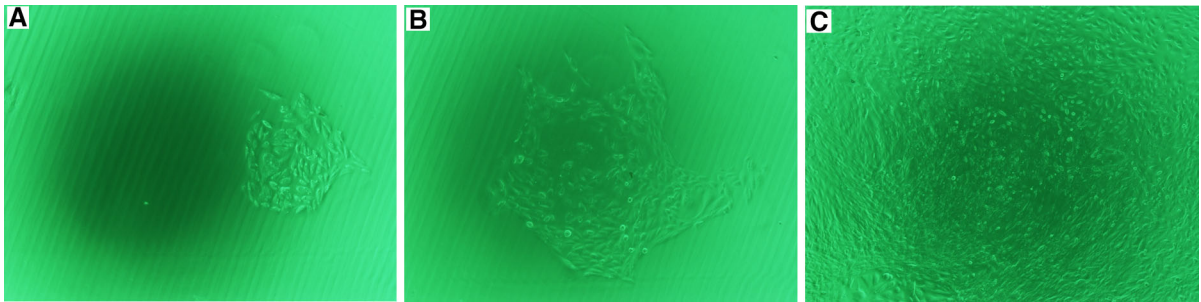
Fig. 1 The morphology of hUSCs (P0) from the middle-aged group was observed by optical microscope. **a** After seeding on day 4, hUSCs attached to the culture plate with a "rice gain"-like and "spindle-shaped" morphology and clone formation. **b** On day 10, the area of hUSCs clone increased with clear boundaries. **c** On day 18, hUSCs reached 90% confluence. Images shown at ×40

Statistical analysis

All statistical analysis was performed with SPSS 16.0 software. When the data were in accordance with the condition of parameters test, ANOVA was used, otherwise, Kruskal–Wallis test was selected. The repeated measure ANOVA was used for data of cell proliferation. Data were expressed as mean ± SD. Statistical significance was indicated as $* P < 0.05$ and $** P < 0.01$.

Results

The morphology, cell surface markers and multiple differentiation potential for identifying hUSCs

Cell morphology

After initial seeding, hUSCs, defined as the first passage, started to attach to culture plates and began to form clones within 3–5 days (Fig. 1a, b), and reached 80–90% confluence in 15–20 days (Fig. 1c). Followingly, hUSCs were split every 2–3 days. hUSCs in primary culture had a "rice gain"-like, "spindle-shaped", and "cobblestone" like morphology. hUSCs at passage 4 (P4) from the children group (Fig. 2a), the middle-aged group (Fig. 2b) and the elder group (Fig. 2c) were elongated compared to the first passage. There was no obvious difference of morphology among those groups at P4.

Cell surface marker expression of hUSCs

Cell surface markers are key parameters to identify stem cells (Dominici et al. 2006). The expression profiles of CD marker of hUSCs (P4) were assessed by flow cytometry. As shown in the Fig. 3, CD73, CD90, CD105 and CD146 were detected, but not for CD34, CD45, or OCT3/4, and weakly positive for STRO-1 (Fig. 3). Our results indicated that hUSCs were mesenchymal stem cells (MSCs), but not hematopoietic stem cells (HSCs), as described in a previous report (Dominici et al. 2006).

Multiple differentiation potential of hUSCs in vitro

To study the multiple differentiation potential of hUSCs, the ability to undergo osteogenic, adipogenic and chondrogenic differentiation was determined.
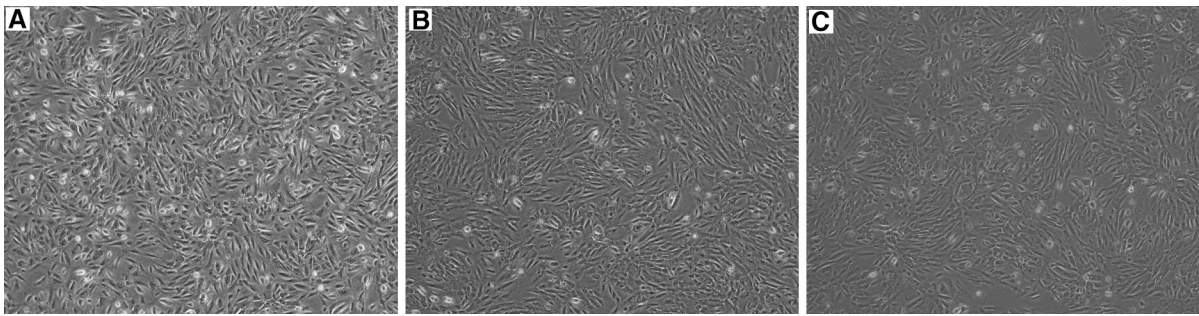
Fig. 2 The morphology of hUSCs (P4) from the children (**a**), the middle-aged (**b**) and the elder **c** groups was observed by optical microscope. The morphology of hUSCs (P4) had no significant differences among all groups, but was much elongated than that at primary culture. Images shown at ×100
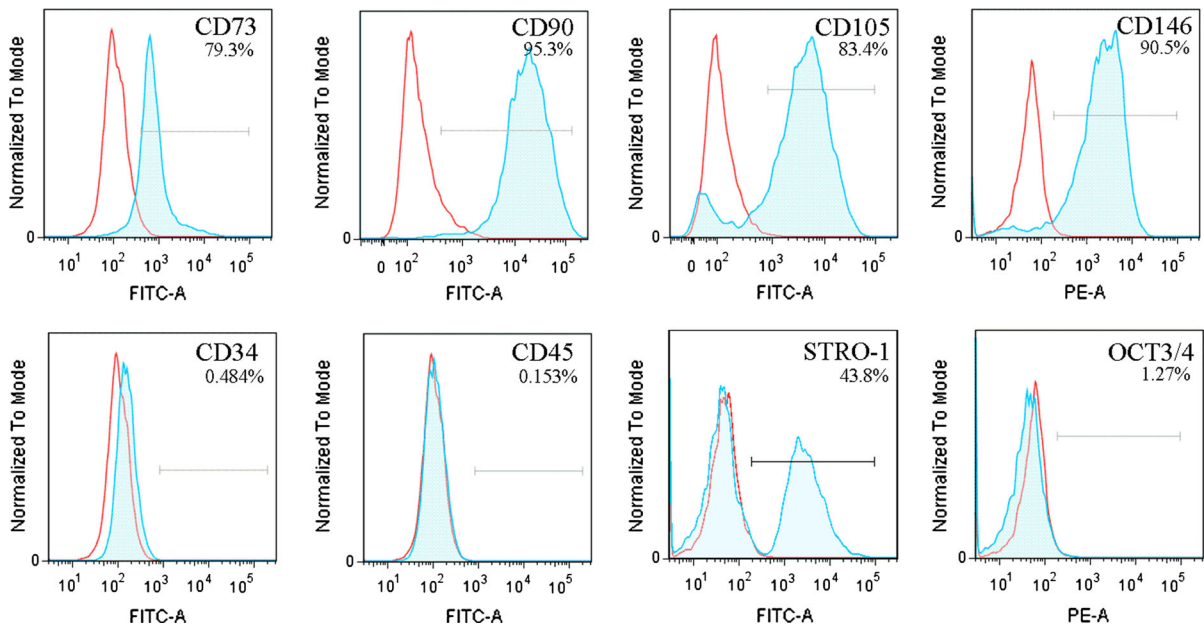

Fig. 3 Cell surface marker expression of hUSCs (P4) was assessed by flow cytometry. The hUSCs (P4) from the middle-aged group were positive for MSC markers (CD73, CD90, CD105), and the pericyte marker (CD146), but negative for HSC markers (CD34, CD45)

After 2 weeks of differentiation, jacinth mineralized nodes were stained by Alizarin Red, indicating the existance of osteoblasts (Fig. 4a). After adipogenic differentiation for 19 days, the formation of tiny lipid droplets was observed by Oil Red O staining, suggesting hUSCs differentiated into adipocytes (Fig. 4b). After 28 days in chondrogenic culture, the pellet grew into spherical masses with shiny surfaces and Alcian blue staining demonstrated the presence of proteoglycans and polysaccharides secreted by chondrocytes (Fig. 4c). Our data showed that hUSCs had a multiple differentiation potential.

The comparison of hUSCs proliferation from different age groups

CCK8 assay was used to evaluate the effects of donor age on hUSCs proliferation. The growth curves in all age groups displayed "S-shape" feature. Cells were in the stationary phase during the first 1–2 days, came into rapid growth phase from day 3 to day 5, then returned to a slow growth phase from day 6 to day 7. The difference of OD values among three groups had statistical significance ($F = 12.970$, $P = 0.007$). The proliferative potential of hUSCs from the children
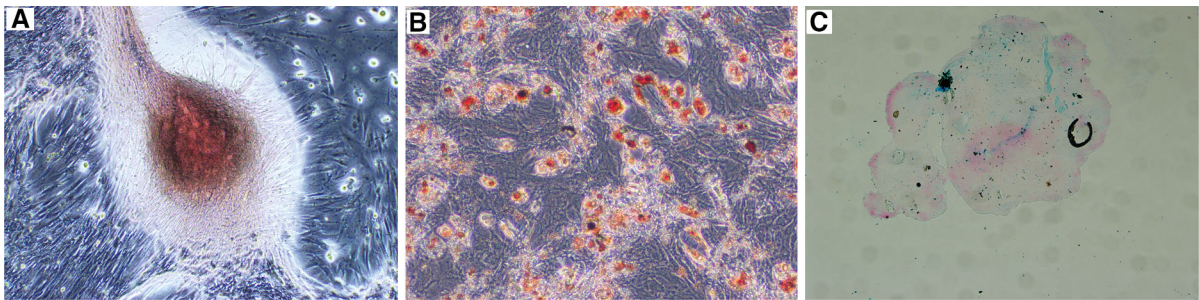
Fig. 4 Multiple differentiation potential of hUSCs. **a** After 14 days of osteogenic induction, mineralized nodes were stained by Alizarin Red, indicated the existance of osteoblasts. Image shown at ×400. **b** After 19 days of adipogenic induction, tiny lipid droplets were stained by Oil Red O, indicating appearance of adipocytes. Image shown at ×100. **c** After 28 days of chondrogenic induction, the pellets grew into spherical mass with shiny-surfaces and were stained by Alcian blue, showing the presence of proteoglycans and polysaccharides secreted by chondrocytes. Image shown at ×200
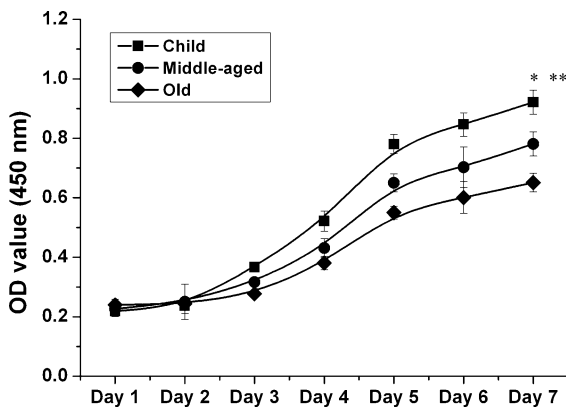
Fig. 5 CCK8 assay for hUSCs proliferation. The growth curve characteristics of hUSCs from all groups displayed similar "S-shape": hUSCs were in the stationary phase during the first 1–2 days, then came into rapid growth phase from day 3 to day 5, and kept a slower growth trend from day 6 to day 7. The proliferation capacity of hUSCs from the children group was higher than that from the middle-aged group (*$P < 0.05$) and much higher than that from the elder group (**$P < 0.01$)

group was higher than that from the elder group ($P = 0.002$) and the middle-aged group ($P = 0.029$). There was no significant difference in proliferation capacity of hUSCs between the middle-aged group and the elder group ($P = 0.068$; Fig. 5).

Senescence of hUSCs from different age groups

Senescence of hUSCs (P4) from different age groups was assessed by senescence-associated β-galactosidase staining (SA-β-Gal). The senescent cells were stained turquoise and the cell morphology became large and flat (Fig. 6a–c). As the result shows, there were significant differences among the three groups ($F = 266.116$, $P = 0.000$). There were significantly more SA-β-Gal positively stained hUSCs from the elder group compared with those from the middle-aged group ($P = 0.000$). Furthermore, the SA-β-Gal positively stained hUSCs from the middle-aged group were also more numerous than those from the children group ($P = 0.001$; Fig. 6d). This may indicate that hUSCs tend to be senescent with increased donor age.

The ALP activity of hUSCs after osteogenic differentiation in different age groups

The ALP activity of hUSCs (P4) after osteogenic differentiation was assessed to evaluate the osteogenic activity among all groups. Significant difference in the ALP activity of hUSCs was observed after osteogenic differentiation among the three groups ($F = 39.119$, $P = 0.000$): the hUSCs ALP activity from the children group was higher than that from the middle-aged group ($P = 0.034$) and hUSCs ALP activity from the middle-aged group was much higher than that from the elder group ($P = 0.001$; Fig. 7). This indicates that the osteogenic capacity of hUSCs might decline with increased donor age.

Expression of RUNX 2 and OCN of hUSCs after osteogenic differentiation in different age groups

According to literature, mRNA expression of osteogenesis-related gene runt-related transcription factor 2 (*RUNX 2*) and osteocalcin (*OCN*) was assessed by quantitative real-time polymerase chain reaction
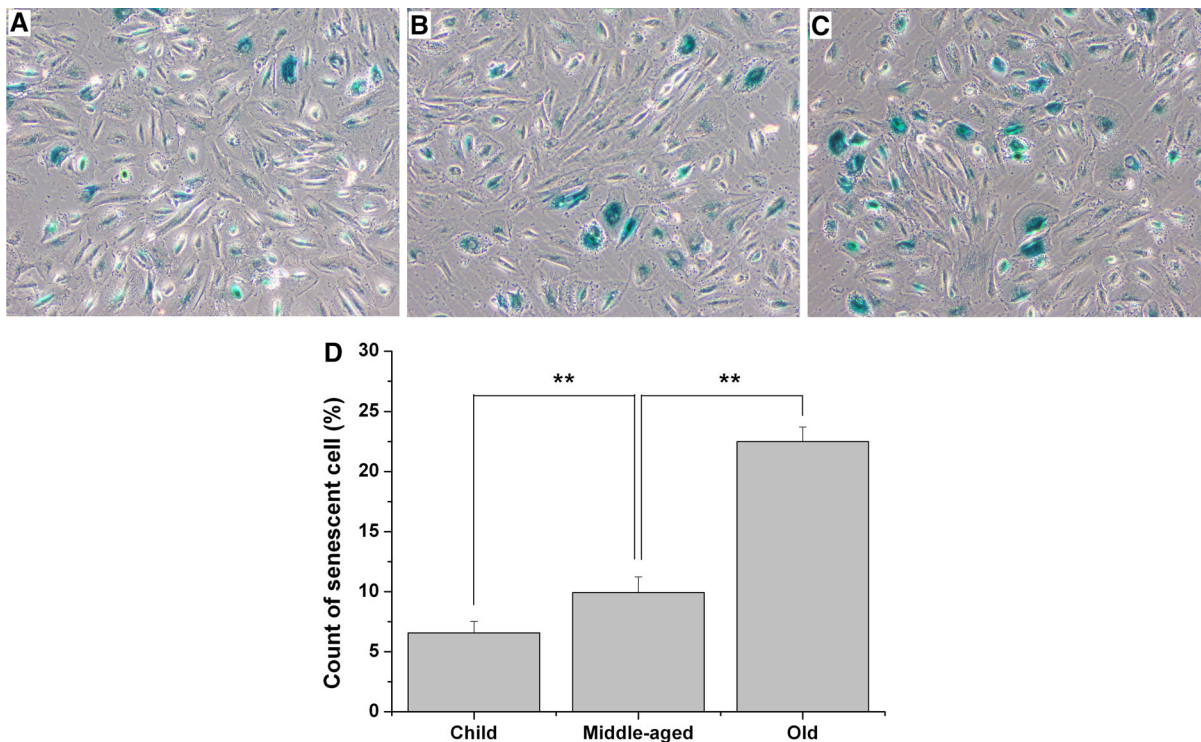
Fig. 6 Compared with normal hUSCs (P4) from all groups, the senescent cells, became large and flat, were stained turquoise by SA-β-Gal. **a** The SA-β-Gal staining of hUSCs from the children group. **b** The SA-β-Gal staining of hUSCs from the middle-aged group. **c** The SA-β-Gal staining of hUSCs from the elder group. **d** The histogram showed the proportion of SA-β-Gal staining-positive cells (senescent cells) relative to the total cells among the three groups. There were much more SA-β-Gal positively stained hUSCs from the elder group than from the middle-aged group (**$P < 0.01$). Furthermore, SA-β-Gal positively stained hUSCs from the middle-aged group were much more numerous than from the children group (**$P < 0.01$). Image shown at ×100
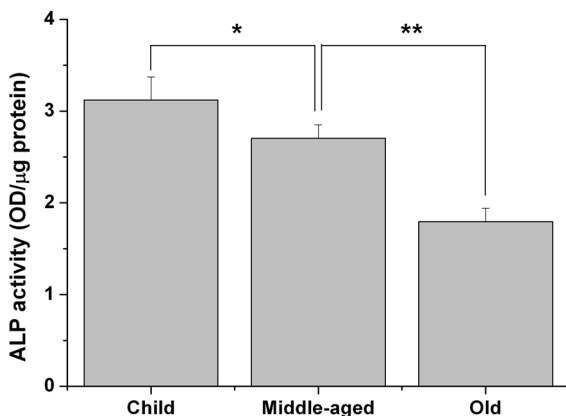

Fig. 7 The ALP activity of hUSCs (P4) after osteogenic differentiation. The ALP activity from the children group was higher than that from the middle-aged group (*$P < 0.05$) and the ALP activity from the middle-aged group was much higher than that from the elder group (**$P < 0.01$)

(qRT-PCR), to evaluate osteogenic capacity of hUSCs (P4) among all groups (Wang et al. 2013; Qin et al. 2014; Tan et al. 2012). qRT-PCR indicated, that the expression of both *RUNX 2* and *OCN* of hUSCs after osteogenic induction among the three groups showed statistically significant difference ($H = 7.200$, $P = 0.027$). The expression of *RUNX 2* and *OCN* from the children group was higher than that from the elder group ($P = 0.007$; Fig. 8a), but there were no significant differences when comparing the middle-aged group with the children group or the elder group ($P = 0.180$; Fig. 8b).

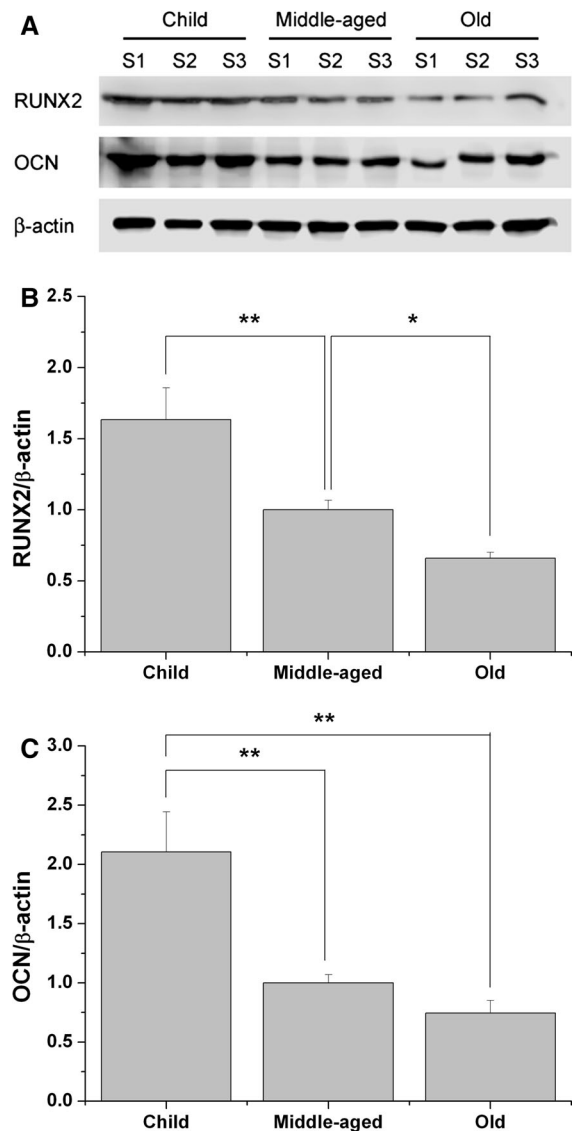The protein expression of RUNX 2 and OCN of hUSCs (P4) after osteogenic induction among all groups was detected by Western blot in triplicate (Fig. 9a). After densitometric quantification, the protein expression of RUNX 2 and OCN among the three
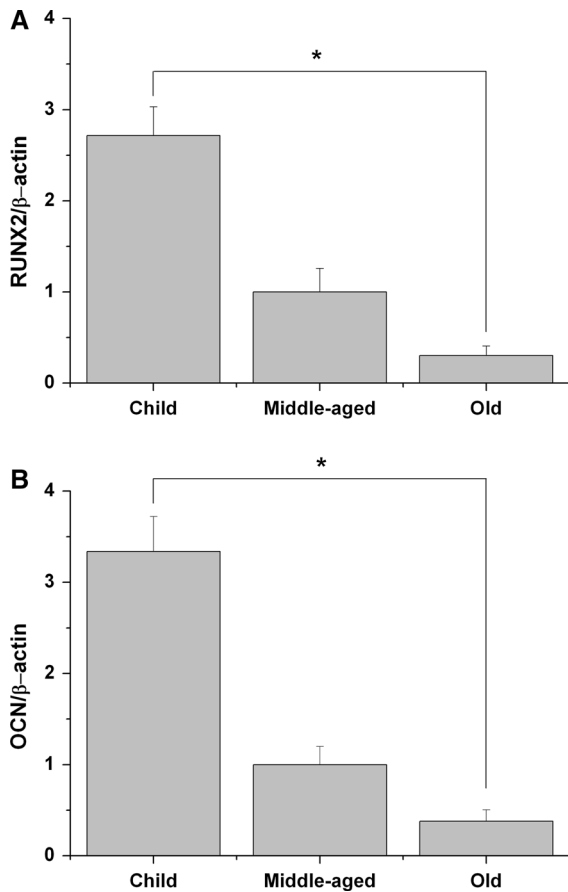
Fig. 8 After osteogenic induction, total RNA was extracted from all groups and expression of *RUNX 2* and *OCN* was assessed by qRT-PCR. The expression of *RUNX 2* and *OCN* of hUSCs (P4) from the children group was higher than that from the elder group (*$P < 0.05$) (**a**, **b**)

groups had statistical difference ($F_{RUNX2} = 38.840$, $P_{RUNX2} = 0.000$; $F_{OCN} = 36.099$, $P_{OCN} = 0.000$). The expression of RUNX2 from the children group was higher than that from the middle-aged group ($P = 0.001$), and that from the middle-aged group was higher than that from the elder group ($P = 0.023$; Fig. 9b). The expression of OCN from the children group was higher than that from both the middle-aged group ($P = 0.001$) and the elder group ($P = 0.000$), but there was no significant difference between the middle-aged group and the elder group ($P = 0.185$; Fig. 9c). The data infer that hUSCs from the children may have a stronger osteogenic capacity so as to be more suitable for basic researches and have better effects on autologous application in bone tissue engineering.

Fig. 9 After osteogenic induction, total protein was extracted and expression of RUNX 2 and OCN of hUSCs (P4) among the three groups was determined by Western blot. **a** Western blot. There were three samples from different donors in each group. **b** Expression of RUNX2 from the children group was much higher than that from the middle-aged group (**$P < 0.01$), and that from the middle-aged group was higher than that from the elder group (*$P < 0.05$). **c** Expression of OCN from the children group was much higher than that from other two groups (**$P < 0.01$)

Discussion

Recently, hUSCs have been utilized in bone tissue engineering because of the following advantages: 1. Easy to isolate and culture. 2. Non-invasive 3. No

rejection or ethical issues. 4. High proliferation efficiency and stable karyotype without tumorigenicity. Even though, there are still gaps in the field of hUSCs, such as what changes of hUSCs phenotype would be with proliferation and passage, whether hUSCs biological characteristics would be changed with phenotype changes, would donor age make hUSCs biological activity decline? etc. More and more reports suggest that hUSCs may be a new source of seed cells in tissue engineering (Zhang et al. 2014; Gao et al. 2016; Jiang et al. 2016). hUSCs were first reported in 2008 with the characteristics of adherent growth, cloning and multiple differentiation potential (Zhang et al. 2008). Researches further identified biological characteristics of hUSCs such as active proliferation, expression of MSC markers (CD73, CD90, CD105) and the peripheral cell marker (CD146), non-expression of HSC markers (CD31, CD34, CD45), unstable expression of some stem cell markers (SOX2, Oct3/4, SSEA-4), multi-potential differentiation into bone, cartilage, fat, endothelial, epithelium, smooth muscle, skeletal muscle and nerve (Chun et al. 2012; Bharadwaj et al. 2011, 2013; Bodin et al. 2010; Guan et al. 2014; Wang et al. 2016). According to the reported method (Frese et al. 2016), we obtained adhesive and clonal growth of cells with "rice gain"-like or "spindle-shaped" morphology. Flow cytometry results showed that the obtained hUSCs were CD73, CD90, CD105, CD146 and STRO-1 positive, but did not express CD34, CD45, OCT3/4. Those cells can differentiate into osteoblasts, adipocytes and chondrocytes, further confirming the mutiple differentiation potential of hUSCs (Zhang et al. 2008; Chun et al. 2012; Bharadwaj et al. 2011, 2013; Bodin et al. 2010; Guan et al. 2014; Wang et al. 2016; Lang et al. 2013).

The active proliferation means stronger self-renewal capacity for seed cells (Zhao et al. 2013; Yamamoto et al. 2013). The proliferation of BMSCs decreased with increased donor age, regardless they came from animals or human-being (Wilson et al. 2010; Artegiani and Calegari 2012; Mueller and Glowacki 2001; Payne et al. 2010; Stolzing et al. 2008). hUSCs have been considered as another source of seed cells in tissue engineering and cell therapy. However, the effects of donor age on proliferation and differentiation of hUSCs are still unknown. In the present experiment, the proliferation and senescence of hUSCs from different age groups have been

compared, in order to explore which donor age group of hUSCs would be more suitable for tissue engineering. As growth curves showed, the proliferation characteristics of hUSCs among all groups suggests the age factor has certain effects on hUSCs proliferation. This indicated more hUSCs might be obtained in a short time for basic research and clinical application if they are derived from children. Many literature references reported that the proliferation of MSCs would decline as donor age increases. However, there is no statistical difference in proliferation of hUSCs between the middle-aged and the elder group. In order to further confirm that phenomenon, we will develop our research by expanding sample sizes in our further work.

Senescence is considered as a way to inhibit formation of tumors and a potential cause of life aging. As literature reported, MSCs from all ages of donors cultured in vitro could be senescent. During cell culture, MSCs morphology changed, such as cell enlargement, flat morphology and cytoplasmic vacuoles, and their proliferation and differentiation capacity decreased or were even lost (Sethe et al. 2006; Zhuang et al. 2015). For example, MSCs from aging mice could be functionally impaired, with high expression of *p16INK4a* and telomere shortening (Janzen et al. 2006; Molofsky et al. 2006; Krishnamurthy et al. 2006). In a rat model of myocardial injury, both differentiation capacity and cardiac recovery of BMSCs from aging rats were lower than those from the young (Jiang et al. 2008; Khan et al. 2011). The number and function of adult stem cells was also related to body aging and some experts thought that this was linked to the *p16INK4A* signaling pathway (Fujita and Tsumaki 2013; Feng et al. 2014). As far as we know, it was not reported whether hUSCs are in accordance with this law. We worked to answer that question and tried to provide an important parameter for hUSCs application in tissue engineering. Senescence-associated β galactosidase (SA-β-Gal) is a metabolic enzyme highly expressed in senescent cells and considered as one of the biomarkers of senescent cells (Debacq-Chainiaux et al. 2009). SA-β-Gal positively stained hBMSCs highly expressed the senescence associated gene *p16INK4a* with significantly decreased osteogenic and adipogenic capacity (Park et al. 2005; Zhou et al. 2008). Therefore, in this experiment, SA-β-Gal staining has been chosen as indicator for senescence of

hUSCs from different donor ages. Our data revealed a phenomenon that "the older donor age, the more cell senescence". This may indicate that the hUSCs from the youth would be more vigorous for tissue engineering application and basic research. Whether the mechanism is related to *p16INK4A* signaling pathway needs to be proven in the follow up work.

The activity of stem cells is decreased with body aging (Baxter et al. 2004). This may be a reason that stem cells from the elder tend to be senescent. In addition, the growth microenvironment of aged donors may be unsuitable for proliferation and differentiation capacity of stem cells. We found that hUSCs activity gradually decreased with increasing donor age. Therefore, more active hUSCs will be acquired from children for tissue engineering.

Seed cells, scaffolds and growth information constitute three elements in tissue engineering. Recently, the ways to improve the ability of osteogenic differentiation of hUSCs by changing the local microenvironment or gene expression were developed, such as gene transfection, incubation with AgNPs and application with artificial materials (Guan et al. 2015a, b, c; Qin et al. 2014). Some experts thought that the function of stem cells might decline, including proliferation, migration and differentiation with increasing age (Kretlow et al. 2008; Lohmann et al. 2012; Hermann et al. 2010), but others thought that this would not (Chen et al. 2012; Fickert et al. 2011). The relationship between donor age and osteogenic capacity of hUSCs is important for hUSCs to be used in bone tissue engineering and it has not been reported yet. In our work, we chose ALP activity, and expression of RUNX2 and OCN as observing targets (Wang et al. 2013; Qin et al. 2014; Tan et al. 2012). ALP is one of the mature osteoblasts markers and its activity can be used to reflect osteogenic capacity of stem cells. Our results indicated that the ALP activity decreased with the increasing donor age, and indirectly implied that there might be a negative correlation between donor age and osteogenic capacity of hUSCs. qRT-PCR and Western blot results showed that the expression of both *RUNX2* and *OCN* from the children group was much higher than that from the elder group, suggesting that hUSCs from children may have stronger osteogenic capacity and may be more suitable for basic research. In addition, higher proliferation and stronger osteogenic capacity of hUSCs may mean the better autologous application effects in bone tissue engineering if they are from children. Although osteogenic capacity of MSCs was thought as declined with age increasing (Zaim et al. 2012), no enough evidence show that osteogenic capacity of hUSCs from the middle-aged was superior than that from the elder in our research.

In our experiment, hUSCs from the elder can be stably proliferating and induced into osteoblasts, which means an application potential for bone tissue engineering. The need for bone defect repair and artificial bone reconstruction in elderly patients is greater. It is meaningful to improve osteogenic efficiency of hUSCs from the elder for autologous application.

Conclusion

With advantages such as non-invasive, convenient obtention, stable culture and high efficiency of proliferation, multiple differentiation potential, and absence of tumorigenicity, hUSCs are considered as a source of seed cells in tissue engineering (Gao et al. 2016; Kang et al. 2015; Yang et al. 2016). We explored the relationship between donor ages and biological characteristics of hUSCs to provide valuable information for basic research and clinical application in bone tissue engineering. Firstly, the proliferation and osteogenic capacity of hUSCs from the children group are much stronger than those from the two other groups, which means applying hUSCs from children in bone tissue engineering may have better clinical effects and be more suitable for basic research. Secondly, hUSCs from all groups can stably proliferate and induce osteogenic differentiation. This implies that hUSCs from the elder could also have potential for application in bone tissue engineering, so as to avoid ethical controversy by application of autologous stem cells. The ways to improve osteogenic capacity of hUSCs from the elder will be explored in further work.

So far, the research of hUSCs in bone tissue engineering has been still in the early stage. There are many blanks need to be explored. How to further improve the osteogenic efficiency of hUSCs? Will the combination of hUSCs with different scaffold materials, such as nano biomaterials, composites, etc., be more effective in the construction of artificial bone (Lee et al. 2015)? Are the multi-potential characteristics of hUSCs helpful to improve the blood supply of

artificial bone? The experimental data of primates in vivo is important for stem cells application in bone tissue engineering. So data of artificial bone constructed with hUSCs in primate models are expected to advance this specific cell therapy domain.

Compliance with ethical standards

Conflict of interest   The authors declare there are no conflicts of interest.

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