In vitro culture and biological properties of broiler adipose-derived stem cells

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Abstract. In the past 10 years, adipose-derived stem cells (ADSCs) have been applied due to their pluripotency. Experimental tissues have been frequently obtained from mammals, including rabbits, mice and humans, but rarely from broilers, *Gallus gallus domesticus*. In the present study, ADSCs were obtained from 20-day-old broiler embryos. Primary ADSCs were sub-cultured to passage 37 *in vitro*. The surface markers of ADSCs, namely CD29, CD31, CD44, CD71 and CD73, were detected by reverse transcription polymerase chain reaction and immunofluorescence assays. The result indicated that CD29, CD44, CD71 and CD73 were expressed on the surface of cells at various passages, but not CD31. The growth curve of cells at the different passages had a typical sigmoidal shape. Furthermore, ADSCs were successfully induced to differentiate into osteoblasts, adipocytes and hepatocyte-like cells. The results denote that the ADSCs isolated from broilers have similar biological properties to those of ADSCs obtained from other animals. The present study provided a theoretical and experimental foundation for the use of poultry as a source of stem cells, and laid a foundation for adipose tissue engineering and strategies in regenerative medicine.

Introduction

Mesenchymal stem cells (MSCs) originate from mesoderm, and are a type of pluripotent cells with a great proliferation and multi-direction differentiation potential *in vitro* (1). The first successful isolation was that of bone marrow mesenchymal stem cells (BMSCs) and later from various other tissue types, including subcutaneous adipose tissue (2-5). Numerous independent studies have demonstrated that BMSCs are multipotent in an artificial environment, with the ability to produce cartilage cells, osteoblasts, supporting hematopoietic cells, adipocytes and hepatocyte-like cells (6-9). Subsequent studies indicated that adipose-derived stem cells (ADSCs) have similar biological properties compared with BMSCs (10). Subcutaneous adipose tissue displays certain advantages over bone marrow, e.g., adipose tissue is easier to collect and enrich, and is located at sites from which patients are more likely to accept biopsies (11,12). Therefore, ADSCs have become a better option for medicinal applications including tissue engineering and replacement treatment.

To date, ADSCs have been mostly isolated from mammals, including humans, rhesus monkeys, mice and rabbits (13-16), but rarely from avian species. Plentiful adipose tissues are present in broilers (*Gallus gallus domesticus*). In the present study, ADSCs were obtained from 20-day-old broiler embryos and cultured *in vitro*, their abilities to proliferate and differentiate into multiple lineages were demonstrated, and they were characterized regarding the expression of specific surface markers. The present study offers a novel source for *in vitro* culturing of ADSCs and lays a foundation for tissue engineering and regenerative medicinal applications.

Materials and methods

Experimental animals. A total of 60 20-day-old male broiler embryos (weight, ~40 g) were provided by the Poultry Experimental Base of the Institute of Animal Sciences (Chinese Academy of Agricultural Sciences, Beijing, China). All procedures conformed to the guidelines established by the Institutional Animal Care and Use Committee at Chinese Academy of Agriculture Sciences (Beijing, China).

Reagents. The following reagents were used in the present study: Dulbecco's modified Eagle's medium (DMEM)/F12, fetal bovine serum (FBS), L-glutamine, and penicillin and

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streptomycin (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) trypsin (dilution, 1:250) and 0.02% (w/v) EDTA (Amresco, Inc., Framingham, MA, USA), rabbit anti-chicken CD29 (bs-0486R), CD44 (bs-2507R), CD71 (bs-1782R) and CD73 (bs-4834R) polyclonal primary antibody, goat serum, EDTA, fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit immunoglobulin (Ig)G (all from Bioss, Beijing, China), ascorbic acid sodium salt, dexamethasone, hepatocyte growth factor, indometacin, insulin transferrin-selenium, 3-isobutyl-1-methylxanthine (IBMX), β-glycerophosphate, oil red O, collagenase I (all from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), basic fibroblast growth factor (bFGF), fibroblast growth factor-4 (FGF-4; both from Peprotech, Inc., Rocky Hill, NJ, USA), alizarin red (Boster, Wuhan, China) and TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.).

Isolation and culture of ADSCs. Adipose tissue samples were collected from 20-day-old broiler embryos under aseptic conditions. The adipose pads were washed with PBS supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin to remove any other cells, including hemocytes and endothelial cells. The adipose pats were fully chopped into small pieces and subsequently incubated with 0.2% (m/v) collagenase I in PBS at 37˚C for 40 min. The enzymatic activity was neutralized by adding an equal volume of DMEM/F12 containing 5% (v/v) FBS. The cell suspension was filtered through a 74-mm-mesh sieve and then centrifuged at 200 x g for 8 min at room temperature. The precipitate was resuspended in complete growth medium composed of DMEM/F12, 10% (v/v) FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10 ng/ml bFGF. Cells were seeded into petri dishes at a density of $1.0x10^5$/ml, and cultured at 37˚C with 5% CO_2. After 24 h, the dishes were washed with PBS to clean out any non-adherent cells, including pericytes, blood cells, endothelial cells and preadipocytes (14,17-19).

The cells were referred to as 'passage 0' (P0) when their confluence reached 80%. Subsequently, the cells were sub-cultured at the ratio of 1:2 after standard trypsin digestion. This generation was referred to as P1. The cells became purified with increasing passages, and were then harvested for other relevant trials.

Morphological observation. The morphology and adhesion of ADSCs prior to and after culture was observed under an inverted microscope.

Reverse transcription polymerase chain reaction (RT-PCR) analysis of cell surface markers. Total RNA was extracted from ADSCs at P5 by using TRIzol and then subjected to RT using an RNA PCR kit (version 3.0; Takara Biotechnology, Co., Ltd., Dalian, China) according to the manufacturer's instructions. Primers were designed in accordance with the sequences of GAPDH (internal control) CD71, CD29, CD31, CD73 and CD44 from GenBank. The template complementary DNA was amplified by PCR using the gene-specific primers listed in Table I. The PCR reaction was performed using the PCR Master Mix kit (Takara Biotechnology, Co., Ltd.), according to the manufacturer's instructions. PCR analyses were performed in 50 μl reactions, containing 25 μl 2X PCR mix, 15 μl ddH_2O, 5 μl template cDNA and 2.5 μl forward and reverse primers. Cycling conditions were as follows: Initialization at 94˚C for 5 min, then 35 cycles of a denaturation at 94˚C for 30 sec, annealing at 50-60˚C for 30 sec, elongation at 72˚C for 30 sec and final elongation at 72˚C for 5 min. PCR products were assessed by 2% agarose gel electrophoresis and bands were visualized with an ultraviolet transilluminator.

Immunofluorescent detection. ADSCs of P5 were fixed in 4% (m/v) paraformaldehyde for 15 min at room temperature, subsequently washed with PBS. The cells were permeabilized using 0.2% (v/v) Triton X-100 for 20 min at room temperature and washed with PBS. The samples were blocked with 10% (v/v) goat serum for 30 min at room temperature, and then incubated in 1% bovine serum albumin (BSA) in PBS containing the polyclonal rabbit anti-chicken antibodies to CD29, CD44, CD71 and CD73 (all at 1:100 dilution) for 12 h at 4˚C. Primary antibody was replaced with PBS for the negative control. The samples were washed with PBS and incubated in PBS containing FITC-conjugated goat anti-rabbit IgG as the secondary antibody for 1 h at room temperature. The samples were washed with PBS after the incubation. They were counterstained with DAPI and were visualized by using a Nikon TE-2000-E confocal microscope with a digital camera system (Nikon, Tokyo, Japan).

Flow cytometry. The expression of cell-associated surface markers was measured by flow cytometric analysis. In brief, ADSCs of P5 were collected by standard trypsin digestion, and the cells were fixed and permeabilized in 70% (v/v) ice-cold ethanol for 12 h. The samples were blocked with 10% (v/v) goat serum for 30 min at room temperature and were incubated in 1% BSA containing rabbit anti-chicken polyclonal antibodies to CD29, CD44, CD71 and CD73 (all at 1:100 dilution) for 1 h at room temperature. Primary antibody was replaced with PBS for the negative control. Subsequently, the samples were washed with PBS and incubated in PBS containing FITC-conjugated goat anti-rabbit IgG as the secondary antibody for 1 h at room temperature. After washing with PBS, the labeled cells were detected with a flow cytometer. Flow cytometric data were analyzed using CXP software version 2.0 (Beckman Coulter, Brea, CA, USA).

Growth kinetics. ADSCs at P3, P16 and P30 were collected and seeded in triplicate in 24-well plates at $1x10^4$ cells/well. Cell viability assessment was performed following trypan blue staining at room temperature for 5 min and cells were counted following detachment over 9 successive days. The population doubling time (PDT) was calculated using the following formula: PDT=(t-t0)xlg2/(lgNt-lgN0), with t0 representing the starting time of culture, t the termination time of culture, N0 the initial number of cultured cells and Nt the final number of cells.

Adipogenic differentiation of ADSCs. ADSCs were divided into a control group and an induction group. When the confluence of cells reached 70-80%, the experimental group was treated with adipogenic medium consisting of DMEM/F12 containing 10% FBS, 200 μM indomethacin, 1 mM dexamethasone, 10 μM insulin transferrin and 0.5 mM IBMX. The control group was cultured in complete growth medium without the factors mentioned above. The medium

Table I. Sequences of primers used for polymerase chain reaction.

Gene	Primer sequence	Tm (˚C)	Cycles (n)	Product size (bp)
CD29	F, 5'-GAACGGACAGATATGCAACG-3' R, 5'-TAGAACCAGCAGTCACCAACG-3'	60	30	300
CD31	F, 5'-CAGGCAAAGGAGACGCACGAT-3' R, 5'-CTTCTGGCAGCTCACAACGT-3'	60	30	221
CD44	F, 5'-CATCGTTGCTGCCCTCCT-3' R, 5'-ACCGCTACACTCCACTCTTCAT-3'	56	30	290
CD71	F, 5'-CTCCTTTGAGGCTGGTGAGG-3' R, 5'-TCAGTGAAGCCACGACCTTC-3'	56	30	293
CD73	F, 5'-AGTGCAAACATTAAGGGAAAA-3' R, 5'-ACGCTCCTGGAAGATAGTGAT-3'	58	30	310
GAPDH	F, 5'-TAAAGGCGAGATGGTGAAAG-3' R, 5'-ACGCTCCTGGAAGATAGTGAT-3'	60	30	244
PPAR-γ	F, 5'-CTGTCTGCGATGGATGAT-3' R, 5'-AATAGGGAGGAGAAGGAG-3'	48	30	199
LPL	F, 5'-AGTGAAGTCAGGCGAAAC-3' R, 5'-ACAAGGCACCACGATT-3'	49	30	477
Collagen I	F, 5'-AAGGATGGTCGCAATG-3' R, 5'-GGTGGCTAAGTCTGAGGT-3'	49	30	310
Osteopontin	F, 5'-CAGAACAGCCGGACTTTC-3' R, 5'-CTTGCTCGCCTTCACCAC-3'	51	30	227
ALB	F, 5'-AGACAGACGCATGGCTTGTT-3' R, 5'-GGGGCTTGCGTTTAATGAGG-3'	60	30	287
AFP	F, 5'-TCGGGCACGCTTGATCTTTA-3' R, 5'-AGCTGTTGCCTTCAACTGGA-3'	62	30	499

Tm, melting temperature; F, forward; R, reverse; PPAR, peroxisome proliferator-activated receptor; LPL, lipoprotein lipase; ALB, albumin; AFP, α-fetoprotein.

was replaced every 2 days. After 1 week, the two groups were evaluated for accumulation of intracellular lipid using oil red O staining for 40 min at room temperature and expression of adipogenic cell-specific genes by RT-PCR.

Osteogenic differentiation of ADSCs. The cells were divided into two groups as above. Upon reaching 60-70% confluence, the cells in the experimental group were cultured in osteogenic medium containing DMEM/F12 supplemented with 10% FBS, 0.1 mM dexamethasone, 10 mM β-glycerophosphate and 50 μg/ml ascorbate. In the control group, cells were cultured with normal growth medium. The medium was replaced every 2 days. After 3 weeks, the formation of calcium nodes was assessed by alizarin red staining for 25 min at room temperature and osteoblast-specific genes were detected by RT-PCR.

Hepatocyte differentiation of ADSCs. ADSCs were divided into two groups as described above. When the confluence reached 60-70%, the induction group was cultured in hepatocyte medium containing DMEM/F12 supplemented with 10% FBS, 20 ng/ml FGF-4, 20 ng/ml hepatocyte growth factor and 1% insulin transferrin. The control group was cultured in normal growth medium. The medium was exchanged every 2 days. After 2 weeks, the capacity of the cells to secrete glycogen was detected by periodic acid Schiff staining for 30 min at 37˚C, and hepatocyte-like cell-specific genes were detected by RT-PCR.

Statistical analysis. Values are expressed as the mean ± standard error of the mean. Comparisons between multiple groups were evaluated by Dunnett's post-hoc test after one-way analysis of variance. SPSS version 19 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Culture and morphological observation of ADSCs. The primary ADSCs obtained from subcutaneous adipose tissue were seeded into 60-mm Petri dishes, and after 24 h cells had begun to stretch into various shapes. After 48 h of culturing, a small number of polygonal cells were also observed under the inverted microscope, and a minority of cells had irregular shapes (Fig. 1A). The cells expanded rapidly and reached 80-90% confluence after 5 days of

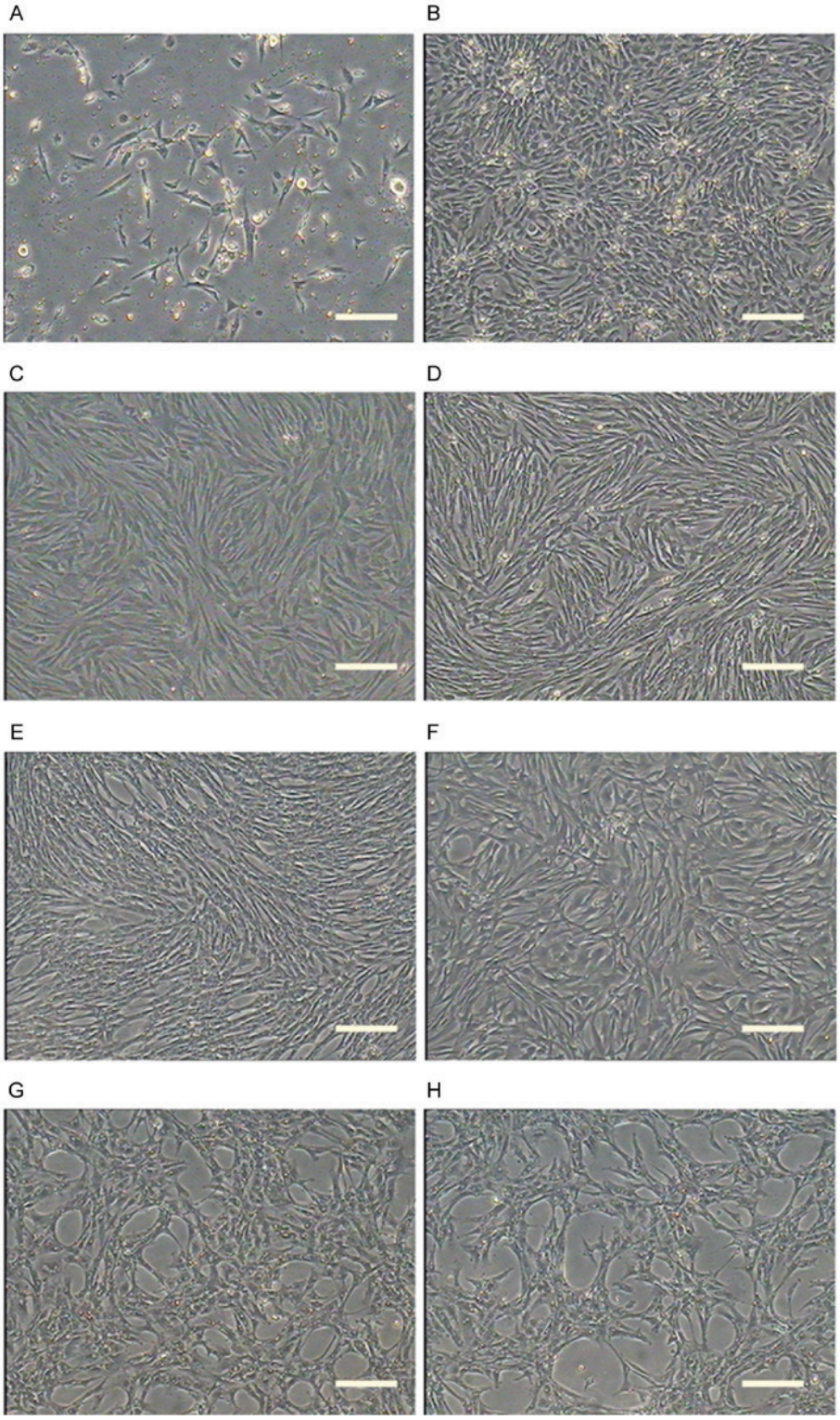

Figure 1. Morphology of primary and sub-cultured ADSCs. (A) Primary cells were cultured for 48 h. Most of cells began to adhere and stretch, and the cell population was inhomogeneous. (B) ADSCs were cultured for 5 days and presented a whirl pattern. (C) At P3, the ADSCs had become pure, and there were no obvious differences in morphology among the subsequent passages. The ADSCs exhibited a fibroblast-like morphology. (D-F) Morphology of ADSCs at (D) P5, (E) P16 and (F) P30. (G and H) The ADSCs at (G) P36 and (H) P37 exhibited typical senescence (scale bars, 100 μm). ADSCs, adipose-derived stem cells; P3, passage 3.

seeding, while being arranged in a whirl pattern (Fig. 1B). In primary culture, numerous hemocytes were admixed with the ADSCs (Fig. 1B). However, from P3, the ADSCs were fully purified, and the shape was also homogenous; the ADSCs exhibited a fibroblast-like morphology (Fig. 1C). No difference in morphology was apparent among the cells of different passages, and the biological properties had remained constant after several cycles of sub-culture (Fig. 1D-F). The cells were cultured to P36 and P37, at which they had a typical senescent appearance, including vacuoles, tabular shapes and

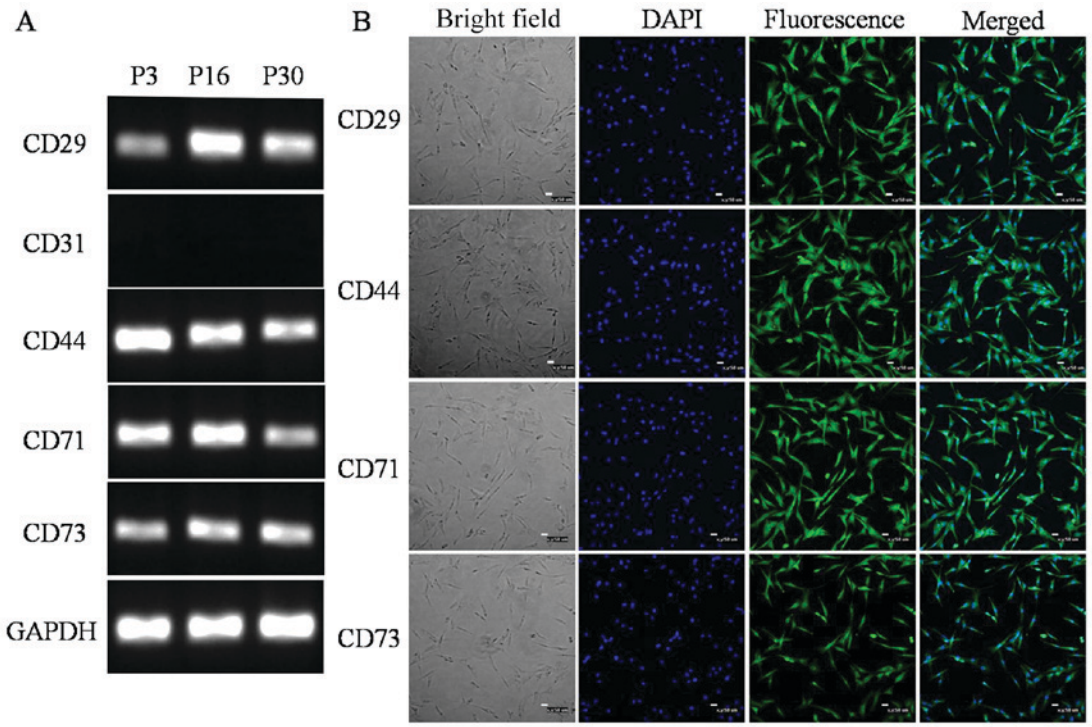

Figure 2. Surface markers of the ADSCs. Surface markers of the ADSCs were similar to those of bone marrow mesenchymal stem cells. The expression of CD29, CD31, CD44, CD71 and CD73 was detected by RT-PCR and immunofluorescence. (A) RT-PCR analysis indicated that the ADSCs expressed CD29, CD44, CD71 and CD73, but not CD31. GADPH was used as an internal control. (B) Immunofluorescence demonstrated immunopositivity for CD29, CD44, CD71 and CD73 (scale bars, 50 μm). ADSCs, adipose-derived stem cells; RT-PCR, reverse-transcription polymerase chain reaction analysis; P3, passage 3.

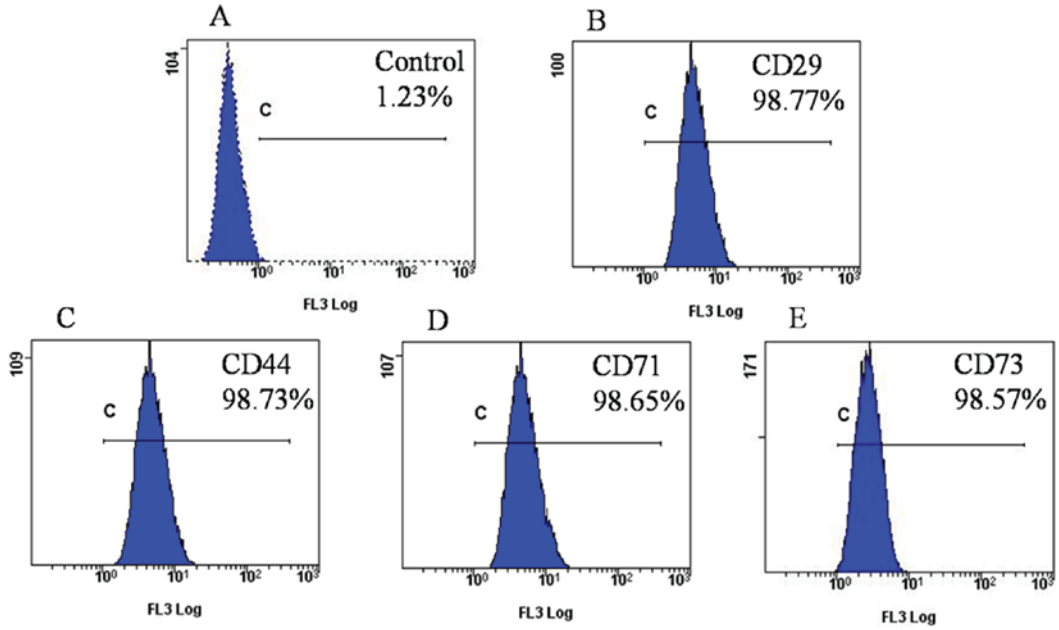

Figure 3. Flow cytometric analysis of ADSCs. Cell surface antigens of the ADSCs were similar to those of bone marrow mesenchymal stem cells. The expression of (A) control, (B) CD29, (C) CD44, (D) CD71 and (E) CD73 was detected by flow cytometry. The results indicated that ADSCs at P5 exhibited high expression of CD29, CD44, CD71 and CD73. ADSCs, adipose-derived stem cells; P5, passage 5.

karyopyknosis in the majority of cells (Fig. 1G and H). With increasing time, they even started to detach from the plates.

Identification of the ADSCs. The specific surface markers of ADSCs were detected by RT-PCR and immunofluorescence. RT-PCR revealed that the ADSCs were positive for CD29, CD44, CD71 and CD73, and negative for CD31, while GAPDH served as an internal control (Fig. 2A). Immunofluorescence microscopy indicated that the ADSC surface antigens CD29, CD44, CD71 and CD73 were expressed by the cells (Fig. 2B). Flow cytometry further confirmed that ADSCs at P5 exhibited high expression of CD29, CD44, CD71 and CD73,

as the positive rates were 98.77, 98.73, 98.65 and 98.57%, respectively (Fig. 3).

Growth kinetics. The proliferation of ADSCs at P3, P16 and P30 was detected by determining the cell number with a hemocytometer, and the data were used to generate a growth curve (Fig. 4). The growth curves suggested that there was a latency phase of ~24 h, followed by rapid proliferation of the cells after entering the logarithmic phase. As the density of the ADSCs increased, the proliferation was inhibited and the cells reached a growth plateau phase at 7-8 days, after which they began to degenerate. The growth curves were all typically sigmoidal and significant differences in the cell growth/kinetics were identified between the different groups ($P<0.05$). The PDT was 38.95, 41.27 and 45.05 h for ADSCs at P3, P16 and P30, respectively.

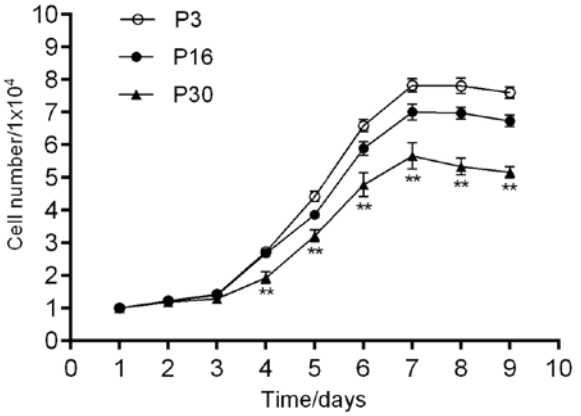

Figure 4. Growth curves of the ADSCs. The proliferation curves of ADSCs at P3, P16 and P30 exhibited typical sigmoidal shapes, with the cell numbers displayed on the vertical axis. The growth curves included a latent phase, a logarithmic phase and a plateau phase. The population doubling time was 38.95, 41.27 and 45.05 h for P3, P16 and P30, respectively. Values are expressed as the mean ± standard error of the mean. $^{**}P<0.01$ for P16 vs. P30. ADSCs, adipose-derived stem cells; P3, passage 3.

Adipogenic differentiation of the ADSCs. Oil Red O staining was used to demonstrate the adipogenic differentiation of the ADSCs (20). After culture in adipogenic differentiation medium for 7 days, the morphology of the ADSCs changed from fibroblast-like to oblate-like and numerous lipid droplets appeared in the cells/dishes (Fig. 5A). With prolonged induction, the number of lipid droplets increased gradually and they assembled into larger droplets. The adipogenic differentiation was confirmed by oil red O staining, as the induced cells were positive (Fig. 5B), while cells cultured in normal growth medium were negative on oil red O staining (Fig. 5C).

To further verify the adipogenic differentiation of the ADSCs, the expression of adipogenic markers was evaluated in the two groups by RT-PCR. In the ADSCs incubated in differentiation medium, the adipocyte-specific genes PPAR-γ and LPL were expressed (Fig. 5D), which did not occur in the control group.

Osteogenic differentiation of the ADSCs. The capacity of broiler ADSCs to differentiate into osteogenic cells was assessed, and the induced cells were subjected to morphological and phenotypic analysis. On the 7th day of induction, the cell shape had changed; the cells became confluent and formed mineralized nodules, whose size increased after further induction. After culture in osteogenic medium for 21 days, morphological changes of the ADSCs were evident and became polygonal (Fig. 6A). Furthermore, the nodules were positive on alizarin red staining (Fig. 6B). However, no change in morphology and no staining with alizarin red were observed in the control group (Fig. 6C).

To determine that differentiation had occurred, the expression of osteogenic markers was assessed in the two groups by RT-PCR. The osteogenic genes OPN and collagen I were expressed in the induced group, but not in the control group (Fig. 6D).

Hepatocyte differentiation. The capacity of broiler ADSCs to differentiate into hepatocyte-like cells was proven by morphological and phenotypic analysis of the induced cells. After culturing in hepatocyte differentiation medium for 7 days, the morphology of certain ADSCs in the experimental group changed to a round shape from the long spindle type. After 14 days, numerous cells in the induction group exhibited a cobblestone-like morphology (Fig. 7A) and were positive on glycogen staining in the cytoplasm (purple; Fig. 7B), indicating that the induced cells had acquired the function of synthesis and storage of liver glycogen. In the control group, no morphological changes and no glycogen staining were observed (Fig. 7C).

In addition, RT-PCR indicated that in the induction group, the genes ALB and AFP, which are specific to hepatocyte-like cells, were expressed, while they were not expressed in the control group.

Discussion

In the past, adipose tissue was considered to be merely a passive energy storage modality (21). However, from the opposite viewpoint, adipose tissue may be regarded as a vital endocrine organ that controls metabolism, immunity and satiety (22). In stem cell research, a step forward was made in 2001 when a novel type of adult stem cell, ADSCs, was first described (1). Later studies proved that ADSCs have similar immunophenotypic properties and multilineage differentiation abilities to those of BMSCs (23). Various factors are responsible for the current lack of studies on avian ADSCs, including insufficient genetic information and progress compared to other fields and limited awareness of the scientific community of their therapeutic potential.

In the present study, ADSCs from the adipose tissues of 20-day-old broiler chick embryos were successfully obtained using type I collagenase digestion. All of the results indicated that the biological characteristics of the newly isolated stem cells were stable. The ADSCs became homogeneous through purification over 3-5 passages. The biological properties of the ADSCs were assessed by cytochemistry, determination of growth dynamics, detection of specific markers and multi-lineage differentiation potency experiments.

Almost all of ADSCs were shuttle-shaped with two ends elongated and proliferated rapidly to form a whirl pattern. Their growth dynamics were assessed by generating growth

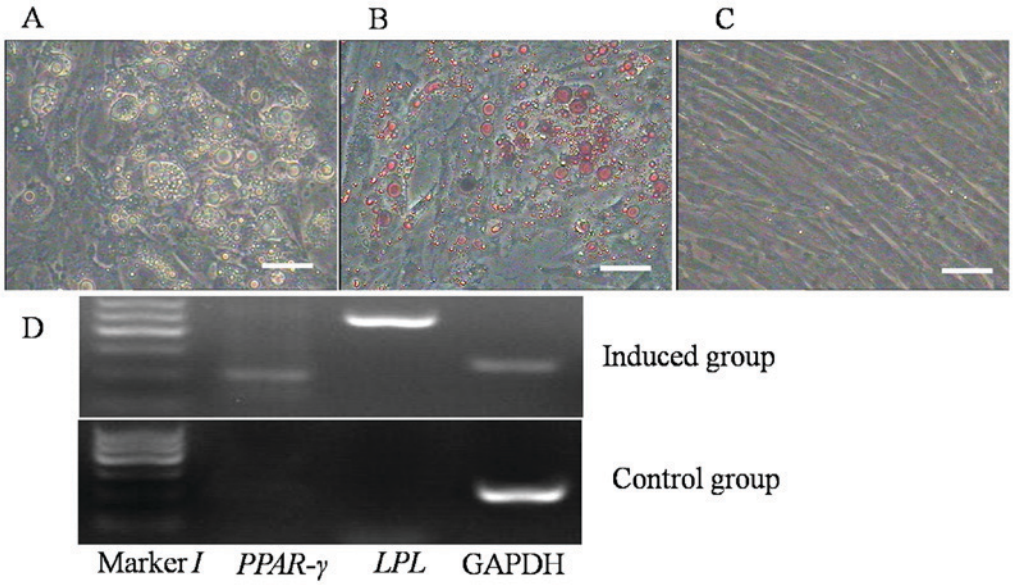

Figure 5. Adipogenic differentiation of the ADSCs. (A) After 1 week of induction, the morphology of the ADSCs began to change from shuttle- to oblate-like, and numerous lipid droplets had formed in the cells. Along with the extension of induction time, the amount of droplets increased and they assembled to form larger ones. (B) Oil red O staining of the differentiated cells visualized the lipid droplets, indicating that they had become adipocytes. (C) The cells cultured in complete growth medium (control group) exhibited no change in morphology and phenotype, and they were negative on oil red O staining (scale bars, 50 μm). (D) Reverse-transcription polymerase chain reaction analysis of the expression of the adipogenic markers LPL and PPAR-γ in the induced group and the control group. The induced cells expressed LPL and PPAR-γ, but the control cells did not. PPAR, peroxisome proliferator-activated receptor; LPL, lipoprotein lipase; ADSCs, adipose-derived stem cells.

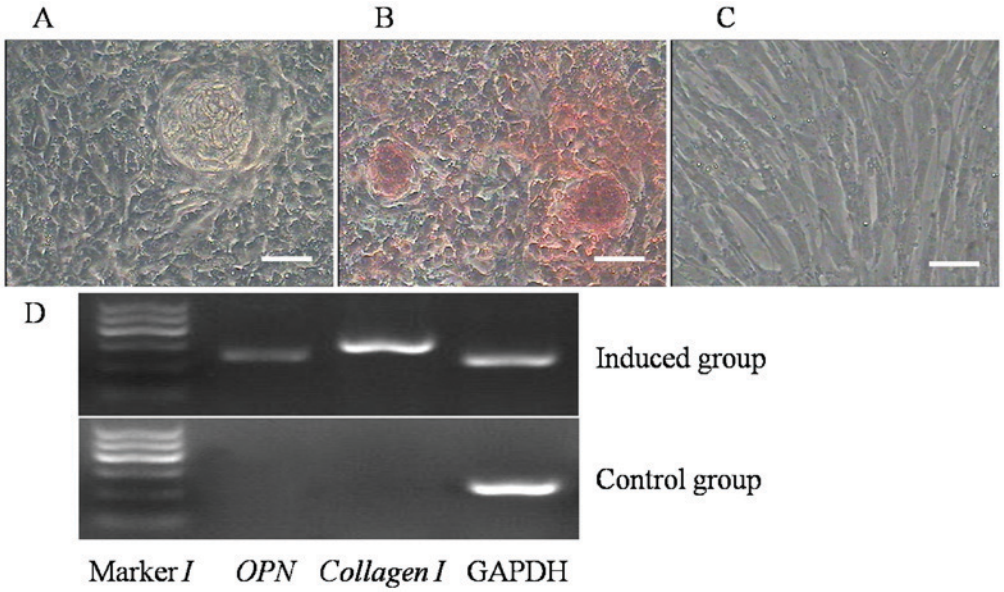

Figure 6. Osteogenic differentiation of the ADSCs. (A) The quantity and size of calcium deposition nodules increased as induction progressed after 21 days. (B) After culture in osteogenic medium for 21 days, the cell shape was changed, and Alizarin Red staining was positive. (C) Cells cultured in complete growth medium (control group) displayed no changes in morphology or staining with Alizarin Red (scale bar, 50 μm). (D) Reverse-transcription polymerase chain reaction analysis of the expression of the osteogenic markers collagen I and OPN in the two groups. Induced cells expressed OPN and collagen I, but the control cells did not. OPN, osteopontin; ADSCs, adipose-derived stem cells.

curves, which had typical sigmoidal and indicated a normal population doubling time.

At present, specific surface markers for ADSCs are lacking. The identity of ADSCs is generally confirmed by the expression of certain MSC-specific surface markers, together with the cell shape and differentiation potential *in vitro* (8). In the present study, the broiler ADSCs expressed CD29, CD44, CD71 and CD73, but not CD31. CD29 is an integrin subunit correlated to very late antigen receptor, and forms a heterodimer binding to the surface and extracellular proteins of MSCs, including CD49 and CD51. It mediates cell-to-cell and cell-to-matrix adhesion. CD44 is a cell-surface glycoprotein, and it mediates cell-cell interactions, cell adhesion and migration (24). It is a receptor for hyaluronic acid. CD44 is involved in numerous cellular activities, including recirculation, homing, lymphocyte activation, metastasis and

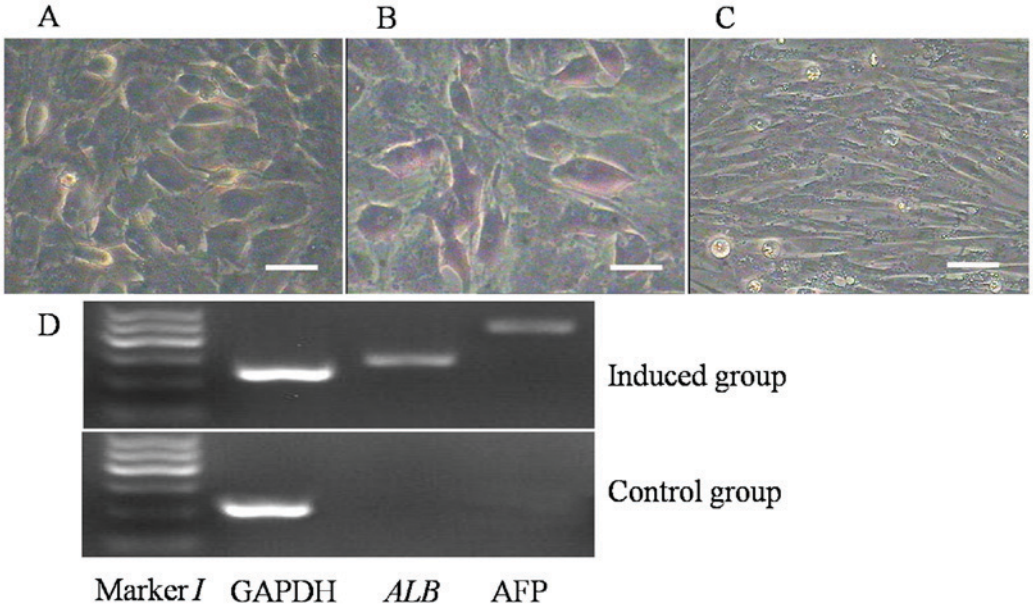

Figure 7. (A) After culture in hepatocyte differentiation medium for 14 days, (B) the cell shape was changed, and glycogen staining was positive. (C) Cells cultured in complete growth medium for 14 days (control group) exhibited no changes in morphology and displayed negative results for periodic acid Schiff staining (scale bar, 50 μm). (D) Reverse-transcription polymerase chain reaction analysis of the hepatocyte differentiation markers ALB and AFP in the induced group and the control group. Induced cells expressed ALB and AFP, but the control cells did not. ALB, albumin; AFP, α-fetoprotein; ADSCs, adipose-derived stem cells.

hematopoiesis (25). CD71, known as a member of the transferrin receptor family, is a carrier for transferrin. It is important for cellular iron uptake by the process of receptor-mediated endocytosis. A low iron concentration promotes increases in the level of transferrin receptor to elevate cellular iron uptake, thereby mediating the iron concentration in MSCs. Thus, the transferrin receptor maintains cellular iron homeostasis (26). CD73 catalyzes the transformation of extracellular nucleotides to membrane-permeable nucleosides (27). As a significant signaling molecule, the protein has been demonstrated to participate in purine salvage and the purinergic cascade that triggers cell metabolism. The results of the present study indicate that broiler ADSCs are a group of undifferentiated stem/progenitor cells different from mesenchymal cells.

The pluripotency of stem cells is their most useful characteristic for cell transplantation therapy. *In vitro*, under the influence of certain induction factors, the expression of certain key genes in the signaling pathways relevant to stem cell differentiation may change. Consequently, differentiation in specific directions may be achieved. In the present study, broiler ADSCs were induced to differentiate into osteoblasts, adipocytes and hepatocyte-like cells and the expression of genes characteristic for the corresponding cell types was assessed. The results demonstrated that various factors were able to induce the ADSCs to differentiate into different directions, and that the ADSCs derived from mesoderm were able to differentiate into endodermal and ectodermal cells. The autologous features of these stem cells, in combination with their distinct pluripotency and easy acquirement, make ADSCs an attractive choice for future tissue engineering and cell-based therapies (28-30).

The above results suggested that broiler ADSCs have a strong growth ability and the potential to differentiate towards mesodermal and endodermal lineages. Although the multilineage differentiation of ADSCs was successful *in vitro*, there are certain technical difficulties regarding the utilization of ADSCs in clinical applications for therapeutic purposes, including a high rate of rejection and instability after cell transplantation. These aspects require being taken into account in future studies and clinical research.

In conclusion, in the present study, ADSCs were obtained from the adipose tissue of 20-day-old broiler chick embryos, and their proliferation and differentiation potential was tested *in vitro*. Considering that male chicks are a waste product of the meat and egg industry, the present study offers an important potential use allowing the sourcing of stem cells and the potential application of ADSCs as a stem cell material for regenerative medicine.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TL, WP and KW analyzed data and drafted the manuscript; WP, FC and YW performed cell culturing and PCR experiments;

TL, SZ and YW performed immunofluorescence and flow cytometry experiments; WG participated in the studies design and coordination. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The protocol of the present study was approved by the Ethics Committee of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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