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In Vitro Culture and Biological Characteristics of Sheep Amniotic Mesenchymal Stem Cells

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Authors' Contribution
TL, WP and SZ analyzed the data and drafted the manuscript. WP, FC and XH performed cell culture and PCR. TL and YW performed immunofluorescence and flow cytometry. WG participated in study design and coordination.

Key words
Amniotic membrane, Mesenchymal stem cells, Sheep, Pluripotent, Differentiation.

ABSTRACT

With the self-renewal capacity and the potentiality of differentiating into various cells, stem cell is a prospective source for tissue engineering. Amniotic mesenchymal stem cells (AMSCs) obtained from amnion do not evoke ethical issues, as they do with the embryonic stem cells. However, a good many experimental tissues and organs were obtained from model animals but rarely from livestock. In this study, AMSCs were obtained from aborted sheep fetuses (1-3 month) under sterile conditions. Primary AMSCs were sub-cultured to passage 25 *in vitro*. The gene of Oct-4, Rex-1, CD29, CD44, CD71, and CD73 were identified by RT-PCR and immunofluorescence technique. The results showed that they were positive in AMSCs. The growth of different passages cells typically appeared in S curve. Furthermore, AMSCs successfully differentiated into osteoblasts, adipocytes, and hepatocyte-like cells after procedure induction. The results showed that the AMSCs obtained from sheep demonstrated the features of multipotent stem cells. Therefore, AMSCs could be a good option for transplantation for treatment and regenerative medical projects.

INTRODUCTION

Mesenchymal stem cells (MSCs) originated from mesoderm, with the ability of great expansion and multipotenntial differentiation *in vitro*. Bone marrow MSCs (BMSCs) is the main material for both animal and clinical research. However, the quantity of BMSCs is not very large, and the procedures for isolating bone marrow involves highly invasive surgery (Stolzing and Scutt, 2006). MSCs can be separated from other tissues such as the amnion. The amnion is a type of cavity membrane filled with liquid. It protects the embryo from attaching to maternal structures and plays an important part in embryonic development. The amnion is formed by five layers such as epithelium, basement membrane, compact layer, fibroblastic layer and spongy layer (Cai *et al*., 2010). Amniotic epithelial cells (AECs) originate from the simple epithelium, and the AMSCs stem from the spongy layer (Roubelakis *et al*., 2012).

AMSCs are isolated from amnion and hence do not evoke any ethical issue on utilizaion of ESCs. AMSCs originate from an early period of embryo, AMSCs can express ESC surface markers (*e.g.,* SSEA-3 and SSEA-4) and pluripotent-specific transcription factors (*e.g.,* Oct-4 and Nanog), and differentiate into cells of all three germ layers (Tsuno *et al*., 2012). Several independent investigators have demonstrated that AMSCs are multipotent in an artificial environment, with the ability to produce osteoblasts, myocardial cells, chondrocytes, and adipocytes, together with vascular endothelial cells (Kim *et al*., 2012, 2013; Paracchini *et al*., 2012). So AMSCs are considered to be suitable seed cells for tissue engineering and for replacement treatment. Most of these studies have however, been done on human and rarely on the mammals.

MATERIALS AND METHODS

Experimental animals and ethics statement

Sheep were provided by the Institute of Animal Science. Fetus samples were collected from induced aborted sheep fetuses (1-3 months) under sterile conditions. In this work, 8 fetal sheep were utilized, which were delivered to the lab within 3–6 h under low temperature. The study was approved by the Chinese Academy of Agriculture

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Sciences (Beijing, China) Institutional Animal Care and Use Committee. All procedures conformed to the regional ethical committee for utilization and care of experimental animals.

Experimental reagents

DMEM/F12 (Gibco, USA), fetal bovine serum (FBS, Gibco), Trypsin (Amresco), non-essential amino acids (Gibco); rabbit anti-sheep CD29 CD44 CD71 CD73 CD90 polyclonal primary antibody (Bioss, China), goat serum (Bioss, China), bovine serum albumin (BSA), EDTA, fluorescein isothiocyanate (FITC) conjugated goat-anti-rabbit secondary antibody IgG (Zhongshan Golden Bridge, China), dexamethasone (Sigma), 4',6-diamidino-2-phenylindole (DAPI), indometacin (Sigma), insulin transferrin (Sigma), hepatocyte growth factor (Sigma), fibroblast growth factor-4 (Sigma), β-glycerophosphate (Sigma), ascorbate (Sigma), alizarin red (Boster, China), isobutyl methylxanthine (IBMX), Oil red O (Sigma), vitamin C, basic fibroblast growth factor (bFGF, Peprotech), collagenase II (Sigma), penicillin and streptomycin (Gibco), Trizol reagent (Invitrogen, USA) and cDNA reverse transcription system (Takara, China).

Isolation and culture of AMSCs

Amniotic tissue samples were collected from aborted sheep fetuses (1-3 month) under sterile conditions. Amniotic membrane is filmy and approximately transparent, and it contains AECs and AMSCs. The amnion was separated and washed seven times with phosphate-buffered saline (PBS) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin to remove blood. The amniotic tissue was finely cut into small pieces with ophthalmic scissors. The small fragments were incubated with 0.05% (w/v) trypsin and 0.02% (w/v) EDTA at 37°C for 10 min to separate AECs from the external layer of the fragments. The following digestion was made by 0.1% (w/v) collagenase II for 30 min. The digestion was subsequently inhibited with DMEM/F12 containing 5 %(v/v) FBS. The miscible liquid was filtered through a 74-mm-mesh sieve. Thereafter, the filtered sample was centrifuged at 1200 rpm for 10 min at room temperature, and the small precipitate was resuspended in prepared medium containing DMEM/F-12, 10% (v/v) FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 ng/mL bFGF and 1% (w/v) non-essential amino acids. Cells were cultured in petri dishes with a density of 1×10^5 cells/ml at 37°C and 5% CO_2. After 24 h, the dishes were washed with PBS to remove non-adherent cells.

The cells could be called P0 generation when the confluence of cells hit 70%–80%. The cells were washed with PBS after the old medium was discarded. Then 0.125% (m/v) trypsin was used to dissociate the cells from the culture dishes, when the vast majority of cells gleamed and retracted into smoother, medium containing required nutrients was added and mixed softly, Then trypsinization was neutralized with fresh complete medium. The cells were sub-cultured at the ratio of 1:2. They were entitled as P1 generation. The morphology and adhesion of AMSCs before and after culture was observed by inverted microscope. With the time going of subculture, the cells were purified after four to five passages. At this moment, the cells expanded could be frozen or directly induced for differentiation.

Growth kinetics

P5, P10 and P15 were collected and seeded in culture dishes with a density of 1.0×10^4/ml. Viability and quantity are counted 3 times to calculate the mean every day and successively for 8 days. The population doubling time (PDT) is calculated by the formula PDT = (t - t0) lg2/(lgNt - lgN0), t0 is starting time of culture; t is terminative time of culture; N0 is initial number of cultured cells; Nt is ultimate number of cultured cells.

Colony-forming cell assay

Cells of P5, P10 and P15 were harvested and seeded in culture plates with a density of 1.0×10^4/well, cultured for 5-7 days, and colony-forming units were counted to calculate colony-forming rate, which is calculated by colony forming unit number/initial cell number per 24-well plates×100%.

RT-PCR analyses

Total RNA was abstracted from AMSCs of P5, P15 and P25 by using Trizol, and subsequently analyzed by reverse transcription method. Primers were designed according to the sequences of Oct-4, Rex-1, CD29, CD44, CD71, CD73, CD90 and GAPDH (internal control) from GeneBank. The template cDNA segments were amplified by PCR using the designed primers listed in Table I. The PCR products were studied by 2% agarose gel electrophoresis, and bands were visualized with a UV transilluminator.

Immunofluorescent detection

AMSCs of P5 were fixed in 4% (m/v) paraformaldehyde for 15 min, thereafter washed twice with PBS. The cells were permeabilized using 0.2% (v/v) Triton X-100 for 20 min and washed twice with PBS. The samples were blocked by 10% (v/v) goat serum for 30 min, then incubated in 1% BSA containing the following polyclonal antibodies: (1) rabbit anti-sheep Oct-4 (1:100);

(2) rabbit anti-sheep CD29 (1:100); (3) rabbit anti-sheep CD44 (1:100); (4) rabbit anti-sheep CD71 (1:100); (5) rabbit anti-sheep CD73 (1:100) and (6) rabbit anti-sheep CD90 (1:100) for 1h at room temperature. The negative control was incubated in PBS instead of primary antibodies. Afterward, the cells were washed twice with PBS, and incubated in PBS containing FITC-conjugated goat anti-rabbit secondary antibody. After incubation for 1 h at room temperature, the cells were washed twice with PBS. They were finally counterstained with DAPI, and were studied by using Nikon TE-2000-E confocal microscope with a digital camera system.

Flow cytometry

The expression of cell-associated markers can be measured by flow cytometric analysis. In order to describe immunophenotype of the collected cells, Oct-4, CD44 and CD90 expression of AMSCs-associated surface markers was analyzed with a flow cytometer (FC500, Beckman Coulter, USA). Briefly, AMSCs of P5 were dissociated with 0.125% trypsin, centrifuged, separated into 1 mL aliquots, and labeled. The cells were fixed and permeabilized in 70% (v/v) ice ethanol for 12 h. The samples were blocked by 10% (v/v) goat serum for 30 min, then incubated in 1% BSA containing the following polyclonal antibodies: (1) rabbit anti-sheep Oct-4 (1:100); (2) rabbit anti-sheep CD44 (1:100); and (3) rabbit anti-sheep CD90 (1:100) for 1 h at room temperature. The negative control was incubated in PBS instead of primary antibodies. Afterward, the samples were washed two times with PBS and were incubated in PBS containing FITC-conjugated goat anti-rabbit secondary antibody. After incubation for 1 h at room temperature, the cells were washed twice with PBS. Finally, cells were detected by using the flow cytometer. Flow cytometric data were analyzed using CXP software (Beckman Coulter).

Table I.- Primer sequences used in RT-PCR assay.

Gene	Primer sequence	Tm (°C)	Cycle	Fragment size (bp)
CD29	F: 5' TTCAGTGCCGAGCCTTCAAT 3'	60	30	393
	R: 5' CCGTGTCCCATTTGGCATTC 3'			
CD44	F: 5' CAGCACCATTTCAACCACAC 3'	54	30	311
	R: 5' GCCTTCCATTCTCAAACCAC 3'			
CD71	F: 5' TGCCAAACATACCAGTCCAA 3'	54	30	394
	R: 5' GCTCCAAAGTCTCCACCACT 3'			
CD73	F: 5' TACACAGGCAATCCACCTTC 3'	53	30	361
	R: 5' TCATAGCATCGCAAATCAGG 3'			
CD90	F: 5' AGGACACAGGAAGCCACAAG 3'	56	30	311
	R: 5' CCCTCACTCTCCATCAGGTC 3'			
Oct-4	F: 5' GCGCCGCAGGTTGGAGTGG 3'	65	30	570
	R: 5' GCTGCTGGGCGATGTGGCTAAT 3'			
Rex-1	F: 5'ACAGCCCCGGCCCGTCCTCTACCC 3'	62	30	297
	R: 5' ACCCCCGCACCCTCCACCCACAA 3'			
PPAR-γ	F: 5' CGGGAAAGACGACAGACAAAT 3'	60	30	147
	R: 5' CGTAGAAGGTCCCCACAGTCA 3'			
LPL	F: 5' CAAGTCGCCTTTCTCCCGAT 3'	60	30	238
	R: 5' AGAGATGAACGGAACGCTCG 3'			
GAPDH	F: 5' TTATGACCACTGTCCACGCC 3'	60	30	216
	R: 5' TCAGATCCACAACGGACACG 3'			
ALB	F: 5' ATCTCCAGCAGTGTCCATTTG 3'	54	30	126
	R: 5' TTCAGGCTCTTGTTTCTCACAG 3'			
AFP	F: 5' CACCATTCTCCGATGACAAGT 3'	56	30	136
	R: 5' GTGCTGGACCCTCTTCTGTAA 3'			
Collagen I	F: 5' CCCAGTTGTCTTACGGCTATG 3'	60	30	323
	R: 5' ACCTCTGTGTCCCTTCATTCC 3'			
Osteopontin	F: 5' TTTCACTCCACCTTTCCCTAC 3'	58	30	489
	R: 5' CCACCCTGCTTTAACATATCC 3'			

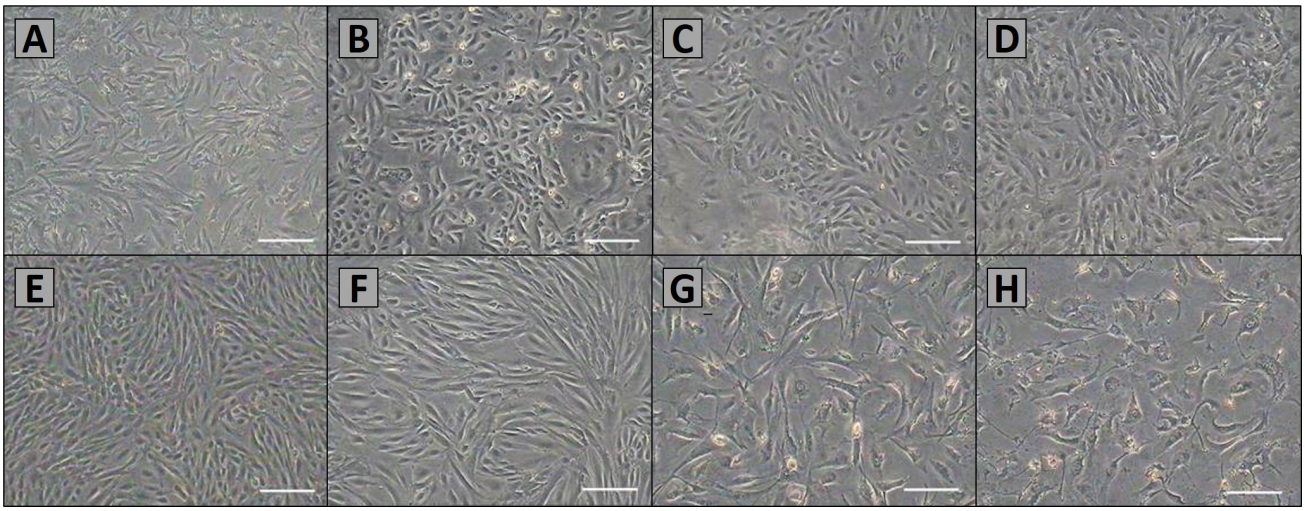

Fig. 1. Morphology of primary and sub-cultured AMSCs. A, primary cells were cultured after 48 h. Most of cells began to adhere and stretch; B, AMSCs were cultured about 4 days, the cells grew to 70%–80% confluence; C, the cells of P1 were non-homogenous; D, after P5, AMSCs were purified, and there was apparently no difference in morphology among successive passages. AMSCs exhibited a fusiform pattern; E and F, morphology of P5, P10 and P15 AMSCs; G and H, the AMSCs of P24 and P25 displayed a typical senescence. Scale bar, 100μm.

Adipogenic differentiation

AMSCs were separated into control group and induced group. When the confluence of cells reached to 60 – 70%, the experimental group was incubated in adipogenic medium (DMEM/F12 containing 10% FBS, 200 μM indomethacin, 1 mM dexamethasone, 10 μM insulin transferrin and 0.5 mM IBMX). AMSCs in the control group were cultured in complete medium without differentiation factors. After 1 week, the two groups were tested for accumulation of intracellular lipid utilizing oil red O staining and expression of adipogenic cells specific genes by RT-PCR.

Osteogenic differentiation

AMSCs were divided into control group and induced group as we did before. At 50%–60% confluence, cells of the experimental group were cultured in osteogenic medium that contained DMEM/F12 supplemented with 10% FBS, 0.1 mM dexamethasone, 10 mM β-glycerophosphate and 50 μg/ml ascorbate. Meanwhile, cells of the control group were cultured in primary medium. The medium was refreshed every 2 days. After three weeks, alizarin red staining was done to determine calcium node formation by induced cells, and osteoblast specific genes were analyzed by RT-PCR.

Hepatocyte differentiation

AMSCs were divided into two groups. When the confluence degree reached 60%–70%, the induced group was cultured in hepatocyte medium that contained DMEM/F12 supplemented with 10% FBS, 20 ng/mL hepatocyte growth factor, 20 ng/mL fibroblast growth factor-4 and 1% insulin transferrin. Simultaneously, cells in the control group were maintained in primary medium. The medium was renewed every 2 days. Two weeks later, the capacity of the cells secreting glycogen was detected by periodic acid Schiff reaction, and specific genes of hepatocyte-like cells were detected by RT-PCR.

RESULTS

Morphological features of AMSCs

The primary AMSCs obtained from amniotic membrane were seeded into 60 mm Petri plate, after about 48 h, some cells began to stretch, a tiny amount of polygonous cells could be also observed under the inverted microscope, and tiny minority of cells showed irregular shape (Fig. 1A). The cells expanded rapidly, approximately 4 days later, the confluence degree could reach 70%–80% (Fig. 1B) and the cells of P1 were non-homogenous (Fig. 1C). From P5, the AMSCs were fully purified, and the shape was also homogenous. AMSCs exhibited fusiform pattern (Fig. 1D). There was no apparent difference in morphology among the cells of different passages. The biological properties were steady after several subcultures (Fig. 1D, E, F). P 24 and P 25 AMSCs exhibited typical senescence feature in the majority of AMSCs, such as vacuole, flatten shape, and karyopyknosis (Fig. 1G, H). Finally, AMSCs would detach from the culture dishes.

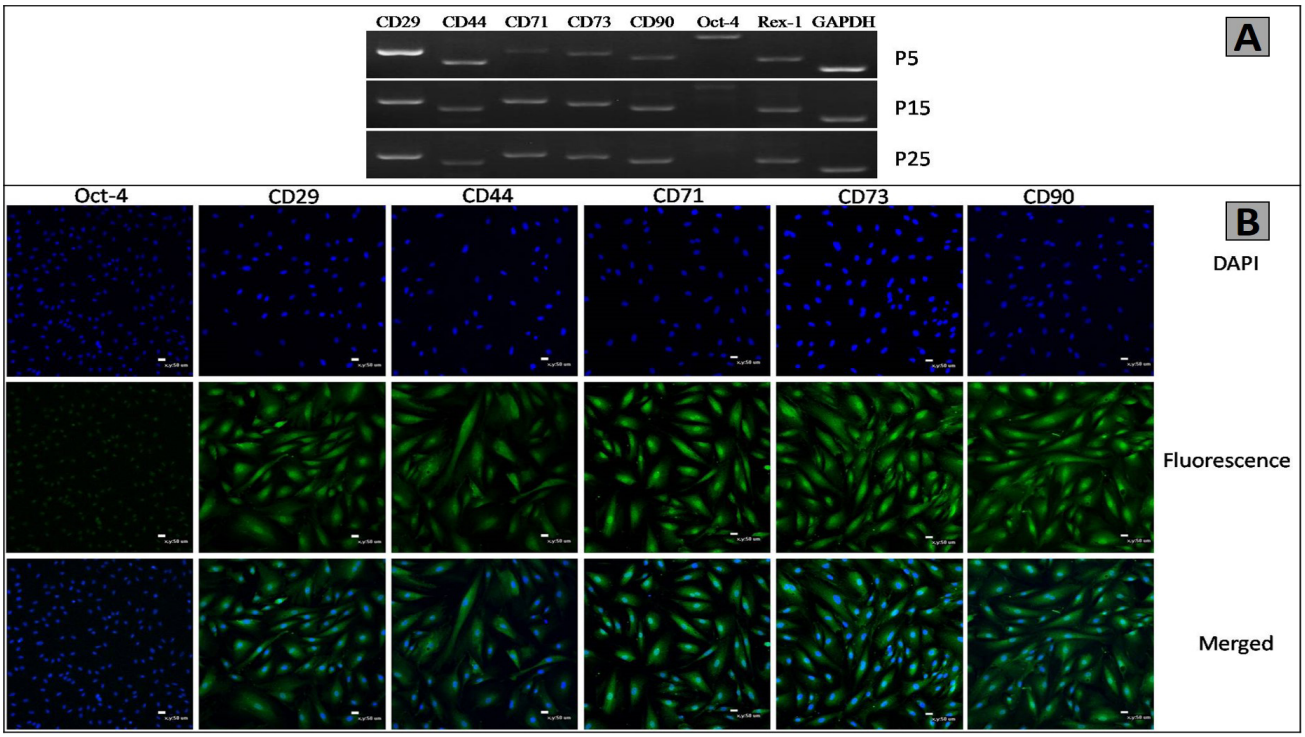

Fig. 2. Specific markers of the AMSCs. Specific markers of the AMSCs are similar with BMSCs. Several surface markers of AMSCs were detected by RT-PCR and immunofluorescence. A, RT-PCR analysis exhibited that the AMSCs expressed CD29, CD44, Oct-4, CD71, Rex-1, CD90, and CD73. and GAPDH was used as an internal control; B, Immunofluorescence exhibited that the expression of CD44, CD29, CD71, Oct-4, CD73 and CD90 were positive. Scale bar, 50μm.

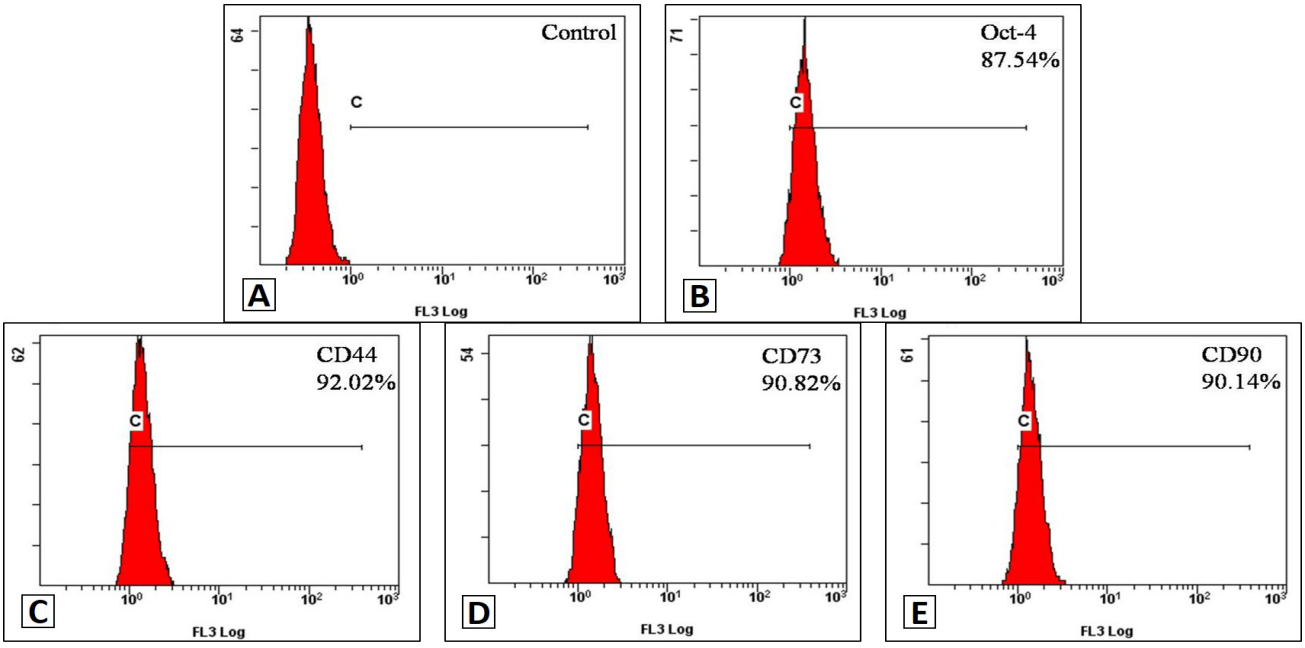

Fig. 3. Flow cytometry analysis of AMSCs. Cell surface antigen of the AMSCs is similar with BMSCs. The expression of Oct-4, CD44, CD73 and CD90 were detected by flow cytometry. The result has shown that P5 of AMSCs were high expression for Oct-4, CD44, CD73 and CD90.

Molecular characteristics of AMSCs

The specific surface markers of AMSCs were detected by RT-PCR and immunofluorescence. AMSCs were found positive for CD29, CD44, CD71, CD73, CD90, Oct-4 and Rex-1. GAPDH served as internal control (Fig. 2A). The immunofluorescence results showed that AMSCs surface antigen Oct-4, CD29, CD44, CD71, CD73 and CD90 were positively expressed under the confocal microscope (Fig. 2B). Flow cytometry has revealed that P5 of AMSCs showed high expression for Oct-4, CD44, CD73 and CD90. The positive rates of Oct-4, CD44, CD73 and CD90 were 87.54%, 92.02%, 90.82% and 90.14%, respectively (Fig. 3) .

Growth kinetics

Growth curves of AMSCs at P5, P10 and P15 (Fig. 4) showed that there was a lag phase of about 24 h; AMSCs proliferated rapidly and came into logarithmic phase. Along with the density of AMSCs increase, proliferation was inhibited and the AMSCs came into the plateau phase after approximately 6-7 days and began to degenerate. The growth kinetics was all typically sigmoidal. The PDT was 31.23 h, 32.92 h and 35.04 h for P5, P10 and P15, respectively, on the basis of the growth curve.

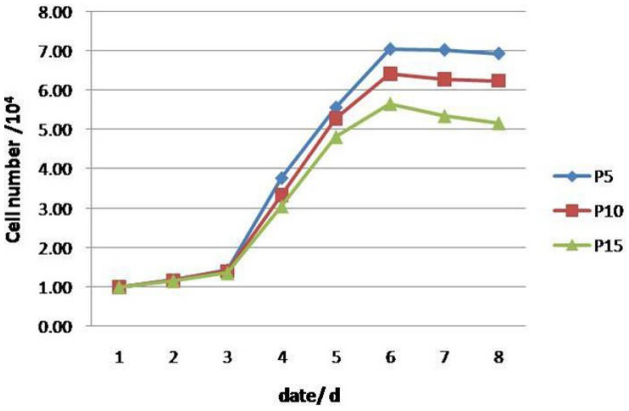

Fig. 4. Growth curves of the AMSCs. The growth curves of P5, P10, and P15 AMSCs were all typical S curve with cell number represented by the vertical axis. The PDT was 31.23 h, 32.92 h and 35.04 h for P5, P10 and P15, respectively.

Colony-forming cell assay

Colony formation was observed after culturing for 7 days. The CFU were 46.36±1.74 %, 41.38±1.72 %, and 34.29±1.83 % for P5, P10 and P15, respectively, showing the ability of obtained AMSCs for self-renewal (Fig. 5)

Adipogenic differentiation of the AMSCs

Oil red O staining could be used to identify adipogenic differentiation of the AMSCs. After culturing in adipogenic medium for 7 days, the AMSCs changed from spindle to

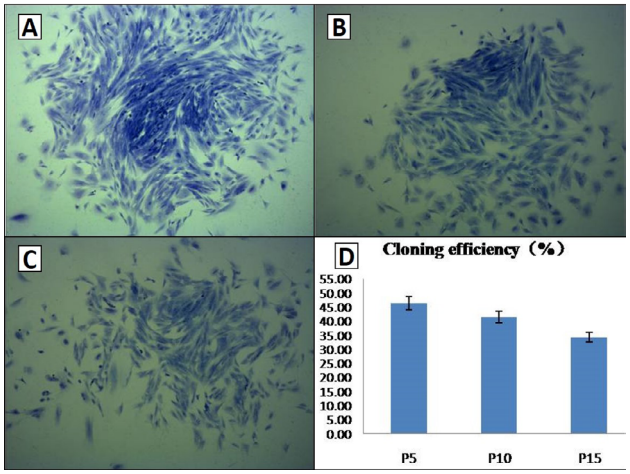

Fig. 5. Colony-forming cell assay. Colony-forming units of P5, P10, and P15 AMSCs were counted, which suggests that colony-forming rates declined with the increase of the passage. A, B and C, colony-forming units of P5, P10 and P15, respectively; D, the bar chart of colony-forming rates for different passages of AMSCs. Scale bar, 100μm.

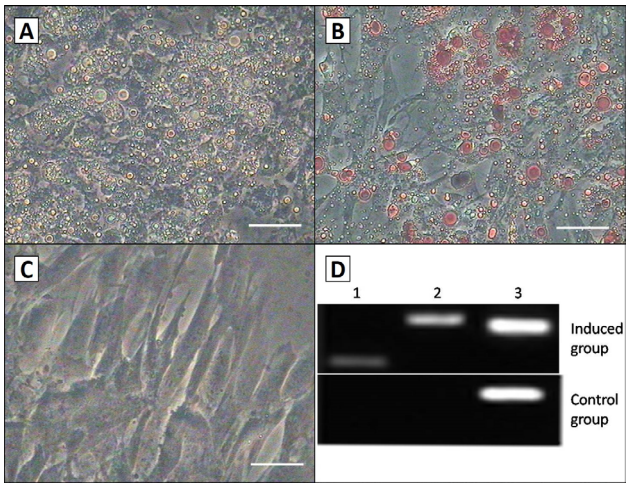

Fig. 6. Adipogenic differentiation of the AMSCs. A, AMSCs were inducted after 1 week, they began to change from fibroblast-like to oblate, and there were a number of lipid droplets in petri dishes. Droplets increased and assembled to form larger ones along with the extension of induction time; B, they were positive for oil red O staining; C, the control group had no difference in the morphology and phenotype, and they were also negative for oil red O staining. Scale bar, 50 μm; D, RT-PCR detection of the adipogenic markers LPL and PPAR-γexpression in the both groups. They were positive in induced cells, but not in control cells. Lane 1, PPAR-γ; Lane 2, LPL; Lane 3, GAPDH.

oblate and produced a large number of lipid droplets in petri dishes (Fig. 6A). With prolonged induction, the quantity of lipid droplets increased gradually and assembled into larger droplets. The differentiation result was determined by the way of oil red O staining, the induced group was positive (Fig. 6B), while cells in control group were negative (Fig. 6C).

To determine differentiation of the AMSCs, the expression of adipogenic markers was tested in both groups by RT-PCR. Adipocyte specific genes PPAR-γ and LPL were positive (Fig. 6D), which was not observed in the control group.

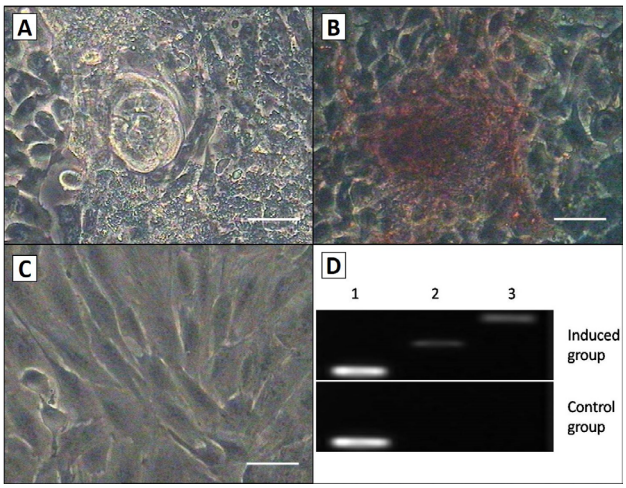

Fig. 7. Osteogenic differentiation of the AMSCs. A, the quantity and size of nodules increased as induction progressed; B, after culturing in osteogenic medium for 3 weeks, cell shape in induced group was changed, and the group was positive by the way of alizarin red staining; C, There was no change in morphology or stained in control group. Scale bar, 50μm; D, RT-PCR detection of the osteogenic markers collage type I and osteopontin expression in the both groups. Induced cells were positive for osteopontin and collage type I, but the control cells were not. Lane 1, GAPDH; Lane 2, collage type I; Lane 3, osteopontin.

Osteogenic differentiation of the AMSCs

The capacity of AMSCs to differentiate into osteogenic cells was verified, and the morphological and phenotypic analysis was carried on for the induced cells. The cell shape altered at the seventh days after treatment; a tiny number of cells became confluent and formed mineralized nodules which were bigger for further induction. After 3 weeks, morphological changes were observed in the AMSCs (Fig. 7A), and the group was positive by alizarin red staining (Fig. 7B). Besides that, after prolonged effects of induction, the nodules increased and became bigger. There was no change in morphology or staining by alizarin red in control group (Fig. 7C).

To determine that differentiation had happened, the specific gene of osteogenic cells, osteopontin and collagen type I, was tested in both groups via RT-PCR. They were positive in the induced group, but not in the control group (Fig. 7D).

Hepatocyte differentiation

The capacity of AMSCs to differentiate into hepatocyte-like cells was proved, and the morphological and phenotypic analysis was carried on for the induced cells. After culturing in hepatocyte medium for 7 days, the shape of some AMSCs in the induction group was gradually transformed from the long spindle type to round shape. After 14 d, many cells in the induction group presented cobblestone-like shape (Fig. 8A), glycogen staining was positive, the cytoplasm was stained purple (Fig. 8B) indicating that the induced cells had the function of synthesis and storage of liver glycogen. However, the control group had no significant morphological change, the glycogen staining was negative (Fig. 8C).

The specific genes of the hepatocyte-like cells, ALB and AFP, were positive by RT-PCR in the induction group, and ALB and AFP were negative in the control group.

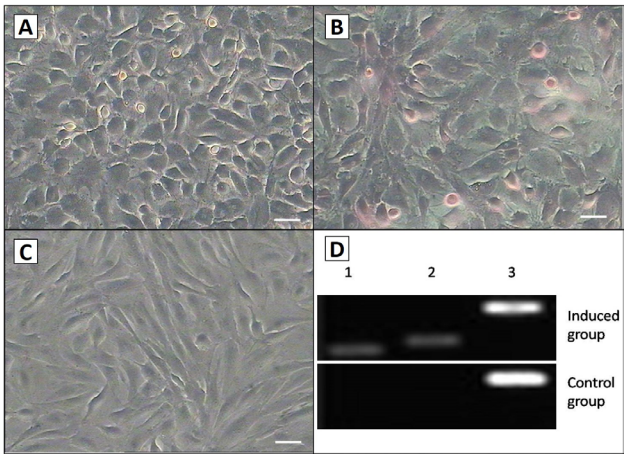

Fig. 8. Hepatocyte differentiation of the AMSCs. A, after cultured in hepatocyte medium for 14 days; B, the cell shape was changed, and glycogen staining was positive; C, there was no change in morphology or stained by glycogen in control group after 14 d. Scale bar, 50μm; D, RT-PCR analysis of the Hepatocyte differentiation markers ALB and AFP expression in induced group and control group. Induced cells were positive for ALB and AFP, but the control cells were not. Lane 1, ALB; Lane 2, AFP; Lane 3, GAPDH.

DISCUSSION

The amnion lies in the innermost layer of the placenta. The amnion formed by five layers including the epithelium, basement membrane, compact layer, fibroblastic layer and spongy layer. AECs originated from the simple epithelium, and the AMSCs stem from the spongy layer. We decided to concentrate on the derivation of stem cells from the mesenchymal sphere of placenta. It was reported that cells isolated from placenta might maintain multipotent phenotype after the differentiation (Chinnici *et al*., 2014). The objective of this research was to characterize AMSCs collected from sheep species for the first time and evaluate them as potential candidates for cellular transplantation therapy and regenerative medical projects.

In this work, AMSCs were obtained from aborted sheep fetuses (1-3 month) under sterile conditions. Primary AMSCs were sub-cultured to passage 25 *in vitro*. The *CD29, Rex-1, CD73, Oct-4, CD71,* and *CD44* genes were detected by RT-PCR and immunofluorescence. The result showed that they were positive in AMSCs. The growth of different passages cells typically appeared in S curve and had a natural PDT. Furthermore, AMSCs were successfully induced to differentiate into osteoblasts, adipocytes, and hepatocyte-like cells. All the results showed that the biological properties of the AMSCs obtained from sheep were stable. Our *in vitro* differentiation studies support the findings already reported in human (Zagoura *et al*., 2012). Therefore, AMSCs may be a good option for cellular transplantation therapy and regenerative medical projects.

At present, many investigators have demonstrated that AMSCs can express ESC surface markers and pluripotent-specific transcription factors. AMSCs are generally confirmed by the expression of some markers of MSCs, together with cell shape and potential of differentiation *in vitro*. The results revealed that sheep AMSCs express *Oct-4, Rex-1, CD29, CD44, CD71, CD73* and *CD90*. Oct-4 (octamer-binding transcription factor 4) is a protein that in humans is encoded by the *POU5F1* gene (Singh *et al*., 2012). As a homeodomain transcription factor of the POU family, the protein is involved in the self-renewal of undifferentiated. Rex1 is a definitized pluripotent marker of undifferentiated ESCs. It also involves in maintaining a pluripotent state of stem cells (Shi *et al*., 2006). CD29, Integrin beta-1, is a protein encoded by *ITGB1* gene in humans (Goodfellow *et al*., 1989). It is an integrin subunit correlated to late antigen receptor, and forms a heterodimer binding to extracellular proteins and surface of MSCs. It mediates cell-cell interactions and cell adhesion (Rahimi *et al*., 2013). CD44 is conducive to a cell-surface glycoprotein, it mediates cell to cell and cell to matrix adhesion and cell migration (Jordan *et al*., 2015). It is a receptor for hyaluronic acid (HA) and can also interact with collage, osteopontin, and matrix metalloproteinases. CD44 involves in many cellular activities including recirculation, homing, lymphocyte activation, metastasis and hematopoiesis (Wu *et al*., 2015). CD71, known as a member of the transferrin receptor family, is a carrier for transferrin. CD71 is important for cellular iron uptake by the process of receptor-mediated endocytosis. Low iron concentration promotes increased level of transferrin receptor to take more iron into the cell, to mediate iron concentration in MSCs. Thus, the transferrin receptor maintains cellular iron homeostasis (Levy *et al*., 1999). CD73 is a membrance-bound glycoprotein, and it catalyses the transformation of extracellular nucleotides to membrane-permeable nucleosides (Gong *et al*., 2011). As a significant signaling molecule, the protein participates in purine salvage and purinergic cascade that triggers cell metabolism. The encoded protein is used to be a marker of lymphocyte differentiation. CD90 is a 25–37 kDa heavily N-glycosylated anchored conserved cell surface protein with a single V-like immunoglobulin domain, originally discovered as a thymocyte antigen (Reif and Allen, 1964). It can be used as a marker for various stem cells and for the axonal processes of mature neurons. The results indicated that sheep AMSCs are a clump of uncommitted stem/progenitor cells distinguishable from mesenchymal cells.

Stem cells' multipotency is the most notable characteristic for cell transplantation therapy. *In vitro*, the expressing of some key genes in the signaling pathway related to stem cell differentiation can change under the action of some inducing factors. Consequently, differentiation in specific directions was achieved. In our research, we induced sheep AMSCs to differentiate into osteoblasts, adipocytes, and hepatocyte-like cells and tested corresponding to genes of the different cells above. The experiments showed that various factors could induce variant differential direction of the AMSCs and that the AMSCs derived from mesoderm could differentiate into endoderm and ectoderm cells. The autologous features of these stem cells, in combination with their distinct multipotentiality and easy acquirement, make AMSCs become an attractive choice for tissue-engineering and cell-based therapies (Capobianco *et al*., 2016; Kim *et al*., 2012). The results above suggested that sheep AMSCs had strong self-renewal ability and the potential to differentiate towards mesodermal and endodermal cells. Although the multilineage differentiation of AMSCs was successful *in vitro*, there are many technical challenges on utilizing AMSCs in clinical application for therapeutic purposes, such as high rate of reduction and instability. These aspects need to be taken into account research and clinical applications in future.

CONCLUSION

In conclusion, in this work, AMSCs were successfully obtained from amniotic tissue from aborted sheep fetuses, and the ability of proliferation and differential potential were tested *in vitro*. This investigation offers an important sense on the research of domestic animals and the potential application of AMSCs as a stem-cell material for cellular transplantation therapy and regenerative medical projects.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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