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ORIGINAL ARTICLE

Isolation and biological characteristics of sheep amniotic epithelial cells

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Abstract Amniotic epithelial cells (AECs), isolated from placenta, have epithelial cells and stem cells characteristics. Most of the previous studies focused on the biological characteristics of human amniotic epithelial cells, which demonstrated amniotic epithelial cells not only had low immunogenicity and potent potential to differentiate into three germ layers, but also could secrete various immunomodulatory factors. However, compared to study on human amniotic epithelial cells, few studies have been done on other animals. In this study, sheep amniotic epithelial cells were successfully isolated and their surface makers were accessed by immunofluorescence assay, and found that AECs were expressed Oct4 and Sox2, which were necessary for maintaining the undifferentiated state of pluripotent stem cells. Based on cloning efficiency and growth kinetics assay, AECs were found to possess self-renewal capacity and the growth curve was S-shaped. In addition, AECs could be induced into adipocytes, osteoblasts and chondrocytes in vitro, showing they had multi-differential ability. Reverse transcription-polymerase chain reaction results showed that AECs expressed CD29, CD44, CD90 and CK19, and didn't expressed CD34, CD45 and the telomerase gene (TERT). Little change in chromosome number was observed in AEC cultures for up to at least the first ten passages. In summary, this study results revealed that sheep AECs possessed more advantages for cell therapy and might play a key role in cell therapy and regenerative medicine in the future.

Keywords Sheep · Amniotic epithelial cells · Stem cell · Differentiation · Biological characterization

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Introduction

Stem cells, derived from embryonic and adult tissues, have the potential for self-renewal, high proliferation, and multi-directional differentiation, and therefore

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have the potential to adjust the structure and treat congenital or acquired diseases (Takahashi et al. 2001; Tejwani et al. 2007; Cargnoni et al. 2009; Venkatachalam et al. 2009; Sakuragawa et al. 2000). According to the differentiation potential, stem cells are divided into three categories: totipotent stem cells, pluripotent stem cells and multipotent stem cells. However, there are some limitations in using these cells for clinical practice, such as embryonic stem cells that are difficultly obtained from adult tissues and have tumorigenicity. Amniotic membrane is a post-production abandonment material that can be obtained non-invasively without ethical restrictions. Therefore, amniotic epithelial cells (AECs) are easily available cellular resources.

The amniotic membrane is located in the innermost layer of the placenta, adjacent to the fetus, which is a smooth, avascular, nerve free, lymphatic free and translucent membrane, and can divided into five layers: an epithelial cells monolayer, an acellular basement membrane layer, a compact layer, a mesenchymal cells layer, and a spongy layer. The amniotic epithelium is composed of a single layer of AECs. Previous studies had shown that biological characteristics of amniotic epithelial cells had lots of advantages, which not only had the potential to differentiate into three germ layers, low immunogenicity and secreted a variety of immunomodulatory molecules, but also had anti-tumor properties, which were needed for the use of regenerative medicine (Niknejad et al. 2014). Under the regulation of different growth factors and microenvironment, hAECs can differentiate into glial cells (Sakuragawa et al. 1996), nerve cells (Shinya et al. 2010), hepatocytes (Marongiu et al. 2011) and hair follicle cells (Fliniaux et al. 2004). In addition, hAECs don't express HLA-A, HLA-B, HLA-C and HLA-DR antigens, and almost no immune response (Akle et al. 1981). Low immunogenicity and anti-inflammatory effects indicate that AECs have important medical value in the field of tissue repair and play a key role in the future of regenerative medicine.

The current researches on amniotic epithelial cells are mostly focused on people, mice, and rabbits, but little on sheep. In the present study, enzyme digestion were used to obtain sheep amniotic epithelial cells, and the expression of CD44, CD90, CK19, E-cadherin, Sox2 and SSEA-4 antibody were confirmed. Self renewal ability and differentiation of AECs were tested. The biological characteristics of the AECs make them particularly well suited for therapy, just like embryonic stem cells.

Materials and methods

All experimental animal protocols were approved and performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the Chinese Academy of Agricultural Sciences. Five-week-old embryo of Small Tail Han sheep was provided by the livestock and poultry Experimental Base Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing.

The materials used in this study were: Dulbecco's modified Eagle's medium/F12 (DMEM/F12), fetal bovine serum (FBS) were purchased from GIBCO BRL (Grand Island, New York, USA); goat serum, trypsin, dexamethasone, β-glycerophosphate, 3-isobutyl-1-methylxanthine (IBMX), vitamin C (VC), indomethacin, L-proline, sodium pyruvate, 4,6 diamidino-2-phenylindole (DAPI), potassium chloride (KCl), ethylene diamine tetraacetic acid (EDTA), and insulin were purchased from Sigma Chemical Co. (St. Louis, MO, USA); basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-transferrin-selenium (ITS) and transforming growth factor-beta 3 (TGF-β3) were purchased from PeproTech Co. (Rocky Hill, TX, USA); rabbit antibody CD44, CD90, CD34, Sox2, Oct4, CK19, SSEA-4 and fluoroisothiocyanate (FITC) conjugated goat anti rabbit secondary antibody IgG were purchased from Bioss (Beijing, China); rabbit antibody Sox2, Oct4, E-cadherin were purchased from Abcam (Cambridge, MA, USA).

Isolation and culture of AECs

Sheep placentas were washed several times with phosphate-buffered saline (PBS) to remove the blood. An intactlayer of the amnion was mechanically peeled off from the chorion, the thin, avascular and almost transparent amnion membrane, which contained AECs and mesenchymal fibroblasts, and then was washed several times to remove blood. In order to isolate AECs, amniotic membrane was cut into approximately 2×2 cm^2 piece and washed 7 times in PBS supplemented with 1% penicillin/streptomycin

and then trypsinized to release the AECs from the supporting connective tissue according to the previous method (Gao et al. 2016). The amnion was incubated with 10 mg/mL Dispase II in PBS at 37 °C for 30 min to generate a loose single layer of amniotic epithelium, and aspirate liquid in the dishes. Isolated epithelial cell sheet was further digested with 0.25% trypsin + 0.02% EDTA (w/v) at 37 °C for 30 min, and then terminate digestion with fetal bovine serum. Another method was also used to isolate AECs: the amnion was incubated with 0.25% trypsin + 0.02% EDTA (w/v) at 37 °C for 30 min, and terminated digestion with fetal bovine serum, then further digested by 0.25% trypsin + 0.02% EDTA (w/v) once. The mixture was filtered through 200 mesh cell strainers. Supernatant were then collected and centrifuged under 200×*g* for 8 min to pellet cells.

Single AEC, isolated from the amnion, was seeded into 60-mm-diameter culture dishes in complete medium, which consisted of DMEM/F12 supplemented with 10% FBS, 10 ng/mL EGF, and 55 μM β-mercaptoethanol. After seeding 48 h, non-adherent cells were discarded and growth medium was changed every 2 days up to confluence. Confluent cells were detached from the substrate by incubation in 0.25% trypsin + 0.02% EDTA (w/v) for 5–10 min and then plated in the same growth conditions at a density of 2×10^4 cells/mL.

Immunofluorescence assay

AECs were fixed in 4% paraformaldehyde for 15 min and then washed three times in PBS. The cells were permeabilized with 0.25% Triton X-100 for 20 min and washed three times in PBS. Cells were blocked with goat serum for 30 min, and subsequently incubated in following polyclonal antibodies (1:200): CD44, CD90, CD34, CK19, E-cadherin, Sox2, Oct4, and SSEA-4 antibody overnight at 4 °C. Then, after washing three times in PBS, the cells were incubated in FITC-conjugated secondary antibody for 1 h at room temperature in the dark. After incubation, the cells were washed three times with PBS in the dark. Finally, nuclei were incubated to label with DAPI. The cells were examined using a Nikon TE-2000-E laser scanning confocal microscope (Nikon Instech Co. Ltd., Tokyo, Japan).

Flow cytometry

AECs were harvested and collected in the centrifuge tubes at a density of 1×10^6 cells per tube. Centrifuged at 400×*g* for 5 min, the cells were fixed 70% (v/v) ice ethanol overnight at 4 °C. Next, the cells were blocked by goat serum after another centrifugation at 400×*g* for 5 min. The cells were incubated with primary antibodies (1:200) overnight at 4 °C. The antibodies are as follows: CD44, CD90, Sox 2 and CK19. Then, the samples were washed with PBS and incubated in FITC-conjugated goat anti-rabbit secondary antibody for 1 h at room temperature. Finally, they were washed twice in PBS and subjected to single channel fluorescently activated cell sorting (FACS) analysis.

RT-PCR analysis

RNA was extracted from cells using the Trizol reagent (Invitrogen, Carslbad, CA, USA), and cDNA was synthesized using a reverse transcription system (Tiangen, Beijing, China). The cDNA targets were used the specific primer pairs shown to amplify by PCR, and the gene specific primers listed in Table 1. The PCR products were visualized by electrophoresis in a 1.5% agarose gel.

Cloning efficiency

AECs from passages 1, 6 and 9 were seeded in 60 mm plates at a density of 100 cells per plate. After 5 days, the numbers of colony-forming units were counted and the cloning efficiencies were calculated as: colony forming unit number/starting cell number × 100%.

Growth kinetics

AECs from passages 1, 6 and 9 were were harvested and seeded in 24-well microplates at a density of 10^4 cells/mL. After plated, the cells from three wells were counted in the same time everyday and successively for 7 days. Cell counting per well was repeated for 3 times to calculate the mean values, which were used to plot growth curves. The population doubling time (PDT) was calculated based on the formula PDT = (t − t0) lg2/(lgNt − lg0). (t0: starting time of culture; t: termination time of culture; N0: initial cell number of culture; Nt: ultimate cell number of culture.)

Table 1 Primer sequences used in RT-PCR assay

Gene name	Primer sequences	Product length (bp)	Tm (°C)
GAPDH	F: 5′-GAAGGTCGGAGTGAACGGATTT-3′	473	56
	R: 5′-TGGTCATAAGTCCCTCCACGAT-3′		
TERT	F: 5′-GAGCGAGTCTCACCTTCACC-3′	552	56
	R: 5′-TCTTGAAGTCTGCGGTCACA-3′		
CD44	F: 5′-ATAGACGGGCATGTGGTGAT-3′	347	56
	R: 5′-GTAGCCTCCTGACGTGCTTC-3′		
CD29	F: 5′-GCAGGGCCAAATTGTGGATG-3′	442	58
	R: 5′-ACGAAAGAGCCAAACCCGAT-3′		
CD34	F: 5′-CAGCAGTCACCTCAGTTCCA-3′	422	57
	R: 5′-CTCTGCCTGTTCCTCCTGAC-3′		
CD45	F: 5′-CCACGGGTATTCAGCAAGTT-3′	513	56
	R: 5′-GAGCAGGTGAGGGTCTTCAG-3′		
CK19	F: 5′-GTGTGGAGGTGGATTCTGCT-3′	445	56
	R: 5′-TTTCGGTAGGTGGCAATCTC-3′		
CD90	F: 5′-CAGAAGGTGACCAGCCTGAC-3′	428	56
	R: 5′-TCACAGGGAGATGAAGTCCA-3′		
OPN	F: 5′-GAGTTGCCACTGCAAACCTG-3′	424	57
	R: 5′-CTGATCGCCATGCTGCTTTC-3′		
ATF4	F: 5′-AGGAGGATGCCCACTCAGAT-3′	359	58
	R: 5′-GGCCAAGGAATCTGCCTTCT-3′		
VIM	F: 5′-TCTGAAGCTGCTAACCGCAA-3′	337	57
	R: 5′-TCCTGCTCTCCTCTCCTTCC-3′		
ACAN	F: 5′-AGAGGCGCGTCTAGAAATCG-3′	424	57
	R: 5′-TCGCACAGCTTCTGGTCAAT-3′		
PPARγ	F: 5′-ACTTTGGGATCAGCTCCGTG-3′	440	58
	R: 5′-AATGGCCATGAGGGAGTTGG-3′		
LPL	F: 5′-AGATCCGGCTGGACCTAACT-3′	450	58
	R: 5′-AGAGATCTCGAAGGCCTGGT-3′		

Differentiation of AECs in vitro

Osteogenic differentiation of AECs

Upon reaching 70–80% confluence, AECs from passage 3 were divided into the induced group and the control group. AECs in the induced group were changed to osteogenic medium, which is consisted DMEM/F12 medium supplemented with 10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerophosphate and 250 μM VC. AECs in the control group were maintained in standard culture medium without any inducers of differentiation. The medium was changed every 2 days. Two weeks later, the cells' capacity for calcium node formation was detected by Alizarin Red staining, and osteoblast specific genes were further detected by RT-PCR.

Adipogenic differentiation of AECs

Upon reaching 70–80% confluence, the passage 3 cells were divided into two groups as described above. AECs in the induced group were incubated in adipogenic medium, consisting of DMEM/F12 medium supplemented with 10% FBS, 1.0 μM dexamethasone, 200 μM indomethacin, 0.5 mM IBMX and 10 mg/L insulin. AECs in the control group were maintained in standard culture medium. After 7 days, intracellular lipid accumulation in the two groups was determined by staining with Oil Red O, and lipocyte specific genes were further detected by RT-PCR.

Chondrogenesis differentiation of AECs

Upon reaching 60–70% confluence, the passage 3 cells were divided into two groups as described above. AECs in the induced group were incubated in chondrogenesis medium, consisting of DMEM/F12 medium supplemented with 5% FBS, 0.1 mM dexamethasone, 1% ITS, 50 μg/mL L-proline, 0.9 mM sodium pyruvate, 50 ug/mL Vc and 10 ng/mL transforming growth factor-beta 3 (TGF-β3). AECs in the control group were maintained in standard culture medium. After 7 days, chondrocytes were determined by staining with Alcian blue. The RNA from the two groups was isolated for RT-PCR experiments.

Karyotype analysis

Standard protocols were used to investigate the chromosomal profiles of the cells (Qu et al. 1998). AECs from passage 10 were incubated in 0.4 μg/mL colcemid for 4 h at 37.5 °C, then the cells were dispersed by 0.25% trypsin. Next, the harvested cells were centrifuged at 200×*g* for 8 min. The pellet were re-suspended in 0.075 M KCl solution and incubated at 37 °C for 30 min. Finally, the cells were fixed in methanol/glacial acetic acid (3:1) and dropped on the prechilled glass slides, stained with Gimesa stain. Then, the chromosome numbers were counted from 100 spreads under an oil immersion objective.

Results

Isolation, culture and morphology of AECs

Under an inverted microscope the morphology, AECs showed a high proliferative potential in culture. After seeded 24 h, mass of cells were observed to adhere to the culture plates, which exhibited oval and cobblestone-like shape with large nuclei. More AECs were obtained by the first treated amniotic membrane with Dispase II (Fig. 1a), compare with 0.25% trypsin + 0.02% EDTA (w/v) twice (Fig. 1b). After 2 day cultured, the cells grew to 80–90% confluence and need to be passed on 2–3 days approximately. According as AEC were digested difficultly by trypsin, cells were differential degested to purify the AECs every time. With the increase of passages, AECs displayed a senescent appearance such as flating and aging in most cells and the number of the mesenchymal stem cells were increased gradually, which were presented long spindle cells. Eventually, the cells from passage 29 stopped proliferating and became detached from the plates gradually (Fig. 1).

Immunofluorescence assay

Specific marker proteins for AECs were detected by immunofluorescence staining, and the results showed that CD44, CD90, CK19, E-cadherin, SSEA-4, Oct4 and Sox2 were positively expressed, and CD34 was negative. These results were similar to previous data on the characterization of AECs in vitro (Fig. 2).

Flow cytometry

A series of cellular antigen markers were labeled with AECs using fluorescence activated cell sorting assays. Analyses of AECs for CD44, CD90, CK19, and Sox 2 antibody, positive expression rates of the viable cell population were 99.6%, 99.4%, 99.6% and 99.7% respectively (Fig. 3b–f). However, expression of control was observed in a distinct population of 0.186%, within the viable AECs population (Fig. 3).

RT-PCR analysis

RT-PCR experiments showed that AECs from passage 3, 10, 15, 25 expressed the pluripotent stem cell transcription factor (Sox2), epithelial cells specific marker (CK19) and the mesenchymal stem cell markers (CD29 and CD44). Hematopoietic stem cells markers (CD34 and CD45) and TERT expressed in ESCs, were not detected in AECs (Fig. 4).

Cloning efficiency

Colony formation was observed by microscopy after 5 days. The colony-forming levels were 32.5 ± 0.22%, 31.3 ± 0.12%, and 22.6 ± 0.23% for passage 1, passage 6, and passage 9 cells, respectively, demonstrating capacity of cultured AECs for self-renewal (Fig. 5).

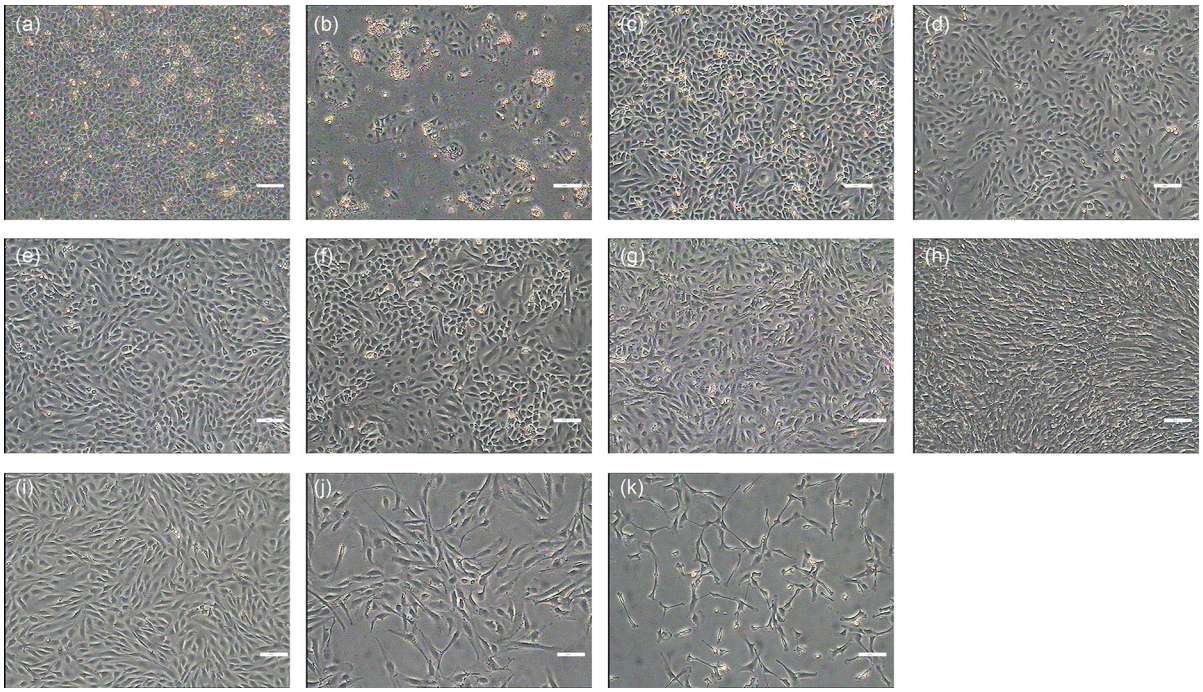

Fig. 1 Morphology of primary cultured and subcultured AECs. **a** After 24 h of primary culture, AECs were obtained by the first treated amniotic membrane incubated with 10 mg/mL Dispase II in PBS at 37 °C for 30 min, then with 0.25% trypsin + 0.02% EDTA (w/v) at 37 °C for 30 min. **b** the amnion was incubated with 0.25% trypsin + 0.02% EDTA (w/v) at 37 °C for 30 min twice. **c**–**k** Morphology of P1, P3, P5, P10, P15, P20, P25, P28, P29 AECs. Scale bar: 50 μm

Growth kinetics

P1, P6 and P9 AECs were counted by blood cell counting plate to analysis the proliferation rules. According to the number of cells per day, the growth curve was drawn by Graph Pad Prism (GraphPad Software, Inc., San Diego, California, USA), as shown in Fig. 6. The proliferation curve appears such a S-shape similar tendency, which contained latentperiod, logarithmic period, stable period and decline period. After growing at a latency about 72 h, the cells began to enter logarithmic growth phase. During this period, the cell proliferation ability was stronger, and the number increased logarithmically. The cell proliferation and death reached equilibrium, and entered the stable phase. Due to the serious contact inhibition phenomenon between cells, the number of cells decreased gradually after stable phase and gradually entered the decline phase. The population doubling time (PDT) was about 40.63 ± 10.63 h, 46.09 ± 8.21 h and 47.09 ± 7.22 h respectively. As the number of cells rises, the cell's ability to proliferate gradually decreases.

Differentiation of amniotic epithelial cells in vitro

Osteogenic differentiation of AECs

After incubation in osteogenic medium for 14 days, morphological changes in the AECs were obvious. Initially, the morphology of the cells changed from orbicular-ovate to tridimensional, and with time the cells aggregated and formed mineralized nodules (Fig. 7a). Alizarin Red staining identified a bright red positive region, showing clear calcium nodules (Fig. 7b, c). As a result of the continued effects of the inducers, the nodules increased in number and size. Osteogenic differentiation of AECs was also determined by RT-PCR. The specific genes including osteopontin (OPN) and activating transcription factor 4 (ATF4) were detected in the induced group (Fig. 7d) by contrast to the control group (Fig. 7e).

Adipogenic differentiation of AECs

After cultured in adipogenic medium for 14 days, fat droplets were showed in the AECs under an inverted

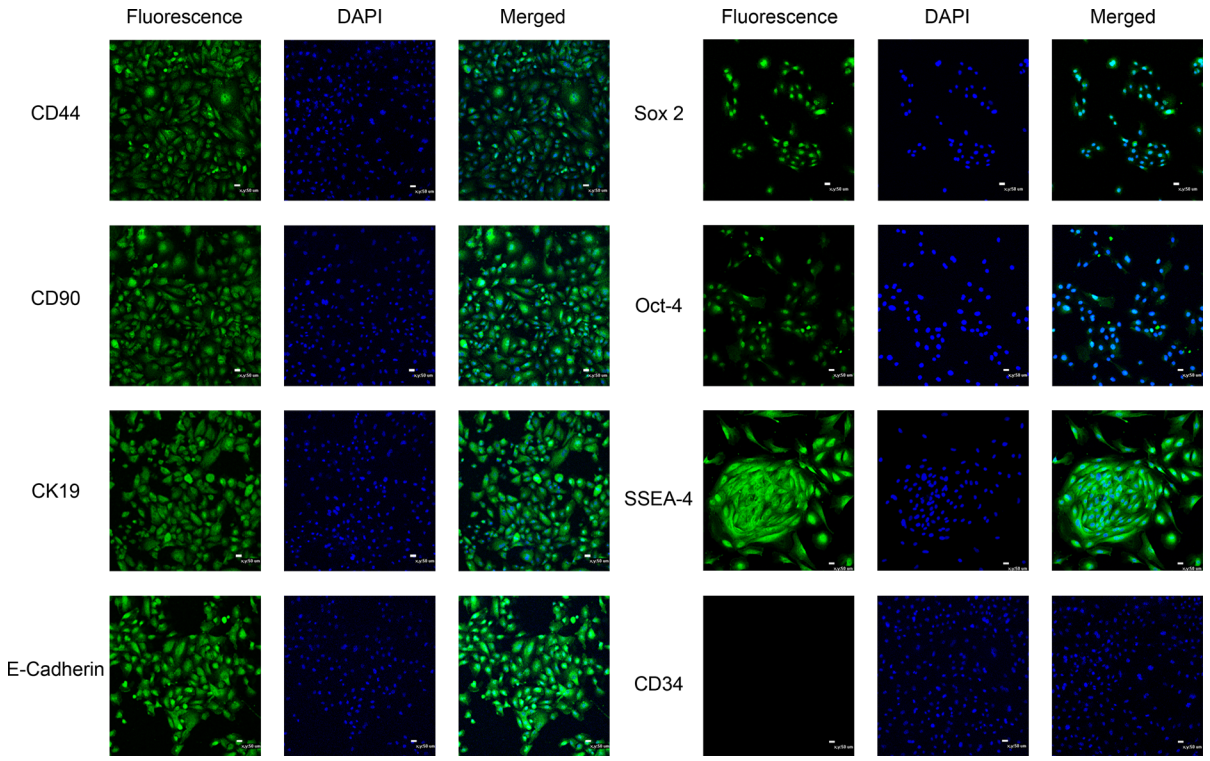

Fig. 2 Surface marker detection of AECs. Cell nuclei stained with DAPI are shown in the middle panels. The pictures shown above indicated that the AECs were CD44, CD90, CK19, E-cadherin, Sox 2, Oct-4 and SSEA-4 positive, and CD34 was negative. Scale bar: 50 μm

microscope. The morphological changes in the AECs were obvious by the contrast to negative control cells. Initially, the cell growth slow down, some cells were separated from cell dishes. Most of the cells changed from spindle into hypertrophy or polygonal in shape after 5–7 days (Fig. 8a). Lipid droplets were evidenced by positive Oil Red O staining (Fig. 8b, c). With continued differentiatial time, the number of droplets increased and small droplets aggregated to form larger ones. Adipocyte specific genes were demonstrated by RT-PCR, and peroxisome proliferator-activated receptor (PPAR-γ) and lipoprotein lipase (LPL) were positive in cells which were cultured in adipogenic medium (Fig. 8d), but not in negative control cells (Fig. 8e).

Chondrogenesis differentiation of AECs

After incubation in chondrogenic medium for 14 days, morphological changes in the AECs were obvious. Initially, the morphology of the cells changed from orbicular-ovate to tridimensional, and with time the cells aggregated and clustered (Fig. 9a). As a result of the continued effects of the inducers, the clusters increased in number and size, which were stained positive with Alcian blue (Fig. 9b, c). The chondrogenic differentiation of AECs was also determined by RT-PCR. The specific genes including vimentin (VIM) and aggrecan (ACAN) were detected in the induced group (Fig. 9d) by contrast to the control group (Fig. 9e).

Karyotype analysis

The chromosome number of sheep is 54, including 26 pairs of macrochromosomes and 1 pair of minichromosomes. The sex chromosome type is XY(♂)/XX(♀) (Fig. 10). In this experiment, 100 representative spreads in the middle of passages 10 were observed to count chromosome numbers, and the average proportion of diploid cells was 97.50 ± 1.29%.

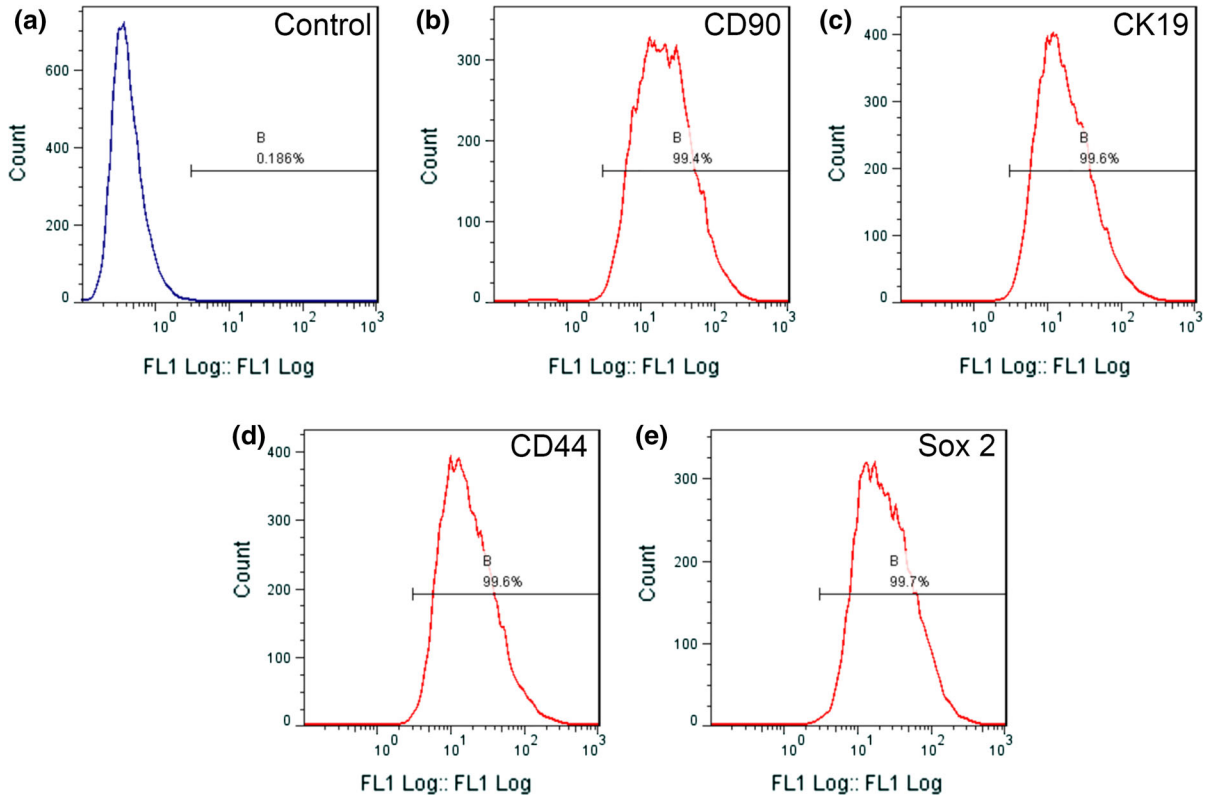

Fig. 3 Detection of AECs markers by flow cytometry. AECs were labelled by markers CD44, CD90, CK19, Sox 2, and positive rates of AECs were 99.6%, 99.4%, 99.6% and 99.7% respectively

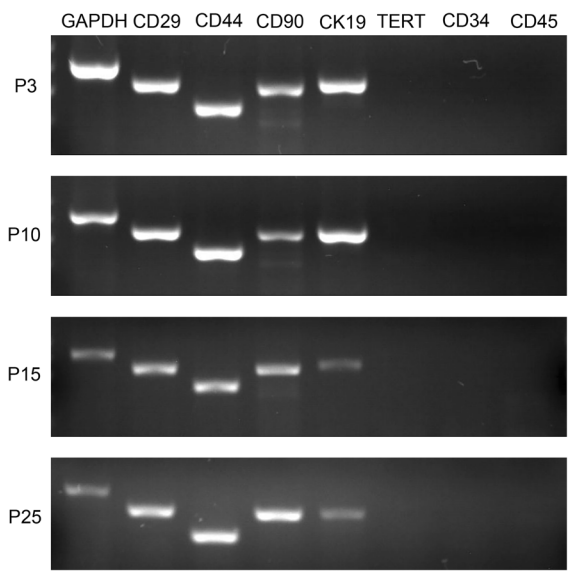

Fig. 4 Detection of AECs markers by RT-PCR. The picture above showed that the expressions of CD29, CD44, CD90, CK19 and GAPDH were all positive, TERT, CD34 and CD45 were negtive

Discussion

Human amniotic epithelial cells are adult stem cells with active substance transport functions, derived from placental amnion tissue. AECs are flat during the pre-pregnancy period, while most of the cells are cuboida in the late period, and located on the fetal surface of the placenta in a cylindrical shape. To isolate sheep AECs, we first treated amniotic membrane with Dispase II for half an hour and with trypsin for another half an hour, which was better than the method of trypsin digestion twice for 30 min. When we usually separate other adult stem cells, we need to cut up the tissue to obtain cells. However, the amniotic membrane does not need to be cut up, and trypsin will act on the surface of the amniotic membrane to digest the cells (Miki and Strom 2006).

AEC cells expressed epithelial cells specific markers, CK19 and E-Cadherin, and did not express hematopoietic stem cells markers, CD34, CD45. AECs could be cultured for 29 generations, and cells

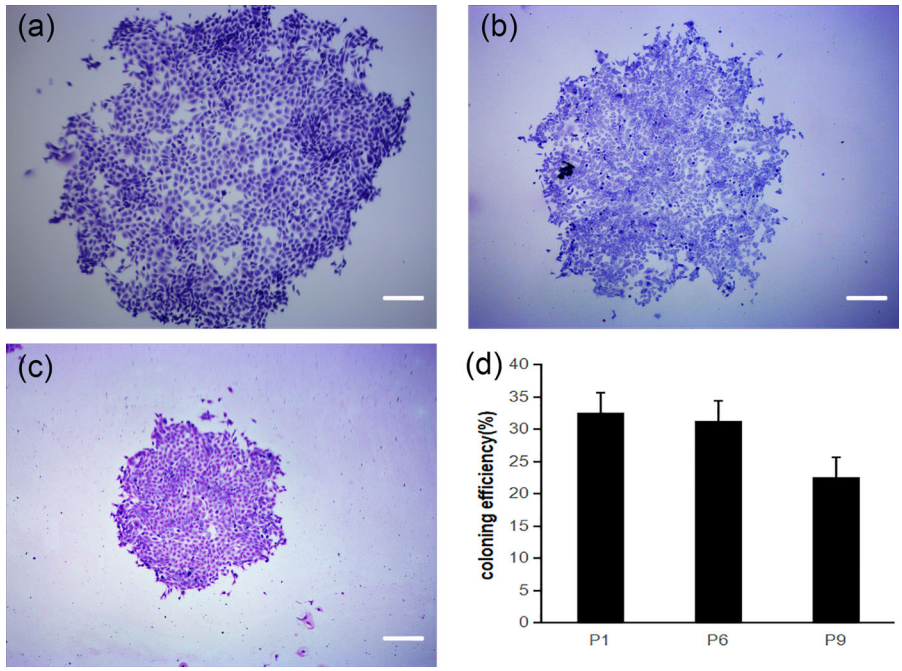

Fig. 5 Clon-genicity of AECs. **a–c** Colonies with the morphology of P1, P6, P9 AECs were cultured for 5 days. Scale bar: 100 μm **d** Bar chart showing the cloning rates for P1, P6, P9 AECs, the colony-forming levels were 32.5 ± 0.22%, 31.3 ± 0.12%, and 22.6 ± 0.23% respectively

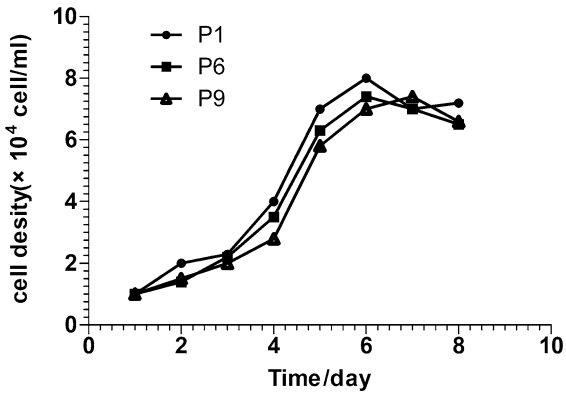

Fig. 6 Growth curves of AECs. The growth curves of P1, P6 and P9 AECs were all typically sigmoidal, with cell density reflected by the vertical axis. Growth curves had a typical sigmoidal shape, indicating the latent, exponential growth, and stationary phases. The population doubling time (PDT) was about 40.63 ± 10.63 h, 46.09 ± 8.21 h and 47.09 ± 7.22 h respectively

gradually became mesenchymal-like cells, which was epithelial-mesenchymal transition (EMT). EMT refers to the conversion of epithelial cells from polar epithelioid cells to mesenchymal-like cells under physiological and pathological conditions (Franzen et al. 2015; Min et al. 2018). During an EMT, epithelial cells lose their polarized organization, lose their typical intercellular connections, acquire migratory and invasive capabilities, and the cell morphology changed from cobblestone-like to fibrocyte-like cells (Diepenbruck and Christofori 2016). In addition, the results of growth kinetics indicated that the AECs had a high proliferative ability in vitro, which was lower at high passages. Previous research showed human placental stem cells expressed markers of pluripotent stem cells, such as Oct4, Nanog, Sox2, SSEA-4, tra1-60, tra1-1-81, and rex-1, which displayed self-renewal and differentiation. Also, human placental stem cells have ability to differentiate to the three germ layers (Battula et al. 2007; Alviano et al. 2007; Miki et al. 2005; Takashima et al. 2004). The maintenance of pluripotency and self-renewal of embryonic stem cells depends on a complex transcriptional regulatory network, while the transcription factors Oct4, Sox2, and Nanog are thought to be the core of transcriptional regulation (Wang et al. 2010).

We used immunofluorescence, FACS analysis, and PCR to detect AECs expressing stem cell marker molecules and related genes, and demonstrated that AECs expressed the stem cells markers, CD44, CD90, SSEA-4, and pluripotent stem cell-specific transcription factors Oct4, Sox2. Additionally, Clonal efficiency was detected, the ability of a single cell to form a colony, which was a very important defining function that demonstrated the self-renewal potential of

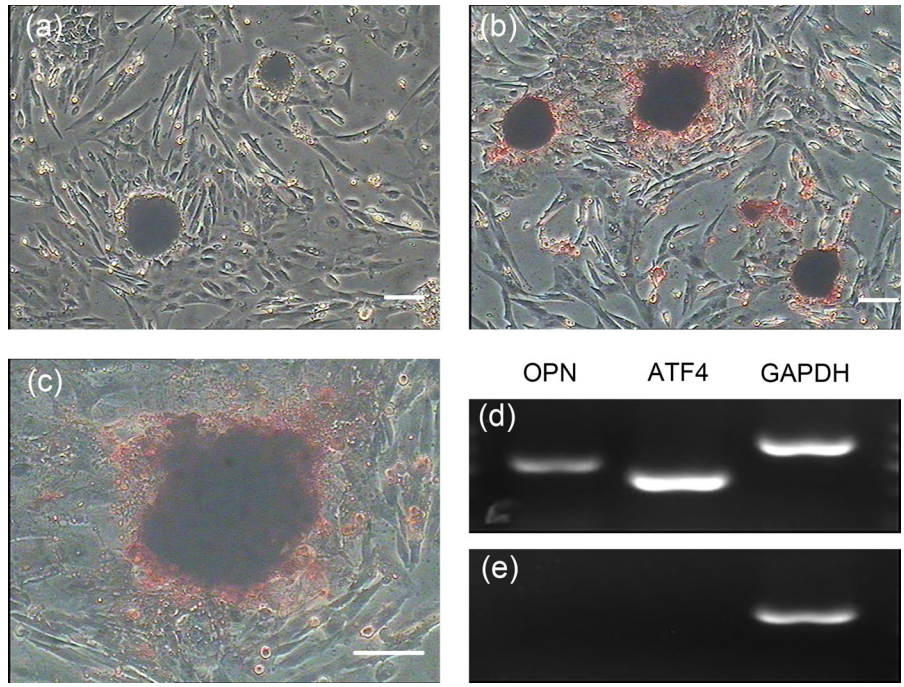

Fig. 7 Osteogenic differentiation of AECs. **a** Day 14 after inducement. **b**, **c** Alizarin Red staining at day 14 postinduction. **d** OPN and ATF4 were positive in the induced group. **e** OPN and ATF4 were negative in the control group. GAPDH served as the internal control. Scale bar: 50 μm. (Color figure online)

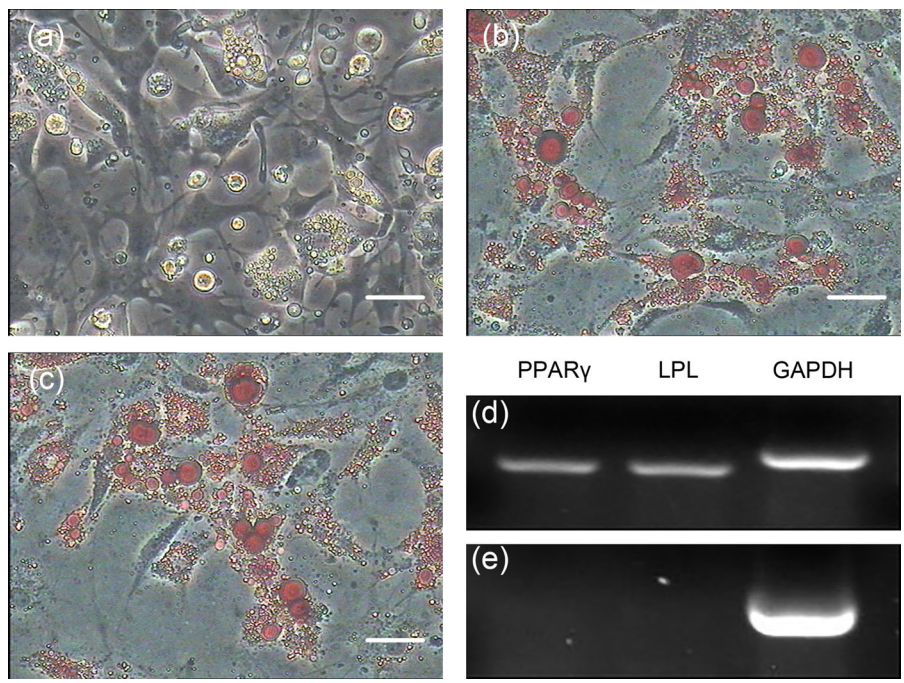

Fig. 8 Adipogenic differentiation of AECs. AECs formed many lipid droplets in cells after induction for 7 days. **a** Day 15 after inducement. **b**, **c** Lipid droplets displayed red through oil red-O staining. **d** LPL and PPAR-γ were positive in the induced group. **e** LPL and PPAR-γ were negative in the control group. GAPDH served as the internal control. Scale bar: 50 μm. (Color figure online)

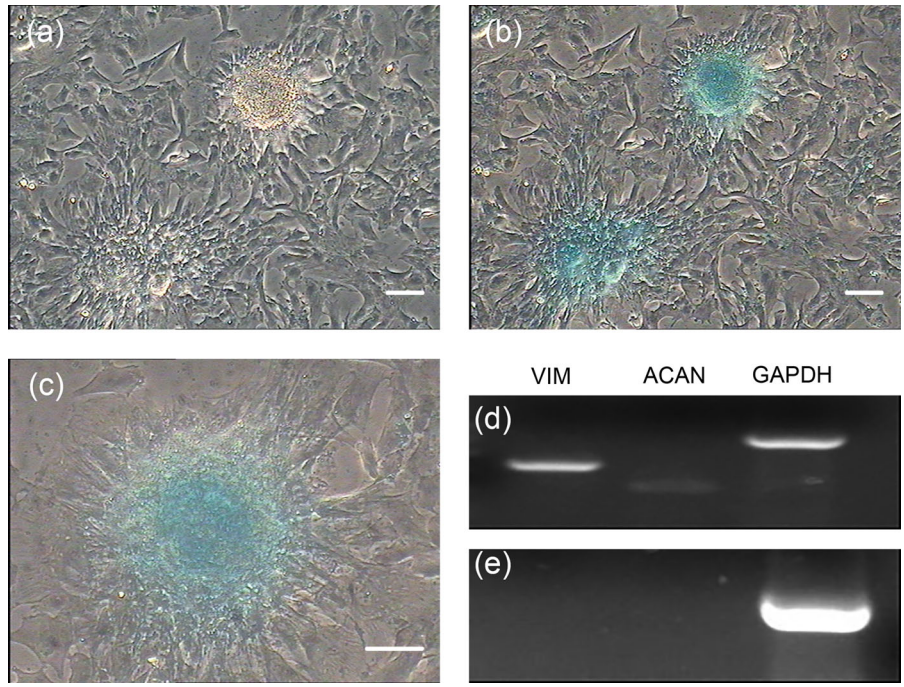

Fig. 9 Chondrogenesis differentiation of AECs. The morphology of the cells changed from orbicular-ovate to tridimensional, the cells aggregated and clustered. **a** Day 14 after inducement. **b–c** The primmorphs were painted blue by alcian blue staining. **d** ACAN and VIM were positive in the induced group. **e** ACAN and VIM were negative in the control group. GAPDH served as the internal control. Scale bar: 50 μm. (Color figure online)

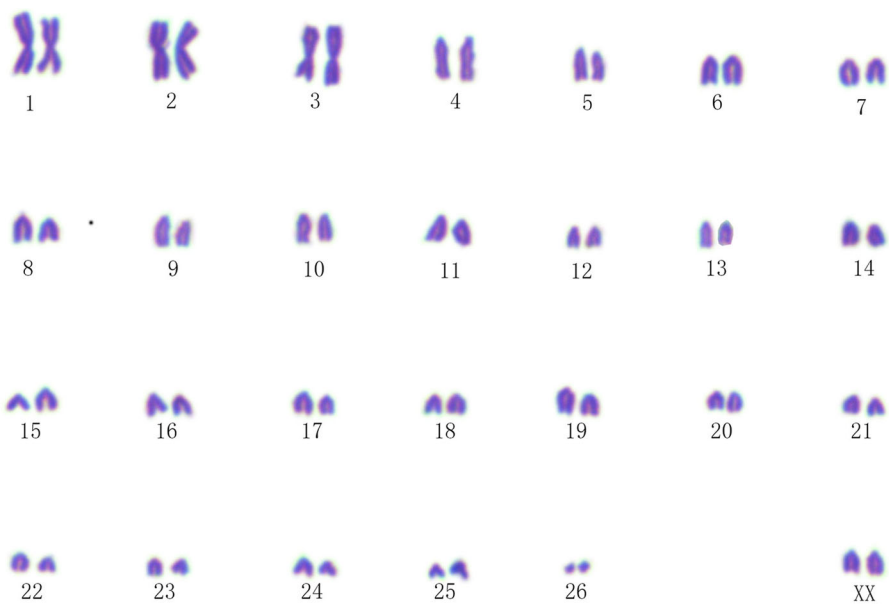

Fig. 10 Karyotype of AECs (♀) XX type. The diploid chromosome number of sheep AECs was $2n = 54$, consisting of 26 pairs of euchromosomes and one pair of sex hromosomes type XY (♂)/XX (♀)

stem cells. As the increases of the generations, the cells colony forming ability gradually declined. Taken together, AEC cells have pluripotency and the ability to self-renew of stem cell.

AECs did not express telomerase genes in stem cells, which protected the ends of the chromosomes, ensuring that DNA was not shortened during replication and immortalizes the cells. Therefore, AECs can

not indefinitely proliferate, having non-tumorigenicity, which have no immunological rejection after allograft transplantation, and also secrete immunosuppressive factors to prevent the occurrence of inflammatory reactions after transplantation. Thus, AECs can be considered as immunonegative cells.

The expression of Oct4 in embryonic stem cells and induced pluripotent stem cells (iPSCs) has also been reported to increase the possibility of teratoma. Although Oct4 was positively expressed in both placenta and fetal stem cells, they did not form teratomas (Fong et al. 2007; Fariha et al. 2011). Although AECs have pluripotent stem cell-like differentiation potential, they are not considered to be tumorigenic cells because precursor cells are not present when injected within immunodeficiency (Miki et al. 2005; Ilancheran et al. 2007). In addition to these studies, human amniotic membranes were transplanted into volunteers' forearms for immunoassays. No serious adverse reactions were found including tumor formation (Akle et al. 1981). Also, the karyotypes of AECs at passage 10 indicated little deviation in chromosome number.

Under the regulation of different growth factors, human amniotic epithelial cells can be differentiated into hepatocyte-like cells, cardiomyocyte-like cells, glial cells, neuronal cells and islet-like cells, and have broad application prospects in cell replacement therapy and tissue regeneration medicine. We induced differentiation of AECs into adipocytes, osteoblasts and chondrocytes in vitro. Embryonic stem cells have two specific properties, self-renewal and differentiation, which make them particularly well suited for therapy. Nevertheless, AECs cannot yet be defined strictly as stem cells, because they cannot be self-renewing for a long time.

Amniotic membrane has been used in the treatment of ophthalmology, surgery, gynecology and other surgical treatment. AECs originate from a large number of abandoned placentas and are not controversial sources of cells in regenerative medicine. Therefore, they can be obtained non-invasively and have a sufficient number of cells. They secrete a variety of immunomodulatory molecules, differentiate into three germinal cell types, have a low immunogenicity and anti-cancer properties (Niknejad et al. 2014). Due to the biological properties of AECs, AECs have been considered for a wide range of medical applications, including ophthalmological diseases, pulmonary fibrosis, hepatic fibrosis, multiple sclerosis, and inborn errors of metabolism such as ornithine transcarbamylase deficiency, familial hypercholesterolemia, spinal cord injury, Parkinson's disease, and allogeneic cell transplantation (Takahashi et al. 2001; Tejwani et al. 2007; Cargnoni et al. 2009; Venkatachalam et al. 2009; Sakuragawa et al. 2000). However, the optimal recovery strategies in AECs and their different types remain to be studied. Future research will be explored to find effective experimental methods of differentiating AECs into different cell types for clinical transplantation. In addition, the differentiation is still a long way to go before this technology really enters clinical application, but there is no doubt that the application prospect of AECs is quite broad.

Conclusions

In conclusion, this study established an optimized method for the isolation of AECs from Small Tail Han sheep 5-week-old embryo, and also suggested that AECs had owning potent self-renewal ability and differential potential. AECs will provide a new type stem cell source and these results have potential utility of AECs as a source of stem cells for regenerative medical therapies. Moreover, the present study provides valuable information for the preservation of genetic resources and the study of cell biology and genomics.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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