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ORIGINAL ARTICLE

Biological characterization and pluripotent identification of ovine amniotic fluid stem cells

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Abstract Mesenchymal stem cells derived from amniotic fluid have become one of the most potential stem cell source for cell-based therapy for the reason they can be harvested at low cost and without ethical problems. Here, we obtained amniotic fluid stem cells (AFSCs) from ovine amniotic fluid and studied the expansion capacity, cell markers expression, karyotype, and multilineage differentiation ability. In our work, AFSCs were subcultured to passage 62. The cell markers, CD29, CD44, CD73 and OCT4 which analyzed by RT-PCR were positive; CD44, CD73, CD90, CD105, NANOG, OCT4 analyzed by immunofluorescence and flow cytometry were also positive. The growth curves of different passages were all typically sigmoidal. The different passages cells took on a normal karyotype. In addition, AFSCs were successfully induced to differentiate into adipocytes, osteoblasts and chondrocytes. The results suggested that the AFSCs isolated from ovine maintained normal biological characteristics and their multilineage differentiation potential provides many potential applications in cell-based therapies and tissue engineering.

Keywords Ovine · Amniotic fluid · Stem cell · Differentiation

Introduction

Mesenchymal stem cells (MSCs) originate from mesoderm and ectoderm in the early development of embryo. They widely exist in the matrix of various tissues and organs (Bobis et al. 2006). For the reason that MSCs have a good ability of self-renewal and multiple differentiation potential, they became one of

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the most promising cell populations for tissue engineering and regenerative medicine (Parekkadan and Milwid 2010).

Amniotic fluid (AF) is a kind of mixed liquid, providing nutrition for the fetus, promoting the growth of the fetus and providing buffer and protection for the fetus (In 'tAnker et al. 2003; Antonucci et al. 2011). A number of studies have proven that stem cells exist in the AF and these cells have the same abilities as MSCs (In 'tAnker et al. 2003; Antonucci et al. 2011; Kaviani et al. 2001; Da Sacco et al. 2011). Researchers began to study AFSCs in the early twenty first century and discovered that these cells possess the ability to extend in vitro (Peister et al. 2009). Priest et al. (1984) found multiple types of cells in the AF. Subsequently, Prusa et al. (2003) discovered that amniotic fluid contained ample stem cells. Amniotic fluid derived cells were named AFSCs by De Coppi et al. (2007) and these cells have properties similar to ES cells. AFSCs were first reported to have the ability of forming embryoid bodies like ES cells and primordial germ cells (PGCs) (Galende et al. 2010). Kim et al. (2014) made use of AFSCs for treatment of neuronal degenerative diseases. Wang et al. (2016) used AFSCs to protect the heart against the ischemia–reperfusion. Recently, AFSCs has become a hotspot in the research of stem cells (Prusa et al. 2003; De Coppi et al. 2007; Tsai et al. 2004; Joo et al. 2012; Gao et al. 2014). AFSCs have been used as seed cells to treat skin wounds, bone defects and breast cancer and so on (Ghafarzadeh et al. 2016; Rota et al. 2012; Da Sacco et al. 2010; Skardal et al. 2012; Zavatti et al. 2015; Young et al. 2016), mainly due to the following reasons: Compared with other stem cells, AFSCs will not cause injury to the donor (Antonucci et al. 2011). It is easy to obtain AFSCs. Furthermore, AFSCs are widely multipotent, express some embryonic stem (ES) cells markers, such as OCT4 and SSEA4 (Tsai et al. 2004; Kim et al. 2007; Pappa and Anagnou 2009). Compared with ES cells, the cultivation of AFSCs does not need feeder layer and the ethical problems are avoided (Delo et al. 2006; De Coppi et al. 2007; Roubelakis et al. 2007). It is a good choice to substitute AFSCs for ES cells. In addition, AFSCs also possess many other properties, such as low immunogenicity (Di Trapani et al. 2015), easy accessibility and inability of form tumors (De Coppi et al. 2007; Tsai et al. 2004). These are the reasons why these cells are considered a candidate for regenerative medicine and tumor targeted therapy.

The ovine is a kind of main livestock and has biological characteristics similar to human. Besides, the ovine is easy to obtain and raise. Moreover, the use of the ovine as experimental animal costs a minimum price. That's the reason why it widely be used in the clinical evaluation and the development of biotechnology. The purpose of this work was to investigate the isolation and culture process of AFSCs in vitro. And exploring the biological characteristics in terms of morphology, growth kinetics, karyotype, cell markers and differentiation potential.

Materials and Methods

Experimental materials

4-week-old ovine embryo was provided by the livestock and poultry Experimental Base Institute of Animal Sciences (Chinese Academy of Agricultural Sciences, Beijing). All animal procedures were in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the Chinese Academy of Agriculture Sciences. Statistical analysis: The statistical software SAS was used for ANOVA variance analysis and variance homogeneity test based on the experimental data. $P < 0.05$ was considered statistically significant.

Isolation and culture of AFSCs

10 mL amniotic fluid samples were extracted with sterile syringes. The sample appeared clear and transparency. If the sample mixed with other impurity in the process of extraction, it will present a faint yellow. The AFSCs isolated from this sample took unnormal karyotype. Then the samples were centrifuged at $500\times g$ for 30 min. The supernatant was discarded and 0.5 mL amniotic fluid at the bottom of the centrifugation tube was retained. The cells were plated in 6 well plates with DF/12 (Gibco Scotland), 10% (v/v) fetal bovine serum (FBS) (Gibco Australia), 10 ng/ml basic fibroblast growth factor (bFGF) (PeproTech USA) and 1% (v/v) GlutaMAX (Gibco New York, USA). Subsequently the cells were incubated at 37 °C, 5% CO_2. About 7 days later, the cell population reached 80% confluence, 0.25% (w/v) trypsin

were added to trypsinized cells. The cells were passaged according to the proportion of 1:2. The medium was refreshed every 2 days.

Kinetics

In order to establish growth characteristics, the ovine AFSCs from passages 5, 20 and 40 were harvested by 0.25% (w/v) trypsin and resuspended in DF/12, 10% (v/v) fetal bovine serum (FBS), 10 ng/ml basic fibroblast growth factor (bFGF) and 1% (v/v) GlutaMAX. Then the cells were plated into a 24-well plate with a density of 10^4/mL, respectively, and three random wells were counted each day for 8 days. The population doubling time was calculated according to the following formula: PDT = (t − t0) lg2/(lgNt − lgN0), t0: starting time of culture; t: termination time of culture; N0: initial cell number of culture; Nt: ultimate cell number of culture.

Karyotype analysis

Standard protocols were used to investigate the chromosomal profiles of the cells. The passage 5 ovine AFSCs were incubated in 0.4 μg/mL colcemid (Baomanbio shanghai, China) 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 was 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 Giemsa stain (Baomanbio shanghai, China). The chromosome numbers were observed under an oil immersion objective.

Immunofluorescent detection of cell markers

When AFSCs reached 60-80% confluence, it could be used for immunofluorescent detection. Firstly, the cells were washed with PBS and fixed with 4% paraphormaldehyde (PFA) (Sigma) for 15 min. Then the cells were permeabilized with 0.25% Triton X-100 for 10 min in order to exclude false positive staining and were washed three times with PBS. Next, The cells were blocked with blocking solution (1% BSA (Biovision USA) in PBS) for 30 min and incubated overnight with primary antibodies at 4 °C. The antibody details are as follows: rabbit anti-CD44 (BIOSS ANTIBODIES, Beijing, China), rabbit anti-CD73 (BIOSS ANTIBODIES), rabbit anti-CD90 (BIOSS ANTIBODIES), and rabbit anti-CD105 (BIOSS ANTIBODIES), rabbit anti-NANOG (BIOSS ANTIBODIES), rabbit anti-OCT4 (BIOSS ANTIBODIES). All of the antibody were diluted with PBS according to the proportion of 1:100. The next day, cells were incubated with FITC-conjugated goat anti-rabbit (BIOSS ANTIBODIES) for 1 h in dark place. Finally, the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Beyotime beijing, China). Nikon TE-2000-E confocal microscope (Nikon, Tokyo, Japan) was used to take photomicrographs.

RT-PCR assay

Passage 5, passage 20 and passage 40 cells were chosen as the low, middle and high passages for RT-PCR analysis. The total RNA was extracted with Trizol reagent from 1×10^6 cells. The Trizol extract was transferred to 1.5 mL EP tube and mixed with 200 μL chloroform. Then the compound was centrifuged at 12,000×*g* for 15 min. After centrifugation, the supernatant was mixed with isopropanol and centrifuged at 12,000×*g* for another 10 min. Next, the RNA pellet was washed with 70% DEPC-ethanol and dried at room temperature. Finally, the RNA concentration was measured by spectrophotometer. The extracted RNA was reverse transcribed to cDNA using an RNA PCR kit version 3.0 (Takara, Shiga, Japan). PCR reaction was conducted by the PCR master Mix Kit (Takara) in a 20 μL mixture that contains 1 μL template cDNA, 0.5 μL each of forward and reverse primers, 8 μL ddH_2O and 10 μL 2 × PCR Mix. The primers which were designed by NCBI primer-blast are listed in Table 1. A thermal cycler was programmed for 35 cycles as follows: 1 cycle at 94 °C for 5 min; 35 cycles including denaturation step at 94 °C for 30 s, annealing step at 50–60 °C for 30 s and elongation step at 72 °C for 30 s; finally 1 cycle at 72 °C for 8 min.

Flow cytometry

According to previously reports, the International Society for Cytotherapy declared that human MSCs must be CD73, CD90 and CD105 positive and CD14, CD34 and CD45 negative (Alsalameh et al. 2004). Flow cytometry analysis was performed to detect the markers of AFSCs including CD44, CD73, CD90,

Table 1 Primer sequences used in RT-PCR assay

Gene name	Primer sequences	Product length (bp)	Tm (°C)
CD29	F:5′-GGCAGGCCGAGAAGGATATTA-3′	406	61
	R: 5′-GGCTCCCCTGATCGTAACTG-3′		
CD44	F: 5′-GACCATGGGGCAAACACAAC-3′	201	60
	R: 5′-TCCGCTAGGCTTTCTCTCCT-3′		
CD73	F: 5′-AAGGGGCCTCTAGCGTCTAA-3′	410	60
	R: 5′-AAAGCATAGGCCTGGACCAC-3′		
OCT4	F: 5′-CCGCCCTATGACTTGTGTGG-3′	527	62
	R: 5′-CGGTTCTCGATACTCGTCCG-3′		
GAPDH	F: 5′-GTCAAGGCAGAGAACGGGAA-3′	232	60
	R: 5′-GGTTCACGCCCATCACAAAC-3′		
ATF4	F: 5′- AGGAGGATGCCCACTCAGAT-3′	359	60
	R: 5′-GGCCAAGGAATCTGCCTTCT-3′		
OPN	F: 5′-GAGTTGCCACTGCAAACCTG-3′	424	60
	R: 5′-CTGATCGCCATGCTGCTTTC-3′		
LPL	F: 5′-CAGGACTCCCGAAGACACAG-3′	203	60
	R: 5′-CCACGATGACGTTGGAGTCT-3′		
PPAR-γ	F: 5′-AAAATACGGCGTGCACGAGA-3′	333	60
	R: 5′-GAGGACTCGGGGTGGTTCAG-3′		
ACAN	F: 5′-AGAGGCGCGTCTAGAAATCG-3′	273	59

CD105, NANOG and OCT4. The cells were harvested by treatment with 0.25% (w/v) trypsin and collected in the FACS tubes at a density of 1×10^6 cells per tube. Then the cells were centrifuged at $200\times g$ for 8 min and the pellet was fixed by 70% ethanol at 4 °C overnight. Next, the cells were blocked by 1% BSA after another centrifugation at $200\times g$ for 8 min. The cells were incubated with primary antibodies at 4 °C overnight. The antibodies are as follows: rabbit anti-CD44 (BIOSS ANTIBODIES), rabbit anti-CD73 (BIOSS ANTIBODIES), rabbit anti-CD90 (BIOSS ANTIBODIES), and rabbit anti-CD105 (BIOSS ANTIBODIES), rabbit anti-NANOG (BIOSS ANTIBODIES), and rabbit anti-OCT4 (BIOSS ANTIBODIES). All antibodies were diluted with PBS according to the proportion of 1:100. Finally, the cells were washed twice in PBS and subjected to single channel fluorescently activated cell sorting (FACS) analysis.

In vitro differentiation of AFSCs

Adipocytic differentiation

In order to verify the ability to differentiate into adipocytes, passage 5 AFSCs were cultured in DMEM/F12, 10% FBS, 0.5 mM dexamethasone, 200 μM indomethacin, 0.5 mM IBMX, and 10 μM insulin (Alsalameh et al. 2004). To test adipogentic differentiation, Oil Red O staining was peformed. Briefly, the cells were washed with PBS for three times and fixed with 4% PFA for 15 min. Then the cells were incubated with Oil Red O for 30 min at room temperature. After that, the cells were rinsed with distilled water. The lipid droplets were observed under an inverted microscope. RT-PCR was used to identify adipocyte specific markers such as proliferator-activated receptor γ (PPAR-γ) and lipoprotein lipase (LPL).

Osteogenic differentiation

The ability of AFSCs to differentiate into osteoblasts was demonstrated by using alizarin red staining and RT-PCR assay. Firstly, the culture medium was replaced with induction medium that consisted of DMEM/F12, 10%FBS, 10 mM β-glycerophosphate, 1 μM dexamethasone, and 0.1 mM L-ascorbic-acid-2-phosphate and the medium was changed every 3 days for 21 days. Then, the cells were fixed with 4% paraformaldehyde (PFA) and stained with Alizarin Red. Finally, the calcium nodules were observed by an

inverted microscope and the specific genes were analyzed by RT-PCR assay.

Chondrogenesis differentiation

The passage 5 AFSCs were induced to differentiate into chondrocytes. After the cells have achieved confluency of 80%, the culture medium was replaced with induction medium that contains 10% FBS, 10 ng/mL transforming growth factor-beta 3 (TGF-β3) and 5 ng/mL bFGF. The cells were cultured under the conditions of 38.5 °C and 5% CO_2 while the medium was refreshed every 3 days. About 3 weeks later, Alcian blue staining was conducted to detect whether chondrocytes were present. RT-PCR was used to analyze the expression of specific genes.

Results

Isolation, culture, and morphology of AFSCs

We successfully isolated AFSCs from a 4 weeks old embryo and subcultured them to passage 62. In primary culture, a few cells were observed in the first three days. About 7 days later, cells spread to more than 80% of 6 well plate. The cell layers were digested by 0.25% trypsin and 0.02% EDTA and the cells were distributed to 60 mm Petri dishes. After passage 3, the AFSCs took on a typical long spindle shape which resembled bone marrow mesenchymal stem cells. After passage 58, cells began to change, cell body increased, cell edge turned burr shaped and they detached from Petri dishes eventually (Fig. 1).

Growth kinetics

The growth curves of AFSCs from P5, P20 and P40 are displayed in Fig. 2. They took on a typical "S" shape. AFSCs entered into logarithmic phase after 3 days, followed by plateau phase after 6 days, and the decline phase took place about 7 days later. The population doubling time (PDT) was calculated with the following PDT formula: $PDT = (t - t0)\,lg2/(lgNt - lgN0)$. The PDT was 33.9, 37.7 and 41.4 h, respectively, for passage 5, passage 20 and passage 40. As the passage numbers increased, the ability of proliferation declined for the cells.

Karyotype analysis

The chromosome number of ovine is $2n = 54$, including 26 pairs of euchromosomes and one pair of sex chromosomes, XX. Normal chromosome number and morphology showed that the cells had normal function. The chromosome numbers per spread were counted for 100 spreads of the passage 5, passage 30, and passage 60, and the ratio of cells with $2n = 54$ was 96.3, 95.1, and 92.7%, respectively. Chromosomal karyotype of ovine AFSCs is shown in Fig. 3.

Detection of AFSCs markers

Cell antigen markers of AFSCs were detected by immunofluorescence and RT-PCR. Experimental immunofluorescence results showed that AFSCs were positive for CD44, CD73, CD90, CD105, NANOG and OCT4 (Fig. 4). The result of RT-PCR demonstrated that AFSCs were positive for CD29, CD44, CD73 and OCT4 (Fig. 5).

FACS analysis

Fluorescent activated cell sorting (FACS) was used for analyzing AFSCs for a series of putative cell antigen markers. Analyses of AFSCs for CD44 (b), CD73 (c), CD90 (d), CD105 (e), NANOG (f) and OCT4 (g) showed that positive rate over 99% of the viable cell population (Fig. 6).

Differentiation of AFSCs

Adipogenic differentiation of the AFSCs

When AFSCs were cultured in adipogenic induction medium, the morphology of cells changed slowly from fibroblast-like to oblate and the cytoplasm contained many tiny lipid droplets after 7 days. With the induction time increasing, the tiny lipid droplets aggregated to larger ones. Adipogenic differentiation of AFSCs was detected by oil red O staining and RT-PCR assay. The differentiated cells were stained with red while the undifferentiated cells were non-stained (Fig. 7). The RT-PCR results showed that differentiated cells expressed adipocyte-specific genes peroxisome proliferator-activated receptor- gamma (PPAR-γ) and lipoprotein lipase (LPL) (Fig. 7e). By comparing the AFSCs with bovine CSPCs, the lipid droplets

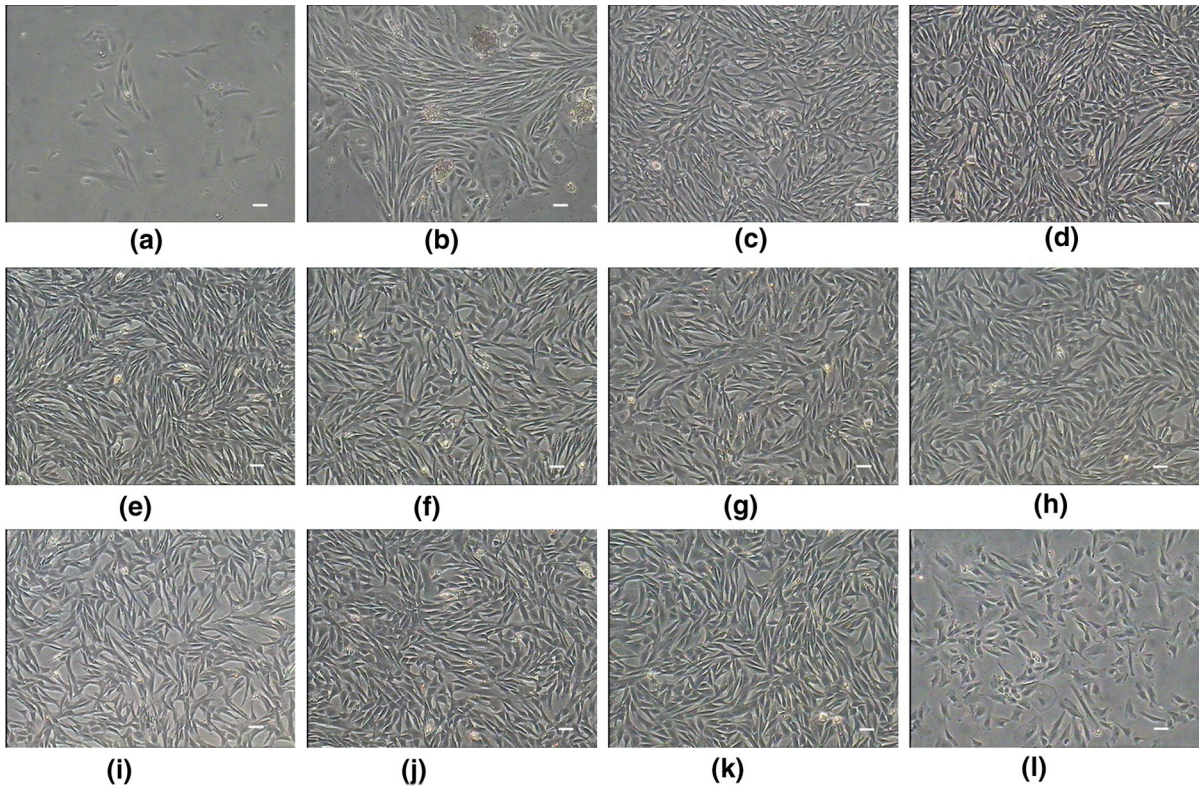

Fig. 1 Morphology of different passages of AFSCs. **a** Ovine cells began to adhere to the Petri dishes after 3 days of cultivation and had a variety of forms. **b** AF-type cells got clustered and there still existed other types of cells. **c** Primary cultured AFSCs covered the Petri dishes. **d**–**k** AFSCs at P5, P15, P22, P34, P45, P53, P55 and P60 grow well and took on a typical long spindle-shape. **l** At passage 62 of AFSCs, the size of cells increased and detached from the Petri dishes. The cells could not continue to grow. *Scale bar* = 100 μm

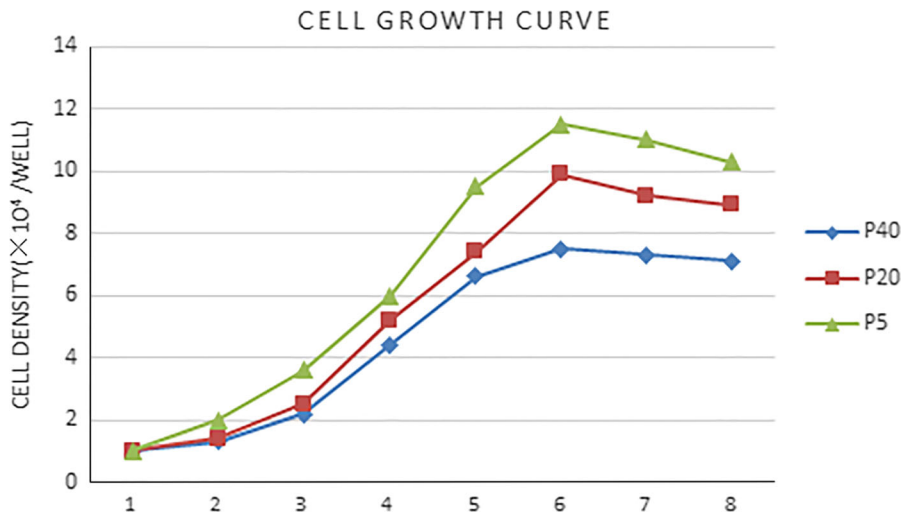

Fig. 2 Growth curves of ovine AFSCs. The growth curves of P5, P20, and P40 of AFSCs displayed a typical "S" shape. Along with the increase of the number of passages, the PDT also increased. PDT was 33.9, 37.7, and 41.4 h for passage 5, passage 20 and passage 40, respectively

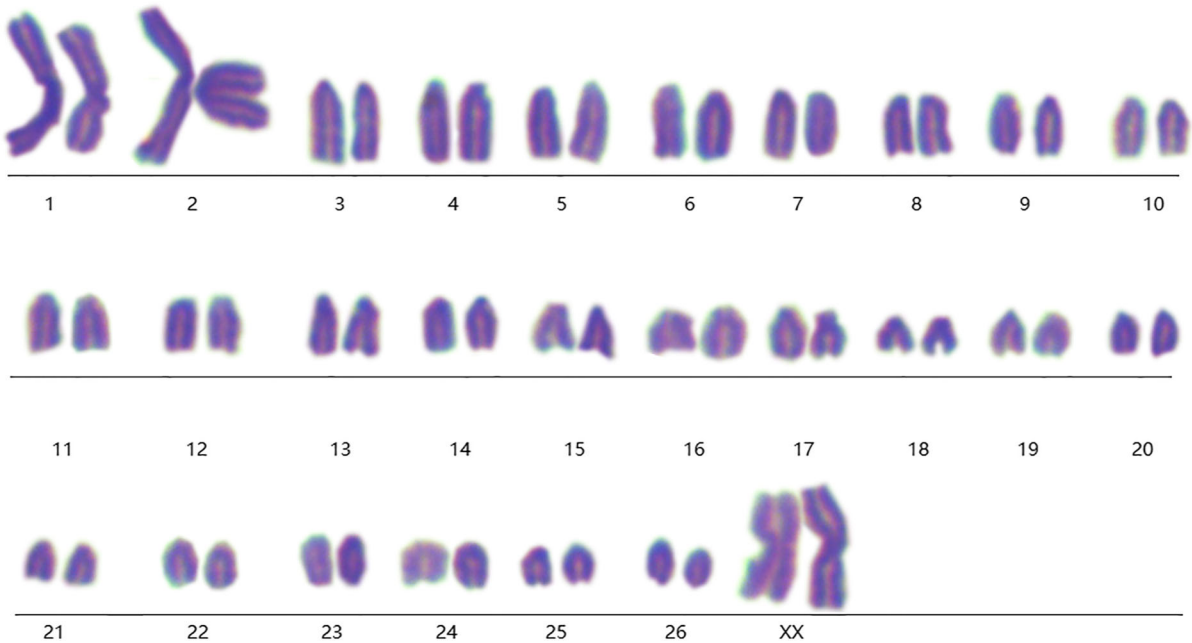

Fig. 3 Karyotype of ovine AFSCs (♀) XX type. The diploid chromosome number of ovine AFSCs was $2n = 54$, consisting of 26 pairs of euchromosomes and one pair of sex chromosomes type XX (♀)

from CSPCs (Fig. 7b, d) are present at higher numbers and have a larger size.

Osteogenic differentiation of AFSCs

After incubating in osteogenic medium for 3 weeks, morphology of AFSCs changed and calcium nodules were visible. With the increasing induction time, the nodules increased in size and number. The nodules were positive for the alizarin red staining (Fig. 8). RT-PCR assay was used for analyzing the osteogenic differentiation of the AFSCs (Fig. 8e). The results showed that differentiated cells expressed osteoblast specific genes osteopontin (OPN) and Activating transcription factor 4 (ATF4).

Chondrogenesis differentiation of AFSCs

AFSCs showed obvious morphological changes after culture in chondrogenic medium for 14 days. After 20 days, the colonies increased in number and size. Then they were stained with Alcian blue (Fig. 9). RT-PCR assay was used for analyzing the chondrogenic differentiation of AFSCs (Fig. 9). The results showed that cartilage-specific genes ACAN and VIM were expressed in the differentiated cells.

Discussion

Amniotic fluid contains at least three types of cells, amniotic fluid specific (AF-type) cells, epitheloid (E-type) cells and fibroblastic (F-type) cells (Miki and Strom 2006; Antonucci et al. 2012). The E-type cells and AF-type cells can form colonies. As the passages increased, E-type gradually disappeared and AF-type became dominant. Thus, we isolated AF-type cells from amniotic fluid and identified the biological characteristics. The AFSCs were differentiate into adipocytes, osteoblasts and chondrocytes in vitro. They were positive for the cell markers CD44, CD73, CD90, CD105, NANOG and OCT4 analyzed by immunofluorescence and flow cytometry. In addition, karyotype analysis and cell growth curve have proven that AFSCs possessed the ability of self-renewal and normal function. The results demonstrated that the biological characteristics of AFSCs were stable.

To date, AFSCs become a suitable candidate for regenerative medicine compared with other stem cells (Bollini et al. 2011; Kang et al. 2012; Cananzi et al. 2009; Rennie et al. 2012). Firstly, the isolation of amniotic fluid stem cells will not cause much damage to the donor (Antonucci et al. 2011). The AFSCs were harvested from amniotic fluid which otherwise will be

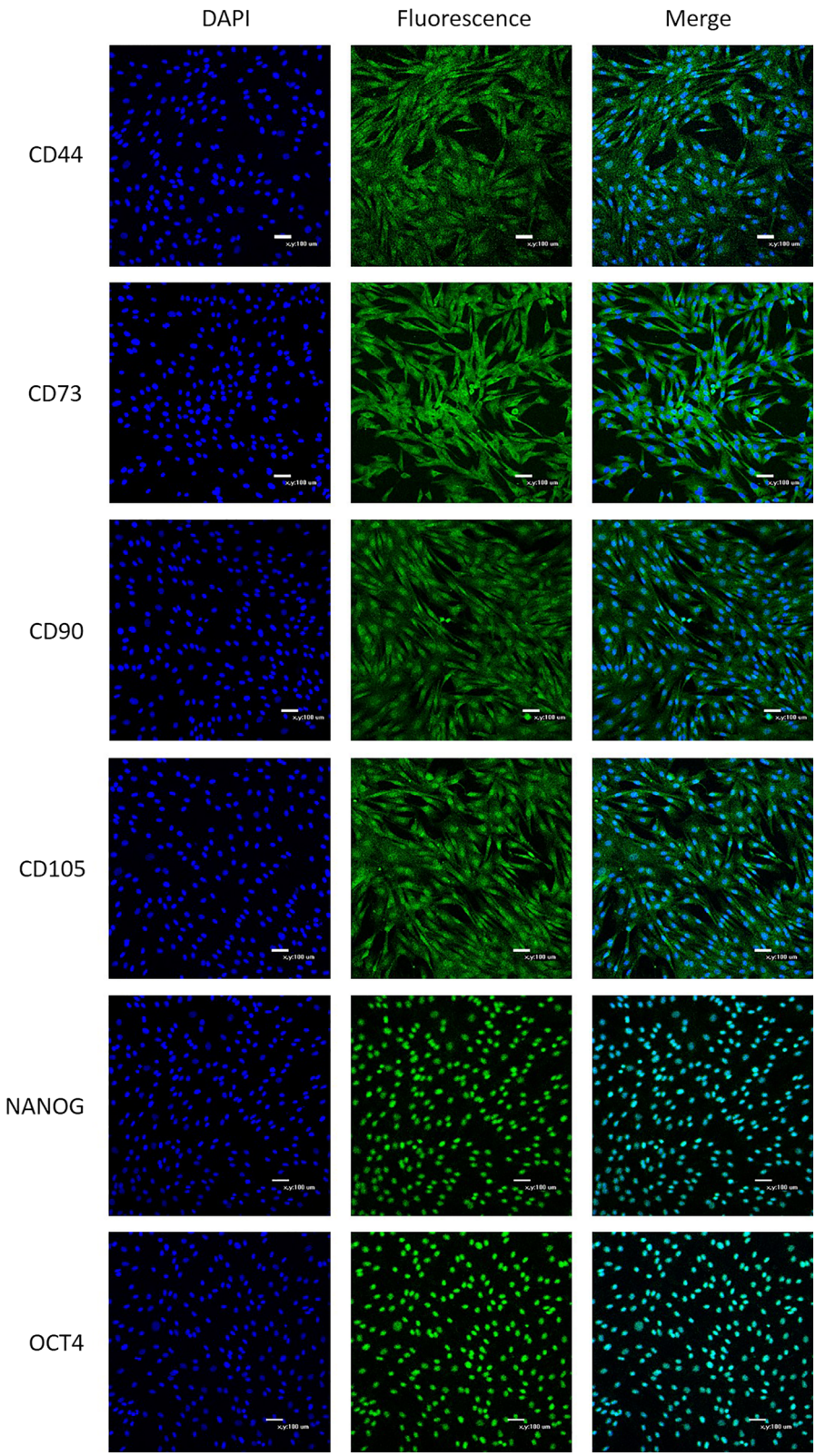

Fig. 4 Detection of AFSCs markers by immunofluorescence staining. The results showed that AFSCs are positive for the mesenchymal stem cell markers CD44, CD73, CD90 and CD105 and they were also positive for the ES cells markers NANOG and OCT4. *Scale bar* = 100 μm

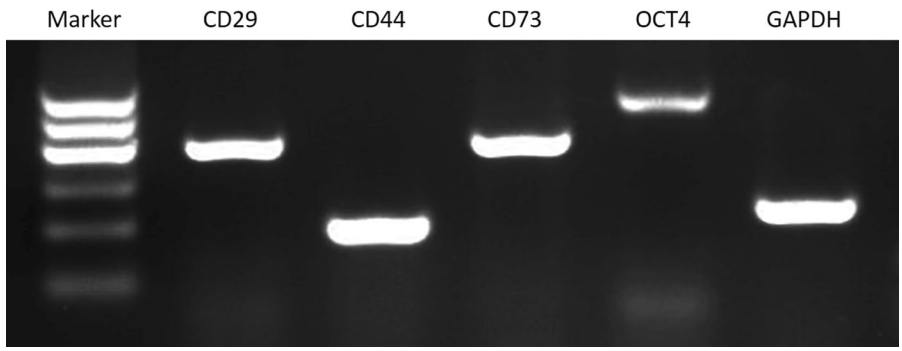

Fig. 5 Detection of AFSCs markers by RT-PCR. The RT-PCR assay also demonstrated that AFSCs were positive both for the mesenchymal stem cell markers and ES cells markers. *Lane1* Marker 600 bp. *Lane 2* CD29; *Lane 3* CD44; *lane 4* CD73; *lane 5* OCT4; *lane 6* GAPDH

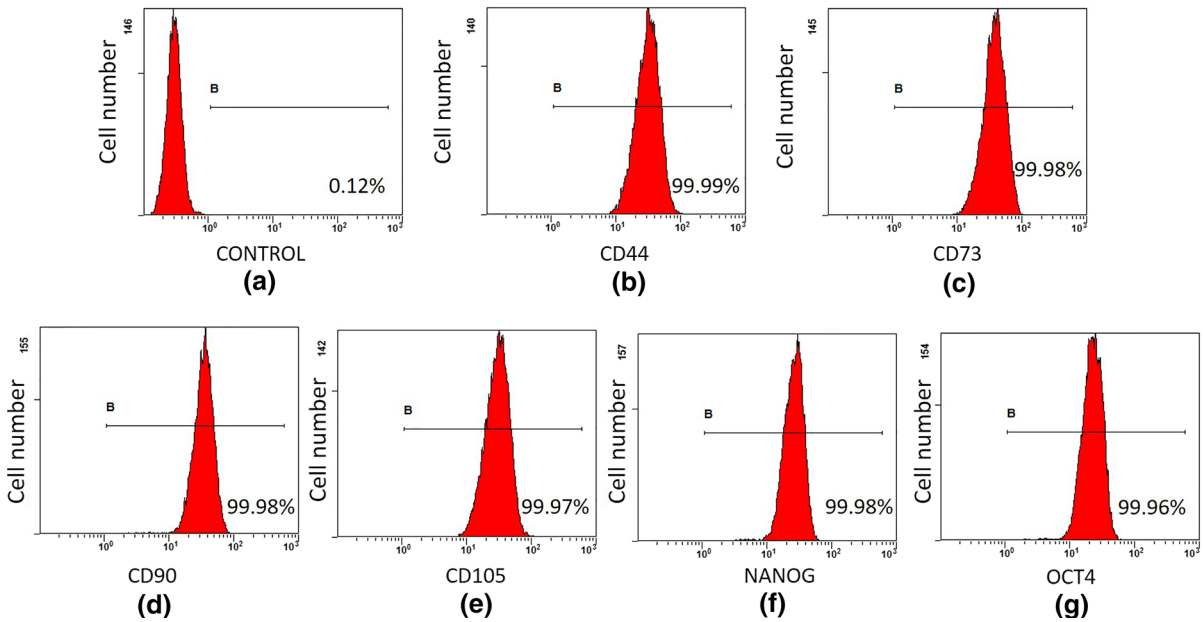

Fig. 6 Detection of AFSCs markers by flow cytometry. The cells were labelled for *markers*. (**a**) stand for control and the positive rate was 0.12%; (**b**) stand for 99.99% detected cells expressed CD44; (**c**) stand for 99.98% detected cells expressed CD73; (**d**) stand for 99.98% detected cells expressed CD90; (**e**) stand for 99.97% detected cells expressed CD105; (**f**) stand for 99.98% detected cells expressed NANOG; (**g**) stand for 99.96% detected cells expressed OCT4

discarded at childbirth. Secondly, the AFSCs possess the ability of strong proliferation and no need for feeder layer, while the embryonic stem (ES) cells cannot live without the feeder layer. Thirdly, AFSCs express pluripotent stem cell markers OCT4 (Colosimo et al. 2013; Di Tomo et al. 2013). The expression of OCT-4 indicates that AFSCs are in an intermediate stage between pluripotent ES cells and MSCs (Kim et al. 2007). Fourthly, because of negative expression of HLA-ABC and HLA-DR, AFSCs have a lower immunogenicity than other MSCs (Di Trapani et al. 2015). Finally, AFSCs will not form tumors in vivo (Cananzi and De Coppi 2012). These are the characteristics which make AFSCs a good source for cell therapy.

According to the previous research, the characterization of the stem cells was mainly based on the surface markers, and stem cell populations possible could be distinguished from other cells depending on cell-surface markers. AFSCs was named by De Coppi et al. (2007) for those expressed CD117 (c-Kit) positive. CD117 is a receptor tyrosine kinase type III and in combination with stem cell factor (a substance that leads certain types of cells to grow), also known as "steel factor" or "c-kit ligand" (Cananzi and De Coppi 2012). The intrinsic tyrosine kinase was

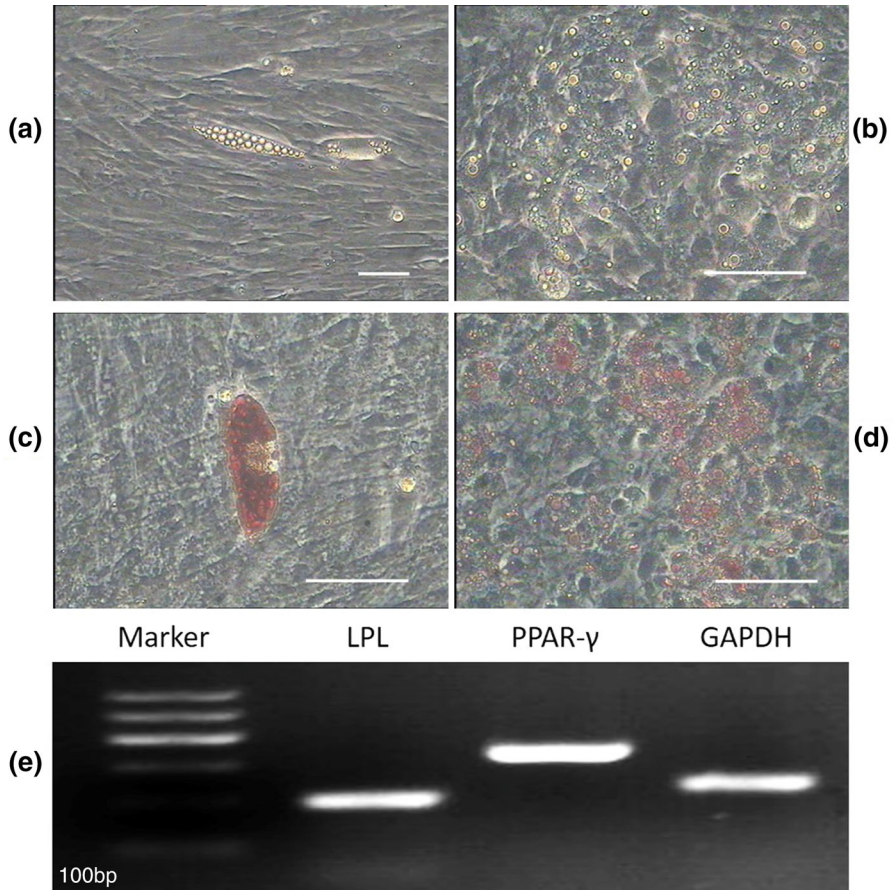

Fig. 7 Adipogenic differentiation of ovine AFSCs and bovine cartilage-derived stem/progenitor cells (CSPCs). **a**, **c** stand for AFSCs while **b**, **d** stand for CSPCs. **a**, **b** The lipid droplets formed after 3 weeks of culture in induction medium. **c**, **d** Oil red O was used to detect the adipocyte. After 21 days induced, AFSCs induced into adipocyte and turn into red after reaction with oil red O. **e** RT-PCR detection of the adipogenic markers LPL and PPAR-γ expression. *Scale bar* = 100 μm

activated once the receptor combined to stem cell factor (SCF). Signalling through CD117 plays an important role in cell survival, proliferation and differentiation. Moreover, as reported by Prusa et al. (2003) OCT-4 is also an important cell antigen in AFSCs. Oct4 is a mammalian POU transcription factor expressed by early embryo cells and germ cells. It is critically involved in the self-renewal of undifferentiated ES cells. Considering the expression of OCT4, AFSCs are at an intermediate stage between ES cells and other MSCs. In addition, whether the AFSCs express NANOG is still in controversy. Phermthai et al. (2010) demonstrated that AFSCs do not express NANOG and SOX2. While Roubelakis et al. (2007) confirmed that AFSCs are positive to NANOG and SSEA4. AFSCs can differentiate in vitro into multiple types of cells, which include adipocytes, osteoblasts, chondrocytes, neurogenic and so on (Mauro et al. 2010). In our study, AFSCs were differentiated into adipocytes, osteocytes and chondrocytes. Indomethacin, IBMX and insulin can promote the differentiation of the AFSCs into adipocytes. β-glycerophosphate, dexamethasone and L-ascorbic-acid-2-phosphate conduct the AFSCs to differentiate to osteoblasts. However, induced into chondrocytes mainly influenced by TGF-β3. It is the multipotential differentiation of AFSCs, which makes them potential candidates for tissue regeneration.

Attributed by the ability of self-renewal, multiple differentiation and low immunogenicity, AFSCs have been used in regenerative medicine and cell-based therapy (Wang et al. 2016; Ghafarzadeh et al. 2016; Skardal et al. 2012; Zavatti et al. 2015). However, there are still many problems to be solved. We have not a clear understanding about AFSCs how to realize the function. For example, AFSCs how to grow under the condition of lacking of feeder layer; What is the regulatory mechanism of AFSCs; How to solve the immune rejection in clinical treatment. If the above problems can be solved, AFSCs could be play an

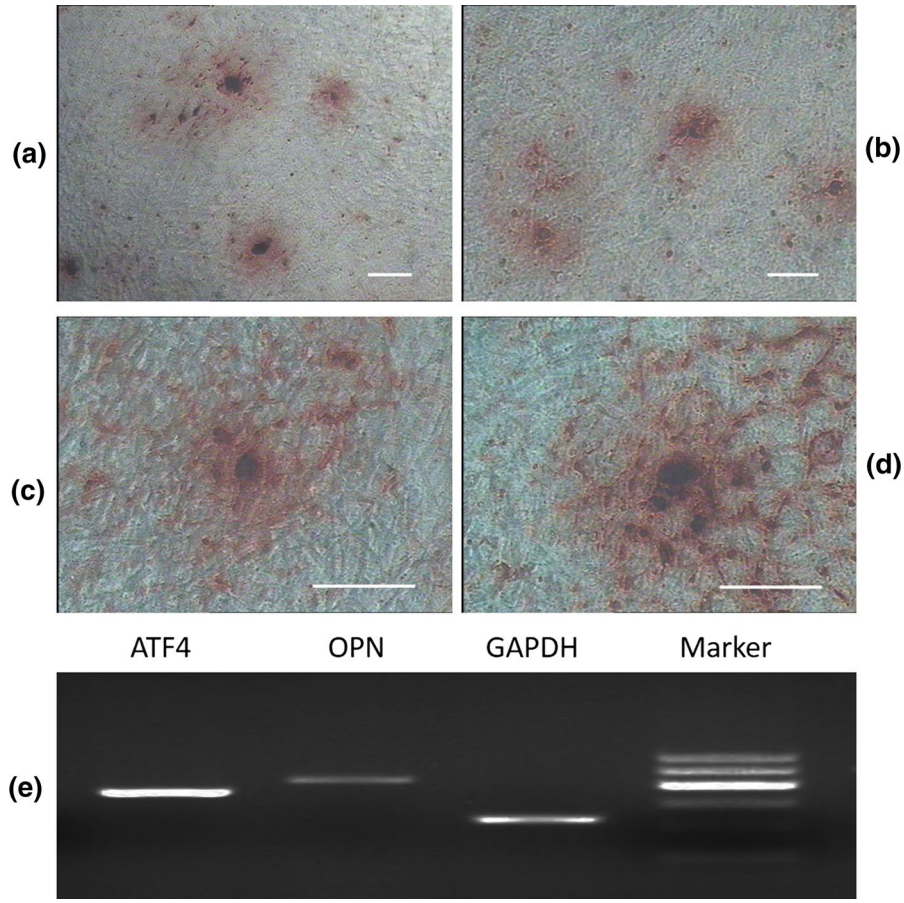

Fig. 8 Osteogenic differentiation of AFSCs. **a**–**d** The calcium nodules were stained by alizarin red. **e** RT-PCR assays revealed the induced cells expressing osteoblast specific genes, including ATF4 and OPN. The GAPDH was used as internal control. *Scale bar* = 100 μm

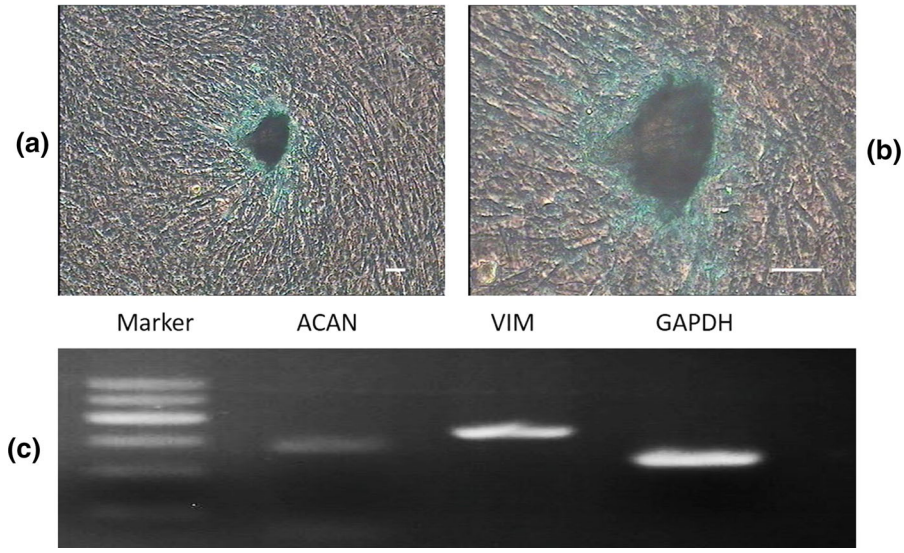

Fig. 9 Chondrogenesis differentiation of AFSCs. **a**, **b** The morphology of AFSCs changed to primmorphs after culture in the induction medium for 3 weeks. The primmorphs were stained blue by alcian blue staining. **c** RT-PCR detection of the chondrogenesis markers ACAN and VIM expression. The marker have 6 lines from the bottom up and respectively represent 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp. *Scale bar* = 100 μm

more important role in tissue engineering, cell therapy and gene engineering.

Conclusion

In conclusion, AFSCs were isolated from amniotic fluid of a 4-week-old embryo. We also evaluated the multipotency of AFSCs that they could be induced to differentiate into adipocytes, osteoblasts and chondrocytes. These results not only provide a technological platform for the establishment of an ovine AFSCs line, but also provide new and abundant seeding cells for cell-based therapy and regenerative medicine.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interests regarding the publication of this paper.

References

Alsalameh S et al (2004) Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage. Arthritis Rheum 50:1522–1532

Antonucci I et al (2011) Amniotic fluid as a rich source of mesenchymal stromal cells for transplantation therapy. Cell Transplant 20:789–795

Antonucci I et al (2012) Amniotic fluid stem cells: a promising therapeutic resource for cell-based regenerative therapy. Curr Pharm Des 18:1846–1863

Bobis S, Jarocha D, Majka M (2006) Mesenchymal stem cells: characteristics and clinical applications. Folia Histochem Cytobiol 44:215–230

Bollini S et al (2011) Amniotic fluid stem cells are cardioprotective following acute myocardial infarction. Stem Cells Dev 20:1985–1994

Cananzi M, De Coppi P (2012) CD117(+) amniotic fluid stem cells State of the art and future perspectives. Organogenesis 8:77–88

Cananzi M, Atala A, De Coppi P (2009) Stem cells derived from amniotic fluid: new potentials in regenerative medicine. Reprod Biomed Online 18:17–27

Colosimo A et al (2013) Prolonged in vitro expansion partially affects phenotypic features and osteogenic potential of ovine amniotic fluid-derived mesenchymal stromal cells. Cytotherapy 15:930–950

Da Sacco S et al (2010) Human amniotic fluid as a potential new source of organ specific precursor cells for future regenerative medicine applications. J Urol 183:1193–1200

Da Sacco S, De Filippo RE, Perin L (2011) Amniotic fluid as a source of pluripotent and multipotent stem cells for organ regeneration. Curr Opin Organ Transplant 16:101–105

De Coppi P et al (2007a) Isolation of amniotic stem cell lines with potential for therapy. Nat Biotechnol 25:100–106

Delo DM et al (2006) Amniotic fluid and placental stem cells. Methods Enzymol 19:426–438

De Coppi P et al (2007b) Amniotic fluid and bone marrow derived mesenchymal stem cells can be converted to smooth muscle cells in the cryo-injured rat bladder and prevent compensatory hypertrophy of surviving smooth muscle cells. J Urol 177:369–376

Di Tomo P et al (2013) Calcium sensing receptor expression in ovine amniotic fluid mesenchymal stem cells and the potential role of R-568 during osteogenic differentiation. PLoS ONE 8:e73816

Di Trapani M et al (2015) Immune regulatory properties of CD117(pos) amniotic fluid stem cells vary according to gestational age. Stem Cells Dev 24:132–143

Galende E et al (2010) Amniotic fluid cells are more efficiently reprogrammed to pluripotency than adult cells. Cell Reprogram 12:117–125

Gao Y et al (2014) Amniotic fluid-derived stem cells demonstrated cardiogenic potential in indirect co-culture with human cardiac cells. Ann Biomed Eng 42:2490–2500

Ghafarzadeh M, Eatemadi A, Fakhravar Z (2016) Human amniotic fluid derived mesenchymal stem cells cause an anti-cancer effect on breast cancer cell line in vitro. Cell Mol Biol (Noisy-le-grand) 62:102–106

In 'tAnker PS et al (2003) Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. Blood 102:1548–1549

Joo S et al (2012) Amniotic fluid-derived stem cells in regenerative medicine research. Arch Pharm Res 35:271–280

Kang NH et al (2012) Potential antitumor therapeutic strategies of human amniotic membrane and amniotic fluid-derived stem cells. Cancer Gene Ther 19:517–522

Kaviani A et al (2001) The amniotic fluid as a source of cells for fetal tissue engineering. J Pediatr Surg 36:1662–1665

Kim J et al (2007) Human amniotic fluid-derived stem cells have characteristics of multipotent stem cells. Cell Prolif 40:75–90

Kim EY, Lee KB, Kim MK (2014) The potential of mesenchymal stem cells derived from amniotic membrane and amniotic fluid for neuronal regenerative therapy. BMB Rep 47:135–140

Mauro A et al (2010) Isolation, characterization, and in vitro differentiation of ovine amniotic stem cells. Vet Res Commun 34:S25–S28

Miki T, Strom SC (2006) Amnion-derived pluripotent/multipotent stem cells. Stem Cell Rev 2:133–141

Pappa KI, Anagnou NP (2009) Novel sources of fetal stem cells: where do they fit on the developmental continuum? Regen Med 4:423–433

Parekkadan B, Milwid JM (2010) Mesenchymal stem cells as therapeutics. Annual Rev Biomed Eng 12:87–117

Peister A et al (2009) Amniotic fluid stem cells produce robust mineral deposits on biodegradable Scaffolds. Tissue Eng Part A 15:3129–3138

Phermthai T et al (2010) A novel method to derive amniotic fluid stem cells for therapeutic purposes. BMC Cell Biol 11:79

Priest et al (1984) Metabolism of [4-14C] androstenedione by cells cultured from human amniotic fluid. J Steroid Biochem 20:935–939

Prusa AR et al (2003) Oct-4-expressing cells in human amniotic fluid: a new source for stem cell research? Hum Reprod 18:1489–1493

Rennie K et al (2012) Applications of amniotic membrane and fluid in stem cell biology and regenerative medicine. Stem Cells Int 2012:721538

Rota C et al (2012) Human amniotic fluid stem cell preconditioning improves their regenerative potential. Stem Cells Dev 21:1911–1923

Roubelakis MG et al (2007) Molecular and proteomic characterization of human mesenchymal stem cells derived from amniotic fluid: comparison to bone marrow mesenchymal stem cells. Stem Cells Dev 16:931–951

Skardal A et al (2012) Bioprinted amniotic fluid-derived stem cells accelerate healing of large skin wounds. Stem Cells Transl Med 1:792–802

Tsai MS et al (2004) Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol. Hum Reprod 19:1450–1456

Wang Y et al (2016) The transplantation of Akt-overexpressing amniotic fluid-derived mesenchymal stem cells protects the heart against ischemia-reperfusion injury in rabbits. Mol Med Rep 14:234–242

Young BK et al (2016) Amniotic fluid as a source of multipotent cells for clinical use. J Perinat Med 44:333–337

Zavatti M et al (2015) Critical-size bone defect repair using amniotic fluid stem cell/collagen constructs: effect of oral ferutinin treatment in rats. Life Sci 121:174–183