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Analysis of the stem cell characteristics of adult stem cells from Arbas white Cashmere goat

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ABSTRACT

Studies have shown that multipotent adult stem cells possess differentiation characteristics similar to embryonic stem cells and pluripotent stem cells. We aimed to explore these similarities further by examining the expression of the pluripotency and stemness biomarkers, AKP, IL-6, Nanog, Oct-4, Rex-1, Sox-2 and TERT, as well as the triploblastic biomarkers, Sox-1, Myod1 and Gata-6 in adipose-derived stem cells (ADSCs), bone marrow stem cells (BMSCs) and muscle-derived satellite cells (MDSCs). These were isolated from adult Arbas white Cashmere goats and cultured *in vitro*. Immunocytochemistry, reverse transcription quantitative PCR and Western blotting were used to analyze the protein and mRNA expression of the markers. To investigate the ability of ADSCs, BMSCs and MDSCs to differentiate and cause tumors *in vivo* they were injected into immunodeficient mice (NOD-SCID). All results were compared to those for mouse embryonic stem cells (mESCs). Immunocytochemistry showed that AKP, IL-6, Nanog, Oct-4, Rex-1 and TERT were expressed in ADSCs, BMSCs and MDSCs, whereas Sox-2 was not. In ADSCs, the expression of *IL*-6 mRNA was relatively high, followed by *Nanog* and *Oct*-4, while *Rex*-1 and *TERT* expression were the lowest ($P < 0.01$). In BMSCs, the expression of *Rex*-1 was relatively high, followed by *IL*-6, while *Oct*-4, *Nanog* and *TERT* were comparatively low ($P < 0.01$). In MDSCs, the expression of *IL*-6, *Nanog* and *Oct*-4 were relatively high, while *TERT* was comparatively low ($P < 0.01$). However, no expression of *Sox*-2 mRNA was detected in any of the three cell lines. The expression of *Sox*-1, *Myod*1 and *Gata*-6 was observed to different degrees in all three cell lines ($P < 0.01$); the expression pattern in MDSCs was different from that in ADSCs and BMSCs. Western blotting indicated that no expression of Sox-2 and Rex-1 protein occurred in ADSCs, BMSCs and MDSCs, while the other five proteins were all expressed to different degrees ($P < 0.01$); the expression pattern was consistent with the mRNA results. In contrast to the mESCs, no teratoma tissue or triploblastic differentiation appendages were formed in the immunodeficient mice after injection of ADSCs, BMSCs and MDSCs. Our results suggest that the three adult goat stem cell types are non-oncogenic and have stemness characteristics similar to embryonic stem cells. Of these, MDSCs were found to exhibit the most ESC-like properties and would make the best candidates for clinical application.

1. Introduction

The establishment of embryonic stem cell (ESC) lines and their combination with genetic engineering techniques has provided a unique technology platform for the study of molecular events in human development and disease. Other areas extensively impacted by such technology include regenerative medicine, drug development and developmental biology [1]. Scientists [2] anticipate the use of human ESCs for the treatment of autoimmune diseases, such as Parkinson's, spinal cord injury and diabetes; however, the tumorigenicity of ESCs, ethical controversy and other factors have hindered research in this field. One effective solution is to isolate and culture adult stem cells from patients, then induce them to differentiate into functional cells for the final cell therapy.

Adult stem cells are undifferentiated, self-renewing cells with a specific differentiation potential corresponding to the tissue from which they are derived. Their main function is to participate in the healing and renewal of adult tissues [3]. Adult stem cells can be conveniently sampled from an extensive array of sources and they bypass the ethical controversy, which is increasingly affecting application of stem cell research and therapy.

ESCs can self-replicate and they can differentiate into the various cell types required for generation of a human body [4]; this is known as pluripotency. Establishing whether adult stem cells

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are similar to ESCs in their ability to maintain pluripotency in long-term *in vitro* culture has become a bottleneck in stem cell research and application [5]. Therefore, a clear understanding of the transcription mechanism regulating ESC pluripotency and self-renewal is required to elucidate their proliferation and differentiation characteristics; this would aid a more in-depth study of adult stem cells. Some transcription factors such as Oct-4, Sox2, Nanog and Rex-1 play a crucial role in the maintenance of pluripotency in ESCs, and genetic modification relating to these can cause transition from one stem cell type to another. This shows how further understanding of key regulatory genes could facilitate more precise control of the cellular differentiation [6].

40-day-old Arbas white Cashmere goat fetuses were chosen as the cell source for this study. ADSCs, BMSCs and MDSCs were isolated and cultured and mouse embryonic stem cells (mESCs) and Arbas white Cashmere goat fetal fibroblasts cells (FFCs) were used for comparison. The expression of pluripotency and stemness biomarkers, including Oct-4, Nanog, Sox-2, Rex-1, AKP, IL-6, TERT, as well as the triploblastic biomarkers, Sox-1, Myod1 and Gata-6 was analyzed in ADSCs, BMSCs and MDSCs. These cells were also injected into immunodeficient mice to assess their *in vivo* differentiation capacity and tumorigenicity. Using these methods, we aimed to gain greater understanding of the transcription regulatory mechanisms of self-renewal and pluripotency of these three adult stem cell types; coupled with examination of their *in vivo* tumorigenicity, this could provide a sound experimental base from which to explore their clinical application.

2. Materials and methods

2.1. Animals

Pregnant Arbas white Cashmere goats were obtained from the Experimental Animal Center at the Inner-Mongolian University. Animals were maintained under pathogen-free conditions. NOD/SCID mice were purchased from Beijing Vital River Laboratory Animals Co., Ltd. (Beijing, China). All studies were performed with the approval of the Experimental Animal Committee of the Inner-Mongolian University.

2.2. ***Cells***

Mouse embryonic stem cells (ESCs) were purchased from Peking University (Beijing, China).

gADSCs were isolated using the method we have described previously [7].

gMDSCs were isolated using the method we have described previously [8].

2.3. Isolation and identification of goat BMSCs

A fetus (40-day) was removed by cesarean section from a pregnant goat. Bilateral lower limbs were cut and muscle and periost were removed to obtain complete femora. The bone marrow plugs were flushed with DMEM/F12 (Hyclone, Logan, UT, USA) containing 3000 U/mL heparin and diluted with DMEM/F12 + 10% fetal bovine serum (FBS) (Hyclone) at a ratio of 1:4. The dilution was loaded onto a Percoll solution (density, 1.083), and separated by centrifugation at 500*g* for 30 min at room temperature. The goat BMSCs (gBMSCs) at the interphase were removed to a new 50 mL tube. The cells were diluted to 1–2 $\times$ 10^6 cells/mL and cultured in DMEM/F12 medium supplemented with 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin at 37 °C and 5% CO_2. At confluence, cells were washed with PBS to remove unattached cells and then treated with 0.25% trypsin containing 0.02% EDTA before passaging. To identify gBMSCs, cells were seeded at 1 $\times$ 10^4/mL in 24-well plates. The cells were fixed with 4% paraformaldehyde at 80% confluence for 30 min, permeabilized with PBS containing 0.1% (v/v) Triton X-100, and incubated with 3% bovine serum albumin (BSA) in PBS for 2 h. Cells were then incubated with the appropriate primary detection antibodies: CD29, CD31, CD44, CD45 and CD90 (1: 500, Abcam, Cambridge, UK) at room temperature for 1 h. After washing in PBS, cells were incubated with a mixture of a fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1: 300, goat anti-rabbit; Abcam, USA) and 4′,6-diamidino-2-phenylindole (DAPI; D9542, Sigma, MO, USA). For negative controls, the primary antibody was replaced with PBS. Cell staining was viewed under an inverted fluorescence microscope [9].

2.4. Immunocytochemistry of ADSCs, BMSCs, MDSCs, ESCs and FFCs

ADSCs, BMSCs, MDSCs, ESCs and FFCs grown on 4-well coverslips were fixed with 4% paraformaldehyde at room temperature for 30 min. Specimens were permeabilized with 0.5% Triton X-100 in PBS for 30 min. After washing (×3), the specimens were blocked in 2% PBS-BSA for 2 h. The cells were incubated with the primary antibodies for 1 h at 37 °C. Antibodies against AKP, IL-6, Nanog, Oct-4, Rex-1, Sox-2 and TERT (Abcam, USA) were diluted 1:400. After washing (×3), cells were incubated with FITC-conjugated goat anti-rabbit (1: 300, Abcam, USA) for 1 h at 37 °C. Finally, the nuclei were stained with DAPI for 5 min at 37 °C. At least three replicates were performed for each sample. The cells were visualized using a confocal microscope.

2.5. Reverse transcription qPCR

AKP, *IL*-6, *Nanog*, *Oct*-4, *Rex*-1, *Sox*-2, *TERT*, *Sox*-1, *Myod*1 and *Gata*-6 mRNA expression levels in ADSCs, BMSCs and MDSCs were measured using reverse transcription qPCR. All primer sequences were determined using established GenBank sequences, which are listed in Table 1 and Table 2. PCR reactions were amplified using the designed primers with GAPDH as the control for assessing PCR efficiency. The PCR parameters were as follows: 40 cycles of 95 °C for 5 s followed by 60 °C for 30 s. To determine if there were multiple PCR amplicons, melting curves were constructed by heating final amplification reactions from 60 to 95 °C for 15 s,

Table 1
Mouse ESCs reverse transcription PCR primers.

Gene name		Sequence	Product length (bp)
GAPDH	F	5′-TTGTGATGGGCGTGAACC-3′	127
	R	5′-CCCTCCACGATGCCAAA-3′	
Oct-4	F	5′-GCCAAGCTCCTAAAGCAGAAGA-3′	122
	R	5′-AAAGCCTCAAAACGGCAGATAG-3′	
Nanog	F	5′-GTCTCTCCTCTTCCTTCCTCCA-3′	116
	R	5′-TCTTCCTTCTCTGTGCTCTCCTC-3′	
Sox-2	F	5′-CATGATGGAGACGGAACTGG-3′	115
	R	5′-CGGGCTGTTCTTCTGGTTG-3′	
IL-6	F	5′-GATGACTTCTGCTTTCCCTACCC-3′	196
	R	5′-TGCCAGTGTCTCCTTGCTGT-3′	
AKP	F	5′-ACGGTCACCATGAAGGCAAAG-3′	125
	R	5′-GTGGTCTGCAGTGGCAAGGA-3′	
TERT	F	5′-GTCACAGAGACCACGTTCCAGAAG-3′	118
	R	5′-ACAGTTCTCGAAGCCGCACA-3′	
Rex-1	F	5′-GGAAGAAAAGGGGAACAACACC-3′	134
	R	5′-CTCATAGCACACATCCTCATCACA-3′	
Sox-1	F	5′-TGGCCCAGGAAAACCCCAAG-3′	114
	R	5′-GTCTCTTGGCCTCGTCGATG-3′	
Myod1	F	5′-CCTGAGCAAAGTGAATGAG-3′	115
	R	5′-ACCTTCGATGTAGCGGATG-3′	
Gata-6	F	5′-TCAGGGGTAGGGGCATCAG-3′	113
	R	5′-GAGGACAGACTGACACCTATG-3′	

Table 2
ADSCs, BMSCs and MDSCs reverse transcription qPCR primers.

Gene name		Sequence	Product length (bp)
GAPDH	F	5′-GGCACAGTCAAGGCTGAGAATG-3′	143
	R	5′-ATGGTGGTGAAGACGCCAGTA-3′	
Oct-4	F	5′-CAGACCACCATCTGTCGCTTC-3′	194
	R	5′- AGACTCCACCTCACACGGTTCTC-3′	
Nanog	F	5′-GCCTCCAGCAGATGCAAGAAC-3′	181
	R	5′-CTGCAATGGATGCTGGGATAC-3′	
Sox-2	F	5′-GTTCTAGTGGTACGTTAGGCGCTTC-3′	80
	R	5′-TCGCCCGGAGTCTAGCTCTAAATA-3′	
IL-6	F	5′-CCACTTCACAAGTCGGAGGCTTA-3′	255
	R	5′-CCAGTTTGGTAGCATCCATCATTTC-3′	
AKP	F	5′-TCCTAAAGGGGCAGTTGGAAG-3′	146
	R	5′-CCCACAGAGATAGGCGGTTG-3′	
TERT	F	5′-CAGCCATACATGGGCCAGTTC-3′	113
	R	5′-AGGCTGCTGCTGCTCTCATTC-3′	
Rex-1	F	5′-GATTTCCACTGTGGCTCTGGGTA-3′	136
	R	5′-GCCACGTGTCCCAGCTCTTA-3′	
Sox-1	F	5′-TGGCCCAGGAAAACCCCAAG-3′	114
	R	5′-GTCTCTTGGCCTCGTCGATG-3′	
Myod1	F	5′-ACGTCTAGCAACCCAAACCA-3′	185
	R	5′-GAGTCGCCGCTGTAGTGTTC-3′	
Gata-6	F	5′-CCTCATCAAGCCGCAGAAG-3′	85
	R	5′-CAGAGTGGTGGTCGTGGTGT-3′	

60 °C for 30 s and 95 °C for 15 s in single degree steps. Primer efficiencies were calculated from readings derived from a standard curve of known DNA concentrations [10].

2.6. Western blotting

Total cellular extracts of ESCs, ADSCs, BMSCs and MDSCs were obtained for the Western blot analyses by lysis of cells in buffer containing 1% Nonidet P-40, 150 mM NaCl, 20 mM Tris, pH 8.0, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL aprotinin. Protein concentrations of the cell lysates were determined by Lowry assay. Aliquots of cell lysates containing 50 μg of proteins were electrophoretically separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with TBST buffer (10 mM Tris-HCl, PH8.0, 0.15 M NaCl, 0.05% Tween 20) containing 5% skimmed milk, incubated with primary antibodies against AKP, IL-6, Nanog, Oct-4, Rex-1, Sox-2, TERT, Sox-1, Myod1 and Gata-6 (Abcam, Cambridge, UK) overnight. This was followed by the addition of horseradish peroxidase-linked anti-rabbit IgG and enhanced chemiluminescence visualization of the protein bands. The intensity of each band was analyzed using ImageJ software.

2.7. In vivo differentiation and oncogenicity

In vivo differentiation and oncogenicity was assessed by investigating teratoma formation in a severe combined immunodeficiency (SCID) mouse. Approximately 1×10^7 morphologically undifferentiated goat ADSCs, BMSCs and MDSCs were injected under the dorsal skin of 7-week-old SCID mice. Mouse ESCs and physiological saline were also injected into different SCID mice serving as the positive and negative controls, respectively. Ten mice were in every group. After 6 weeks, teratomas were excized and fixed in 4% paraformaldehyde for histological examination (hematoxylin and eosin staining) [11].

2.8. Statistics

All data were analyzed using the SPSS 19.0 statistical package for Windows. One-way analysis of variance (ANOVA) was used to determine statistically significant differences among the groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Growth, morphology and identification of BMSCs and MDSCs

The growth of BMSCs was relatively fast and no morphological signs of cell aging were observed after 50 passages (Fig. 1 A–C). MDSC growth was also relatively fast and cell morphology did not indicate aging after 50 passages (Fig. 1D–F). Immunocytochemistry showed that BMSCs in long-term culture were positive for CD29, CD44 and CD90 and negative for CD31 and CD45 (Fig. 1). The MDSCs expressed Desmin, α-Sarcomeric Actinin, MyoD1, Myf5 and PAX7 (Fig. 1). Thus, these two cell types were confirmed to be BMSCs and MDSCs and were, therefore, suitable for the use in subsequent experiments.

3.2. Expression of stemness marker proteins in mESCs, ADSCs, BMSCs and MDSCs (immunocytochemistry)

All the stemness biomarkers tested (AKP, IL-6, Nanog, Oct-4, Rex-1, Sox-2 and TERT) were expressed in the mESCs. In the isolated ADSCs, BMSCs and MDSCs, AKP, IL-6, Nanog, Oct-4, Rex-1 and TERT were expressed, but Sox-2 was not. In FFCs, only IL-6 was expressed (Fig. 2).

3.3. Differential expression of AKP, IL-6, Nanog, Oct-4, Rex-1, Sox-2, TERT, Sox-1, Myod1 and Gata-6 mRNA in ADSCs, BMSCs and MDSCs (RT-qPCR)

The pattern of expression in mESCs was used as a reference, but because of the differences between species, the actual quantities of expressed mRNA and protein could not be compared between mouse ESCs and goat adult stem cells.

At the mRNA level, all seven stem cell biomarkers were expressed in mESCs; the four transcription factors, Nanog, Oct-4, Rex-1 and Sox-2 showed high expression, TERT moderate expression and IL-6 low expression ($P < 0.01$). High expression (relative to other markers) of IL-6, moderate expression of Nanog and Oct-4, and low expression of TERT and Rex-1 were found in ADSCs ($P < 0.01$). In contrast, high expression of Rex-1, moderate expression of IL-6, and low expression of Oct-4, Nanog and TERT was found in BMSCs ($P < 0.01$). High expression of IL-6, Nanog and Oct-4, and low expression of TERT was found in MDSCs ($P < 0.01$). However, no *Sox*-2 mRNA expression was detected in any of the cell types (Fig. 3).

We compared mRNA expression of the same genes in the three adult stem cell types and found differences in the levels of *Oct*-4, *Nanog*, *Rex*-1, *AKP*, *IL*-6 and *TERT* mRNA (Fig. 3A). IL-6 showed high expression in ADSCs: 826.28 and 0.88 times those in BMSCs and MDSCs, respectively ($P < 0.01$). Rex-1 was highly expressed in BMSCs: 294.12 times and 71.43 times those in ADSCs and MDSCs, respectively ($P < 0.01$). AKP, IL-6, Nanog, Oct-4 and TERT all exhibited high expression in MDSCs: for AKP, 17.02 and 23.32 times that in ADSCs and BMSCs, respectively ($P < 0.01$); for IL-6, 1.13 and 933.97 times, respectively ($P < 0.01$); for Nanog, 30.96 and 1794.22 times, respectively ($P < 0.01$); for Oct-4, 73.90 and 2052.87 times, respectively ($P < 0.01$); for TERT, 192.79 and 277.62 times, respectively ($P < 0.01$).

Also at the mRNA level, the mESCs expressed the ectoderm marker, Sox-1, the mesoderm marker, Myod1, and the endoderm marker, Gata-6, of which Sox-1 expression was the highest ($P < 0.01$). In ADSCs, Gata-6 had the highest expression, followed by Myod1 and Sox-1 ($P < 0.01$). In BMSCs, Gata-6 and Sox-1 were

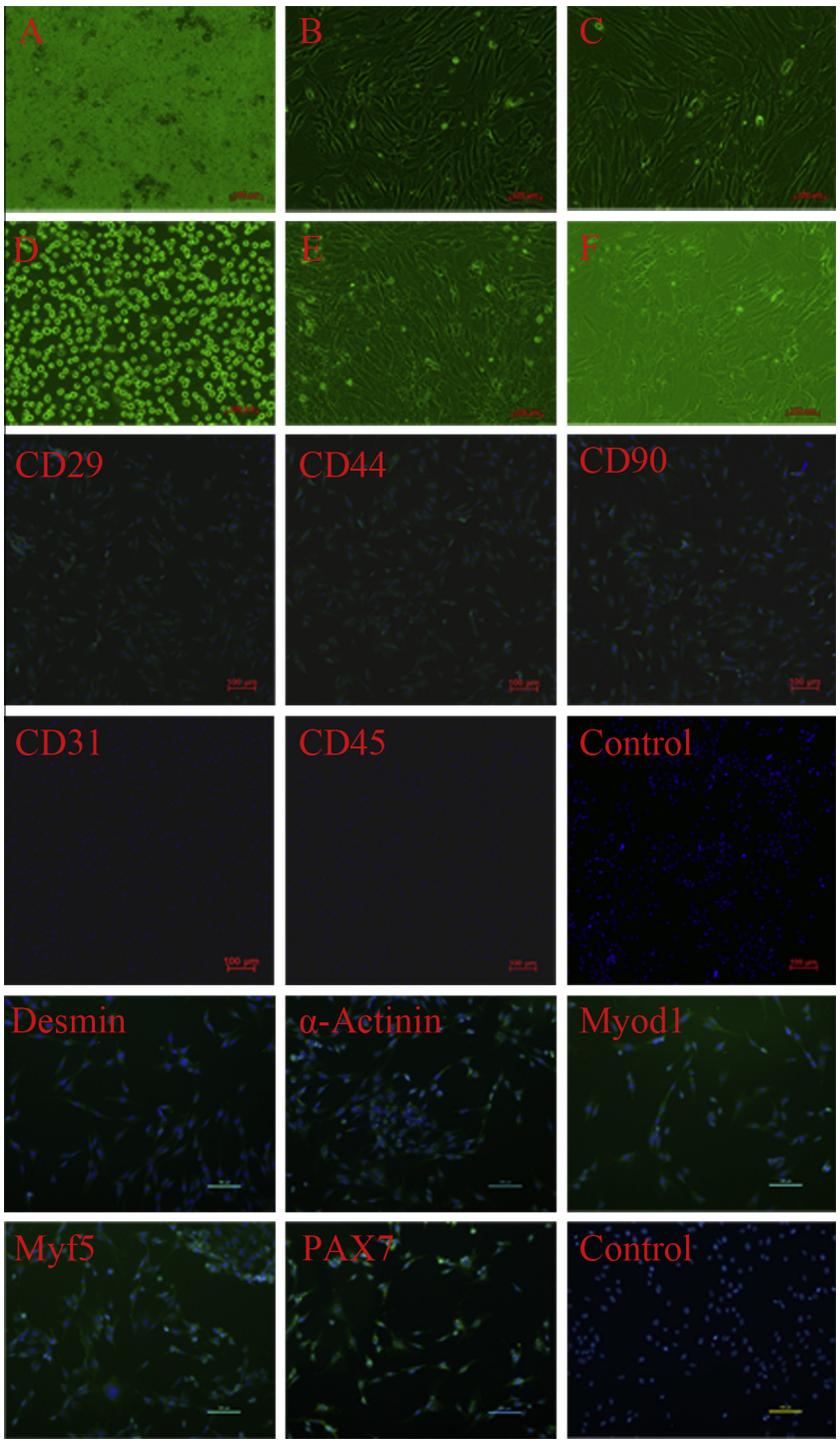

Fig. 1. Morphology and identification of BMSCs and MDSCs. (A) Non-adherent round BMSCs at the early stage of separation. (B) The second generation of BMSCs. (C) The 50th generation of gBMSCs. (D) Non-adherent round MDSCs at the early stage of separation. (E) The second generation of MDSCs. (F) The 50th generation of MDSCs.

more highly expressed than Myod1 ($P < 0.01$). In MDSCs, Myod1 and Gata-6 had comparatively high expression, with almost no Sox-1 expression ($P < 0.01$) (Fig. 3B). Considering all 10 markers, the characteristics of MDSCs were closest to those of ESCs.

After sequencing and sequence alignment, we found that the AKP, IL-6, Nanog, Oct-4, Rex-1, TERT, Sox-1, Myod1, Gata-6, in this study, respectively, showed approximately 99%, 99%, 97%, 98%, 98%, 97%, 92%, 100% and 100% similarity with the NM_001046242, HM565937, JQ801747, JQ284053, XM_004021847, NM_001046242, NM_009233.3, JF829005.1 and AB612145.1 sequences in the NCBI database. This showed that our reverse transcription qPCR products of *Oct*-4, *Nanog*, *Rex*-1, *AKP*, *IL*-6 and *TERT* were consistent with the targeted sequences and thus were not the result of non-specific amplification (Table 3).

3.4. Differential expression of AKP, IL-6, Nanog, Oct-4, Rex-1, Sox-2 and TERT proteins in ADSCs, BMSCs and MDSCs (Western blot)

All seven proteins were expressed in mESCs. No Sox-2 and Rex-1 protein expression was detected in ADSCs, BMSCs and MDSCs,

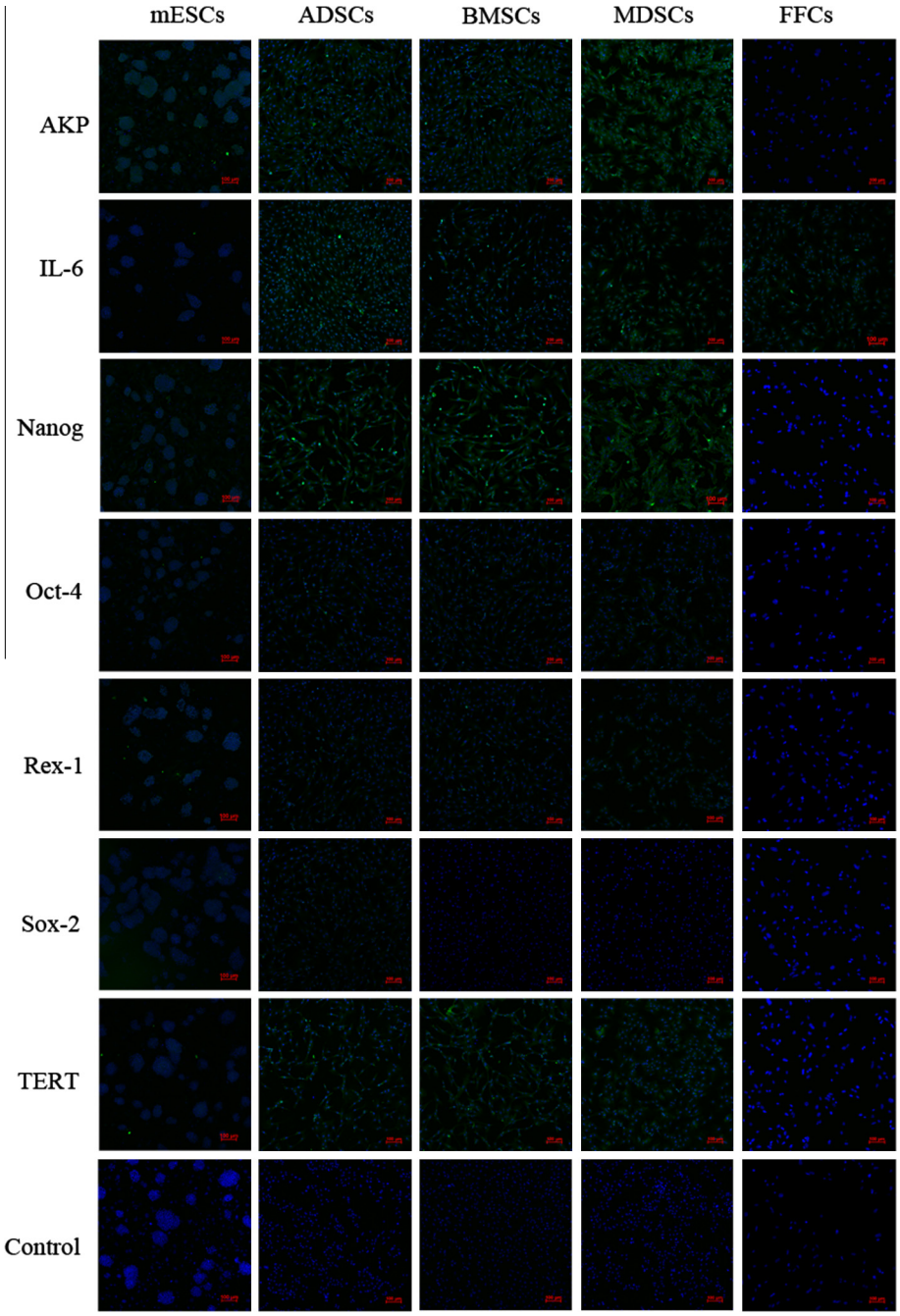

Fig. 2. Expression of AKP, IL-6, Nanog, Oct-4, Rex-1, Sox-2 and TERT in mESCs, ADSCs, BMSCs, MDSCs and gFFCs; detected via immunocytochemistry (FITC labeled antibodies).

while the other five proteins, Oct-4, Nanog, AKP, IL-6 and TERT were expressed to different degrees ($P < 0.01$). Among these, IL-6 showed significantly higher expression in ADSCs, 1.26 and 1.10 times that in BMSCs and MDSCs, respectively ($P < 0.01$). AKP expression was significantly higher in BMSCs, 1.47 and 1.29 times that in ADSCs and MDSCs, respectively ($P < 0.01$). Nanog, Oct-4 and TERT all had higher expression levels in MDSCs, being 1.48 and 1.38 times ($P < 0.01$), 5.94 and 1.53 times ($P < 0.01$), and 1.65 and 1.09 times ($P < 0.01$) those in ADSCs and BMSCs, respectively. Finally, when compared with ADSCs and BMSCs, Oct-4, Nanog, AKP, IL-6 and TERT showed higher protein expression in MDSCs. Thus, these Western blot results indicated that, at the protein level too, MDSCs had the strongest expression of stem cell specific markers (Fig. 4A and B).

3.5. In vivo differentiation and oncogenicity in ADSCs, BMSCs and MDSCs

None of the NOD/SCID mice injected with the goat ADSCs, BMSCs, MDSCs and physiological saline had formed teratomas or triploblastic differentiation appendages 6 weeks after injection ($n = 1$). In contrast, the NOD/SCID mouse injected with mESCs developed six teratomas 6 weeks after injection. The excized teratoma measured more than 4 cm in diameter. Histological examination showed that it contained a variety of tissue types, including epidermis tissue-like structures (ectoderm; Fig. 3C left), skeletal muscle (mesoderm Fig. 3C middle) and gland-like tissue (endoderm, Fig. 3C right), all originating from the three germ layers.

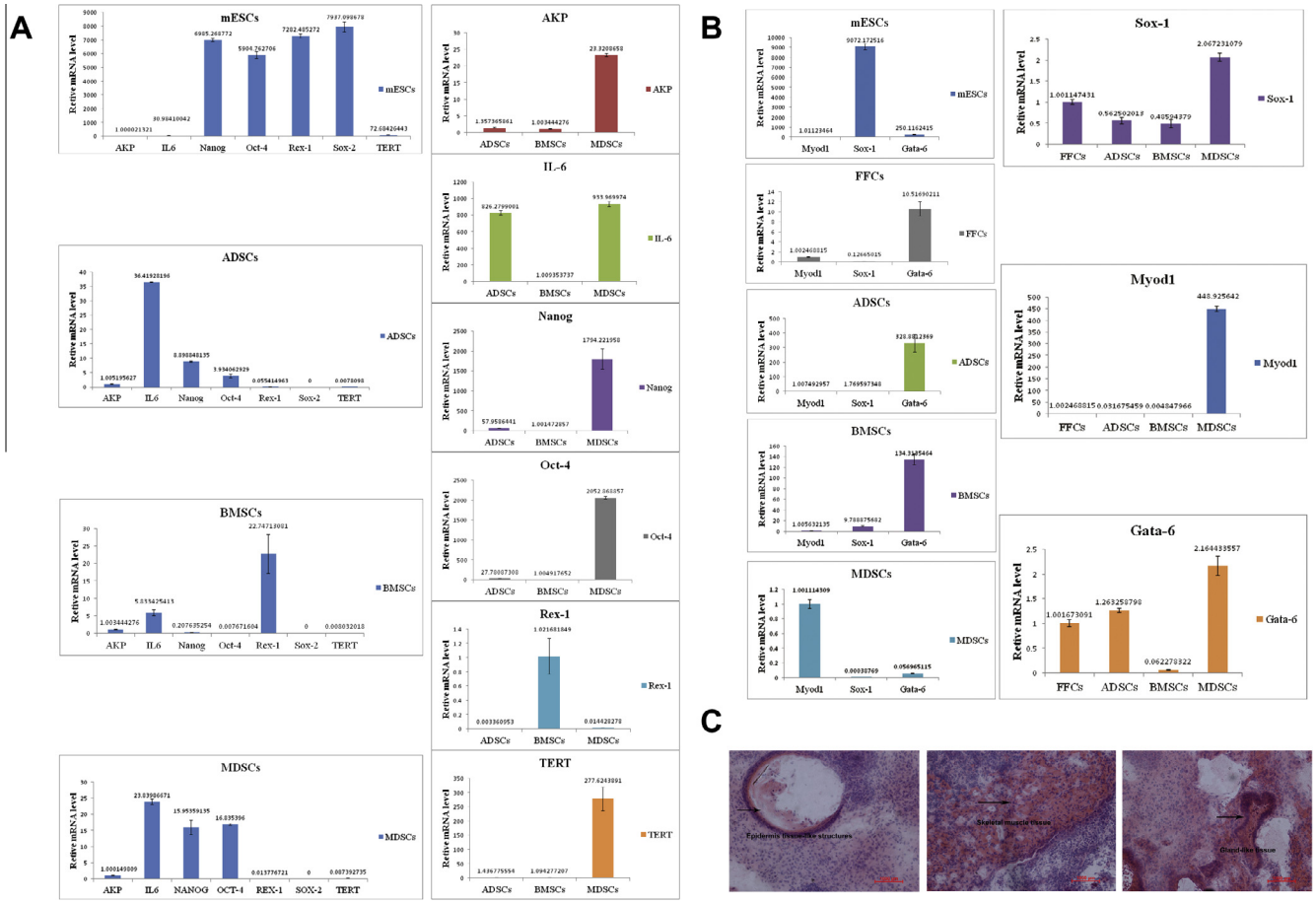

Fig. 3. Relative expression of (A) *AKP*, *IL*-6, *Nanog*, *Oct*-4, *Rex*-1, *Sox*-2, *TERT*, (B) *Sox*-1, *Myod*1 and *Gata*-6 mRNA in mESCs, ADSCs, BMSCs and MDSCs; measured by RT-qPCR and (C) teratoma formation.

Table 3
Sequence alignments.

Gene name	qPCR production sequences	Targeting sequences	Similarity (%)
AKP	TGTGGTCTGCAGTGGCAAGGATCAGCGTGTCCAGTTCGCTAGTGAGCTCG TTAGCCTTGGCGATGGCATTGTCAAACATGACTGTATCAGTCAGGGCCAT ATAAGCTTTGCCTTCATGG	NM_001046242	99
IL-6	TGATGACTTCTGCTTTCCCTACCCCGGGTCCCCTGGGAGAAGATTTCAAA AATGACACCACCCCAAGCAGACTACTTCTGACCACTCCAGAAAAAACCGA AGCTCTCATTAAGCACATCGTCGACAAAATCTCTGCAATAAGAAAGGAGA	HM565937	99
Nanog	TATGTGAAAAGAATGACGAGTGTGAAAACAGCAAGGAGACACTGGCA GTCTCTCCTCTTCCTTCCTCCATGGATCTGCTTATTCAGGACAGTCCTGA TTCTTCCACAAGCCCCAGAGTGAAACCACTGTCCCCATCTGCGGAGGAGA GCACAGAGAAGGAAGA	JQ801747	97
Oct-4	GCCAAGCTCCTAAAGCAGAAGAGGATCACTCTAGGATATACCCAGGCCGA TGTGGGGCTCACCCTGGGGGTTCTGTTTGGAAAGGTGTTCAGCCAAACGA CTATCTGCCGTTTTGAGGCTTTA	JQ284053	98
Rex-1	GCACACATCCTCATCACATAAGATCCGCGTCGTGTCAGGAGGCTCCTGCT GGGCTGGCAGTGACTTGTCTGTACGGGCAGCTCTTCTACCCAGGCCTTTC TGGCTAGGTGTTGTTCCCCTT	XM_004021847	98
TERT	GTCACAGAGACCACGTTCCAGAAGAACCGGCTCTTTTTCTTCCGGAAGCG CGTCTGGAGCCAGTTGCAGCGCCTGGGCGTCAGACAACACTTAGAGCGTG TGCGGCTTCGAGAACTGTA	NM_001046242	97
Sox-1	GTCTCTTGGCCTCGTCGATGTACGGCCGCTTCTCTGCCTCGGACAGAAGC TTCCACTCGGCACCTAGGCGTTTGCTGATCTCTGAGTTGTGCATCTTGGG GTTTTCCTGGGCCA	NM_009233.3	92
Myod1	ACGTCTAGCAACCCAAACCAGCGGCTGCCCAAGGTGGAGATCCTGCGCAA CGCCATCCGCTATATCGAAGGCCTGCAGGCGCTGCTTCGCGACCAGGACG CCGCGCCTCCCGGCGCTGCCGCTGCCTTTTACGCGCCTGGCCCGTTGCCC CCCGGCCGCAGCGGCGAACACTACAGCGGCGACTC	JF829005.1	100
Gata-6	CCTCATCAAGCCGCAGAAGCGCGTGCCTTCCTCCCGGCGGCTTGGATTGT CCTGTGCCAACTGTCACACCACGACCACCACTCTG	AB612145.1	100

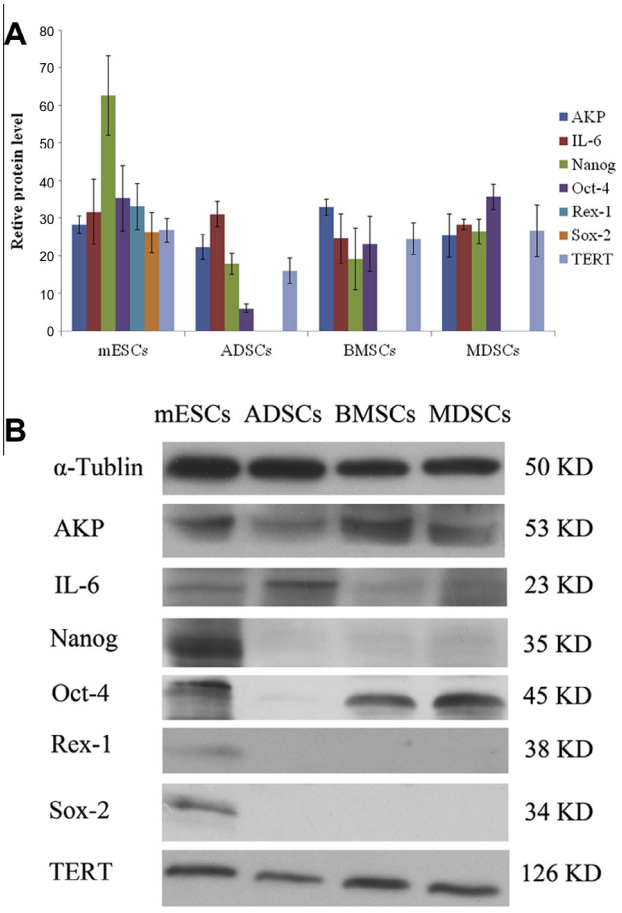

Fig. 4. (A) Relative expression of AKP, IL-6, Nanog, Oct-4, Rex-1, Sox-2 and TERT in mESCs, ADSCs, BMSCs and MDSCs, respectively. (B) Western blot results.

4. Discussion

In this study, the expression of several genes relating to the proliferation and differentiation of embryonic stem cells (AKP, IL-6, Nanog, Oct-4, Rex-1, Sox-2 and TERT) was investigated in adult stem cells at the mRNA and protein level. Our results showed that adult goat stem cells (ADSCs, BMSCs and MDSCs), expressed all of the above stemness markers, except Sox-2, suggesting that they may share the pluripotency and *in vitro* proliferation characteristics of ESCs.

Higher expression of TERT reflects an increased ability of cells to proliferate; telomere length plays a key role in deciding how many times a cell can replicate and extension of telomeres by telomerase is primarily determined by the expression of TERT [12,13]. In this study, TERT was highly expressed in ADSCs, BMSCs and MDSCs, indicating that these three isolated adult stem cells possessed a strong ability to proliferate *in vitro*, making them suitable for continuous subculture.

Nanog gene expression occurs in undifferentiated ESCs, embryonic carcinoma cells and embryonic germ cells, but it disappears in differentiated ESCs [14]. It has been shown that no *Nanog* mRNA expression occurs in differentiated fibroblasts and hematopoietic cells [15], and at present, expression has not been reported in any differentiated cells. This expression pattern indicates that *Nanog* is a key gene maintaining the proliferation and pluripotency of stem cells. Oct-4 is another core regulatory factor maintaining these characteristics of stem cells. In this study, Oct-4 and Nanog were expressed in ADSCs, BMSCs and MDSCs, indicating that these cells had characteristics similar to ESCs.

Sox-2 also plays a key role in maintaining stemness and is often used as a marker for pluripotent cells [16,17]. In this study, no Sox-2 expression was detected at the mRNA or protein levels; this may have been due to the absence of expression or to very low, undetectable expression in adult stem cells, at least in those derived from goat. Whatever the case, insufficient Sox-2 may be responsible for the loss of pluripotency in ADSCs, BMSCs and MDSCs.

Recently, successive studies have reported the expression profile of Rex-1 in cells of different tissues [18–20]. As well as adult pluripotent stem cells derived from bone marrow and heart, Mongan et al. [21] and Beltrami et al. [22] detected the expression of Rex-1 in human epidermal keratinization cells with strong renewal capacities, and in epithelial cells derived from prostate and lung. Expression disappeared gradually with increasing passage number, indicating that Rex-1 expression was closely related to the self-renewal capacity of the cell. However, the Niwa laboratory found that Rex-1$^{-/-}$ ESC lines could be established, and could differentiate normally *in vivo* and *in vitro*; implying that Rex-1 is not necessary for maintaining the self-renewal capability of stem cells and for embryonic development [23,24]. The status of Rex-1 as a core stemness marker remains unclear [25]. In our study, the three adult stem cell types all expressed *Rex*-1 mRNA to different degrees, with expression in BMSCs being the highest. However, no Rex-1 protein expression was detected in ADSCs, BMSCs and MDSCs, the cause of which might be related to the lack of Sox-2 expression. Therefore, the absence of Rex-1 might have affected the proliferation of the adult stem cells, so that they could not be passaged indefinitely compared with ESCs. In addition, whether the lack of Rex-1 also has an effect on the pluripotency of adult stem cells needs to be further investigated.

ADSCs, BMSCs and MDSCs are pluripotent stem cells derived from the mesoderm; all expressed the ectoderm marker gene, *Sox*-1, the mesoderm marker gene, *Myod*1, and endoderm marker gene, *Gata*-6, to different degrees. Mouse ESCs were used as a reference (not control), and the expression patterns of the three genes in ADSCs, BMSCs and MDSCs were different from that in mESCs. The expression pattern of the genes in ADSCs was similar to that in BMSCs, but different from that in MDSCs. The expression of the *AKP*, *IL*-6, *Nanog*, *Oct*-4 and *TERT* genes was highest in MDSCs at both the mRNA and protein levels, showing that among the three adult stem cell types, MDSCs exhibited a strongest stem cell characteristics, making them more suitable as seed cells for developmental biology research, cell engineering, and the development of novel functional gene-based drugs.

In conclusion, examination of the expression profiles of stemness marker proteins and mRNA, along with cell morphology and growth, suggests that goat adult stem cells possess similar characteristics to embryonic stem cells. Among ADSCs, BMSCs and MDSCs, the characteristics of MDSCs were clearly the closest to those of ESCs and thus, these cells offer the greatest potential for research and clinical application. Furthermore, such applications may be safer with adult stem cells, since, unlike the embryonic cells, all of the adult cells were found to be non-oncogenic in an immunodeficient (NOD/SCID) mouse over six weeks.

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