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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].

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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′	