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Biological Characteristics and Multilineage Differentiation of Kidney Mesenchymal Stem Cells Derived from the Tibetan Mastiff

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Of all the significant researches that have taken place in isolation, culture and characterization of mesenchymal stem cells (MSCs), the field of kidney-derived mesenchymal stem cells (KMSCs) in Tibetan mastiff is still a blank. Therefore, the purpose of this study is to isolate, culture and characterize the Tibetan mastiff KMSCs. The KMSCs were successfully isolated from one-day year old Tibetan mastiff kidney, cultured for 16 passages and distinguished by two methods: immunofluorescence staining and RT-PCR. The Tibetan mastiff KMSCs expressed specific surface marker genes (VIM, CD44, FN1, CD90, CD109, CD73, FN1) and kidney marker gene PAX2. The proliferation ability of Tibetan mastiff KMSCs was measured through cell count and clonality. Furthermore, cells differentiated into different cell types (hepatocellular cells, osteogenic cells, adipogenic cells and chondrogenic cells) under special induced medium, and the marker genes of induced cells were identified with Immunofluorescence staining and RT-PCR. All of these results indicated that the Tibetan mastiff KMSCs were obtained successfully, which possessed certain characteristics of multipotent stem cells. Therefore, MSCs in Tibetan mastiff kidney hold potential for clinical applications for regenerative therapy and their further studies are waiting to be required to investigate their functions.

Keywords: Tibetan Mastiff, Kidney, Mesenchymal Stem Cells, Biological Characteristic, Induce.

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1. INTRODUCTION

Mesenchymal stem cells (MSCs), also called multipotent mesenchymal stromal cells, exist in almost all tissues.[1] MSCs had been isolated from numerous different tissues, including bone marrow,[2–7] liver,[8] lung,[9] kidney,[10] nerve,[11] spinal cord,[12] amniotic fluid,[13] dermis,[14] adipose tissue,[15] amniotic membrane,[16] umbilical cord,[17] and MSCs are also a significant important key cell source for repair and regeneration of tissues and organs. Recently, there are several studies showed MSCs have come to be recognized as one type of adult stem cells actively participating in repair of tissues function.[18] Especially, Under the pathological case of acute injury, MSCs either in the immediate vicinity or those derived from bone marrow are believed to migrate into the damaged tissue.[1] Normally, apoptosis as a programmed cell death mode, does not trigger inflammatory reaction in the body. After apoptosis occurs, macrophages will quietly devour cell fragments. However, recently numerous researches suggest that even in the non-immune and the non-infectious injuries, pathological tissue damage usually involves the activation of immune cells and inflammatory cells, MSCs are mobilized towards the site of damage and release the corresponding cytokines to help the body repair injury.[1, 19–20] There are also some studies have shown that MSCs can affect a variety of physiological and pathological responses, such as immunity and inflammation.[21]

Mesenchymal stem cells have some properties that had distinguished them from other type of stem cells.[1] First, they are mostly unspecialized and are self-renewing. Second, they can be induced to differentiate into various specialized cell types, and thus hold promise for

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regenerative medicine.[22] Though of all the significant studies that have taken place in the MSCs, especially in the bone mesenchymal stem cells, there are no studies have taken place in the kidney mesenchymal stem cells of Tibetan Mastiff up to now. Tibetan Mastiff known as one of the oldest and most ferocious dog breeds in the world, and has been introduced and domesticated into many other countries as pet.[23] Tibetan mastiff shares many diseases common to human beings, w can be used as an animal model to provide theoretical support for human regenerative treatment in addition to treating diseases of Tibetan mastiff.

2. MATERIALS AND METHODS

2.1. Experimental Animals

Two one-day old male Tibetan mastiffs, weighting for 480–500 g, were supplied by Beijing Tibetan Mastiff breeding base (Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, China), maintained at a temperature of 22 °C under a 24 h light/dark cycle and had free to obtain food and water. All cell culture media were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA), unless otherwise provided. Animals were narcotized by intraperitoneal injection of sodium pentobarbital (100 mg/kg; Sigma-Aldrich; Merck Millipore). Animal experimental procedures were consistent with the Institutional Animal Care guidelines. The present study was approved by the Institute of Animal Science, Chinese Academy of Agricultural Sciences (Beijing, China).

2.2. Isolation and Culture of the MSCs from Fetal Kidney in Tibetan Mastiff

The one-day old Tibetan mastiff fetus were sacrificed by intraperitoneal injection of sodium pentobarbital, and then soaked with 75% alcohol for 10 min, transferred to the sterile operation table for 15 min by ultraviolet irradiation, and the bilateral kidney tissue of Tibetan mastiff was taken out. The bilateral kidney tissues were isolated from the fetal Tibetan mastiff and were washed 4–6 times by phosphate-buffered saline (PBS) without calcium and magnesium. The kidney tissues were cut into 1 cm^3 per piece with scissor into 60-mm dish and extensively wash these pieces with PBS until the tissue suspension is free of hemoglobin. The kidney tissues were digested with 0.2% (w/v) type IV collagenase (Gibco; Thermo Fisher Scientific, Inc., Grand Island, NY, USA) in DMEM/F12 with 10% fetal bovine serum (FBS; Gibco, Carslbad, CA, USA) and 1% penicillin/streptomycin for 30 min at 37 °C. Then use the 0.25% (w/v) trypsin and 0.02% (w/v) EDTA (Gibco; Thermo Fisher Scientific, Inc., Grand Island, NY, USA) to incubate the undigested kidney tissues for 15 min. By the end of digestion, neutralize the collagenase and trypsin activities with a constant volume FBS. The undigested tissue debris was removed by filtering single cell suspensions through a sterile 200 mesh screen, and the filtered single-cell suspension was centrifuged at 200× g for 8 min at temperature. After centrifugation, the supernatant of the cells suspension was removed, and then resuspended the bottom cells with the complete medium (DMEM/F12, 10% (v/v) FBS and 10^4 IU/ml penicillin/streptomycin, 10 ng/mL EGF, 10 ng/mL bFGF). After counting, the cells were inoculated on the 60-mm culture dish with the destiny of 1×10^6 cells/mL and incubate at 37 °C in 5% CO_2. After 24 h later, to observe whether the cells adhere to the wall, wash the cell dish twice with PBS, and then replace the complete medium. When the density reached about 80%, the cells were digested with warm trypsin (0.25% trypsin and 0.02% EDTA) and then seeded on a new culture plate. After 3–4 passages, the kidney mesenchymal stem cells in Tibetan mastiff were purified.

2.3. Immunofluorescence Detection of Kidney Cell Markers

Kidney mesenchymal stem cells of different generations were inoculated in 6-well plates with a density of 0.5×10^4. When the cells were fused to over 80%, the culture medium was removed and washed with calcium-magnesium-free PBS for 3 times. Cells were fixed with 4% paraformaldehyde solution for 30 min, fixed solution was removed, and cells were washed with calcium-magnesium-free PBS solution three times (5 min/time). Every hole added 1 mL 2 mol/L HCl, incubated in 37 °C, 30 min of incubation cell degeneration. The cells were washed with 0.01 mol/L sodium borate solution (5 min/time) to neutralize the cells. Cells were washed with PBS three times for 5 min each time. Add 1 mL 0.25% Triton X-100 for each well, let it through for 15 min. Cells were washed with PBS 3 times, 5 min each time. Before adding antibodies, 500 uL 10% goat serum was added to each well to block cells for 30 min. The cells were incubated with the following primary antibodies: Anti-CD109 (Ab199283, Abcam, Cambridge, MA, USA), anti-PAX2 (cat. no. bs-1187R), anti-VIM (cat. no. bs-3472R) and anti-CD90 (cat. no. bs-0778R) (all 1:100; Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) at 4 °C overnight or room temperature for one hour. Cells were washed three times (5 min per wash) with PBS, followed by with fluorescein isothiocyanate-conjugated goat anti-rabbit (cat. no. ZF-0314) secondary antibodies (1:100; Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) in dark for 1 h at room temperature or at 37 °C for 30 min. Removed the secondary antibody, washed the cells three times (5 min per wish) with PBS. Finally, useed 10 μg/mL DAPI (Solarbio Biotechnology Co., Ltd., Beijing, China) to label the cell nuclei in dark at room temperature for 15 min or at 37 °C for 15 min, and then, the images were captured using a Nikon TE-2000-E confocal microscope with an attached Nikon ZE-1-C1 3.70 digital camera system (Nikon Corporation, Tokyo, Japan).

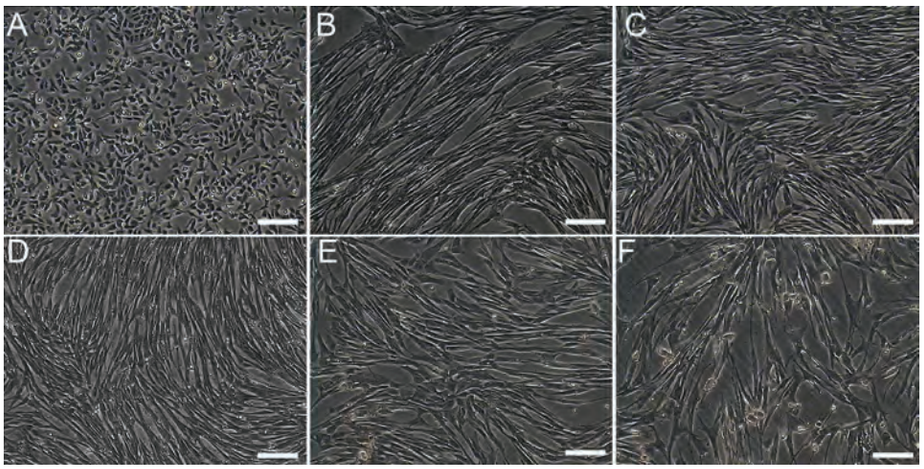

Fig. 1. Morphology of cultured KMSCs. (A) Passage 1, (B) passage 3, (C) passage 5, (D) passage 10, (E) passage 15, (F) passage 20, respectively. All cells were homogeneous and exhibited a spindle-shaped morphology during culture. KMSCs, kidney mesenchymal stem cells (scale bar = 100 μm).

2.4. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Different passages of mesenchymal stem cells were collected and used Trizol to extract total RNA from three generations of renal mesenchymal stem cells, and then used 2.0 μg total RNA as a template for reverse transcription into cDNA. The transcribed cDNA was amplified by PCR. Two percent agarose gel was prepared, and the productions of PCR amplification were analyzed by agarose gel electrophoresis.

2.5. Colony-Forming Cell Analysis

The Tibetan Mastiff KMSCs were arose from P5, P10 and P15, and were cultured in 60-mm plates at a density of 50–100 cells/well for about two weeks. When the clonal units were formed, fixed them with 4% paraformaldehyde for 15 min, then stained with 10% Giemsa solution at room temperature for 30 min, after that, washed them with flow water for 5 min, and then counted the numbers of colony-forming units. The cloning efficiencies were calculated as follows: Colony-forming unit number/$100 \times 100\%$.[2]

2.6. Growth Kinetics

The P5, P10 and P15 Tibetan mastiff KMSCs were plated in 6-well microplates at a density of 1×10^4 cells/well for growth kinetics analysis for eight days. The proliferation rules about KMSCs development of Tibetan mastiff were explored through observation on the growth curves. Hence, P5, P10 and P15 KMSCs were counted with the blood cell counting plate, and the growth curve was drawn according to the counting data. According to the mean values of cells, to graph growth curves.[10] Computed the population doubling times (PDT) as follows: $PDT = (t - t0)\log 2/(\log Nt - \log N0)$, where t = termination time of culture, $t0$ = start time of culture, Nt = the final number of cells in culture and $N0$ = initial number of cells in culture.

2.7. Cell Differentiation Assays

2.7.1. Hepatocellular Differentiation

The Tibetan mastiff KMSCs were seeded in 6-well plates at a density of 2×10^4 cells/well and separated into two groups: experimental group (induced MSCs into other kind of cells) and control group. When the cells reached 60–70% confluence, cells in the experimental group were replaced with the induced medium, while the cells in control group maintained in culture medium. The induction medium that induced MSCs differentiate into hepatocellular include DMEM/F12, 5% FBS, 10^4 IU/mL penicillin/streptomycin, 20 ng/mL HGF, 40 nmol/mL dexamethasone, 1% ITS liquid media supplement, 10 ng/mL interleukin-6 and 20 ng/ml EGF. The changes of cell morphology were observed every day and the new induction medium were replaced every two days. Two weeks later, cells in the two groups were fixed with 4% paraformaldehyde and detected by staining with periodic acid-Schiff stain (PAS) and hepatocellular genes (marker genes: AFP, ALB) were detected with RT-PCR.

2.7.2. Osteogenic Differentiation

The Tibetan mastiff KMSCs were seeded in 6-well plates at a density of 2×10^4 cells/well and separated into two groups as described above. When the cell density reached 70% after cell inoculation and the cell culture medium was replaced one kind of special functional inducer. So, the osteogenic differentiation was induced in 2-D monolayer cultures using osteogenic medium that consisted of DMEM/F12, 10% FBS, 10 mM β-glycerophosphate, 10 nM dexamethasone and 0.1 mM l-ascorbic-acid-2-phosphate. The osteogenic medium was changed every 2 days and cell morphology was observed. About three

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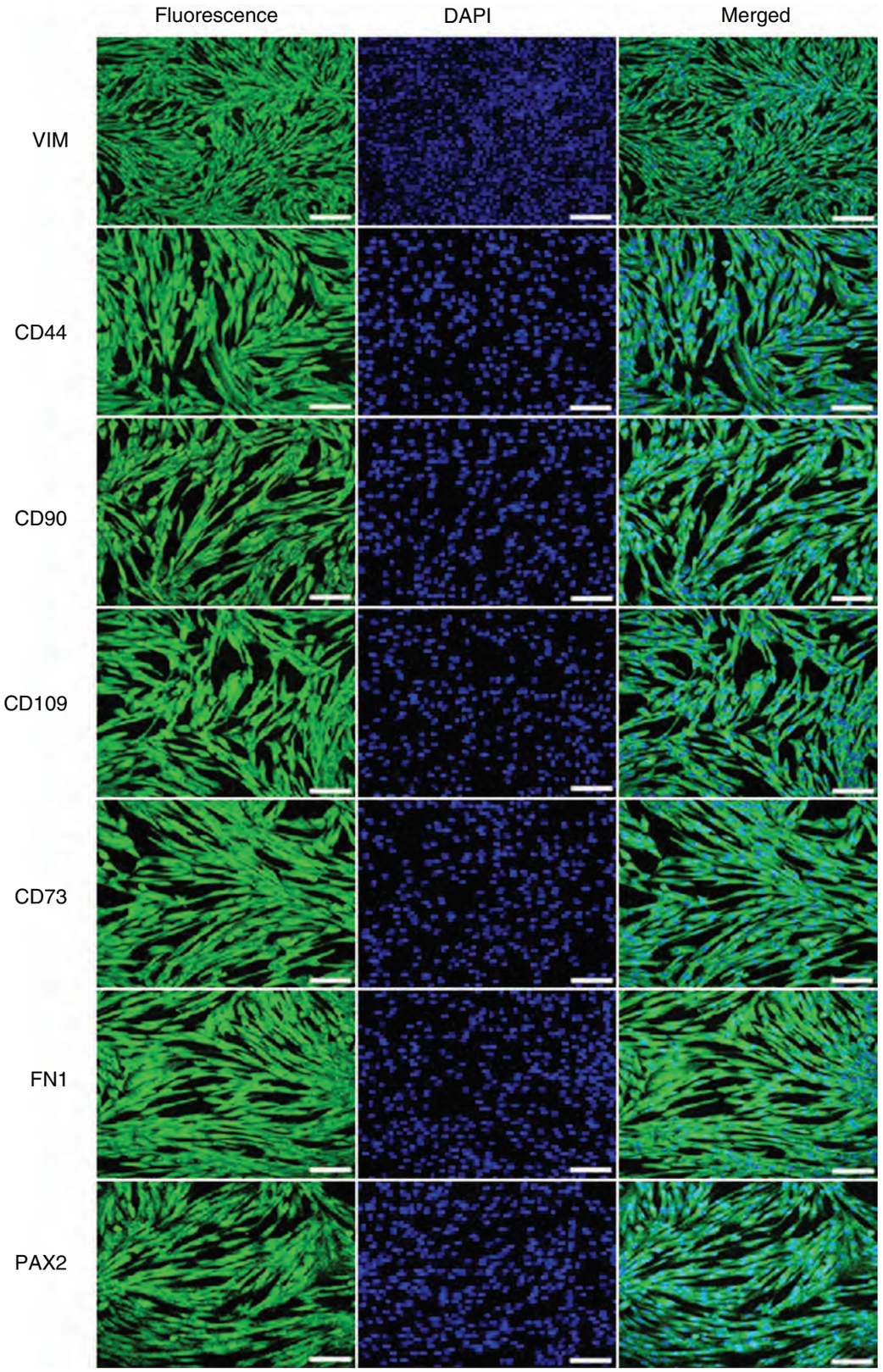

Fig. 2. Detection of kidney mesenchymal stem cell markers by immunofluorescence staining. The results show that VIM, CD44, CD90, CD109, CD73, FN1, PAX2 are positively expressed (scale bar = 100 μm).

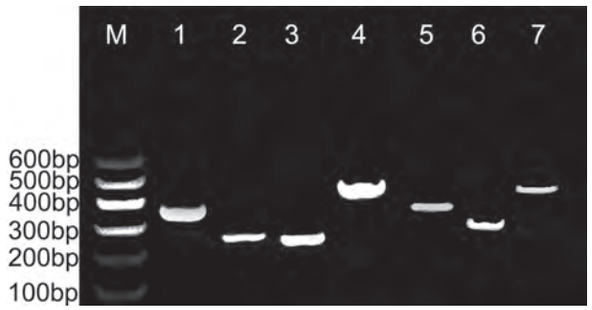

Fig. 3. Semi-quantitative polymerase chain reaction analysis of specific markers in kidney mesenchymal stem cells (KMSCs). The results revealed that R-MSCs expressed VIM, CD44, CD90, CD109, CD73, FN1, PAX2. M, Marker; 1, VIM; 2, CD44; 3, CD90; 4, CD109; 5, CD73; 6, FN1, 7, PAX2.

weeks later, the capacity of the cells for calcium node formation was determined by alizarin red staining, and osteoblast specific genes (marker genes: RUNX2, SPP1) were detected further using RT-PCR.

2.7.3. Chondrogenic Differentiation

The Tibetan mastiff KMSCs were seeded in 6-well plates at a density of 2×10^4 cells/well and separated into two groups as described above. When the cells reached 60–70% confluence, cells in the experimental group were replaced with the induction medium, while the cells in control group maintained in culture medium. The induction medium that induced MSCs differentiate into chondrogenic cells include DMEM/F12, 10% FBS, 1% ITS, 50 μg/mL L-proline, 0.1 μm dexamethasone, 0.9 mM sodium pyruvate, 50 ug/mL L-ascorbic acid and 10 ng/mL transforming growth factor-β3. Observed and recorded the cells morphology every day and exchanged the induced

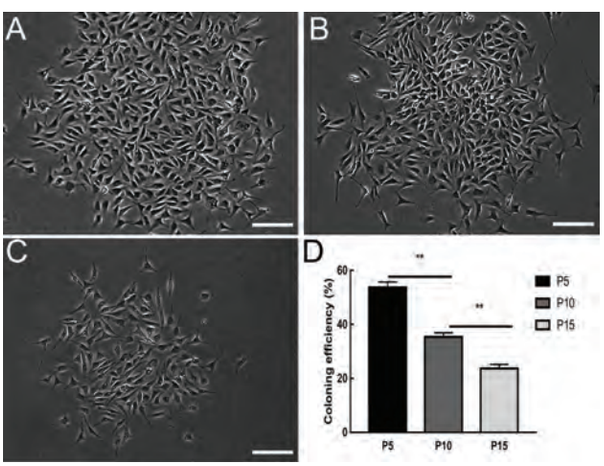

Fig. 4. Colony forming efficiency of kidney mesenchymal stem cells (KMSCs). Colonies with the morphology of KMSCs were cultured for 14 days. (A) P5, (B) P10 and (C) P15 (scale bar = 100 μm). (D) Bar chart showing the cloning rates for different passages of R-MSCs ($^{**}P < 0.01$).

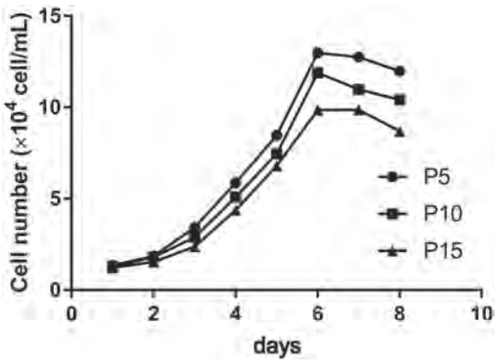

Fig. 5. Growth curves of kidney mesenchymal stem cells (KMSCs). The growth curves of P5, P10 and P15 MMSCs were all typically sigmoidal, with cell density reflected by the vertical axis. The growth curve consisted of a latent, logarithmic and plateau phase. The population doubling times were 33.32, 35.33 and 47.04 h at P5, P10 and P15 respectively.

culture medium every two days. Two weeks later, cells in the two groups were fixed with 4% paraformaldehyde and detected by staining with Alcian blue and chondrogenic cells genes (marker genes: SOX9, COL-2) with RT-PCR.

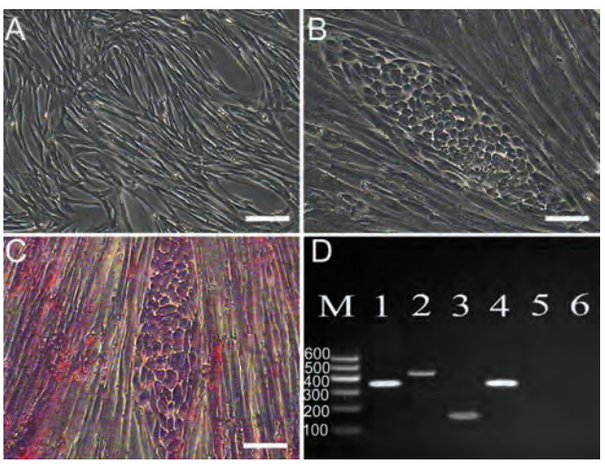

Fig. 6. Hepatocellular differentiation of kidney mesenchymal stem cells (KMSCs). (A) As a negative control, cells cultured in complete medium showed no changes in morphology and were negative for periodic acid-Schiff staining (PAS) staining. (B) After 14 days of differentiation, the cells displayed a rising/piled morphology with dark cytoplasm and light nuclei that were stained with PAS (scale bar = 100 μm). (C) Expression of hepatocyte-specific genes AFP and ALB were detected by reverse transcription-quantitative polymerase chain reaction in the induced group after induction for 14 days. Hepatocyte-specific genes were not expressed in the control group. Lane 1: GAPDH served as the internal control in the inducted group; lane 2: AFP was positive in the inducted group; lane 3: ALB was positive in the inducted group; lane 4: GAPDH served as the internal control in the control group; lane 5: AFP was negative in the control group; lane 6: ALB was negative in the control group. M, Marker 100–600 bp, molecular ladder.

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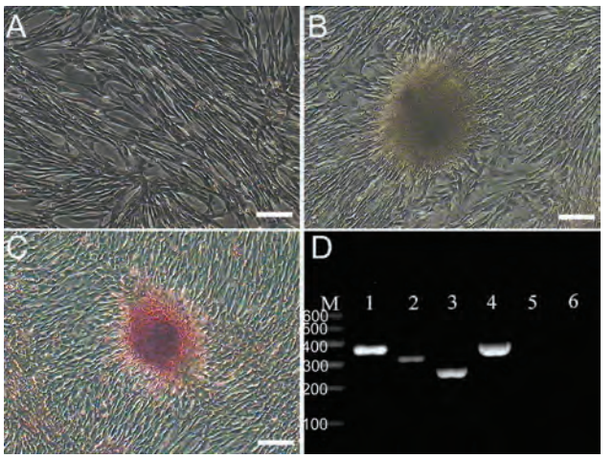

Fig. 7. Osteogenic induction of KMSCs. Morphological analysis of the osteogenic induction of R-MSCs was performed. The cells were incubated in inducing medium for 4 weeks. Following induction, cells were positively stained with Alizarin Red S: (A) Control group; (B) cells were induced for 14 days and without Alizarin Red S staining; and (C) cells were induced for 14 days and stained with Alizarin Red S positively. (D) Semi-quantitative-polymerase chain reaction analysis of osteoblast-specific genes. Lanes 1 and 4 were GAPDH for inducted group and control group, respectively. The induced group expressed the osteogenic-specific genes RUNX2 (lane 2) and SPP1 (lane 3), whereas the control group (lanes 5 and 6) didn't express. KMSCs, kidney mesenchymal stem cells. RUNX2, runt related transcription factor 2. SPP1, secreted phosphoprotein. M, Marker 100–600, molecular ladder.

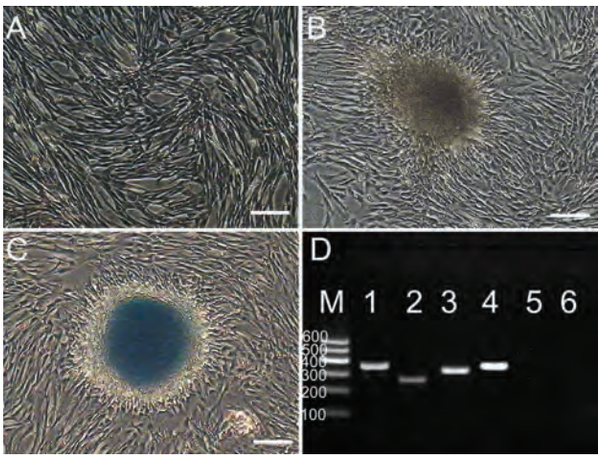

Fig. 8. Chondrogenic differentiation of kidney mesenchymal stem cells (KMSCs). (A) As a negative control, cells cultured in complete medium showed no changes in morphology and were negative for Alcian blue staining. (B) After 20 days of differentiation, the cells showed obvious morphological changes and formed colonies that didn't be stained with Alcian blue (scale bar, 100 μm). (C) Chondrogenic colonies were stained with Alcian blue (scale bar = 100 μm). (D) Expression of cartilage-specific genes SOX9 and COL-2 were detected by reverse transcription-quantitative polymerase chain reaction in the induced group after induction for 20 days. Cartilage-specific genes were not expressed in the control group. Lane 1 and lane 4: GAPDH served as the internal control respectively in the control group and induced group. Lane 2: SOX9 was positive in the inducted group. Lane 3: COL-2 was positive in the inducted group. Lane 5: SOX9 was negative in the control group. lane 6: COL-2 was negative in the control group. M, Marker 100–600 bp, molecular ladder.

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3. RESULTS

3.1. Isolation, Culture and Morphology of KMSCs in Tibetan Mastiff

After the isolated KMSCs were cultivated in a petri dish for 24 h, it was found that some cells had adhered to the well, the cells were round, polygonal or irregular shapes, and some cells and blood cells had not adhered to the well. After primary cultured for 48 h, the cell morphology gradually showed long spindle shape, most of the cells were fibrous. After five days of primary cultured, the cell coverage at the bottom of the petri dish reached 80–90%. A variety of hybrid cells were mixed in the primary cells. After subculture for 3–4 generations, the hybrid cells were basically removed, and the remaining cells had uniform morphology without significant morphological difference. *In vitro* culture of MSCs lasted for 16th generations. With the improvement of generation, cells gradually aged, showing slow cell proliferation, poor cell morphology, flat growth, cell cavitation and apoptosis disintegration.

3.2. Analysis of Self-Renewal, Proliferation and Differentiation

Three different generations (P5, P10, P15) of KMSCs in Tibetan mastiff were inoculated on the petri dish and observed them experience incubation period, logarithmic growth period, plateau period and decline phase, and the growth curve presented a typical "S" shape.

After the incubation period of the first two days, the cells entered the logarithmic growth phase on the third day, reached the plateau on the sixth day, and began to decline on the seventh day. Population doubling time in the three generations of P5, P10 and P15 were 33.38, 35.90 and 48.09, respectively. As shown in the results, the proliferation rate of cells decreased with the increase of cell sub generations.

The liquid was changed every two days after the inoculation of KMSCs (P5, P10, P15). KMSCs were didn't treated with 4% paraformaldehyde and Giemsa staining until the cells grew into a clone group. After Giemsa staining, the clone formation rate was counted. The clonal formation rate of P5 generation was $56.80\% \pm 2.52\%$, that of P10 generation was $34.58\% \pm 3.06\%$, and that of P15 generation was $20.89\% \pm 2.08\%$. The clone formation rate of P5 generation was significantly higher than that of P10 generation and P15 generation, and that of P10 generation was significantly higher than that of P16 generation.

3.3. Characterization of MSCs in Tibetan Mastiff

3.3.1. Markers of MSCs

The specific markers of KMSCs of Tibetan mastiff were detected by immunofluorescence staining and RT-PCR analyses. The results of immunofluorescence staining

showed that the KMSCs expressed CD44, CD90, CD109, VIM and PAX2. Similarly, the results of RT-PCR showed that KMSCs positively expressed CD44, CD90, CD109, VIM, PAX2, CD73.

3.3.2. Hepatocellular Differentiation of MMSCs

Mesenchymal stem cells of Tibetan mastiff kidney were induced with hepatocellular medium. After 7 days, only a few round cells appeared, the cells displayed obvious morphological changes. 14 days later, the cells showed a three-dimensional "pavers" morphology with light nuclei and dark cytoplasm that were stained with PAS. Cells cultured in complete medium were used as invisible control, and the cells stained with PAS were not stained. Cells were identified with RT-PCR after inducing for 14 days, and the results indicated that the cells expressed the alpha-fetoprotein (AFP) and albumin (ALB) genes specially labeled by hepatocytes, while the control group didn't express the specific genes.

3.3.3. Chondrogenic Differentiation of MMSCs

The mesenchymal stem cells of Tibetan mastiff kidney were cultured in chondrogenic medium and grew slowly. The KMSCs were induced for 7 days, the morphology of MSCs changed and presented clonal growth. 12 days later, the KMSCs showed obvious morphology changes and presented numerous colonies. While, after differentiation for 20 days, Alcian blue stained the colonies and counted the colonies. However, the control group cells cultured with total medium showed no significance. The results of RT-PCR represented that cartilage-specific genes SOX9 and COL-2 were optimistic expressed in the differentiated group, but negatively in the control group.

3.3.4. Osteogenic Differentiation of RMSCs

The KMSCs were cultured with osteogenic medium and presented significant alterations in appearance. With the prolongation of inducing time, significantly changes in the cells morphology and the aggregates increased gradually. 4 weeks later, cells were fixed with 4% paraformaldehyde and stained with Alizarin Red S to observe. The results of RT-PCR presented that osteogenic-specific genes RUNX2 and SPP1 were expressed in the induced group, but negatively in the control group.

Table I. Primer sequences used for reverse transcription-polymerase chain reaction.

Gene	Primer sequences	T_m (°C)	Product length (bp)
VIM	F: CTCTCTGAGGCTGCTAACCG R: TGGAAGAGGCAGAGCAATCC	59	357
CD44	F: CTGCCCAATGCCTTTGATGG R: GGCTGGAGTCCATATTGGTAA	58	283
CD90	F: TGTGGGACATGCCTCAACAG R: GGGAGTTCAAGGTGGCAGTT	60	265
CD109	F: CGTCTCGGATTGCTAAACGC R: AATCTCATCCTGGGCACGTC	59	465
CD73	F: GCGTGTTTTGTCAGGGTTCC R: AGTGGCCCCTTTGCTTTGAT	60	275
FN1	F: AGGAAAAAGACAGGACAAGAAGC R: GGTCGAAGCACGAGTCATCT	59	304
PAX2	F: CACGGGGGTGTGAACCAG ATCATTGGAGGCGCTGGAAA R: ATCATTGGAGGCGCTGGAAA	60	492
GAPDH	F: CCGCGTCTTCTGGTGCTG CTCCATGGTGGTGAAGACCC R: CTCCATGGTGGTGAAGACCC	60	354
AFP	F: CAGCCACTTGTTGCCAACTC R: CCCAAAGCAGCACGAGTTTT	53	451
ALB	F: TGCCACCGATGATCCTCCTA R: TTGGGGTGCTTTCTTGGTGT	60	169
sspp1	F: TGGCTAAAGCCTGACCCATC CACACTATCACCTCGGCCAT R: CACACTATCACCTCGGCCAT	55	318
RUNX2	F: ATCTTGCAACCACGAGAGGG GAGGGCGTGTGGTTTCAAAG R: GAGGGCGTGTGGTTTCAAAG	60	258
SOX9	F: CTCCGGCATGAACGAGGTG R: GCGGCAGGTACTGGTCGAAT	62	254
COL2A1	F: ACCAGATTGAGAGCATCCGC GCCAGGTTGTCATCACCGTA R: GCCAGGTTGTCATCACCGTA	60	310

4. DISCUSSION

The firstly isolated cells contain a lot of other types of cells, included epithelial cells and blood cells, which could be removed by fluid exchange because they didn't adhere to the well. Epithelioid cells were flat in shape and closely adhered to the culture dish. In the process of cell passage, the digestion time of epithelioid cells was longer than that of renal mesenchymal stem cells. Therefore, the digestion time can be controlled. In the process of culture, the survival time of other hybrid cells *in vitro* was shorter due to the unsatisfactory conditions. Pure kidney mesenchymal stem cells were obtained after subculture for 3–4 generations. In the case of low cell sub generation, the cells increased rapidly, and the cell morphology was plastically. However, with the increase of culture sub generation, the cell growth raised slowly, the cell started to grow like a flat, and the contour became worse. When the cells were cultivated up to more than 20 generations, it could be seen that the cell had a cavity and the growth rate was slow. The results of clonal formation analysis and growth curve showed that the cell growth activity decreased with the increase of generation. Gradually, cells became large flat, grew slowly, and the expansion ability decreased.

International Society for Cellular Therapy (ISCT) defined the criteria for the identification of mesenchymal stem cells as follows: Firstly, MSCs must grow adherently. Secondly, MSCs must express the proteins of VIM, CD44, CD90, CD109, CD73 and not express the protein of CD34 and CD45. Thirdly, MSCs must differentiate into other special kinds of cells, such as hepatocytes, osteocytes and chondrocytes-like cells by special inducers.

Immunofluorescence staining and RT-PCR were used to identify the surface marker genes of KMSCs (genes: VIM, CD44, CD90, CD109, CD73, FN1, PAX2). VIM as a type IV intermediate filament protein that is expressed in mesenchymal stem cells,[24] plays an excellent role in maintaining and regulating cell functions. CD90 also known as Thy-1, the progenitor marker gene CD90 exhibited strong expression,[25] just like CD109. CD44 is a widely distributed cell surface adhesion molecule involved in cell proliferation, differentiation and migration, and CD44 as a receptor for hyaluronic acid and a protein coding gene, encodes a cell-surface glycoprotein involved in cell adhesion, cell–cell interactions and migration such as collagens, osteopontin and matrix metalloproteinases.[10, 26–28] CD44 as a significant receptor of MSCs released can repair the trabecular meshwork of damaged eyes, under the effect of low immunogen of MSCs, intracameral stem cells injections appear to be helpful in preserving trabecular architecture and cellularity after acute trabecular injury.[29] CD73, other known as ecto-5′nucleotidase, is a glycosyl-phosphatidylinositol-linked 70-kD molecule expressed on different cell types,[30] and also is an extremely important type of stem cell surface marker. CD73 is a signaling molecule and has been shown to participate in purine salvaging and purinergic cascades that lead to cell metabolism.[31] With the development of studies in CD73, it was found that CD73 is widely present in most tissues and controls a variety of physiological functions, such as epithelial ion exchange, liquid transport, platelet function, tissue hypoxia and avascular leakage.[32] From what has been discussed above, CD73 plays an extremely important role in the transformation of ATP into adenosine, especially, it can make AMP into adenosine,[33–35] and the role of CD73 is particularly important in cell proliferation and differentiation. FN1 is one type of gene that encodes fibronectin, a soluble dimeric glycoprotein present in extracellular matrix and in a dimeric or multimeric from at the cell surface, can promote the connection between cell and fiber matrix.[36] In addition to the above description, moreover, fibronectin is present on the surface of myeloma-derived exosomes and enhances exosome-cell interactions and involves in cell adhesion and migration processes that including embryogenesis, wound healing, blood coagulation, host defense and metastasis.[37] Paired Box2 (PAX2), encodes a nuclear protein that binds with DNA, serving as a transcription factor.[38] PAX2, presented in fetal kidneys, numerous studies found that PAX2 plays a significant regulatory factor in embryonic kidney development, including nephrogenic cord induction and duct formation, ureter germination, nephrogenic branch configuration and nephron establishment.[38, 39] Moreover, loss and overexpresses of PAX2, all of abnormal expression of PAX2 results in hereditary and acquired kidney diseases.[40, 41] The extraordinary markers were detected by immunofluorescence staining and RT-PCR to identify the identity of KMSCs.

As mentioned earlier, MSCs also known as pluripotent mesenchymal stromal cells, exists in almost tissues and are the key cells sources for tissue repair and regeneration.[1] Role of MSCs in tissue repair and acute inflammation, such as under acute immune response pathological conditions, just like tissue injury,[20] lymphocytes,[18, 19] monocytes and neutrophils release some of inflammatory cytokines and immunoregulatory factors to the site of injury, which activates MSCs migrate the site of injury and release trophic factors, such as endothelial cells and fibroblasts.[1, 8, 9, 18–21] KMSCs, as one of the types of mesenchymal stem cells, will also drive themselves to transfer to the damaged site and release nutritional factors in the case of acute kidney injury. Moreover, KMSCs are immunodeficient cells with low immunogenicity and immunoregulatory characteristics.[1] This feature will play an important role in clinical kidney transplantation and acute inflammatory response. It has been confirmed that KMSCs have multipotent and could differentiate into other special kinds of cells.[2, 10, 12] For example, the KMSCs not only have ability to self-renew, but also have the potential to differentiate *in vitro* into hepatocytes, osteocytes and chondrocytes-like cells by special inducers.[2] It is the distinct characteristic distinguish them from other kinds of stem cells. It's proved that using the "homing ability" of KMSCs, inject specific MSCs to the site of injury for future clinical treatment once more.

5. CONCLUSION

In conclusion, the KMSCs were successfully isolated from fetal Tibetan mastiff and identified by immunofluorescence staining and RT-PCR. The self-renewal ability and differentiation potential of Tibetan mastiff KMSCs were analyzed *in vitro*. Emerging data presented that KMSCs have an multidirectional differentiation potential, and thus these cells will be crucial for developing potential therapeutic applications and for more possibilities for regenerative medicine.

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