Differentiation Potential Analysis of Bone Marrow Mesenchymal Stem Cells from Guizhou Miniature Pigs

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Abstract. In this study, different growth characteristics of hematopoietic stem cells and bone marrow mesenchymal stem cells were used to isolate and culture BMSCs by whole bone marrow adhesion method, and RT-PCR was used to detect cell surface markers CD29, CD31, CD34, CD44, CD71, CD73, CD90. The differentiation of osteocytes into adipocytes and osteoblasts was induced *in vitro*, and the differentiation ability was observed by staining with oil red O and rubinin. To investigate the isolation and culture methods of BMSCs from Guizhou miniature pigs (*Sussa Ivanius*) *in vitro*, and to study their biological characteristics and potential for multi-differentiation. The results showed that a small number of adherent cells could be seen in the primary BMSCs cultured *in vitro* within 48 hours, and the confluent cells reached 90% in 5~6 days. The cell growth state was good, and the shape was spindle and polygonal. RT-PCR was used to detect the positive expression of CD29, CD44, CD71, CD73 and CD90 on the cell surface, and the negative expression of CD31 and CD34. Oil red O staining showed the formation of lipid droplets in the cells, and alizarine red staining showed calcium nodules in the cells, indicating that the cells isolated and cultured in this experiment had the ability of multidirection differentiation. It is consistent with the characteristics of BMSCs. The growth of BMSCs cultured *in vitro* was stable, and the expanded BMSCs had multidirectional differentiation potential, which was an ideal seed cell for tissue engineering.

Keywords: Guizhou miniature pig; Bone marrow mesenchymal stem cells; Isolation culture; Induced differentiation.

1. Introduction

Bone marrow mesenchymal stem cells (BMSCs)[1] have the potential of self-replication, regeneration and multi-differentiation, and are easy to obtain, prolifically rapid in vitro proliferation, good gene stability through passage, and low immune rejection. It has always been a hot spot in the research of induced differentiation of stem cells [2-5]. The most important characteristic of BMSCs is their multidirectional differentiation potential. BMSCs can differentiate into osteoblasts with aggregates or nodules and increase alkaline phosphatase activity[6]. The use of semasone, insulin and growth factor β1 can promote the differentiation of BMSCs into chondrocytes[7-8]. In addition, BMSCs were successfully induced to transform into neuron-like cells by using α-merhydryl alcohol, dimethyl sulfoxide, and buhydroxyanisole, and specific neuronal markers were expressed[9]. The addition of 1-methy-3-isobutylxanthine, dexamethasone and insulinase in the medium can rapidly differentiate BMSCs into adipocytes[10].

BMSCs have good osteoblast morphology, proliferation and osteogenic ability[11]. The relative number of BMSCs in the bone marrow tissues of normal people or animals is relatively small. Therefore, the cultivation and isolation of sufficient number and high purity BMSCs can provide necessary seed cells for the successful development of subsequent in vivo and in vitro experiments[12-13]. Domestic pigs are genetically closer to humans than other commonly used laboratory animals such as mice, which is of great significance for the application of domestic pigs in medical research and drug testing[14]. In this study, through the isolation and purification of porcine BMSCs in vitro, bone marrow adhesion culture, differentiation of bone marrow mesenchymal stem cells by cell cycle, identification of surface markers and induction of differentiation (osteoblasts, lipoblasts, neuro-like cells and islet cells), new seed cells were provided

for bone tissue engineering research. It also provides theoretical basis for further study on the clinical application of BMSCs.

2. Materials and Methods

2.1 Experimental Animal

Healthy miniature pigs were selected from the experimental base of Laboratory Animal Research Institute of Guizhou University of Traditional Chinese Medicine, aged three months.

2.2 Experiment Reagent

L-DMEM base medium and trypsin (Gibco); Fetal bovine Serum (FBS) (Gibco); PBS buffer salts (Hyclone); 100X streptomycin (Solarbio); EGF and BFGF(Peprotech); PI, DMSO, TritonX-100 and L-glutamine (Sigma Corporation); Paraformaldehyde (Beijing Chemical); Oil red O staining solution; Ruarubin S staining solution; 2×Taq PCR-MIX (Beijing); Reverse transcription kit (Beijing Chemical); All other reagents were domestic analytical pure reagents.

2.3 Experimental Method

2.3.1 Isolation of pig bone marrow cells

The piglets were anesthetized, bloodied and killed, soaked in 75% alcohol for 15 minutes, then the surface hair was scraped, killed and dissected under sterile conditions, and the femur and tibia were isolated. The adherents were rinsed repeatedly with PBS containing 1% streptomycin and removed. The exposed bone marrow cavity was rinsed repeatedly with basic medium for 5 to 6 times. The rinsing solution was collected into a sterile centrifuge tube, centrifuged at 1200 rpm/ min for 8 minutes, the supernatant was discarded, and the cells were suspended with L-DMEM medium (containing 10% FBS+10ng/mL bFGF+10ng/mL EGF+2mM glutamine + penystreptomycin). Inoculated into 6-well culture plates. It was cultured in a 37℃, 5% carbon dioxide incubator. After 3days, half of the liquid change. After 5 days, the whole fluid was changed, and then the consumption of cells and culture medium was observed.

2.3.2 Cell culture and proliferation

When the cell fusion rate reached 80%-90%, the subculture was carried out. Discard the old medium, rinse it with PBS buffer for 2 to 3 times, add 0.25% trypsin, gently shake the dish to make the cells fully in contact with trypsin, put it in an incubator at 37℃ for 2 to 3 minutes, observe it under an electron microscope, when most of the cells shrink into balls and suspend in the medium, Add 2 ml L-DMEM whole culture solution to terminate digestion, and gently blow dispersed cells. The passage was carried out at the ratio of 1:2, and the cell growth was observed and the fluid was changed every day. Let's call the primary cell P0, the first cell P1, and so on.

2.3.3 Cell reverse transcription and RT-PCR detection

RNA extracted from generation P3, P8 and P10 were selected as 6 uL templates, and cDNA libraries were synthesized according to the instructions of reverse transcription kit. The surface antigen molecules of CD29, CD31, CD34, CD44, CD71, CD73 and CD90 were identified by RT-PCR. The PCR system is shown in Table 1.

Table 1. The RT-PCR reaction system

Reaction system	Dosage
forward primer（10μm/L）	1μL
reverse primer（10μm/L）	1μL
*Taq*DNA polymerase	10μL

cDNA template	2μL
ddH_2O	6μL

Take GAPDH as the internal parameter. Primer references[14] are shown in Table 3, and RT-PCR amplification conditions are shown in Table 2.The PCR products were detected by 1% agarose gel electrophoresis.

Table 2. RT-PCR amplification conditions

Temperature	Time	Cycle index
94℃	Predegeneration 5min	35 cycles
94℃	Degeneration 30s	
55℃	Backout 30s	
72℃	Primer extension 10min	

Table 3. Primer information of gene amplification

Gene	Primer sequence	Temperature/℃	Product size/bp
CD90	F:5-GGCATCGCTCTCTTGCTAAC-3 R:5-CCCCTCATCTTTGGTAGTGAAG-3	60	285
CD73	F: 5-GAGAGTCATTTATCCAGCAGTTGA-3 R: 5-TCTTTTCCTGTGCGTTCTGTAA-3	59	319
CD71	F: 5- CTCGGCAGGTAGATGGTGATA-3 R: 5-GGTGTTTGAGGGAAGTTTTCTG-3	60	304
CD44	F: 5-AAATGGGTTTCTTGGCATCT-3 R: 5-CTGGGGTGTTTGTCTCTTTCAT-3	59	128
CD29	F: 5-TGATTGCTGGTTCTACTTCACA-3 R: 5-TTCCCTCATACTTCGGATTGAC-3	58	302
CD34	F: 5-CGAAACCGTGAACTCTTCTGT-3 R: 5-GCATCAACTTCTCTCCATTGTC-3	60	323
CD31	F: 5-GCCCATTTCCTACCAACTTTTA-3 R: 5-GGCTTGTTCTCCTTTTCTTTGT-3	59	318
GAPDH	F: 5-GCTCTGGAGACTGCTGAACGA-3 R: 5-CTAACGACAAGGCGGTGATGC-3	60	364

2.3.4 Induced differentiation of cells *in vitro*

The eighth generation cells with good cell growth were selected, and when the confluent rate reached 80%-90%, digestion was performed with 0.25% trypsin, and terminated with L-DMEM full medium. The cells were inoculated into 6-well plates at a density of 1×105, with three replicates per well. The control group was inoculated with L-DMEM medium containing 10%FBS. When the cell fusion rate reached 60%, it was replaced with lipogenic and osteoblast induction solution, and the solution was changed every 3 days. Stain with alizarin red at about 10, 15, 20 days after osteogenic induction, and stain with improved oil red O at about 7, 10, 14 days after lipogenic induction.

3. Result and Analysis

3.1 Primary culture of cells

Early observation of the morphology of pig bone marrow cells showed that there were a large number of mixed suspended cells, including blood and stroma. After 24 hours, a small number of cells can be seen attached to the wall, in the shape of bars, stars or fusiform, irregular shape. After 48 hours, the full dose of fluid was changed again to remove all the cells that did not stick to the

wall. In the first 72 hours, the growth rate of primary cultured cells was slow, and after 24 hours, the proliferation rate of cells became fast and the cells became elongated. As the number of fluid changes increased, the suspended cells were expelled from the body, and after about 7 days, the fusion rate of the cells reached 90% and filled the entire petri dish(Fig.1).

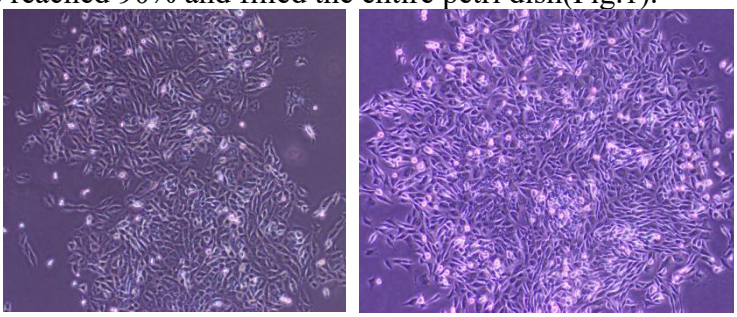

Fig. 1 Morphology diagram of BMSCs (100×)

3.2 Observation of cell culture

After passage, the cells quickly adhered to the wall and prolifed rapidly, and the whole dish could be filled with cells after about 2-3 days, with uniform distribution, orderly cell arrangement and few stray cells. The cells of the 2-4 generations grew the most vigorously and had a short cell passage cycle with regular shape. When the cells were transmitted to the 6th generation, the cell proliferation rate slowed down and the cell passage cycle became longer. After the eighth generation, the phenomenon of aging gradually appeared(Fig.2).

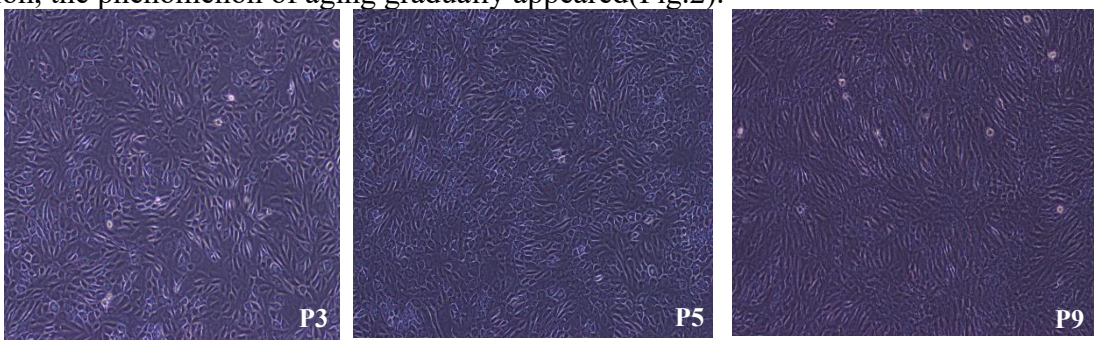

Fig. 2 Morphology diagram of BMSCs (100×)

3.3 Cell surface antigen identification

Cell surface markers were detected by RT-PCR. It can be seen from the results that CD29, CD44, CD71, CD73 and CD90 of the five MSCs specific porcine bone marrow surface markers were positive, while endothelial cell specific markers CD31 and hematopoietic stem cell specific markers CD34 were negative (Fig.3).

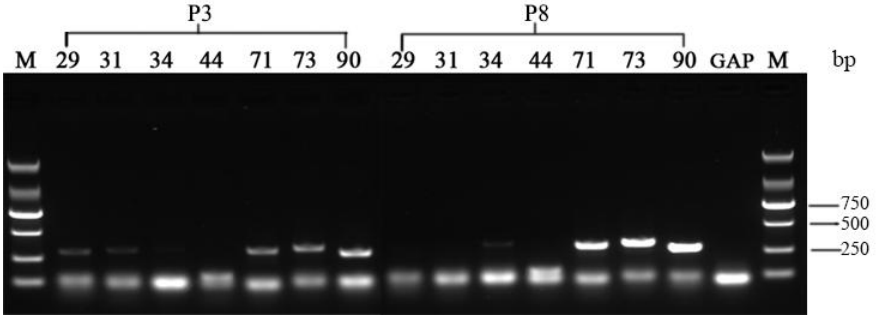

Fig. 3 Electrophoresis results were determined by PCR of bone marrow marker gene expression in P3 and P8 generation pigs

3.4 Cell induced differentiation

During the induction process of in vitro osteogenesis, the cell morphology of P5 generation BMSCs gradually became shorter. After 21 days of induction, the induced cells were stained with rubitin, and the calcium nodules formed became red and eventually formed red dense nodules (Fig.4A). The results showed that the cultured porcine bone marrow cells could be successfully induced to develop into osteoblasts. After induction into fat, the cell shape on the cell surface also showed some irregular changes. Fifteen days after successful induction, the cells were stained with modified oil red O, and bright fat droplets were found in the cells, but no fat droplets appeared without induction, and the fat droplets in the cells could be dyed red by the modified oil red O (Fig.4B). Fig.4B shows that porcine bone marrow cultured in vitro can induce adipocytes.

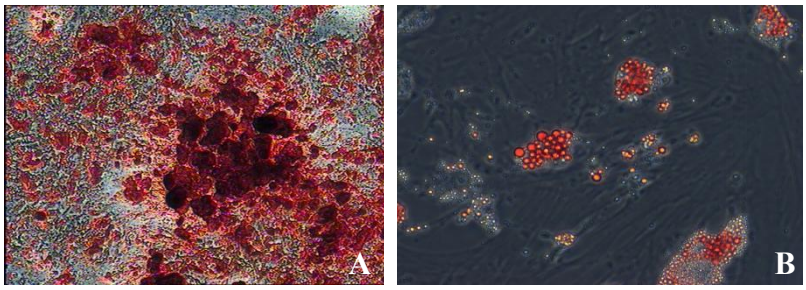

Fig. 4 A. Alibibin staining after induction of BMSCs in P 5 generation pigs (×100)
B.O staining in modified oil after induction of BMSCs in P 5 generation pigs (×100)

4. Summary

In this experiment, the porcine bone marrow cells isolated by the adherent separation method were used for morphological observation. The results showed that a small amount of porcine bone marrow cells were adherent to the wall when cultured for 24 hours in primary culture, and the adherent cells were basically adherent to the dish around the 7th day. When the cells were inverted under the microscope, it was observed that most of the cells were spindle-shaped and elongated, and most of them were distributed like whirlpools. In the 1:2 ratio of passage, the primary cells expanded more slowly than the post-passage cells, and the bottom of the petri dish was filled in about 3 days. In this experiment, it was found that bone marrow cells transmitted to more than the sixth generation, the growth and proliferation of cells became slow, the cell passage cycle became longer and the cell shape was not so regular, if the passage culture continued, the cells could not completely stick to the entire petri dish and there would be some vacuoles, and the cells gradually began to age after the eighth generation. Morphological analysis of cell adhesion growth showed that it was consistent with the characteristics of BMSCs[15].

Studies have shown that the surface of bone marrow expresses CD29, CD44, CD71, CD73, CD90 and other cell surface antigens, but does not express CD31, CD34 and other cell surface markers[16], among which CD34 is a typical surface antigen of hematopoietic cells. The results showed that CD29, CD44, CD71, CD73 and CD90 in pig bone marrow were positive for the surface markers of these five different types of mesenchymal stem cell specific markers, while endothelial cell specific markers of CD31 and hematopoietic stem cell specific markers of CD34 were negative in vitro. After differentiation into bone and adipocytes, osteoblasts were identified by alizarin red staining, and adipocytes were stained by modified oil red O staining. The results showed that osteoblasts showed red dense nodules after staining, and fat cells formed red fat drops after staining, which provided experimental basis for further in vitro culture. These results showed that mesenchymal stem cells could be induced to differentiate into osteoblasts, lipoblasts, and multidirectional differentiation, which laid a good foundation for further in vitro induced differentiation and clinical application.

Acknowledgments

The author contributed equally to this work.

References

[1] Duan Changwei, Chai Yanjie, Zhao Jiangdong, et al. Isolation and culture of bone marrow mesenchymal stem cells. Ningxia Medical Journal, 2019, 43(6):573-576.

[2] Yu Haisheng, Wang Ning. Research progress of methods for promoting the proliferation of bone marrow mesenchymal stem cells. Chinese Pharmacy, 2014, 25 (37) : 3532-3534.

[3] Watanabe K, Ikeda K. Osteoblast differentiation and bone formation. Nippon Rinsho, 2009, 67(5):879-886.

[4] Agha-Hosseini F, Jahani MA, Jahani M, et al. In vitro isolation of stem cells derived from human dental pulp. Clin Transplant, 2010, 24(2):E23-28.

[5] Cen HH, Han CM, Lai PP, et al. Isolation, culturation and adipogenisis committed differentiation of adult human mesenchymal stem cell. Zhejiang Da Xue Xue Bao Yi Xue Ban, 2003, 32(2):137-140.

[6] Tseng Y, Chen CJ, Sheu CC, et al. Spontaneous differentiation of adult rat marrow stromal cells in a long-term culture. J Vet Med Sci, 2007, 69(2):95-102.

[7] Han Liqiang, Jiang Yi. Research progress on the origin of bone tissue engineering seed cells. International Journal of Osteology, 2006, 27(1): 28-9

[8] Chiu CH, Chang TH, Chang SS, et al. Application of bone marrow-derived mesenchymal stem cells for muscle healing after contusion injury in mice. Am J Sports Med, 2020, 48(5) : 1226- 1235.

[9] Cortes Y. Ojeda M. Araya D, et al. Isolation and multilineage differentiation of bone marrow mesenchymal stem cells from abattoirderived bovine fetuses. BMC Vet Res, 2013, 9(1) : 133-146.

[10] KOBOLAK J, DINNYES A, MEMIC A, et al. Mesenchymal stem cells: identification, phenotypic characterization, biological properties and potential for regenerative medicine through biomaterial microengineering of their niche. Methods, 20l6, 99(12): 62-68.

[11] Wei ZJ，Fan BY，Liu Y, et al. MicroRNA changes of bone marrow- derived mesenchymal stem cells differentiated into neuronal-like cells by Schwann cell-conditioned medium. Neural Regen Res, 2019, 14(8) : 1462-1469.

[12] Chiu CH，Chang TH，Chang SS,et al. Application of bone mar- row-derived mesenchymal stem cells for muscle healing after con- tusion injury in mice. Am J Sports Med, 2020, 48(5) : 1226- 1235.

[13] Zhang J, Cheng T, Chen Y, et al. A chitosan-based thermosensi-tive scaffold loaded with bone marrow-derived mesenchymal stem cells promotes motor function recovery in spinal cord injured mice. Biomed Mater, 2020, 15(3) : 035020.

[14] Shan ZQ. Study on isolation and biological characteristics of Bama miniature pig bone marrow mesenchymal stem cells. Shanxi Agricultural University, 2015.

[15] Cao Y, Vacanti JP, Paige KT, et al. Transplantation of chondrocytes utilizing a polymer-cell construct to produce tissue-engineered cartilage in the shape of a human ear. Plast Reconstr Surg, 1997, 100(2):297-302.

[16] Pontikoglou C, Delorme B, Charbord P, et al. Human bone marrow native mesenchymal stem cells. Regen Med, 2008, 3(5) : 731-741.