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In vitro cultivation of rat bone marrow mesenchymal stem cells and establishment of pEGFP/Ang-1 transfection method

Xiu-Qun Zhang[1,2], Long Wang[2], Shu-Li Zhao[2], Wei Xu[1*]

[1]*Department of Hematology, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China*

[2]*Department of Hematology, Nanjing First Hospital, Nanjing Medical University, Nanjing 210006, China*

PEER REVIEW

Peer reviewer

Malinee Pongsavee, Ph.D., Associate Professor, Head of Medical Technology Department, Faculty of Allied Health Sciences, Thammasat University, Rangsit Campus Patumthani 12121, Thailand.
Tel: 662-9869213 ext.7252
Fax: 662-5165379
E-mail: malineep@tu.ac.th

Comments

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ABSTRACT

Objective: To obtain the bone marrow mesenchymal stem cells (BMSCs), complete phenotypic identification and successfully transfect rat BMSCs by recombinant plasmid pEGFP/Ang-1.
Methods: BMSCs were isolated from bone marrow using density gradient centrifugation method and adherence screening method, and purified. Then the recombinant plasmid pEGFP/Ang-1 was used to transfect BMSCs and the positive clones were obtained by the screen of G418 and observed under light microscopy inversely. Green fluorescent exhibited by protein was enhanced to measure the change time of the expression amount of Ang-1.
Results: BMSCs cell lines were obtained successfully by adherence screening method and density gradient centrifugation. Ang-1 recombinant plasmid was transfected smoothly into rat BMSCs, which can express Ang-1 for 3 d and decreased after 7 d.
Conclusions: Adherence screening method and density gradient centrifugation can be effective methods to obtain BMSCs with high purity and rapid proliferation. Besides, the expression of transfected recombinant plasmid pEGFP/Ang-1 in rat BMSCs is satisfactory.

KEYWORDS
Bone marrow mesenchymal stem cells, Angiopoietin-1, Transfection method

1. Introduction

Bone marrow mesenchymal stem cells (BMSCs), obtained from a variety of sources, are expolited for autologous transplantation which can entirely circumvent the problem of immune rejection and do not cause the formation of teratomas. Besides, BMSCs have the ability of self-renewal and multi-directional differentiation. As BMSCs are considered to be hypoimmunogenic and have low capacities of antigen presentation, they can also be used for allotransplatation with good tolerance and may avoid host humoral and/or celluar immunity. Under different conditions, they may further differentiate into all mesoblast-derived tissue cells such as adipose cells, vascular endothelial cells, osteoblast cells and neuroglial cells[1-4]. It can be concluded that transplantation of BMSCs provides a new idea to repair the damages of various systematic diseases and cell replacement therapy in clinical practice[5,6]. Based on the aboved mentioned advantages, it is evident that BMSCs can not only be used for the replacement of defective or absent cells in defective tissues and organs, but also serve as the effective vectors of gene fragment. According to the studies by Lou *et al*. and Yoo *et al*.[7,8], as BMSCs have higher rates of gene transfection

*Corresponding author: Wei Xu, Department of Hematology, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China.
Tel: +86-25-83781120
Fax: +86-25-83781120
E-mail: xuwei10000@hotmail.com
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and may further differentiate into corresponding tissues after they enter different tissues through transfection, they can be used as the effective vectors for gene therapy, which therefore provoke the interests of many reaserchers in the fields of hepatic cells researches worldwide.

2. Materials and methods

2.1. Materials

Four to six Sprague-Dawley (SD) rats about 4-6 years old with the average weights of (110.1±10.2) g were selected for BMSCs cultivation. All the SD rats were purchased from Comparative Medical Center of Yangzhou University and fed in Animal Experiment Center, Nanjing First Hospital, Nanjing Medical University. Eukaryotic expression vector pEGFP-N1 was constructed by GenScript (Nanjing) Co., Ltd.

2.2. Reagents

Dulbecco's modified Eagle's medium with L-glutamine (L-DMEM) medium was the product of Invitrogen corporation. ethylene diamine tetraacetic acid, fetal calf serum (FBS), peocoll and Hanks' balanced salt solution were obtained from Hyclone. FuGENE® HD transfection reagents were purchased from Ebiotrade. Rabbit anti-rat CD antibody including 31 polyclonal antibody (CD 31), 44 polyclonal antibody (CD 44), 45 polyclonal antibody (CD 45) and 105 polyclonal antibody (CD 105).

2.3. Isolation, cultivation and identification of BMSCs

2.3.1. Isolation and cultivation of BMSCs

Adherence screening method proposed by Franklin *et al*.[9] and peocoll density gradient centrifugation[10] were adopted to obtain BMSCs. Two 4 week-old SD rats were selected and executed by neck shot. Then the bilateral femurs were chosen, stripped off muscle and fascia and cut off compact bone substances at both ends of femurs to fully expose the marrow cavities, which were then washed by L-DMEM containing 15% FBS until the color of bone substances turned grey repeatedly. Then the aliquots were transferred to the centrifuge tube, centrifuged at 1 000 r/min for 5 min and added fresh medium to mix completely. Finally, single cell suspensions were prepared. It should be noticed that cell suspensions were gently added to the upper layer of peocoll-Paque separation medium (density 1.077), centrifuged at 2 500 r/min for about 20 min, then the mononuclear cells layer was transferred to L-DMEM containing 15% FBS and centrifuged at 1 000 r/min for 5 min. Cell sediment was diluted with L-DMEM containing 15% FBS after washed twice, then cell counting was conducted. Cells were inoculated in the culture flask at the density of $1\times10^7/cm^2$ and cultured at 37 °C in 5% CO_2 cell incubator. Medium were changed after 2 days and every 3 days thereafter until the cells reached 70%-80% confluence. Then 0.25% trypsin with 0.02% ethylene diamine tetraacetic acid solution was used to dissociate the adherent cells, washed the cells with D-hanks liquid and subcultured at the proportion of 3:1.

2.3.2. Indentification of rats BMSCs

Cells were innoculated at the density of 1×10^4/mL after two to three times of passages (P2-P3) in 6-well plates containing coverslip and CD 31, CD 44 ,CD 45 and CD 105 were determined by immunological histological chemistry (IHC). Detailed steps were as following: when the degree of cell fusion reached 70%-80%, the cells were washed with phosphate buffer for three times for about 10 min each time, then they were fixed with 10% paraformaldehyde for 0.5 h divided by three times. At the same time, cells were immersed in the mixture of 30% H_2O_2 and pure methanol (1:50) for 15 min and washed with distilled water for three times. About 3% bovine serum albumin was added, and 0.5 h later, diluted rabbit anti-rat CD 31 polyclonal antibody, CD 44 polyclonal antibody, CD 45 polyclonal antibody and CD 105 polyclonal primary antibody were added and stood in the condition of 4 °C overnight. The aliquots were washed with phosphate buffer solution (PBS) for three times, labled with phycoerythrobilin and florescein isothiocyanate and added the secondary antibody simultaneously to coincubate at 37 °C for 0.5 h. Then the aliqouts were washed with PBS for three times repeatedly. According to the manufacture's instruction of DAB Horseradish Peroxidase Color Development Kit, slides can be dehydrated, cleared and permanently mounted. When the color decayed, the slides were observed under microscope carefully. The observed brownish yellow particles in the cytoplasm were considered as positive cells. PBS was used to replace the primary antibody as the negative control group.

2.4. Transfection of recombinant plasmids into BMCSs by FuGENE® HD transfection reagents

After P2-P3, BMSCs were inoculated in 6-well plates at the density of 1 to 2×10^5/mL and cultivated for 19-24 h untill the cells reached 50%-80% confluence. Preparation of solution A in the polystyrene tube: DNA containing 2 μg plasmids were fused into 100 mL serum-free medium. Preparation of solution B: 8 μL HD reagent was diluted and then transfered into serum-free medium with the total volume reached to 100 μL. Solutions A and B were homogenized and kept at room temperature for 15 to 30 min. Then DMEM was removed, and the cells were washed with serum-free medium only one time and added into 0.8 mL serum-free medium again. The aliquots were transferred into the mixture of HD transfection reagent and plasmid DNA, mixed evenly and gently titrated into cells, which were cocultivated in 5% CO_2 at 37 °C incubator. One or two days after cultivation, the transfecting liquid was removed, and 2 mL complete

medium was added for subculture. Two or three days after transfection, when close to the fusion state, cells were subcultured at the proportion of 1:4 until the cells density reached 50%-70% confluence. Then DMEM was removed and substituted by 500 μg/L G418 medium (the G 418 medium could be qualified for the following trials for G 418 concentrations only when the death rates of cells about 13 d decreased to the lowest level under the condition that the diluted cell density was 1 000/mL and the concentration of G 418 medium was at the range of 100 to 10 000 μg/mL). In our study, the selected concentration of G 418 medium was 500 μg/mL and cells without transfecting liquid (blank control group) were compared. If a large proportion of cells death occurred in the blank control group, the medium was changed every 3 to 5 days and its concentration might decrease to 150-250 μg/mL to sustain the function of screening. Ten to twenty days later, resistant clone was formed and increased in volume until it was transferred to 24-well plates.

2.5. Detection of Ang-1 expressions in BMSCs by western blot

Transfected BMSCs and non-transfected BMSCs were separately collected, washed twice with PBS and added 60-100 μL cell lysis buffer. The aliquots were oscillated for several minutes, cooled on ice for 0.5 h, centrifuged at 12 000 r/min for 5 min and the supernate was discarded. Bradford assay was used to determine concentrations of protein samples in the extracted medium. Electrophoretic separation was conducted in sodium dodecyl sulfate polyacrylamide gel electrophoresis (120 g/L) and then protein was transferred to polyvinylidene difluoride membrane which contained 50 g/L defatted milk powder. The membrane was blocked in the Tris-buffered saline with Tween 20 buffer for about 1.25 h at 37 °C, sequentially rabbit anti-rat Ang-1 antibody was added which was diluted to 1:1 000 prematurely and co-incubated overnight at 4 °C. While the aliquots were washed for three times about 10 min each time with Tris-buffered saline with Tween 20 solution, it was developed by chemical fluorescence method. Gel images were obtained and analysed.

3. Results

3.1. BMSCs morphology and identification by IHC

Firstly from the perspective of cells morphology, BMSCs were round mononuclear cells suspended in the medium to exhibit high refractivity under light microscopy. As shown in Figure 1, BMSCs were found to be of varioius morphology such as irregular polygon, short shuttle-like shape 4 to 5 days later. After P3, BMSCs were found to be in fusiform or spindle shape. The cells had only a few impurities from bone marrow, and cell arragements were obviously spiral or radial shaped (Figure 2). On the other hand, immunohistochemistry (results showed that basically all CD 44 and CD 105 in BMSCs were expressed as positive (Figures 3 and 4), and CD 31 and CD 45 were expressed as negative (Figures 5 and 6). It can be concluded that the observed cells were definitely BMSCs.

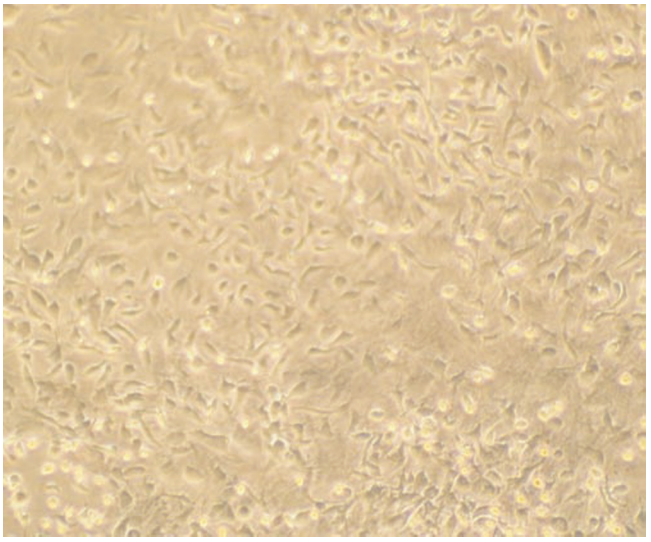

Figure 1. BMSC P1 under light microscopy (×100).

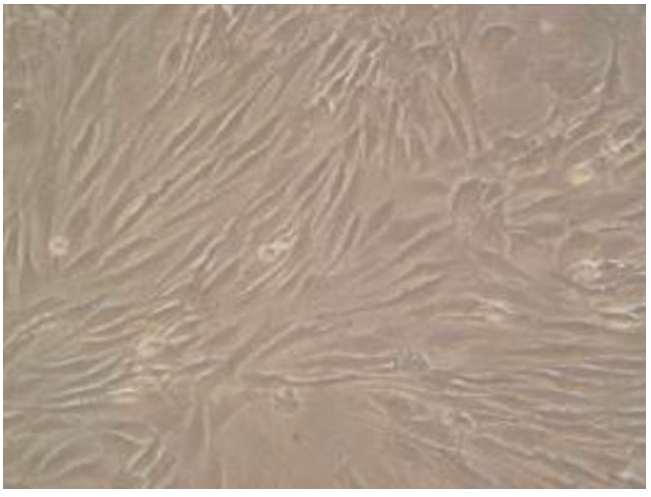

Figure 2. BMSC P3 under light microscopy (×200).

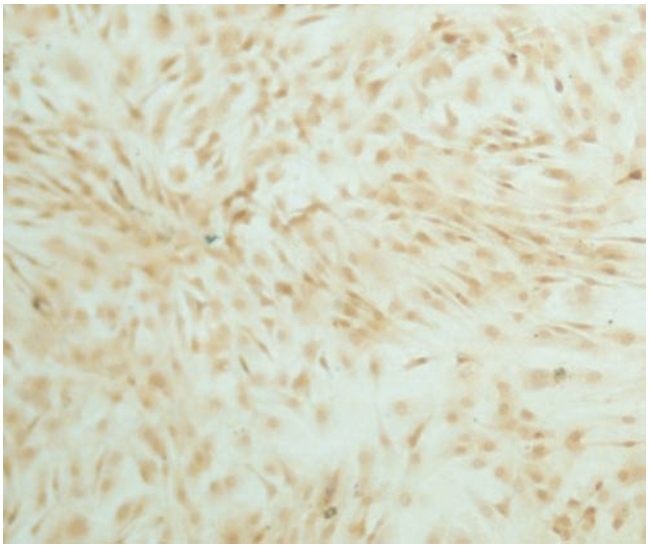

Figure 3. Immunohistochemical staining CD 44 (×200).

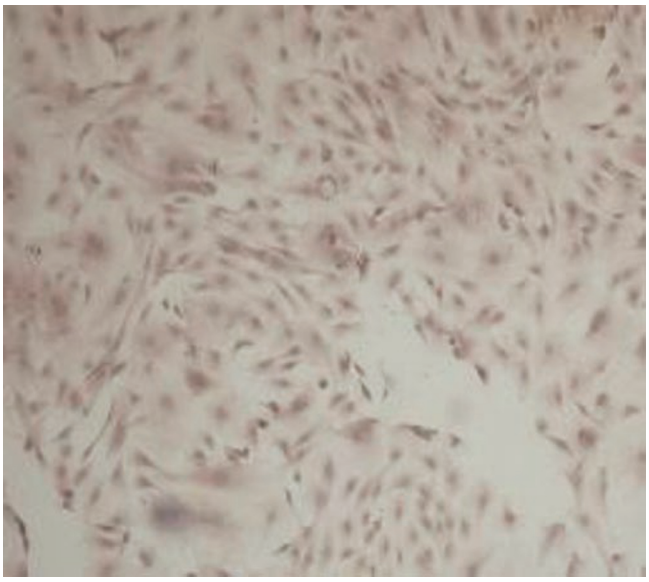

Figure 4. Immunohistochemical staining CD 105 (×200).

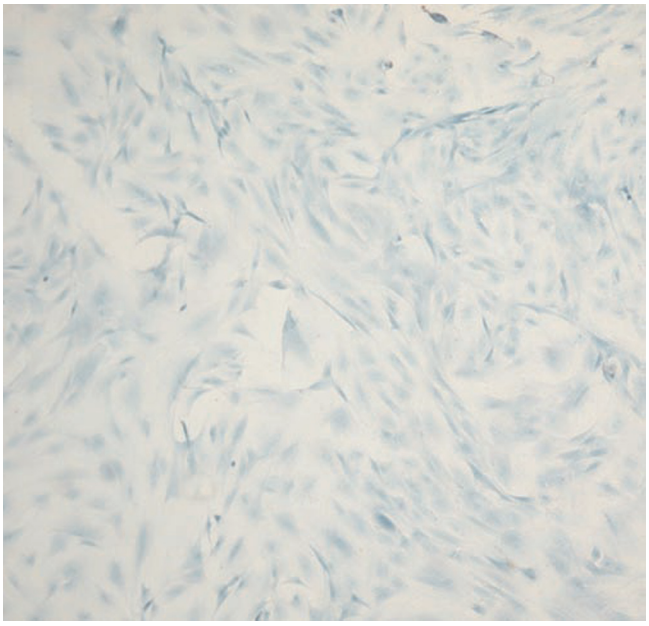

Figure 5. Immunohistochemical staining CD 31 (×200).

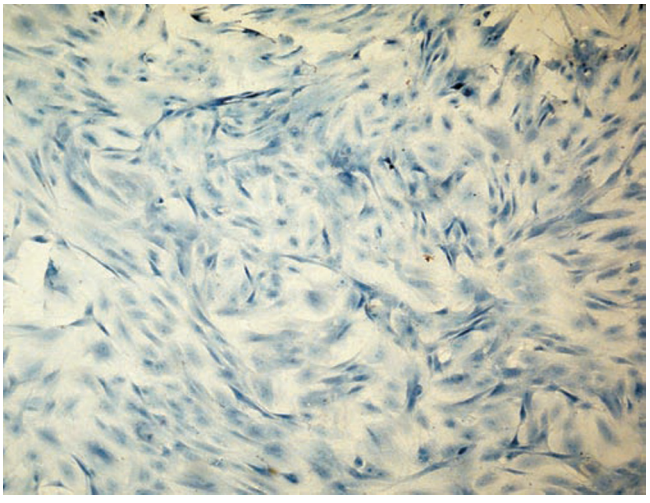

Figure 6. Immunohistochemical staining CD 45 (×200).

3.2. Western blot results

Total protein of transfected pEGFP/Ang-1 cells was extracted for western blot analysis with β-actin as the internal control. Figure 7 shows that BMSCs which were transfected with Ang-1 had correspondingly expressed Ang-1 protein in cells, while no Ang-1 protein was found to be expressed in non-transfected cells.

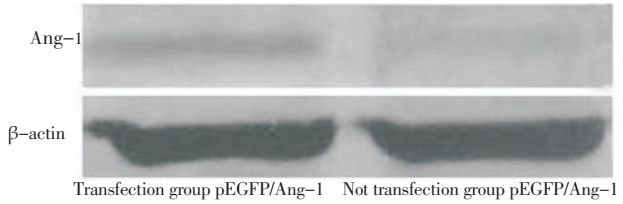

Figure 7. Expression of recombinant fusion protein identified by Western blot.

3.3. BMSCs morphology after transfection

It is shown in Figures 8 and 9 that the pEGFP/Ang-1 plasmids had been successfully transfected into BMSCs. On Day 1, the transfected BMSCs showed light green fluorescent, while the intensity reached the maximum on Day 3.

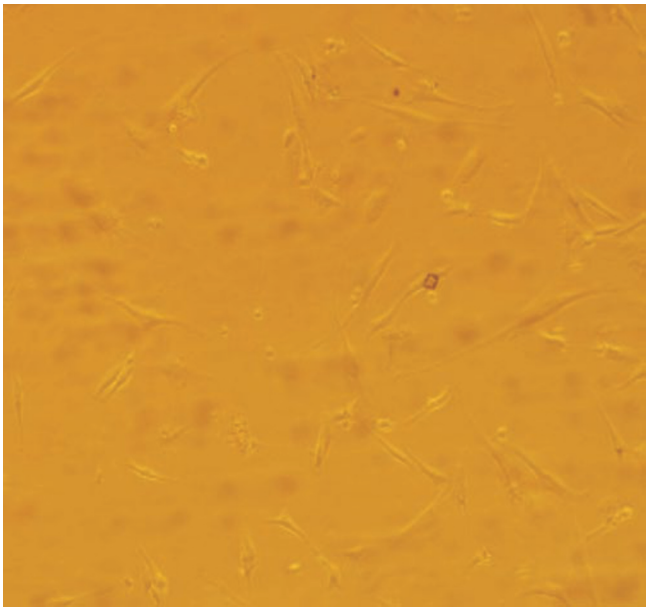

Figure 8. pEGFP/Ang-1 under an inverted microscope (×100).

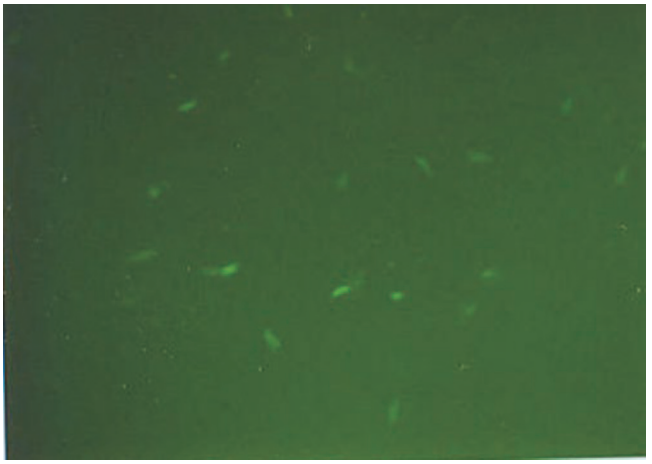

Figure 9. Fluorescence of pEGFP\Ang-1 gene transfection (×100).

4. Discussion

BMSCs possess the potential of self-replication and multiple directional differentiation, which are common features of all stem cells. Under different circumstances, BMSCs may differentiate all mesoderm-origined tissues, such as adipocytes, osteoblasts, vascular endothelial cells and neurogliocytes[1,4]. Currently, our commonly used methods for the separation of cultured primary cells include flow cytometry sorting, adherence screening method, magnetic cell sorting, and density gradient centrifugation. 1. The advantages of flow cytometry sorting are relatively higher purity of cell separation and recovery rate, fully enclosed operating environment and greatly reduced the posibility of contamination. However, it is a costly and complex equipment that requires operators with high technical level, thus it is suitable for very high purity and recovery of cells. 2. Firstly developed in the late nineteenth century by Franklin *et al*.[9], adherence screening method is a routine cultivation method for BMSCs separation, which has been demonstrated as a quick assay *in vitro* from a large quantities of experiments and is widely used in most hospitals. 3. Magnetic cell sorting could conveniently separate BMSCs with high purity through specific antigen at cell surface to combine with the monoclonal antibody and screened BMSCs with the highest purity. Phinney adopted the technology of debilitation of immune system and precisely separated target cells such as hematopoietic and endothelial cell lines from stroma cells[11], thus it provides a new method for the separation and purity of BMSCs in clinical practices. As a complex separation and purification method, it is not routinely used in laboratory. 4. The basic idea behind density gradient centrifugation is that gradient centrifugation exacts monocytes based on different components and proportion of BMSCs. Specialists have demonstrated in large quantities of researches that the purity of BMSCs could reach surprisingly 95% from density gradient centrifugation. In our study, we combined the adherence screening and density gradient centrifugation method to reasonably avoid the drawbacks of single method and obtained higher purity of BMSCs. In accordance to the morphology observation of BMSCs, we found that the shape of cells at P3 turned to fusiform and spindle and arrangement of cells was radial and spiral shape with little other impurities. As cells at the stage of mitosis metaphase with satisfactory state, it may be much easier for the expression of exogenous gene transfected by lipidosome. If we choose to transfected Ang-1 recombinant plasmids into rats BMSCs at this stage, Ang-1 could be stablely expressed in BMSCs in 3 days and the expression level decreased after 7 days. In recent years, scientists have conducted detailed studies on the plasticity of tissue stem cells and made great breakthrough in the fields such as development potential, isolation and culture, purification and induced differentiation. As BMSCs possess easier separation, quick amplification and stable biological features, they have broad prospects in various fields such as tissue engineering, cell transplantation and gene therapy. Obtaining quickly amplified BMSCs with higher purity will greatly shorten the duration of process.

Among the above mentioned methods in the process of gene therapy, determining which kind of methods to choose mainly depends on foreign genes transformed into stem cells and finally entered the cell nucleus through cell membranes. The most prominent advantage of gene therapy is that it can effectively avoid the costly expense of recombinant proteins by self-secreted genetically modified products. Either *in vivo* or *in vitro* experiment where the foreign genes are inserted into cells, a relatively safer carrier is a guarantee. In the past few decades, exciting breakthroughs have been made in carriers used in modern gene therapy, of which the most widely used are virus vectors, plasmid vectors and liposome vectors. Liposome vectors are characterized by low toxicity, simple to use and a certain degree of transfection rate[12], and the gene transfection technique is in rapid growth rate with satisfactory safety and high quality commercialized products, thus it is widely used in the fields of gene transfection[13,14]. In our study, pEGFP-N1 containing green fluorescence protein is a kind of reporter gene applied in mammalian cells to continuously, efficiently and lastingly express proteins without any cytotoxicity or damages. G 418 is successfully used to transfect the targets cells with pEGFP-N1 and strong replication function of plasmid lead to its effective and stable expression in transfected BMSCs at early stage. Whenever gene coded green fluorescent protein in pEGFP-N1 is challenged, it could send out green fluorescent without the involvement of exogenous substances and no cytotoxicity, thus we could directly observe the expression of foreign genes in viral cells. As an ideal reporter gene to observe dynamic changes of foreign gene expression under electron microscopy, eukaryotic expression vectors are much more advantageous than virus vectors, including safety, very little probability of carcinogenicity or teratogenicity, realizing transient expression of carriers, the products of which can also be quickly detected[14]. Yuan *et al*. proved that green fluorescent protein can be successfully transfected into BMSCs, which may continously send out green fluorescent[15]. While the proportion of transfection agents and plasmids reach 8:2 (μg) during the process of transfecting pEGFP/Ang-1 plasmids to BMSCs, the transfection efficacy reaches the highest level. If the proportion is lower than 8:2, then excessive plasmids may neutralize cationic liposome so that liposome is hard to combine with negatively charged membrane. On the contrary, if the proportion is higher than 8:2, then insufficient plasmids may decrease the combination rates and excessive liposome may cause cytolysis mainly because negatively charged proteins blended in serum combining with positively charged liposome may further prevent the combination between liposome and plasmids and transfection efficiency further decrease. Therefore, we used serum-free medium in the process of transfection. In order to obtain satisfactory transfection efficiency, plasmids and liposome were homogenized, kept still for 0.5 h at room temperature until plasmids were completely encapsulated liposome. It could be observed under fluorescence microscope that the intensity of green fluorescence reached the top on Day 1 and gradually decreased 2 days later indicating that plasmids have been successfully inserted into BMSCs. In conclusion, as the transfection efficiency of BMSCs is high and it can be transformed into different tissues after reaching various tissues, BMSCs can

be regarded as an effective carrier for gene therapy and provide theoretical basis for relevant animal experiments.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

BMSCs which can be obtained from a variety of sources, are exploited for autologous transplantation. BMSCs have the ability of self-renewal and multi-directional differentiation. BMSCs can not only be used for the replacement of defective or absent cells in defective tissues and organs, but also serve as the effective vectors of gene fragment.

Research frontiers

BMSCs were isolated from bone marrow using density gradient centrifugation method and adherence screening method, and purified. The recombinant plasmid pEGFP/Ang-1 was used to transfect BMSCs and the positive clones were obtained by the screen of G 418. Observed under light microscopy inversely, green fluorescent exhibited by protein was enhanced to measure the change time of the expression amount of Ang-1.

Related reports

Yuan Wei *et al*. proved that green fluorescent protein can be successfully transfected into BMSCs, which may continously send out green fluorescent.

Innovations and breakthroughs

In this study, pEGFP-N1 containing green fluorescence protein is a kind of reporter gene applied in mammalian cells to continuously, efficiently and lastingly express proteins without any cytotoxicity or damages. G 418 is successfully used to transfect the targets cells with pEGFP-N1 and strong replication function of plasmid lead to its effective and stable expression in transfected BMSCs at early stage. Whenever gene coded green fluorescent protein in pEGFP-N1 is challenged, it could send out green fluorescent without the involvement of exogenous substances and no cytotoxicity. It could directly observe the expression of foreign genes in viral cells.

Applications

BMSCs are an effective carrier for gene therapy and provide theoretical basis for relevant animal experiments.

Peer review

It is an interesting idea that adherence screening method and density gradient centrifugation can be effective methods to obtain BMSCs with high purity and rapid proliferation. Besides, the expression of transfected recombinant plasmid pEGFP/Ang-1 in rat BMSCs is satisfactory.

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