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Research Article

Exogenous Bmscs Migrate to the Inflammation Territory of Dental Pulp and Participate in the Recovery of Dental Pulp *In Vivo*

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Abstract

Dentin defects in teeth and pulpitis have long been challenging clinical issues in the field of dentistry. Meanwhile, the number and activity of dental pulp stem cells decline at an inflammatory state, which limits their ability to repair pulpitis-related damage. Bone marrow mesenchymal stem cells (BMSCs) were widely used in the regeneration field. Whether exogenous could migrate to inflammatory sites *in vivo* and contribute to pulp tissue repair has become an important question. In this study, BMSCs from green fluorescent protein (GFP) mice were extracted *in vitro*. By tail vein injection of these cells, BMSCs-GFP cells were observed directed migration toward to pulpitis tissue at early stage and could differentiation into dental cells expressing DSP, DMP-1at middle stage of pulpitis, it suggests that BMSCs-GFP participated in the post-inflammatory repair of dental pulp.

Keywords: BMSCs; Dentin defect; GFP; DSP; DMP-1

Introduction

Dental defects are usually caused by caries, attrition and trauma, causing pulpitis and periapical periodontitis [1]. When the defect degree is within a certain range, the tooth will self-repair by the differentiation of pulp stem cells into odontoblast cells [2]. Current studies have found that the directed differentiation of stem cells is closely related to the stem cell niche [3-5], which is the local three-dimensional environment in which stem cells exist and function, maintaining and regulating the self-renewal and proliferation of stem cells [6]. Many scholars have studied the differentiation of mesenchymal stem cells (MSCs) into tissue cells and participating in tissue defect repair under the regulation of specific stem cell niches [7,8]. The source of odontogenic stem cells is scarce [9] and the number and proliferation of stem cell in the inflammatory state were declining [10], so non-odontogenic MSCs need different into odontoblast cells and participate in the repair of dentin defects.

Bone marrow-derived mesenchymal stem cells (BMSCs) have strong self- proliferation ability and have the potential to differentiate into a variety of tissue cells derived from mesoderm and neuroectoderm [11,12] including bone cells, chondrocytes fibroblasts, cardiomyocytes, skeletal muscle cells, liver cells, epithelial cells and neurons and glial cells [13]. Because of abundant source, large amount *in vitro* and little damage to the body, BMSCs become a research hotspot to repair various tissue defects. Whether BMMSCs differentiate into odontoblasts to enhance the restorative power of inflamed pulp tissues *in vitro* remained controversial, there have been no relevant reports on the differentiation of BMSCs into odontoblast cells *in vivo* and their involvement in the repair of dentin defects.

In this study, the tail vein injection method of mice was used, and it was observed that exogenous bone marrow mesenchymal stem cells labelled by fluorescent protein could survive for more than 2 weeks *in vivo*. Not only that GFP-BMMSC migrate to the inflammatory area, but also different into odontoblast-like cells expressing DSP, DMP-1. Exogenous bone marrow mesenchymal stem cells may participate in the repair of the inflammatory dental pulp tissue, providing an experimental basis for the use of BMSCs for injured pulp tissue repair *in vivo*.

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Materials and Methods

Primary Culture of BMSCs-GFP

The GFP mice, aged 3-4 weeks and weighing 10-15g, were sacrificed with the excessive anesthetic, the femur was soaked in 75% alcohol for 5-10 min, the whole femur was removed under aseptic conditions, and the soft tissue was removed and placed in a sterile petri dish. The bone marrow cavity was repeatedly rinsed with α-MEM medium after carefully femur cut, the bone marrow was flushed out and transferred to a centrifuge tube, and the bone marrow cell suspension was completed by repeated blowing, centrifuged at 1000r/min for 5min. The whole bone marrow cell suspension after cracking was inoculated in a 25 cm^2 culture bottle at a density of 1×10^5/ml, and 5ml a-MEM culture solution containing 15% FBS was added for routine culture. When the cell proliferation reached 80% confluent, pancreatic enzyme /EDTA (0.25/0.1, PH=6.4) was digested and passed. Observations were made under fluorescence microscope and photographs were taken.

Flow Cytometry (FCM) was used to Detect Stem Cell Markers

The 3rd generation of adhesion cells were digested with pancreatic enzyme /EDTA (0.25/0.1, PH=6.4), then re-suspended in a-MEM medium and divided into three 1.5ml EP tubes, and incubated with Rhodamine-labeled anti-mouse antibody including CD29, CD45 and CD90 for 30 min. Flow cytometry was used immediately after washing with 1×PBS. The 3rd generation BMSCs of ordinary C57BL/6 mice were used as a negative control.

Identification of multidirectional differentiation ability of BMSCs

Identification of Bone-Forming Ability of Bmscs-GFP: Ability of osteogenesis of BMMSCs was stained alizarin red after osteogenic induction. When the 3rd generation BMSCs-GFP were cultured into 40% to 50% confluent, the mineralized induction medium (90% a-MEM+ 10% FBS +100 U /ml penicillin +100 mg/L streptomycin +2 mmol/L glutamine + 10 mmol/L dexamethasone +0.3 mmol/L vitamin C + 10mmol/ LB-sodium glycerol phosphate) were added to induce osteogenesis. After induction for 3 weeks, the culture medium was removed, the cells were washed with 1×PBS, fixed with 10% formaldehyde for 30 min, and stained with alizarin red at room temperature. The images were taken under a fluorescence microscope to detect the expression of fluorescence.

Identification of Lipid-forming ability of BMSCs-GFP: Ability of adipogenesis of BMSCs was stained oil red after adipogenic induction. When the 3rd generation BMSCs-GFP were cultured into 40% to 50% confluent, the adipose-cell induction solution (90% a-MEM+ 10% FBS +100 U /ml penicillin +100 mg/L streptomycin +1umol/L dexamethasone+0.5mmol/IBMX) were continued under normal conditions. After 2 weeks, 10% formaldehyde was fixed and oil red O was dyed at a room temperature. The images were taken under fluorescence microscope to detect the expression of fluorescence.

Establishment of Pulpitis of C57BL/6 Mouse

Pulpectomy of mandibular molars in rats was established as a model of pulpitis. The experiment was divided into four time points: D0, D5, D10 and D15, which represented the acute stage of pulpitis, the early stage of pulpitis restoration, the middle stage of pulpitis restoration, and the late stage of pulpitis restoration, respectively, C57BL/6 mice aged 4-6 weeks were sacrificed, soaked in 75% alcohol for 5min. The whole femur was removed under sterile conditions, and the soft tissue was removed and placed into a sterile petri dish. The blood was washed with PBS containing 3% FBS. Take a pair of scissors and cut off both ends of the femur, the bone marrow cavity was repeatedly rinsed with a-MEM medium from a 1ml disposable syringe, the bone marrow was flushed out and transferred to a centrifuge tube, and the bone marrow cell suspension was completed by repeated blowing, centrifuged at 800r/min for 5min, gently blown for several times, and then left for 3min away from light. Centrifuge 1000r/min for 5min. After washing with PBS twice, the cell concentration of 1×PBS was adjusted to 1×10^5/ml.

Construction Model of Pulpitis of Tooth

Fluorescent BMSCs were randomly selected as the normal control group, The other 6 mice with fluorescent BMSCs were anesthetized by intraabdominal injection of 0.1% pentobarbital sodium (3ml/kg). Both maxillary molar areas were wiped and disinfected with 75% alcohol cotton balls. High speed turbine and 1/2# ball drill were used to create dentine defects on both maxillary first molar surfaces, respectively. The defect depth of each surface was 0.3~0.5mm, and all operations were performed by the same surgeon. After operation, the mice were fed with 4% paraformaldehyde heart perfusion at 5d, 10d and 15d respectively. 4% paraformaldehyde heart perfusion was performed. The maxillae of all mice were separated immediately after death, placed in 4% paraformaldehyde, fixed at 4°C for 24h, decalcified at 10% EDTA for 15 days, and rinsed with water for 24h, then 5um frozen sections were prepared in the sagittal direction to staining with HE staining.

Bmscs can Proliferate and Survive in the Body more than 2 weeks, which can be Observed in the Liver and Kidney Organs Early, Gradually and Eventually Reaches Area Attribution to the Inflammation of the Pulpitis

Frozen slicing procedure is the same as above. After slicing, it is fixed with acetone at 4°C for 10~15min, washed with 1×PBS 3 times, immersed in 37°C and 1×PBS containing 75% H_2O_2 for 30min, washed with 1×PBS 3 times for 1 0~15min each time, and add 1: 10 dilute sheep serum in a 37°C wet box for 30 min, then added with moderately diluted primary antibody, placed in a 37°C wet box for 1h, washed in PBS 3 times for 5 min each time, then added with Rhodamine labeled secondary antibody, placed in a 37°C wet box away from light for 30 min. After washed with 1×PBS twice, moderately diluted Hoechst was added, observe under fluorescence microscope and take photos.

Results

Identification of BMSCs-GFP

At the early stage of primary culture, BMSCs-GFP attached to the wall which were mostly irregular or short spindle with a few cell prominences and large nuclei. After that, the adherent cells gradually divided and proliferated, gradually became long spindles, the cells were cloned, and the fluorescence microscope showed stable green

fluorescence continuously. Flow cytometry was used to detect the third generation of BMSCs-GFP, and the positive rate of green fluorescence was over 95%. The cells with positive expressions of CD29 and CD90 accounted for 86.1% and 92.8% respectively, and the cells with negative expressions of CD45 accounted for 1.4%. After 3 weeks of osteogenic induction, BMSCs-GFP could differentiate into osteoblasts. Alizarin red staining showed deposition of orange-red mineralized matrix, and the differentiated cells also expressed green fluorescence and maintained the original intensity. After 2 weeks of lipogenic induction, BMSCs-GFP had hypertrophy and red oil. When the staining was positive, a large number of orange fat vacuoles could be seen in the cytoplasm, and the differentiated cells also expressed green fluorescence and maintained the original intensity.

Efficacy of Establishment of Chimeric Mice of BMSCs-GFP

Results of flow cytometry was used to detect BMSCs in peripheral blood cells of chimeric mice, and the results of proportion of spontaneous green fluorescence cells in the three random samples were 5.7%, 6.6% and 7.9%, respectively. GFP was detected by several organs of chimeric mice of BMSCs-GFP through HE stanning. Scattered green fluorescent cells were found in the jaw and pulp cavities; Green fluorescent cells were found in the heart. A small number of green fluorescent cells were found in the liver and all appeared around the capillaries in the liver; A small number of green fluorescent cells were found in all mice, and appeared around the capillary mouth in the kidney; 4 out of 7 mice were found to have a distribution of green fluorescent cells in the spleen; No green fluorescent cells were found in the thymus of 7 mice.

Establishment Model of Dentin Defect of Chimeric Mice of BMSCs-GFP

Dentin defect of dentin defect was established in the mandibular first molar of chimeric mice of BMSCs-GFP. Result of HE stanning showed that the pulp tissue was composed of odontoblast cell layer, multicellular layer and intrinsic pulp cells, and the odontoblast cells were columnar arranged in the inner wall of pulp cavity in control group. In the sample group of 5d, the pulp tissue was partially necrotic into the dentin cell layer, and the small blood vessels of the pulp were dilated. In the sampling group at 10d, a large number of narrow and long cell layers appeared in the pulp wall corresponding to the defect. In the 15d sampling group, dentin appeared in the inner wall of the pulp cavity corresponding to the defect site. In the normal control group, there were a small number of cells expressing green fluorescence in the periodontal tissue and pulp cavity. In the 5d sampling group, the number of cells carrying green fluorescence increased in the periodontal tissue and pulp cavity. In the 10d sampling group, the number of cells carrying green fluorescence in periodontal tissue and pulp cavity continued to increase and were distributed in the pulp wall corresponding to the defect. In the 15d sampling group, the number of green fluorescent cells in the periodontal tissue and pulp cavity decreased compared with that in the 10d group.

Relative Expression of DSP, DMP-1 and SDF-1 of Chimeric Mice of BMSCs-GFP

In the normal control group, there were a small number of cells expressing green fluorescence in the periodontal tissue and pulp cavity, and the fluorescence staining of DSP, DMP-1 and SDF-1 was negative. In the 5d sampling group, the number of cells stained with SDF-1 positive, DSP and DMP-1 were negative. In the 10d sampling group, the number of cells stained with DSP, DMP-1 and SDF-1 fluorescence were distributed in the pulp wall corresponding to the defect. DSP, DMP- 1 and SDF-1 fluorescence staining of green fluorescent cells in periodontal tissues were negative. In the 15d sampling group, DSP, DMP-1 and SDF-1 fluorescence staining were still positive in some cells carrying green fluorescence in pulp cavity, but the number decreased compared with that in the 10d group.

Discussion

The pulp tissue is ridiculous in neurovascular, and once it invaded by bacteria or damage and thus caused to result in pulpitis, it is usually treated with pulp removal or root canal treatment. Recently, researchers have found that dental pulp stem cells can differentiate into periodontal ligament stem cells, and ameloblasts [14]. However, the number and viability of dental pulp stem cells declined in the inflammatory microenvironment [15], and it is necessary using exogenous stem cells to increase the number of stem cells at the area of inflammation dental pulp and to improve the repair ability of pulp tissue.

BMSCs have a strong ability to proliferate and can differentiate into various tissue cells [16]. Tail vein injection of GFP-BMSCs could proliferate and survive in the body more than 2 weeks, which can be observed in the liver and kidney organs first, gradually and eventually reached to the inflammation area of the pulpitis. We observed strong SDF-1 expression in GFP-BMSCs at the early stage of inflammation at 5 days, then decreased SDF-1 expression reversibly increased DMP-1 and DSP in the middle of inflammation at 10 days, indicating differentiation of BMSCs into pulp cells. Although it could not confirm that exogenous stem cells can completely recover pulp damage, we saw that BMSCS reached the pulp inflammation zone accompanied by SDF-1 strongly expressed, and subsequently of DMP-1 and DSP-1, suggesting that BMSCs could migrant directed early stage and differentiate into pulp-like cells at the middle stage of pulpitis *in vivo*. That is to say, BMSCs could not help to inhibit inflammatory response at early but take part in repair at the recovery stage of pulp inflammation. The biological activity of BMSCs depends on improving the dental pulp inflammatory microenvironment.

Fluorescent protein labelling technology is a cell labelling technology that has been rapidly developed in recent years [17]. At present, the fluorescent green protein gene has been widely used *in vivo* through tracing of transplanted stem cells, and the timeliness of its cell labeling is extremely unstable, and the fluorescence is easy to quench [18]. Fluorescent protein transgenic animals do not experience the issue of unstable labeling. In this experiment, we used GFP mice as cell donors, and the 3rd generation of BMSCs-GFP, which obtained by the whole bone marrow cells after repeated adhesion screening. Results of flow cytometry showed the specific expression of CD29 on the cell surface, CD90 positive and CD45 negative. The 3rd generation cells were induced *in vitro* with osteogenic and lipogenic induction solution. The results showed that the 3rd generation cells could differentiate into osteoblasts and adipocyte. After induction of lipid formation, lipid droplets were formed in the cells, and green fluorescence was continuously and stably expressed after differentiation. The multidirectional differentiation of BMSCs-GFP

was verified by bone and lipid induction experiments. It can function as a highly efficient and convenient tracer for stem cells, enhancing research and treatment possibilities. In this study, we transplanted 3rd BMSCs-GFP cultured *in vitro* into 6-Co-y treated C57BL/6 mice by tail vein injection to obtain a chimeric mouse model of fluorescent BMSCs that can be used for stem cell tracing. The immune repulsion of C57BL/6 mice treated with 6-Co-y rays is reduced, which reduces the occurrence of graft versus host disease GVHD (Graft versus host disease) [19]. Because GFP mice are genetically modified mice derived from C57BL/6 mice, they belong to the same genus of mice [20]. Therefore, our chimeric mouse model of fluorescent BMSCs greatly reduced the mortality caused by graft-versus-host disease and host immune rejection [21].

The original mesenchymal stem cells in C57BL/6 mice treated with 6-Co-y rays were subjected to radiation. In this experiment, the number of fluorescent cells detected by flow cytometry in the peripheral blood of chimeric mice was much less than the number of injected fluorescent stem cells, indicating that the number of stem cell niches in the body is fixed, and when all stem cell niches in the host are occupied, other non-homing stem cells will gradually lose their stem cell characteristics as time goes by [22]. Therefore, the stem cell environment of the fluorescence BMSCs chimeric mouse model we constructed is very similar to the original stem cell environment of the host. The chimeric mouse model of BMSCs should be used to trace stem cells and study the molecular mechanism of cell niches. From this experiment, we also saw that BMSCs do not only exist in bone marrow, but also exist in circulating peripheral blood or inhabit around micro-vessel, and it has been reported that BMSCs can repair spinal cord injury through cerebrospinal fluid circulation. The chimeric C57BL/6 mouse model of BMSCs is of great significance for studying the repair of defects, the treatment of inflammation and the regulation mechanism of stem cell niche. The dentin defect model established in this experiment observed the process of dentin defect repair and the migration and differentiation of BMSCs carrying green fluorescence during the repair process which could be observed under a fluorescence microscope. Therefore, BMSCs have the ability to differentiate in the direction of odontoblast cells under the regulation of specific stem cell niches, and can be used as seed cells for dental tissue engineering.

Conclusion

In conclusion, we successfully constructed a model of a GFP transgenic chimeric mouse with dentin defect. We studied the regulation of the migration and differentiation of BMSCs during the processes of dentin defect formation and repair using fluorescence tracer localization. Our next research focus will be on utilizing the BMSCs-GFP chimeric C57BL/6 mouse model to investigate the regulatory mechanisms of the stem cell niche during the repair of dentin defects by BMSCs and verify whether BMSCs could be a promising target to repair pulpitis.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Ethics Approval and Consent to Participate

The animal surgical procedures were approved by the Animal Ethics Committee of the Stomatology of the Fourth Military Medical University (Shaanxi, China) (kq-021).

Authorship Contribution Statement

Liu Xiangdong: Conceptualization, methodology, writing original draft. Duan Qingyue: Methodology, formal analysis, writing, review and editing. Bai Qingxia: Writing review & editing. Yang Qianjuan: Material synthesis and characterization. Ma Wei: revision of manuscript and funding. Lu Qun: Printing scaffolds, revision of manuscript and funding.

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