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Differentiation of transforming growth factor β1-induced mesenchymal stem cells into nucleus pulposus-like cells under simulated microgravity conditions

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Abstract

This study aims to observe the differentiation effects of transforming growth factor β1 (TGF-β1)-transfected bone marrow mesenchymal stem cells (BMMSCs) into the nucleus pulposus-like cells under simulated microgravity conditions. BMMSCs were isolated from the adult rabbits, then cultured and proliferated to the third generation. After transfected by TGF-β1, these MMSCs were incubated in the calcium alginate gel microspheres and rotary cell culture system. The content changes of TGF-β1 inside BMMSCs, as well as the proliferation abilities of BMMSCs, were detected. The immunohistochemistry and toluidine blue staining were performed to detect the type II collagen. The RT-PCR method was performed to detect the expressions of proteoglycan and type II collagen mRNA. Results found that, on the 14th day, the polygonal nucleus pulposus-like cells could be observed. The TGF-β1 content inside the supernatant and the DNA content inside BMMSCs were significantly increased ($P<0.05$). The immunohistochemistry staining exhibited the positive results about the type II collagen. RT-PCR showed the expressions of proteoglycan and type II collagen mRNA inside BMMSCs. The TGF-β1-transfected BMSCs exhibited the increased synthesizing abilities of proteoglycan and type II collagen under simulated microgravity conditions than the control group.

Key words: BMMSCs, nucleus pulposus, TGF-β1, microgravity.

Introduction

The intervertebral disc degeneration is a kind of disease with high incidence, which is mainly expressed with the number reduction and functional degradation of nucleus pulposus cells. The nucleus pulposus is dehydrated and calcified, thus its synthesizing abilities of proteoglycan and type II collagen are diminished (1, 2). Gruber et al. (3) re-implant the autologous nucleus pulposus cells into the gerbil disc degeneration model, which effectively treats the intervertebral disc degeneration. However, the nucleus pulposus is the tissue lack of cells, and it will be much more difficult to obtain sufficient nucleus pulposus cells from a degenerative disc to meet the re-implantation needs of autologous nucleus pulposus cells (4). In addition, the in vitro culture of nucleus pulposus cells exhibits the characteristics of slow proliferation (5). The mesenchymal stem cells (MSCs) are the pluripotent cells (6). The latest researches show that, under different induction conditions, MSCs have the abilities of differentiating into a variety of tissue cells in vivo and in vitro, such as bone tissue, cartilage tissue and intervertebral disc tissues (7-10). The rotary cell culture system (RCCS) can simulate the microgravity environment on the ground, through producing low-shear and low vortex towards the cultured cells. It reduces the mechanical damages produced by the culture medium to the cells, increases the cellular nutrient transferring roles, and accelerates the elimination of metabolites, thus contributing to the proliferation of cells.

The transforming growth factor β1 (TGF-β1) has multiple biological effects, and is one of the major growth factors that can regulate the MSCs proliferation and direct differentiation into the cartilages. Steck et al. (11) have cultivated bone marrow mesenchymal stem cells (BMMSCs) in the TGF-β1-mediated media and confirm that, the phenotypes of induced BMMSCs are similar to those of nucleus pulposus cells. However, the TGF-β1's biological half-life is short, and the biological half-life of active TGF-β1 is only 2-3 min. It can only play a short biological effect inside cells, and the continuous stable TGF-β1 actions are needed towards such chronic disease as disc degeneration.

This study intended to introduce TGF-β1 into BMMSCs and culture under the simulated microgravity conditions. The objective is to explore whether TGF-β1 can induce BMMSCs to differentiate into the nucleus pulposus-like cells, and increase the contents of proteoglycan and type II collagen, thus providing a new treatment method for disc degeneration.

Materials and methods

Isolation and cultivation, grouping and cell identification of BMSCs

The tibial bone marrow blood of 2-month-old New Zealand rabbits was paracentetically extracted, and was mixed with Hanks solution with the ratio as 1:1, centrifuged at 800 r/min for 5 min to eliminate the blood lipids. Then it was repeatedly washed with dulbecco's modified eagle medium (DMEM), and centrifuged at 1500 r/min for 15 min. The interface cells were then collected, and washed twice with DMEM, followed by centrifugation. 10%-FBS containing DMEM was used to pipet the cells into the single cells, followed by the culture at 37°C (5% CO2, saturated humidity). 72 h later, the fresh medium was replaced, and the non-wall-adherent cells were removed. When the colony formed and the cells grew to 85% fusion, the cells were

passaged. 0.25% trypsin was added for the digestion. When the fusiform BMMSCs became round, the digestive fluid was decanted, followed by adding the medium and pipetting the cells. The cells were seeded into 50 ml culture flasks, with the density as 1×10^7 Pml. An inverted microscope was used to observe the cell morphology daily and take photographs for the reford. When the cell density was more than 90%, 0.25% trypsin was used for the digestion. The cells were subcultured according to the ratio of 1: 2. The 3rd generation of cells were identified by the flow cytometry. The following fluorescent-labeled rabbit anti-mouse monoclonal antibodies (CD29-FITC, CD44-FITC and CD45- FITC) were divided into 3 tubes. The isotype negative control used the mouse anti-human IgG1-FITC/IgG2a-FITC (20 μl each tube). 100 μl cell suspension was added into each tube, followed by reaction at room temperature for 20 min. Each tube was added with 1.5 ml PBS, followed by mixing. After centrifugation at 1800 r/min for 5 min, the supernatant was discarded. Each tube was added with 500 μl PBS for detection by flow cytometry. After isolation and cultivation, 3 BMMSCs culture groups were established. The microgravity-induced culture group (group A) was performed with dynamic culture of TGF-β1 transfected BMMSCs within the calcium alginate gel microspheres and rotary cell culture system and under simulated microgravity conditions. The inducted culture group (group B) was performed with culture of TGF-β1 transfected BMMSCs within the calcium alginate gel microspheres. The microgravity-natural-differentiation group (group C) was performed with culture of BMMSCs within the calcium alginate gel microspheres and rotary cell culture system. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Harbin Medical University.

BMSCs transfection

1 μg plasmid was taken and transformed into the competent cells of E.coli for the monocloning. The monoclonal cells were picked and cultured, and the plasmids were extracted, folowed by enzyme digestion and sequencing. The reagent kit was used to largely extract the plasmids and stored at -20°C for future use. 1×10^7 MMSCs were passaged into 100 mm dishes, and cultured at 5% CO_2 and saturated humidity. When the cells were fused to 90% to 95%, the transfection was performed. The transfection method and procedures were in accordance with the instructions of LipofectamineTM2000 kit (Invitrogen, USA). 24 h later, the transfected cells were observed under an inverted microscope.

Preparation of cells and stent complex

After digested by the trypsin, the 3rd generation BMMSCs were centrifuged. After discarding the supernatant, the obtained cells were added to 12 g/L sodium alginate solution to form the single cell suspension. This cell suspension was dropwisely added into 35 g/L $CaCl_2$ solution, followed by soaking for 10 min for generating the MMSCs-wrapped alginate gel microspheres. After that, the alginate gel microspheres were soaked in 0.5 g/L poly-lysine solution for 10 min to prepare the poly-lysine micro-capsule film. Meanwhile, the Ca^{2+} inside the alginate gel microspheres was replaced out. Whenthe culture in above alginate gel microspheres was finished, MMSCs inside the microspheres were recovered, and 22 g/L sodium citrate solution was used to dissolve the microspheres.

Cells culture under simulated microgravity conditions

The RCCS high aspect ratio vessel (HARV) was used for the culture. The BMMSCs/alginate gel microspheres in each group were placed in 50 ml HARV. The entire container was filled with the culture medium that was used to passage the cells. After draining the bubbles, the container was fixed onto the rotating brake and placed into the incubator. The bioreactor was then rotated at 37°C, 5% CO_2 and 25 r/m. In the early stage of BMMSCs cultivation, the situations of bubble generation inside the reactor should be regularly observed, and the reactor could be stopped at any time to supplement the culture medium and eliminate the bubbles. 48 h later, the whole medium was replaced, the culture durations were 3 days, 7 days, 14 days and 21 days before each testing.

Detection indexes

The ELISA method was used to detect the TGF-β1 content inside the supernatant of cell culture solution (the related reagents were provided by Shanghai SANGON Biological Engineering Co., Ltd., Shanghai, China). Hoechst33258 method was used to detect the DNA content. The cells at different time points were collected, followed by digestion with papain (Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China). The absorbance of sample was determined using F-7000 fluorescence spectrophotometer (Hitachi High-Technologies Corp., Tokyo, Japan). The calf thymus DNA (Shanghai SANGON Biological Engineering Co., Ltd., Shanghai, China) was used to plot the standard curv. The DNA content of sample was calculated according to the standard curve. The immunohistochemistry and toluidine blue staining were performed to detect the type II collagen, and RT-PCR method was performed to detect the expressions of proteoglycan and type II collagen mRNA (the related reagents were provided by Shanghai SANGON Biological Engineering Co., Ltd., Shanghai, China).

Statistical analysis

The results were analyzed by the SPSS11.5 statistical software. The data were expressed as ($\bar{x}\pm s$). The multi-group comparison was performed with the analysis of variance. t-tes and q test were used for intergroup comparison and intragroup comparison, respectively. $P<0.05$ was considered as statistically significant.

Results

Cell morphologic and identification

After culturing for 48 h, a small amount of BMMSCs presented fusiform and wall adherence. 7 day later, the cell colony formed. 14 d later, the dense wall-adherent layer appeared, totally covering the bottom of culture flask. After passage to the third generation (the begin-

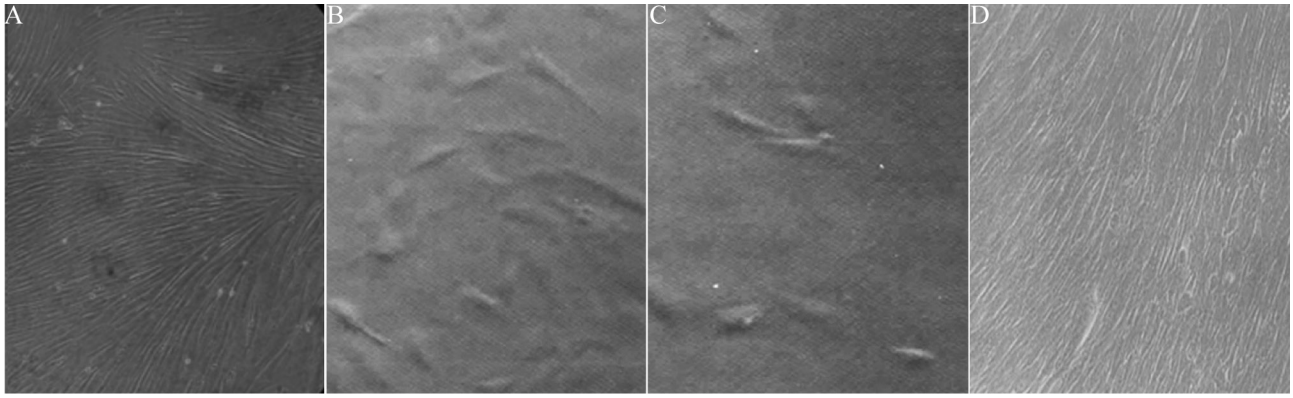

Figure 1. Observation of cellular morphology of each group.
A: The cells were fusiform (×100); **B:** The A group exhibited the active cell proliferation, the cellular morphologies were fusiform, polygonal and irregular, and the cell numbers were large (×100); **C:** The B group appeared polygonal, fusiform and irregular cells (×100); **D:** The C group appeared the irregular cells, which cover the whole culture flask.

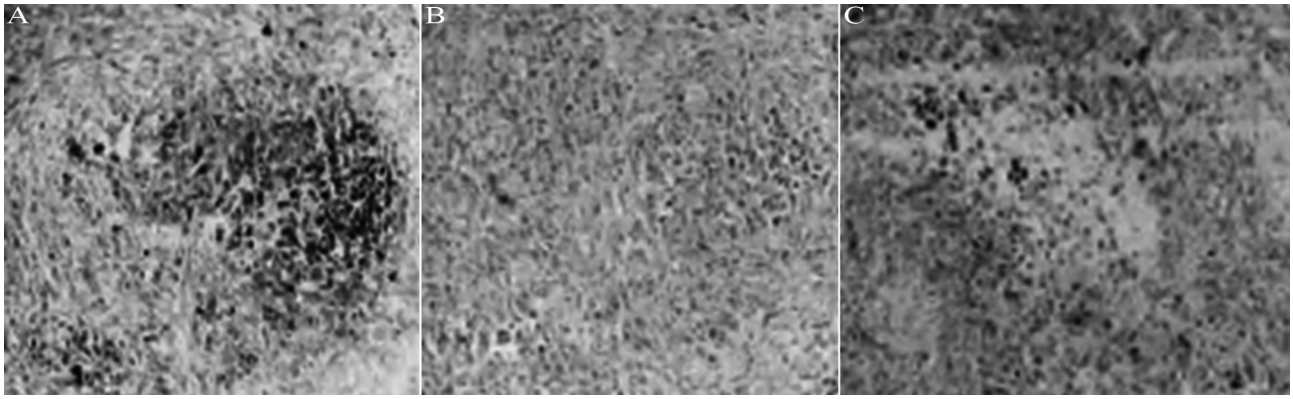

Figure 2. Conditions of type II collagen toluidine blue staining of each group after 21-day MMSCs cultivation.
A: After the 3D dynamic induction, MMSCs inside the calcium alginate gel of the A group on the 21st day (toluidine blue staining, ×100); **B:** MMSCs inside the calcium alginate gel of the B group on the 21st day (toluidine blue staining, ×100); **C:** MMSCs inside the calcium alginate gel of the C group on the 21st day (toluidine blue staining, ×100).

ning time point), the cells were fusiform (Fig. 1A). 24-48 h later, the cell clones appeared, which arranged tightly and presented as whirlpool. 7 days later, the dense wall-adherent layer appeared, and the cell volume increased, with grass-bundle arrangement. The flow cytometry revealed that, the 3rd generation BMMSCs highly expressed CD44, while lowly expressed CD14, which was consistent with the characteristics of BMMSCs. On the 14th day, the polygonal nucleus pulposus-like cells could be observed in group A and B, while group C presented irregular shape. On the 21st day, group A exhibited the active cell proliferation. The cellular morphologies were fusiform, polygonal and irregular, with large cell number (Fig. 1B). Group B presented polygonal, fusiform and irregular cells (Fig. 1C). Group C presented the irregular cells, which covered the whole culture flask wall (Fig. 1D).

Determination of cytokine contents

Group A exhibited higher TGF-β1 content in the supernatant than group B from the 3rd day, and also significantly higher than group C (P<0.05). The TGF-β1 content was gradually increased with time prolonged, which reached the highest on the 21st day. The expressions of TGF-β1 at different time points exhibited the statistically significant differences (P<0.05) (Table 1).

DNA content determination

The DNA content of group A was gradually higher than group B from the 3rd day, which increased with time prolonging, and significantly increased than group C (P<0.05). The DNA contents at different time points exhibited the statistically significant differences (P<0.05) (Table 2).

Table 1. Detection of TGFβ1 content in the culture supernatants among different experimental groups at different time points. ($\bar{x}$ ±*s, n=6, pg/ml*)

Time	C group	B group	A group
3d	101.13±7.16	203.62±12.33	313.82±15.81*#
7d	114.24±5.32	264.26±13.35	425.93±17.10*#
14d	145.12±6.27	268.35±14.61	515.81±20.69*#
21d	173.53±10.45	252.91±18.26	616.75±21.37*#

Note: *Comparison between the A and B group, <0.05,Compared with the C group, P<0.05; #Comparison within the same group while at different time points, P<0.05

Table 2. Detection of DNA content in the culture supernatants among different experimental groups at different time points ($\bar{x}$ ±*s, n=6, pg/ml*)

Note: *Compared with the C group, P<0.05, #Comparison within the same group while at different time points, P<0.05; ΔCompared with the B group, P<0.05

	C group	B group	A group
3d	0.255±0.025	0.319±0.011	0.337±0.016*#Δ
7d	0.261±0.036	0.277±0.05	0.447±0.015*#Δ
14d	0.311±0.055	0.365±0.013	0.550±0.016*#Δ
21d	0.245±0.065	0.438±0.016	0.618±0.030*#Δ

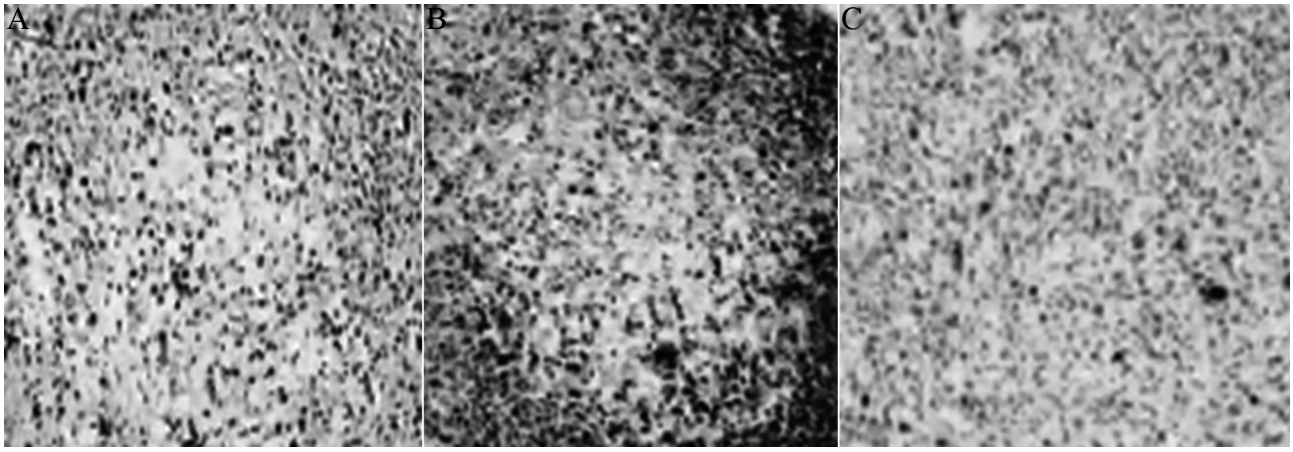

Figure 3. Conditions of type II collagen immunohistochemical staining of each group after 21-day MMSCs cultivation.
A: After the 3D dynamic induction, MMSCs inside the calcium alginate gel of the A group on the 21st day (type II collagen immunohistochemical staining, ×100); **B:** MMSCs inside the calcium alginate gel of the B group on the 21st day (type II collagen immunohistochemical staining, ×100); **C:** MMSCs inside the calcium alginate gel of the C group on the 21st day (type II collagen immunohistochemical staining, ×100).

Table 3. Expressions of proteoglycan and type II collagen mRNA among the experimental groups at different time points ($\bar{x} \pm s$, *pg/ml*)

Time	C group		B group		A group	
	Proteoglycan	type II collagen	Proteoglycan	type II collagen	Proteoglycan	Type II collagen
3d	0	0	0	0	0	0
7d	0	0	0	0	0.907±0.013$^{\#}$	0.706±0.026$^{\#}$
14d	0.016±0.011	0	0.516±0.026	0.706±0.031	0.990±0.011$^{*\#\Delta}$	0.800±0.039$^{\#\Delta}$
21d	0.134±0.017	0	0.706±0.031	0.706±0.031	1.113±0.024$^{*\#\Delta}$	0.883±0.037$^{\#\Delta}$

Note: *Compared with the C group, $P<0.05$; $^{\#}$Comparison within the same group while at different time points , $P<0.05$; $^{\Delta}$Compared with the B group, $P<0.05$

Immunohistochemistry

The toluidine blue staining showed that, on the 14th day, BMMSCs in group A became polygonal, and the cytoplasm became purple-blue. On the 21st day, BMMSCs in group A became polygonal or circular. The cytoplasm exhibited clear violet blue, and the metachromasia was obvious. The nucleus was clear, and the nucleolus was obvious (Fig. 2A). Group B exhibited the pale purplish-blue stained cytoplasm. The metachromasia was not obvious, with clear nucleus and nucleolus (Fig. 2B). Group C exhibited fusiform cells, without obvious metachromasia (Fig. 2C). The type II collagen immunohistochemical staining showed that, in group A, on the 7th day there were polygonal cells. The nucleus was clear, and the cytoplasm exhibited the brown-stained particles. On the 14th day, BMMSCs were round or polygonal, with clear nucleus and brown-colored particles in cytoplasm. On the 21st day, BMMSCs were similar to those on the 14th day (Fig. 3A). In group B, on the 21st day, BMMSCs exhibited the beige-stained particles inside the cytoplasm, and the nucleus was clear (Fig. 3B). In group C, on the 21st day, most BMMSCs presented fusiform, with clear blue-stained nuclei, while no brown particles was observed inside the cytoplasm (Fig. 3C).

RT-PCR

The detection results of proteoglycan and type II collagen mRNA expressions among the experimental groups were shown in Table 3. From the 14th day, the expressions of proteoglycan and type II collagen in group A were significantly different from group B, and gradually brightened with the incubation time prolonging. The absorbance values between the group A and B had the significant difference ($P<0.05$) (Table 3), while there was no significant change in group C, and there was almost no expression of proteoglycan or type II collagen in group C at any time point.

Discussion

The nucleus pulposus cells are the para-chondrocytes. The phenotypic changes of chondrocytes depend on the selection of stents, mechanical stimulation, as well as extracellular environmental changes such as adding certain growth factors (12-16). The growth factors and BMMSCs can promote the intervertebral disc's proliferation and differentiation in the in vitro culture (17). It was found that, before BMMSCs are seeded and cultured, the differentiation-related markers such as type I, II and III collagen, alkaline phosphatase and osteopontin are not expressed (18). Based on these characteristics, the gene transfection technology can introduce the genes of some bioactive factors to the target cells through the tissue engineering method, so the cells can relatively stably synthesize and secrete certain biological factors, thus achieving the purposes of promoting the cellular proliferation and differentiation (19). Risbud et al. (19) have confirmed that, BMMSCs can be differentiated into the nucleus pulposus-like cells. Under the conditions of low oxygen and TGF-β1 existence, the differentiation of BMMSCs can be made to trend to the consistent direction of nucleus pulposus's phenotypes, and these induced BMSCs can promote the regeneration of degenerated tissues when applied into the animal models of injured and degenerated discs. The experimental results of this research showed

that, after 21days of culture, the cell morphologies of group A and B had been changed significantly, which was changed from the initial long spindle and spindle to the latter polygonal. Their shapes were already very close to those of nucleus pulposus. This study also set the TGF-β1 expression in the cell culture supernatant as the indicator of BMMSCs transfection effects. The results showed that, on the 3rd day of culture, the TGF-β1 content of group A was higher than group B, and significantly higher than group C ($P<0.05$). It was gradually increased with the culture time prolonging, and reached the highest on the 21st day. Meanwhile, the DNA content was set as the marker to detect whether BMMSCs exhibited the biological activities. On the 3rd day, the DNA content of group A was gradually higher than group B, with the time prolonging, and it was significantly higher than group C ($P<0.05$). This indicated that, BMMSCs in group A exhibited higher biological activities. Therefore, its abilities of proliferation and synthesizing the extracellular matrix were stronger. In the immunohistochemical staining, BMMSCs of group A exhibited the positive type II collagen staining. The BMMSCs bodies were stained, exhibiting the typical phenotype of para-nucleus pulposus cells. The group B exhibited the weak positive staining, and only a few cells expressed the type II collagen. The group C had no significant metachromasia, suggesting that there already existed the synthesis of type II collagen inside the cell bodies of BMMSCs. We also set the expressions of proteoglycan and type II collagen mRNA inside BMMSCs as the indicator to identify its differentiation into the nucleus pulposus-like cells. The RT-PCR method was performed. It was confirmed from the gene level that, the type II collagen and proteoglycan mRNA existed inside BMMSCs of group A and B. These substances, which mainly existed inside the nucleus pulposus cells, were significantly expressed in BMSCs. This suggests that, the TGF-β1-transfected BMMSCs had differentiated towards the nucleus pulposus cells. With the time prolonging, this differentiation would be increasingly apparent.

The dynamic 3D culture can maximize the functions of cells, and provide a new model for in vitro culture of BMMSCs. Sikavitsas et al. (20) compared the dynamic and static 3D culture systems and found that, the mouse BMMSCs, which were cultured on the dynamic 3D biodegradable materials, could proliferate and differentiate. The functional cells could effectively grow in 3D cell culture system, and maintain strong activities. In order to provide a better 3D cell culture system, this experiment wrapped BMMSCs within the alginate gel microspheres, which could not only provide a 3D micro-environment for the in vitro BMMSCs culture, but also conducive towards its growth (21). On the other hand, the RCCS invented by National Aeronautics and Space Administration (NASA) could simulate the microgravity environment on the ground. The combination of these two technologies could provide suitable environment for the simulated microgravity 3D culture of wall-adherent growing BMMSCs. The tissue cells could be in a stable fluid-suspension state, and the fluid power could counteract the gravity-caused settlement. Therefore, the cell growth could be sustainably stimulated by the low shear, and the gravity vector that continuously changed its directions might directly affect the gene expressions, or indirectly promote the cellular autocrine/paracrine. This would be conducive to the intercellular signal transmission, thus improving the quality of cell differentiation (22). The results of this study displayed that, under the simulated microgravity 3D culture conditions, the TGF-β1 content in TGF-β1-transfected BMMSCs, as well as the contents of proteoglycan and type II collagen mRNA detected by RT-PCR, were significantly higher than other two groups, suggesting that the simulated microgravity conditions could promote BMMSCs to synthesize the proteoglycans and type II collagen.

The mechanisms of microenvironment impacts on BMMSCs are complex. The results of this study suggested that, under the simulated microgravity conditions, the stimulation of TGF-β1 was a necessary factor towards the direct differentiation of BMMSCs into the nucleus pulposus cells. However, how can this stimulus be converted into the intracellular signaling pathways still needs to be further investigated. With further studies, the model that in vitro simulats the microenvironment of intervertebral disc will help to explore the conditions under which the stem cells can differentiate into the nucleus pulposus. In addition, through simulating these environmental factors, more and more BMMSCs can be differentiated into the nucleus pulposus-like cells, even the mature nucleus pulposus cells. This study has provided a foundation for the clinical treatment of intervertebral disc degeneration.

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