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Differentiation of Mesenchymal Stem Cells into Nucleus Pulposus Cells *In Vitro**

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Summary: To find a new source of seed cells for constructing tissue-engineered intervertebral disc, nucleus pulposus (NP) cells and mesenchymal stem cells (MSCs) were isolated from New Zealand white rabbits. The nucleus pulposus cells population was fluorescence-laelled and co-cultured with MSCs with or without direct contact. Morphological changes were observed every 12 h. Semi-quantitaive reverse transcriptase-polymerase chain reaction was performed to assess the expression levels of Sox-9, aggreacan and type Ⅱ collagen every 24 h after the co-culture. MSCs treated with direct contact rounded up and presented a ring-like appearance. The expression of marker genes was significantly increased when cells were co-cultured with direct contact for 24 h. No significant change was found after coculture without direct contact. Co-culture of NP cells and MSCs with direct contact is a reliable method for generating large amount of NP cells used for cell-based tissue engineering therapy.
Key words: mesenchymal stem cells; nucleus pulposus cells; co-culture

MSCs isolated from bone marrow aspirates provide a nearly unlimited cell source with extremely high proliferation activity and the potential to differentiate into several mesenchymal cell lineages, including chondrogenic differentiation[1-4].

MSCs are now under intensive investigation for applications in tissue engineering and regenerative medicine because they are relatively easy to isolate and can differentiate down into mesoderm-derived lineages[5-7]. But the study concerning the ability of MSCs to differentiate into NP cells is scanty. In this study, we detected the gene expression of MSCs after co-culture with NP cells *in vitro*.

1 MATERIALS AND METHODS

1.1 Materials

Agarose, Hepes, DTT, EDTA, DAPI were purchased from Sigma Chemical, USA. Transwells were bought from Becton Dickinson, USA. Collagenase Ⅱ, trypsin, DMEM, Trizol reagent were procured from Invitrogen, USA. PCR primers, Taq DNA polymerase, DNA ladder were obtained from Sangon, China. FBS was purchased from Sijiqing Co., China. New Zealand rabbits were from the Animal Center of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

1.2 Methods

1.2.1 MSCs and NP Cells Collection Bone marrow was collected from New Zealand white rabbits by flushing femurs and tibias with phosphate-buffered saline with 2 mmol/L EDTA. The cells were washed with PBS/EDTA and plated into a 75-cm^2 flask with DMEM/F12 and 10% FBS. After 3 days, non-adherent cells were removed by medium change, and adherent cells were further cultured in DMEM/F12, 10% FBS for an additional 4 days. On day 6, the adherent cells were passaged into a 6-cm dish. The medium was changed two to three times a week, and cell density was maintained at $2–15\times10^3$ cells/cm^2. MSCs were used in the experiments only after 2 to 3 expansion passages to ensure depletion of monocytes/macrophages. NP cells were taken from thoracolumbar spines including T_5 to L_7 levels of 20 New Zealand white rabbits weighing an average of 1.5 kg while under inhalation anesthesia with 2.5% isoflurane. The gel-like nucleus pulposus was separated from the annulus fibrosus, by using a dissecting microscope, and treated with 0.1% collagenase Ⅱ for 30 min. The partially digested tissue was maintained as an explant in DMEM/F12. All co-cultures were conducted in bipartite in 6-well plates, with a pore-size of 0.4-μm.

1.2.2 DAPI Staining and Co-culture of the Nuclei of NP Cells NP cells were incubated with DAPI diluted in a cell culture medium at a final concentration of 10 μg/mL for 15 min at 37°C. A fluorescent signal was monitored and representative images were taken after 1 h, 12 h, and 24 h. The DAPI incubation started 30 min before each observation. Images were acquired by using a fluorescent microscope equipped with a Kodak digital camera system. Image acquisition was performed by using the MetaMorph 4.1 program. The micrographs were taken at central parts of the wells, where cellular density was most uniform. DAPI-labeled NP cells and control wells of unlabeled MSCs were prepared using of

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$5\times10^3/cm^2$. Each group had 20 samples of MSCs with or without direct (cell-to-cell) contact with NP cells. In the experimental group, the primary NP cells were directly added into the monolayer of the third-passage MSCs. The co-cultured cells were maintained for 24 h in DMEM/F12 at 37°C and in 5% CO_2 in a humidified atmosphere. In the control group, the primary NP cells were seeded onto the transwell membrane of the inner chamber 1 to 2 h before the beginning of the co-culture, and then MSCs were cultured in the lower chamber of 24-mm diameter.

1.2.3 RT-PCR RNA was isolated from cells by using Trizol reagent. Total RNA was isolated from mono-layer differentiated MSCs. The PCR amplification was conducted as follows: 1 cycle at 94°C for 1 min, 30 cycles at 94°C for 30 s, 55°C for 10 s, 72°C for 40 s and 1 cycle at 72°C for 5 min. Target genes included Sox-9, aggrecan, type Ⅱ collagen and GAPDH. The primers of Sox-9 were 5'-GACTTCCGCGACGTGGAC-3' (upstream) and 5'-CAGTACCTGCCGCCCAAC-3' (downstream). The primers of type Ⅱcollagen were 5'-GGCAATAGCAG GTTCACGTACA-3'(upstream) and 5'-CGATAACAG TCTTGCCCCACT-3'(downstream). The primers of aggrecan were 5'-CGCGAGACCTGGGTGGATGC-3' (upstream) and 5'-GAAGGGGCAGGCTGGATATTGC-3' (downstream). The primers of GAPDH were 5'-TGAAG GTCGGAGTCAACGGATTTGGT-3' (upstream) and 5'-CATGTGGGCCATGAGGTCCACCAC-3' (downstream). The RT-PCR products were electrophoresed and quantified by scanning with an optical densitometer. The ratios of the target genes/GAPDH were calculated and used as indicators of relative levels.

2 RESULTS

2.1 Morphological Findings

When MSCs (black arrow) differentiated into NP cells (white arrow), they experienced a rapid morphologic change. The MSCs co-cultured without direct contact remained fusiform, whereas cells co-cultured with direct contact rounded up and presented a ring-like structure when viewed at high magnification (×200) (fig. 1). After cultured separately for 8 days, MSCs underwent fusion and showed refraction, like the primary NP cells (fig. 2).

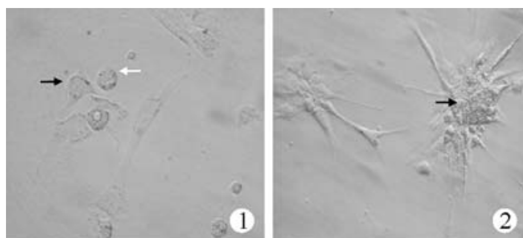

Fig. 1 Co-culture for 24 h with the direct contact
Fig. 2 Culture separately on the 8th day

2.2 Relative Gene Expression

Semi-quantitative RT-PCR was employed to study the changes in gene expression between control and co-cultured samples. Relative gene expressions of aggrecan, type Ⅱ collagen and Sox-9 mRNA were significantly increased in differentiated MSCs on the 4th day ($P<0.01$). After co-cultured without direct contact, almost no change in gene expression was noted in these genes ($P<0.01$) (fig. 3). And we found that gene expression continued to increase 6 days after be induced and cultured separately. The gene expression reached a peak on the 4th day.

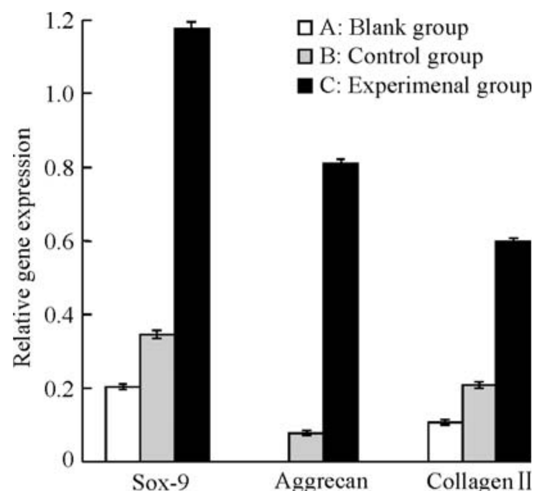

Fig. 3 Relative gene expression on the 4th day

3 DISCUSSION

Low back pain is extremely common, with 60% to 80% of people affected at some point during their lives and has a massive impact on economies due to both health care spending and loss of productivity[5]. Several studies have shown that degeneration of intervertebral disc (IVD) is one reason for a multiplicity of cases of low back pain[6,7]. Surgical procedures such as herniotomy and vertebral fusion are the most widely used and effective current treatments for IVD herniation and spondylolisthesis, conditions that can arise from IVD degeneration[8]. However, these procedures sacrifice the function of the IVD and increase the mechanical load on the adjacent discs. A new approach aiming to repair degenerated discs is tissue engineering of IVDs. For tissue engineering of IVD, one major aim is to identify suitable cell populations with the capacity to generate IVD tissue[9].

MSCs have been attracting increasing attention and may be an ideal seed cells for the construction of tissue-engineered IVD. Due to milieu-induced-differentiation, MSCs experienced corresponding changes upon receiving chemical or physical stimuli from microenvironment. Many researchers investigated the influence of direct contact on the differentiation of MSCs by using co-culture system. Yamamoto *et al* suggested that co-culture with direct contact induced growth factor expression, including transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF), which is not seen in the co-culture without contact[10], but they failed to observe the morphological change of MSCs. Our findings were consistent with those of Ball *et al*, who demonstrated that co-culture influences bone marrow mesenchymal stem cell fate [11].

Co-culture of MSCs with direct contact showed substantial increase in gene expression of Sox-9, aggrecan and type Ⅱ collagen, indicating that direct contact is essential for MSC differentiation to NP cells. MSCs co-culture without contact did not show any significant changes in gene expression. It is concluded that the cells with which MSCs are co-cultured determine their fate and that co-culture of MSCs with NP cells with direct contact produces NP cells differentiated from the stem cells. Moreover, like NP cells, MSCs presented a ring-like appearance.

In conclusion, NP cells could induce MSCs to differentiate into NP cells and MSCs promise to be used as seed cells for the construction of tissue engineered intervertebral disc for the treatment of IVD disease.

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