Construction of Sox9 Gene Eukaryotic Expression Vector and Its Inductive Effects on Directed Differentiation of Bone Marrow Stromal Cells into Precartilaginous Stem Cells in Rats*

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Summary: Sox9 gene was cloned from immortalized precartilaginous stem cells and its eukaryotic expression vector constructed in order to explore the possibility of bone marrow-derived stromal cells differentiation into precartilaginous stem cells induced by Sox9. A full-length fragment of Sox9 was obtained by RT-PCR, inserted into pGEM-T Easy clone vector, and ligated with pEGFP-IRES2 expression vector by double digestion after sequencing. The compound plasmid was transfected into born marrow-derived stromal cells by Lipofectamine 2000, and the transfection efficacy and the expression of Sox9 and FGFR-3 were observed. Flow cytomtery was used to identify the cell phenotype, and MTT was employed to assay proliferative viability of cells. Sequencing, restrictive endonuclease identification and RT-PCR confirmed that the expansion of Sox9 and construction of Sox9 expression vector were successful. After transfection of the recombinant vector into bone marrow-derived stromal cells, the expression of Sox9 and FGFR-3 was detected, and proliferative viability was not different from that of precartilaginous stem cells. It was concluded that Sox9 gene eukaryotic expression vector was successfully constructed, and the transfected bone marrow-derived stromal cells differentiated into the precartilaginous stem cells.

Key words: precartilaginous stem cells; Sox9; bone marrow-derived stromal cells; transfection

Precartilaginous stem cells (PSCs) are the adult stem cells that control the growth of animal extremities in growing phase, and can differentiate directionally. As tissue engineering seed cells, PSCs can be used as an alternative for repairing the defects of cartilages and bones. PSC differentiation starts on the 5th generation. The sampling of the cells is difficult, and a large number of PSCs with identical phenotypes can be obtained by primary cell culture technique. This difficulty can be overcome by inducing differentiation of marrow stromal cells (MSCs) into PSCs. Sox9 inhibits the differentiation of precartilaginous cells at end-stage, prolongs the maturing process of cartilage and suppresses its apoptosis. In this study, eukaryotic expression vector pEGFP-IRES2-Sox9 was constructed, and transfected into MSCs to induce differentiation of PSCs in order to provide stable cell supply for the study of the differentiation of precartilaginous cells and for tissue engineering.

1 MATERIALS AND METHODS

1.1 Materials
Immortalized PSCs (IPSCs) line, and E. coli-DH5α strain were stored in the Laboratory of Orthopedics, Tongji Hospital, Tongji Medical College, HUST, China. DMEM/F12 medium and fetal bovine serum were purchased from Gibco, USA. Trizol reagent and RT-PCR kit were the products of Toyobo, Japan. Rabbit monoclonal antibody FGFR-3/c-15 was bought from Santa Cruz, USA. BamHI, EcoRI, and pGEM-T Easy were from Promega, USA, pEGFP-IRES2 and Lipofectamine2000 were from Invitrogen (USA), type II collagen and type X collagen antibody from Promega (USA), rabbit anti-mouse antibody FGFR-3/c-15 was bought from Santa Cruz, USA. Alexa Fluor goat anti-rabbit IgG (secondary antibody) from Sigma (USA).

1.2 Design of Primers
According to GeneBank (NM012636) sequence coding region, the primers containing BamHI and EcoRI enzyme digestion sites respectively were synthesized by Shanghai Yingjun Biologic Co., China. The upper stream sequence was 5'-ATG GAA ATC ACG GAA GAG CGT C-3', and the downstream sequence was 5'-GTG CTG CD44, CD45 and CD34 (primary antibodies), and alexa Fluor goat anti-rabbit IgG (secondary antibody) from Sigma (USA).

1.3 Methods
1.3.1 Construction of Sox9 Eukaryotic Expression Vector Total RNA was extracted from about 10^6 IPSCs from the third generation by one-step method according to the instructions of Trizol Reagent Kit. After measurement of RNA concentration, about 2-μg total RNA was used as a revere transcription template to synthesize the cDNA first strand according to the instructions of reverse transcription kit. The conditions of PCR were as follows: pre-denaturation at 95°C for 5 min, denaturation at 94°C for 1 min, annealing at 62°C for 45 s,
extension at 72°C for 50 s, 35 cycles, and final extension at 72°C for 10 min. Five μL PCR product was electrophoresed on 0.2% agarose gel, and primarily identified. By using a routine method, pGEM-T Easy was constructed, and screened out by blue and white screening. The positive clones were identified by bacterial PCR and single enzyme cutting, and sequenced by Shanghai Yingjun Co., China. The correct pGEMT-Sox9 plasmids were cut by BamHI and EcoRI, and the fragments containing Sox9 were recovered, and ligated with pEGFP-IRES2 fragments recovered by the same double enzyme cutting, which were screened out with kalamycin and identified by double enzyme cutting.

1.3.2 Isolation, Purification, Transfection and Identification of MSCs According to the method described by Friedenstein[8], primary rat MSCs were obtained, cultured at 37°C with 5% CO2 and 100% humidity. Forty-eight h later, the medium was replaced, once every 3–4 days, and MSCs were passed within 7–19 days, purified by adsorption separation method, and expanded.

After MSCs in 24-well plates grew to 60% confluency, pEGFP-IRES2-Sox9 was transfected into the cells according to the instructions of Lipofectamine™2000. Four h later, serum-free medium was removed, and complete culture medium was added. Forty-eight h later, the MSCs were screened with G418 at a concentration of 300 μg/mL for 12–14 days, and the resistance gene clones were expanded with culture medium containing 200 μg/mL G418. After culture for 48 h, the nuclei of MSCs were labeled with DPI, and observed under the fluorescent microscope. By using Image-Pro Plu, the transfection efficiency was calculated. Transfection efficiency=Number of cells with green cytoplasm/Number of cells with red nuclei.

The transfected MSCs were lysed with RIPA, and protein was quantitatively measured by BCA method, electrophoresed, transferred to the membrane. The membrane was blocked for 2 h, incubated with primary rabbit anti-mouse Sox9 IgG (1:4000) overnight at 4°C, rinsed with TBS-T thrice, incubated with secondary goat anti-rabbit (1:500) at room temperature for 1 h, and developed by ECL. The expression of Sox9 in the transfected MSCs was detected by using RT-PCR.

1.3.3 Detection of FGFR-3 Expression in the Transfected MSCs by Immunohistochemistry and Western Blotting The MSC smears at the 12th day after transfection were examined by using immunohistochemical kit with rabbit multiple clonal antibody as primary antibody, and goat anti-rabbit IgG-HRP as secondary antibody, respectively. The expression of FGFR-2 was detected by using Western blotting.

1.3.4 Flow Cytometrical Identification of Phenotypes On the 30th day after transfection of MSCs, the expression and proportion of antigens were flow cytometrically detected with rabbit multiple clonal antibody FGFR-3 (c-15) and IgG-HR as primary antibodies, and PE- and FITC-labeled goat anti-rabbit IgG as secondary antibody.

1.3.5 Assay of Proliferative Viability of MSCs by MTT Method MSCs were digested with trypsin and transfected for 30 days, and PSCs at 2nd generation (PSCs 2) were inoculated into 96-well plates at a density of 3×10^4/well. Before absorbance (A) measurement, 20-μL (5 g/L) MTT solution was added, medium was removed, and 150-μL DMSO was added. The A values at 490 nm were measured 5 times.

2 RESULTS

2.1 Construction and Identification of Recombinant Eukaryotic Expression Vector The extracted total RNA was subjected to PCR, and a specific band of about 2000 bp was obtained after the electrophoresis of the PCR product (fig. 1).

2.2 Detection of FGFR-3 Expression in the Transfected MSCs by Western Blotting A target band of 2000 bp and a linearized plasmid band of about 3.0 kb were obtained (fig. 3), suggesting that Sox9 gene has been successfully linked to the region between BamHI and EcoRI at the pEGFP-IRES2 site, and the recombinant eukaryotic ex-