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Original Article

In-vitro simulation of BMSC implantation into inner ear via co-culture with basal membrane

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Abstract: Objective: Loss of spiral ganglion neurons (SGNs) can lead to sensorineural hearing loss (SSHL). Bone marrow mesenchymal stem cell (BMSC) is one of promising stem cell sources for cell replacement therapy. Here, we modeled the BMSC implantation with co-culture systems in vitro to provide a basis for cell replacement therapy. Methods: The study includes two parts: First, basal membranes and BMSCs were isolated separately, and indirectly co-cultured in Transwell system. Second, GFP-BMSCs were added into basal membranes to perform direct co-culture. Results: BMSCs exhibited expression of tublin, a marker of SGNs in the indirect co-culture system. GFP-BMSCs formed a line just in the location of SGNs in the direct co-culture system. Conclusion: BMSC can differentiate into SGN-like cells in the micro environment with basal membranes, and trend for the site of injured SGNs. This may provide an in-vitro theoretical basis for cell replacement therapy.

Keywords: Cell replacement therapy, co-culture, basal membrane, BMSC

Introduction

BMSCs have a strong ability of differentiation and can be get from patient's own bone marrow [1], making it a promising stem cell source for cell replacement therapy. Previously, reports have shown that MSCs could be induced and perform neuronal differentiation in vitro by application of basic fibroblast growth factor (bFGF), and ciliary neurotrophic factor [2, 3]. In this study we used a new method to induce the neural differentiated of BMSCs in vitro.

Cell replacement therapy is supposed to replace the injured spiral ganglion neurons with stem cells to for hearing loss [4]. While stem cells were transplanted into inner ear without various factors in cell replacement therapy, the fate of these stem cells remains unclear. Here, we used a co-culture system to mimic the status of BMSCs transplanted into inner ear at the same time.

Materials and methods

Isolation and culture of BMSCs

All animal studies were performed in accordance with guidelines established by the Central South University Committee on Animal Care and Use. BMSCs were isolated according to the previous reports [2, 5-7]. Rat weighing 150-200 g were anesthetized with pentobarbital sodium 30 mg/kg body weight intraperitoneally. A skin incision was made to expose the femur and tibia. Epiphyses of femurs and tibias were removed, and the marrow was flushed out with Dulbecco's Modified Eagle Medium/F12 (Gibco, USA) into a 25 mm^2 culture flask. The isolated bone marrow, composed of hematopoietic and stromal cells, was maintained in DMEM/F12 supplemented with 10% FBS (Gibco, USA) and 100 units/ml penicillin (Sigma) at 37°C with 5% CO_2 atmosphere. Hematopoietic and nonadherent cells were removed by changes of medium. Only the cells

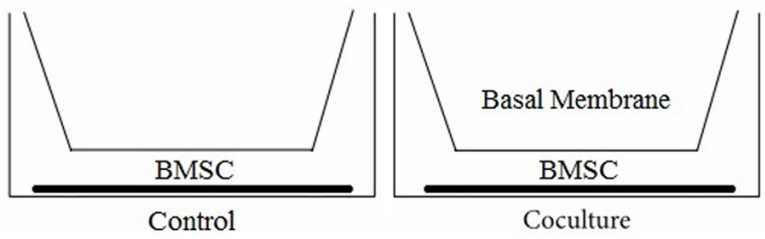

Figure 1. The indirect co-culture system (right) are consist of lower compartment, coverslip, BMSCs, upper compartment, basal membrane (from lower to upper). Left is control group without basal membrane in the upper compartment.

that attached to the culture flask were isolated and rinsed. These cells were continuously cultured in DMEM/F12 containing 10% FBS and 100 U/ml penicillin. The culture medium was replaced at 3-day intervals. Cell were trypsinized (Trypsin, Hyclone, USA) at 80%-90% confluence. BMSCs subcultured to the third generation were used for the experiments. GFP-BMSCs were purchased from Cyagen Biosciences at passage three. They were cultured in the same condition of BMSCs isolated from bone marrow of rats.

Isolation of basal membrane

After SD rat pups were anesthetized, cochleaes were dissected in ice-cold PBS. The skull was opened and the temporal bones were harvested. The membranous labyrinth was separated from the modiolus and the stria vascularis and spiral ligament were carefully removed under the microscope. With the organ of Corti and the spiral ganglion kept intact, Reissner's membrane and tectorial membrane were removed with fine forceps. All the turns of basal membrane were used for each culture in our studies.

Indirect co-culture of BMSCs and basal membrane

In transwell assays, BMSCs were trypsinized (Trypsin, Hyclone, USA) and plated on the coverslip prepared in lower compartment beforehand with basal membrane placed in the upper compartment. Both compartments were filled with DMEM/F12 media containing 10% FBS and 100 U/ml penicillin. In control group, no basal membrane was placed in the upper compartment. Only BMSCs were cultured in lower compartment in DMEM/F12 media containing 10% fetal bovine serum FBS and 100 U/ml penicillin. The culture medium was changed every 3 days. BMSCs and basal membrane were co-cultured for 2 weeks.

Judgment and count of positive staining BMSCs: under the microscope, BMSCs whose cytoplasm presenting red fluorescence light was positive staining cells. Randomly select 10 high power visual fields to count the number of positive staining cells and the total cell number for further analysis.

Direct co-culture of BMSCs and basal membrane

The cochleae was removed and placed in Leibovitz's medium (Gibco, USA). After removing the lateral wall, the whole basal membrane containing the organ of Corti and spiral ganglion neurons was isolated, transferred onto a collagen gel matrix in a 35 mm culture dish containing a solution consisting of 20 μl of rat tail collagen (Type 1, BD, USA, 10× basal medium eagle, Sigma, USA, 2% sodium carbonate, 9:1:1 ratio and 1.3 ml DMEM/F12 supplemented with 1% FBS and 100 units/ml penicillin) and incubated overnight at 37°C with 5% CO_2. On day 2, the culture medium was removed and replaced with DMEM/F12 containing 10% FBS and 100 units/ml penicillin. And on day 2, GFP-BMSCs were trypsinized and placed in the same 35 mm culture dish as basal membrane. The culture medium was changed every 3 days. BMSCs and basal membrane were co-cultured for 4 days.

Fluorescent labeling of cells and basal membrane

All procedures were performed in the culture well with cells and basal membrane attached to the coverslip. After fixation with 4% paraformaldehyde for 15 min, cells and basal membrane were permeabilized with 0.25% Triton X-100 (Molecular Probe, Life technology, USA) in PBS for 30 min, incubated for 60 min in 5% normal goat serum (Gibco, USA) in PBS. The cells and basal membrane were incubated sequentially in primary and secondary antibod-

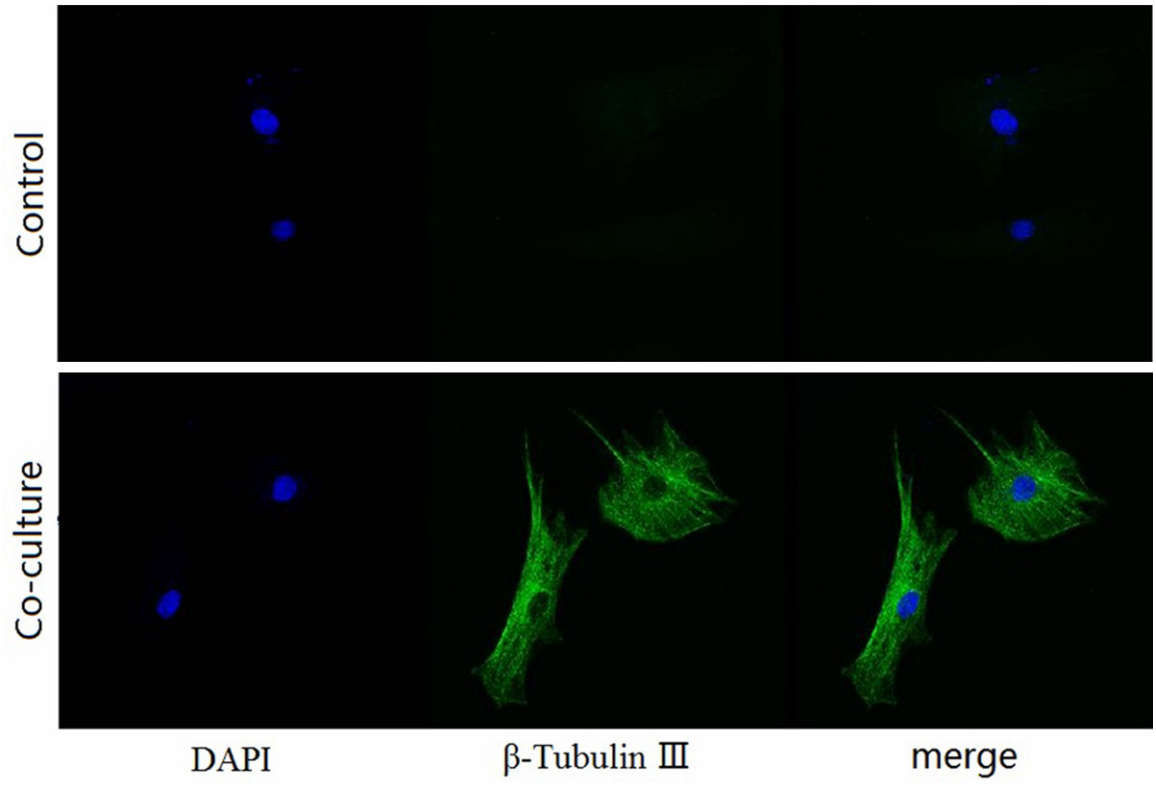

Figure 2. The first row is control group with negative β-Tubulin III staining of BMSCs. The second row is indirect co-culture group with positive β-Tubulin III staining of BMSCs (green).

Table 1. Positive rate of β-Tubulin III staining BMSCs (%, n=5)

Group	β-Tubulin III	*p* value
Co-culture	24.96±5.65	0.00
Control	0.00±0.00	

ies diluted in PBS, primary antibodies overnight at 4° and secondary antibodies for 1 h at room temperature, with three intervening PBS washes. Primary antibodies used were as follows: β-Tubulin III antibody was a monoclonal antibody from the rabbit (Sigma, USA; dilution 1:1000); Neurofilament (NF-200) antibody was a polyclonal antibody from mouse (Millipore, Billerica, MA, USA; dilution 1:200). Cells and basal membrane were visualized using a Zeiss confocal laser scanning microscope.

Culture of GFP-BMSCs

GFP-BMSCs were purchased from Cyagen Biosciences Inc. (USA). They were cultured in DMEM/F12 supplemented with 10% FBS (Gibco, USA) and 100 units/ml penicillin (Sigma) at 37°C with 5% CO_2 atmosphere. Cell were trypsinized (Trypsin, Hyclone, USA) at 80%-90% confluence. The culture medium was replaced at 3-day intervals. BMSCs were subcultured to the third generation, and then used for the experiments.

Statistical analysis

Measurement data are presented as mean ± SEM. Comparisons between measurement data were made by χ^2 test between enumeration data. *P* value <0.05 was considered statistically significant.

Results

Neural differentiation of BMSCs in the indirect co-culture system

Transwell system (**Figure 1**) was employed to perform indirect co-culture of BMSCs and basal membranes. BMSCs co-cultured with basal membranes exhibited expression of β-Tubulin III (**Figure 2**), a marker of SGNs. Positive rate of β-Tubulin III staining BMSCs was shown in **Table 1**. And there is significant difference between positive rate of β-Tubulin III staining in co-culture and control group (P<0.05).

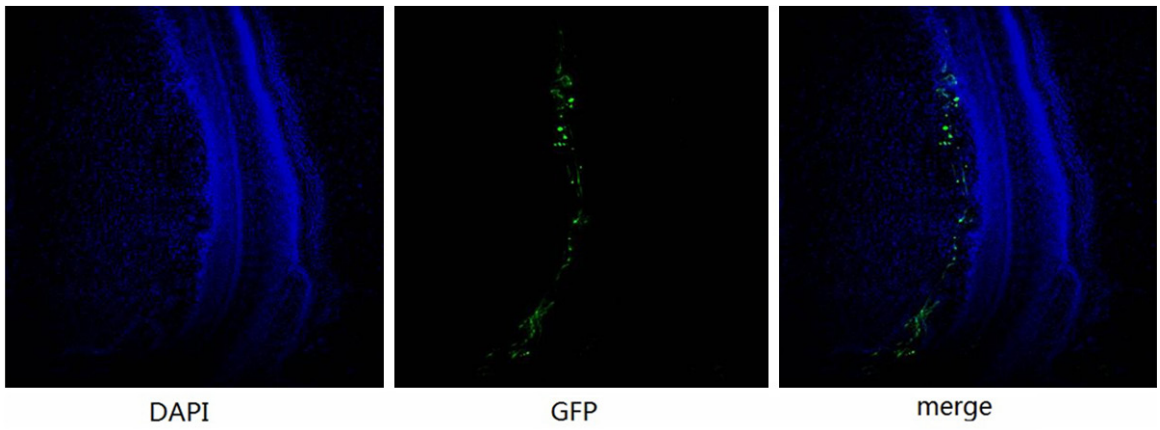

Figure 3. GFP-BMSCs formed a line just in the location of original SGNs in the inner part of direct co-culture basal membrane. DAPI (blue), GFP-BMSCs (green).

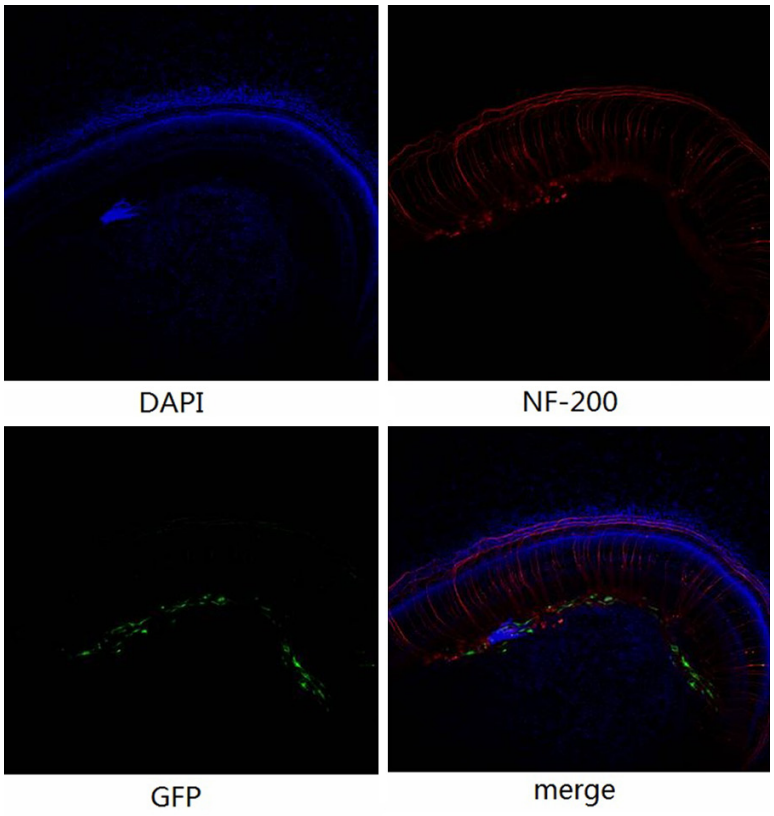

Figure 4. GFP-BMSCs (green) and NF-200 (red) overlap in the direct co-culture system of BMSCs and basal membrane. DAPI (blue).

Aggreation of BMSCs in basal membranes in direct co-culture system

In the direct co-culture system, GFP-BMSCs and basal membrane were cultured together formed a line just in the location of original SGNs (**Figure 3**). GFP-BMSCs and NF-200 marked spiral ganglion neurons overlapped in the same site (**Figure 4**).

Discussion

Restoration of SGNs is one of the targets for stem cell therapy in the treatment of hearing loss. Neural differentiation and migration of stem cells may help to restore SGNs.

Previously, Dezawa et al [1] have induced BMSCs to differentiate towards neurons by application of some neural factors. Deng et al [8] treated MSCs with isobutylmethylxanthine/dibutyryl cyclic AMP for 6 days, finding that MSCs may have been differentiated into early progenitors of neural cells in vitro under conditions that increase the intracellular level of cAMP. Here, we used Transwell system to indirectly co-culture basal membrane and BMSCs. Ideally, the indirect co-culture system simulated that BMSCs transplanted into inner ear were separated from basal membrane and spiral ganglion neurons. The semi permeable membrane separated basal membrane, but allowed various factors to go freely from one side to the other. Some neural factors [6, 9, 10] has been proved to have effect on

BMSCs transplanted into inner ear. So, we can observe the differentiation of BMSCs in the stimulant co-culture environment. The result of indirect co-culture revealed undifferentiated BMSCs performed neural differentiation with co-culture of basal membrane. The above data predicted that basal membrane may release some neural factors to induce neural differentiation of BMSCs in vitro. This was consistent with the result in vivo of Wei Chen, etc [11] that transplanted stem cells formed an ectopic spiral ganglion, which had undergone differentiation by β-tublin III staining representing the neural differentiation.

To further simulate the migration of BMSCs in inner ear, we directly co-cultured basal membrane and GFP-BMSCs to observe the migration in vitro. Results showed that BMSCs formed a line just in the location of original SGNs. This showed that BMSCs had an ability of migration towards the location of SGNs. This is consistent with the report of Ahn et al [12-14] that stem cells transplanted into inner ear were found in the spiral ganglion area.

In conclusion, our present findings revealed a new method to induce neural differentiation of BMSCs. This may present in-vitro evidence that BMSCs transplanted into inner ear trended for the site of SGNs, and could differentiate into SGN-like cells in the micro environment with basal membranes and spiral ganglion neurons. These may provide an in-vitro theoretical basis for cell replacement therapy.

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Disclosure of conflict of interest

None.

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