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Impregnation of plasmid DNA into three-dimensional PLGA scaffold enhances DNA expression of mesenchymal stem cells *in vitro*

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Current efforts had been made to undertake a three-dimensional (3-D) reverse transfection of bone marrow-derived mesenchymal stem cells (BM-MSCs) in PLGA scaffolds. As a kind of multipotent stem cells, BM-MSCs show great potential and tremendous capacity in the gene transfection field and PLGA 3-D scaffold has been shown to be a biomaterial that provides structural support to cells proliferation and tissue engineering. The objective of this study was to assess the transfection efficiency of BM-MSCs with a 3-D reverse transfection method by using PLGA scaffold and observe the SEM photographs of BM-MSCs cultured on PLGA scaffold. BM-MSCs were cultured in 3-D PLGA scaffold which was incorporated with pullulan-spermine/pGL3. It was shown that the gene expression duration of BM-MSCs transfected using 3D reverse method with pullulan-spermine/DNA in the presence of serum maintained 12 days at high levels as compared with the plasmid DNA in medium, and scanning electronic microscopy (SEM) photographs of BM-MSCs cultured on PLGA scaffold exhibited robust cell attachment and viability when cultured in PLGA scaffold *in vitro*. This study demonstrates that the 3-D reverse transfection method of BM-MSCs using PLGA scaffold could achieve long gene expression in a relatively high level, therefore this transfection system is promising in gene transfection and tissue engineering.

1. Introduction

In gene transfection research and regenerative medicine, stem cells are promising for their proliferation capacity and differentiation potential. Nowadays, BM-MSCs have been experimentally used to demonstrate their *in vivo* potential to induce the regeneration of mesenchymal tissues (Niklason et al. 1999) and they have been intensively investigated alone or in combination with scaffolds which could promote cell proliferation and differentiation (Tabata 2003). Since it is reported that stem cells are effective in inducing the regeneration of tissues other than cells (Griffith et al. 1999), their feasibility in the cell source for regenerative medicine is highly probable as they could be isolated easily. The viability and maintenance of cells in the scaffold materials is a critical step for the cell proliferation and underlying differentiation (Goh et al. 2003), as the properties of the scaffold material for cell attachment play a major role in the cell morphology, proliferation, function and the subsequent tissue organization (Ingber et al. 1995). Cells were firstly attached to the material surface of the scaffold and then the cells will be spread and proliferate. Cell attachment to the surface of the scaffold is a critical step during cell proliferation. The 3-D PLGA scaffold could offer a mimic physical circumstance and a large surface area to promote the adhesion and growth of cells.

The current study done was to examine the gene transfection efficiency of BM-MSCs in this 3-D structure. 3-D PLGA scaffold were functionalized with Pronectin to promote the adhesion and growth of cells on their surfaces. The BM-MSCs attached into the 3-D PLGA scaffold was investigated with SEM. BM-MSCs were seeded into the 3-D PLGA scaffold which was incorporated with pullulan-spermine/pGL3 to evaluate the gene expression of BM-MSCs in 3-D PLGA networks.

2. Investigations, results and discussion

From Fig. 1, it was shown that expression of BM-MSCs transfected using the reverse method maintained at a relatively high level after 12 d of transfection, we hypothesize that the reverse transfection system has reduced the negative influence of serum while better mimic microenvironment for cells give a higher rate of cell adhesion and similar morphology as *in vivo*.

Fig. 2 shows SEM photographs of BM-MSCs cultured on PLGA scaffold. Scanning electron microscopy images showed that PLGA scaffold was a porous 3-D matrix (Fig. 2A and 2B) with the seeded cells homogeneously distributed and the PLGA scaffold possessed an interconnected porous structure and their intrastructural appearance was similar.

This article describes the development of an *in vitro* culture system to enhance the expression of a plasmid DNA for BM-MSCs which were cultured in the PLGA scaffold by impregnation of plasmid DNA into 3-D scaffolds. Results clearly

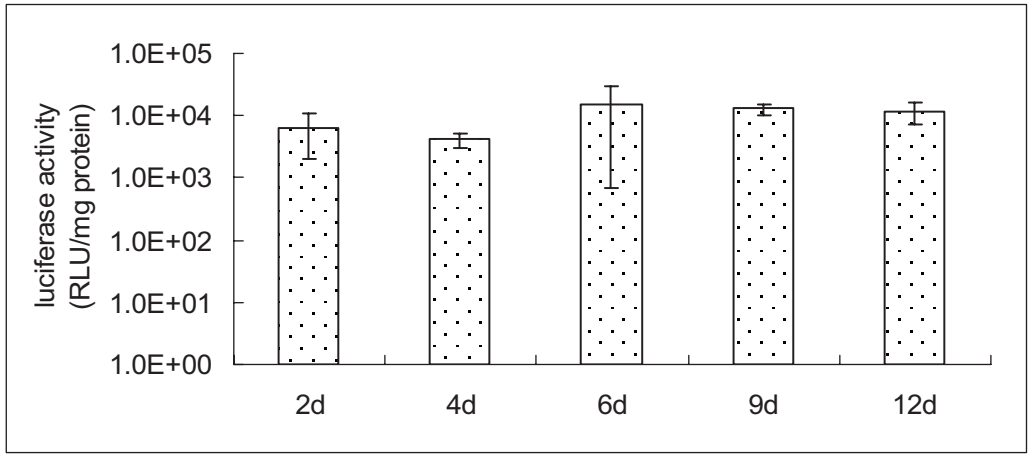

Fig. 1: Time course of luciferase expression level of BM-MSCs transfected using 3D reverse method with pullulan-spermine/DNA in the presence of serum

demonstrated that the reverse transfection method was more effective in enhancing the level and duration time of gene expression from plasmid DNA impregnated in scaffold than by plasmid DNA in medium (He et al. 2011).

Gene therapy has captured the scientific and public interest with the promise to deliver genes and proteins to specific tissues or to replace deficient host cell populations. Gene transfection is a powerful and promising technique to introduce exogenous genes into cells for experimental and therapeutic purposes *in vitro* or *in vivo*. Irrespective of the final goals, it is of vital importance to enable plasmid DNA to internalize into cells as efficiently as possible and to facilitate DNA expression for an extended time period. BM-MSCs are multipotent stem cells that have shown tremendous therapeutic potential and have been widely investigated for use by themselves or combined with scaffolds for applications in regenerative medicine (He et al. 2011). One future approach with BM-MSCs is combinational therap with genes. The cells not only function as a vehicle carrying target genes to the site of action, but also positively participate in the process of tissue repair. Therefore, a lot of research on tissue regeneration by genetically engineered BM-MSCs has been performed (He et al. 2011).

Biomaterials can serve as delivery vehicles that mediate cell alignment, cell migration and growth factor release (Richardson et al. 2001). A biocompatible porous scaffold plays an important role in seeding cells and serves as a template for tissue regeneration. The scaffold used should be biocompatible, biodegradable. Because BM-MSC proliferation is substrate-dependent, it is preferable to increase the surface area of culture substrate. Several 3-D substrates, so-called scaffolds, have been designed to demonstrate their feasibility in the enhancement of proliferation (Takahashi et al. 2004), as the 3-D scaffold provides a larger surface area for cell attachment and spreading than a 2-D scaffold (a tissue culture plate). Therefore the cell growth in the 3-D scaffold was sustained for longer time periods than that of 2-D substrate. Several researchers have demonstrated that cell proliferation was superior in the 3-D scaffold than in the 2-D substrate (Mueller-Klieser 1997). In the reverse transfection method, cells are cultured in the presence of serum. It is possible that the presence of serum enables cells to proliferate more efficiently under better culture conditions, resulting in enhanced gene transfection. Cell viability after reverse transfection culture was significantly higher than with conventional transfection culture in the absence of FBS. This indicated that the

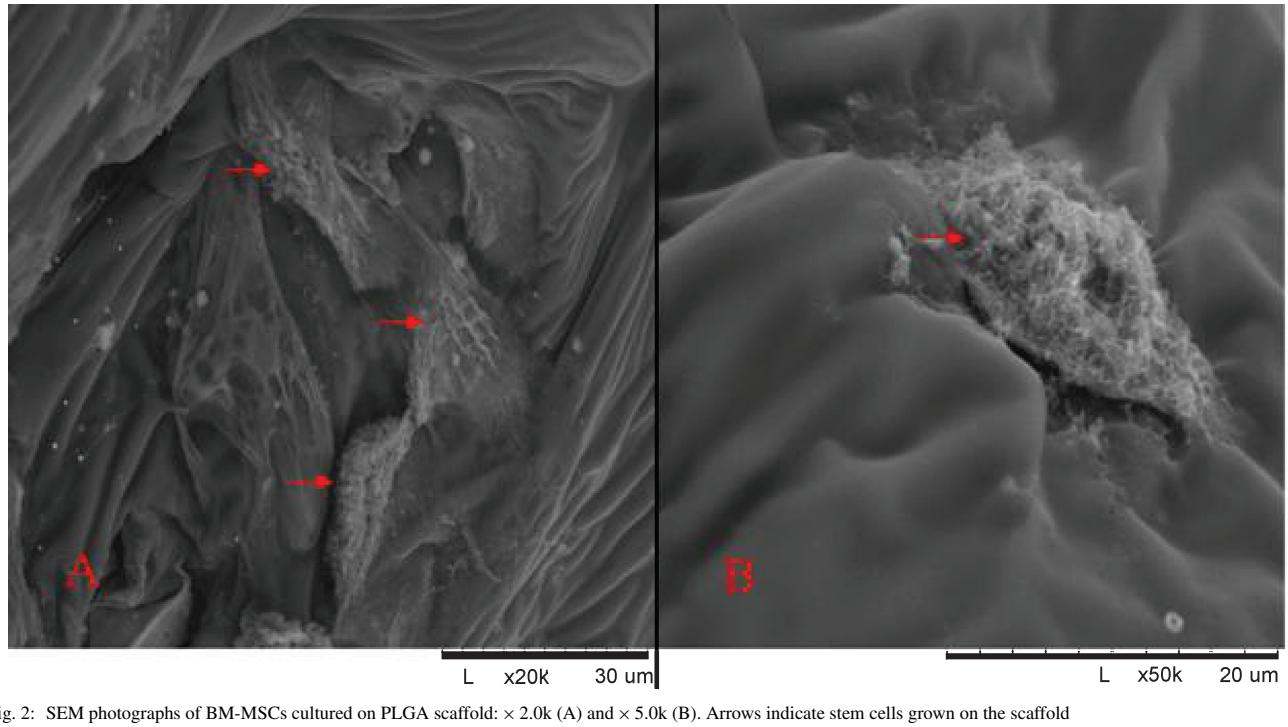

Fig. 2: SEM photographs of BM-MSCs cultured on PLGA scaffold: × 2.0k (A) and × 5.0k (B). Arrows indicate stem cells grown on the scaffold

reverse transfection is good for cell activity. Cells always make contact with the complex in reverse transfection culture, which is different from the contact time of 6 h in conventional transfection culture (Okazaki et al. 2007). Gene transfection with 3-D tissue constructs is a new field and few studies have been published on the *in vitro* transfection of genes into 3-D tissue constructs (Zimmermann et al. 2000; He et al. 2011). The goal of our research is to enhance the level of gene expression by combining a 3-D scaffold impregnated with plasmid DNA and reverse transfection for BM-MSCs. To evaluate the efficiency of gene delivery based on the constructed reverse 3-D systems, pDNA coding for luciferase was delivered to BM-MSCs.

The present study clearly demonstrates that BM-MSCs transfected with non-viral vector and pDNA coding for luciferase by reverse transfection method show their capacity to maintain a longer gene expression duration at a higher level than that of 2-D conventional method. We observed that BM-MSCs had robust cell attachment and viability when cultured in PLGA scaffold *in vitro*, a finding in concurrence with published literature (Catelas et al. 2006). It is highly conceivable that the continuous exposure of complex to cells and the adhesion between cells and the substrate, which minimizes the serum infiuence on the transfection activity of complex, results in the enhanced and prolonged gene expression.

In conclusion, the reverse transfection method combined with the stirring cell culture method in the presence of the adhesion substance is a promising technology to enhance the efficiency of gene expression for stem cells. This technology is applicable to any type of cell. It is expected that this transfection method with the non-viral pullulan carrier can be applied to genetic engineering for cell therapy as well as basic research into stem cell biology and medicine.

Future studies using an *in vivo* animal model are necessary to validate the feasibility of the BM-MSCs reversely transfected by non-viral vector and cultured in the 3-D scaffold in the tissue engineering.

3. Experimental

3.1. Materials

Pullulan-Spermine and anionic gelatin were synthesized by our laboratory. Pronectin (Sanyo), Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin and trypsin were obtained from Gibco BRL (U.S.A.). 0.05% Trypsin-EDTA (Invitrogen, Grand Island, NY). Plasmid DNA coding for luciferase was kindly provided by the Institute of Pharmacology & Toxicology, College of Pharmaceutical Sciences, Zhejiang University (China). Fetal bovine serum (FBS) was purchased from Sijiqing Biologic Co., Ltd. (China). PLGA scaffold was purchased from Shandong Medical Instruments Institute (China), Plasmid DNA coding for pGL3 gene was purchased from Origene Co. (China). The luciferase assay and BCA Protein Assay Kit were purchased from Beyontime Co.(China). All other chemicals were of analytical grade.

3.2. Animals

Sprague-Dawley (SD) male rats (3 weeks old, 50∼60 g) were supplied by Zhejiang University Experimental Animal Center, China. All animals were maintained under constant conditions (temperature 25 ± 1 °C) and had free access to a standard diet and drinking water. All of the experimental procedures were in accordance with the Zhejiang University guidelines for the welfare of experimental animals.

3.3. Procedures

3.3.1. Preparation of pullulan-spermine/DNA complexes

Pullulan-spermine/DNA complexes were prepared by mixing the aqueous solution of pullulan-spermine with that of plasmid DNA coding for luciferase gene at N/P ratios•the ratios of moles of the amine groups of pullulan-spermine to those of the phosphate ones of DNA••3. Briefly, same volume pullulan-spermine and pDNA which have been dissolved with phosphate-buffered saline solution (PBS, pH 7.4) were mixed together and incubated for 20 min at room temperature and then diluted with PBS with the ultimate concentrations of pDNA of 100 μg/ml.

3.3.2. Preparation and culture of BM-MSCs

Bone marrow-derived mesenchymal stem cells (BM-MSCs) were isolated from the bone shaft of femurs of 3-week-old SD male rats according to the technique reported by Lennon et al. (1995). Briefly, both ends of rat femurs were cut away from the epiphysis and the bone marrow was flushed out by a syringe (21-gauge needle) with 1 mL of Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 15% FBS L-glutamine and penicillin–streptomycin (50 U/mL). The cell suspension (5 mL) was placed into two 25 cm^2 flasks and cultured at 37 °C in a 95% air–5% CO_2 atmosphere. The medium was changed on day 4 of culture and every 3 days thereafter. When the cells of the first passage became subconfluent, usually 7–10 days after seeding, the cells were detached from the flask by treatment for 5 min at 37 °C with PBS solution containing 0.25 wt% trypsin and 0.02 wt% ethylenediaminetetraacetic acid (EDTA). Cells were normally subcultured at a density of 2×10^4 cells/cm^2. Second-passage cells at subconfluence were used for all experiments.

3.3.3. 3-D reverse transfection

PLGA scaffolds were pre-sterilized with 75% (v/v) ethanol followed by repeated washing with PBS to remove any residual alcohol. Then, a similar coating procedure with the 80 μl anionic gelatin containing Pronectin incubated at 37 °C for 2 h in a 95% air–5% CO_2 atmosphere. Replaced the supernatant and washed twice with PBS, then pullulan-spermine/pDNA complex (pGL3) was performed for the scaffolds. After the coating of the complex, 100 μl of BM-MSCs (2×10^6 cells/ml) were seeded into the scaffold and incubated for 2 h for cell attachment. Then 2 mL of medium contained serum was added slowly to the plate wells and the seeded scaffolds were transferred to fresh 24-well culture plates containing medium in the next day, and exchanged medium twice a week thereafter. The luciferase assay was performed as described above in different time point (2–4–6–9–12 d respectively).

3.3.4. Morphology observation

The morphology of 3-D scaffold and MSC attached to network were observed with a scanning electron microscope, PLGA scaffolds with cells which reverse transfected 9 d later were washed with PBS solution and incubated in paraformaldehyde at 4 °C overnight. Samples were then rinsed in 0.1 M phosphate-buffered saline solution (PBS, pH 7.0) and immersed in 1% OsO_4 solution for 1–2 h, then dehydrated in 50%, 70%, 95%, and 100% ethanol for 20 min for each respective ethanol change. Thereafter the samples were treated with a solution of ethanol and the same volume isoamyl acetate for 30 min, then samples in pure isoamyl acetate overnight. Set the temperature point at 35 °C, when the pressure reached 72.8 Pa to dry it at critical point for 3 min. Then samples were mounted on specimen holders and dried from CO_2. The dried samples were finally sputter coated with gold for SEM observation (Hitachi X-650, Tokyo, Japan).

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References

Catelas I, Sese N, Wu BM, Dunn JC, Helgerson S, Tawil B (2006) Human mesenchymal stem cell proliferation and osteogenic differentiation in fibrin gels *in vitro*. Tissue Eng 12: 2385–2396.

Goh KL, Hukins DW, Aspden RM (2003) Critical length of collagen fibrils in extracellular matrix. J Theor Biol 223: 259–261.

Griffith M, Osborne R, Munger R, Xiong X, Doillon CJ, Laycock NL, Hakim M, Song Y, Watsky MA (1999) Functional human corneal equivalents constructed from cell lines. Science 286: 2169–2172.

He CX, Li N, Hu YL, Zhu XM, Li HJ, Han M, Miao PH, Hu ZJ, Wang G, Liang WQ, Tabata Y, Gao JQ (2011) Effective gene delivery to mesenchymal stem cells based on the reverse transfection and three-dimensional cell culture system. Pharm Res. 2011 Feb 24.[Epub ahead of print].

Ingber DE, Prusty D, Sun Z, Betensky H, Wang N (1995) Cell shape, cytoskeletal mechanics, and cell cycle control in angiogenesis. J Biomech 28: 1471–1484.

Lennon DP, Haynesworth SE, Dennis JE, Caplan AI (1995) A chemically defined medium supports *in vitro* proliferation and maintains the osteochondral potential of rat marrow-derived mesenchymal stem cells. Exp Cell Res 219: 211–222.

Mueller-Klieser W (1997) Three-dimensional cell cultures: from molecular mechanisms to clinical applications. Am J Physiol 273: C1109–C1123.

Niklason LE, Gao J, Abbott WM, Hirschi KK, Houser S, Marini R, Langer R (1999) Functional arteries grown *in vitro*. Science 284: 489–493.

Okazaki A, Jo JI, Tabata Y (2007) A reverse transfection technology to genetically engineer adult stem cells. Tissue Eng 13: 245–251.

Richardson TP, Peters MC, Ennett AB, Mooney DJ (2001) Polymeric system for dual growth factor delivery. Nat Biotechnol 19: 1029–1034.

Tabata Y (2003) Tissue regeneration based on growth factor release. Tissue Eng 9: S5–S15.

Takahashi Y, Tabata Y (2004) Effect of the fiber diameter and porosity of non-woven PET fabrics on the osteogenic differentiation of mesenchymal stem cells. J Biomater Sci Polym Ed 15: 41–57.

Zimmermann WH, Fink C, Kralisch D, Remmers U, Weil J, Eschenhagen T (2000) Three-dimensional engineered heart tissue from neonatal rat cardiac myocytes. Biotechnol Bioeng 68: 106–114.