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Non-viral oligonucleotide antimiR-138 delivery to mesenchymal stem cell sheets and the effect on osteogenesis

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ABSTRACT

Cell-sheet technology has already constituted an important part in the regenerative medicine. Nonetheless, oligonucleotide delivery that has been widely performed on isolated stem cells to foster specific function is rarely conducted on the cell sheets. This study is designed with the two-fold aims of verifying the feasibility of non-viral oligonucleotide delivery for the cell sheets and confirming the osteogenesis enhancing effect of antimiR-138 on the cell sheets composed of bone marrow mesenchymal stem cells (BMSCs). The BMSC sheets are fabricated by a vitamin C inducing method, which can be successfully delivered with the oligonucleotides with a high delivery efficiency of nearly 100% by the properly adapted and optimized Lipofactamine2000 based formulation. The antimiR-138 delivery significantly enhances the *in vitro* osteogenic differentiation of BMSC sheets, indicated by the higher alkaline phosphatase (ALP) production, denser extracellular matrix mineralization and up-regulated osteogenesis related genes including runt-related transcription factor-2 (RUNX2), osterix, ALP, osteocalcin and bone morphogenetic protein-2 at both mRNA and protein levels, compared to controls. Regarding the underlying mechanism, the antimiR-138 delivery down-regulates the endogenous miR-138 levels in the BMSC sheets, consequently activates the extracellular signal regulated kinases 1/2 pathway and enhances the RUNX2 expression. The *in vivo* results indicate a robust enhancing effect of the antimiR-138 delivery on the bone regeneration ability of BMSC sheets. Massive bone with good vascularization is regenerated by the antimiR-138 delivered BMSC sheets, showing immense clinical significance for bone defect repair/regeneration applications. More importantly, the feasibility of non-viral oligonucleotide delivery system for the cell sheets as verified by our study shall hold a general significance for the cell sheets of various cell type and therapeutic purposes.

1. Introduction

The cell-sheet technology that can generate high density cells with abundant endogenous deposited extracellular matrix (ECM) and intact cell-to-cell contact is a valuable extension of current initiatives in regenerative medicine [1,2]. The cell sheets can be easily layered and well attach to tissue beds, which allows either direct *in vivo* transplantation or *in vitro* tissue engineering application. The *in vivo* cell sheet transplantation has been applied in a broad range, and some of them have already gone to the clinic stage, including cardiac patches [3], corneal reconstruction [4], esophageal stricture prevention after endoscopic submucosal dissection [5], periodontal tissue regeneration [6] and so on. Besides, the cell-sheet technology provides a unique scaffold-free approach for engineering three dimensional tissues by layering the individual cell sheets of either same or different cell types [7]. The scaffold-free tissue engineering approach is beneficial to obviate many complex side effects related to artificial scaffold, such as unfavorable inflammatory reactions [8].

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Delivery of DNA or RNA into target cells is a fundamental technology for studying the basic biology of stem cells as well as achieving enhanced clinical therapeutic and regenerative results [9]. Various therapeutic agents including DNA, antisense oligonucleotides, small interfering RNAs (siRNAs), microRNAs (miRNAs, miR) are delivered to cells. Usually, vectors are needed to protect DNA or RNA from degradation and aid in their intracellular transport. Although viral vectors can mediate the intracellular transport of DNA and RNA with relatively high efficiency and long-term expression, non-viral vector based formulations are more favorable in terms of safety concern [10]. Even though the oligonucleotide delivery has been widely conducted on isolated stem cells, it is rarely conducted on cell sheets only with some relatively complex approaches such as viral delivery approach aided by magnetic force [11,12]. It is of high significance to explore a simple and practical oligonucleotide delivery approach for the cell sheets to further improve their specific performance.

In the present study, we fabricate the cell sheets composed of bone marrow mesenchymal stem cells (BMSCs), one of the most widely used stem cells in regenerative medicine, based on a vitamin C (Vc) inducing method [13]. The delivery efficiency of a Lipofectamine2000 based non-viral formulation that has been broadly used for isolated cell culture in respect of the BMSC sheets is assessed with several adjustment and optimization. Our study has the two-fold purposes of verifying the feasibility of the modified non-viral Lipofectamine2000 based oligonucleotide delivery formulation for cell sheets and confirming the osteogenesis enhancing effect of antimiR-138 that shows an osteogenesis enhancing effect on isolated BMSCs by inhibiting the intracellular endogenous miR-138 level [14] on the BMSC sheets.

2. Materials and methods

2.1. In vitro isolation and culture of BMSCs

The animal procedures were approved by the University Research Ethics Committee of The Fourth Military Medical University. Primary rat BMSCs were isolated and cultured according to a previously described method [15]. Briefly, after euthanasia, the hind limbs were aseptically removed and the tibias and femurs were dissected free of soft tissues. Marrow cavities of the bones were flushed with basal medium containing α minimum essential medium (α-MEM, Gibco) supplemented with 15% fetal bovine serum (FBS, Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.) and 1% penicillin and streptomycin. The marrow contents with cells were seeded in 75 cm^2 tissue culture flasks and cultured in a humidified atmosphere of 5% CO_2 at 37 °C. The medium was changed every 2–3 days to remove non-adherent cells. The adherent cells were cultured until confluence and then BMSCs were passaged after digestion with 0.25% trypsin/1 mM EDTA. BMSCs at passages 1–2 were used for cell sheet engineering.

2.2. Cell sheet engineering and oligonucleotide delivery

The procedures for the BMSC sheet engineering and oligonucleotide delivery were shown in Fig. 1. Briefly, BMSCs were plated on 6-well plates at a density of 3×10^5 cells/well and cultured for 24 h to allow the cells reaching 100% confluence. Then the cell culture medium was shifted to cell sheet-inducing medium (α-MEM supplemented with 10% FBS, 1% penicillin and streptomycin and 50 μg/mL Vc) and after 6 days of culture the cell sheets initially formed.

The antimiR-138 and negative control oligonucleotides were purchased from GenePharma, Shanghai, China. Four groups of oligonucleotide delivery formulations were prepared with a given quantity of Lipofectamine2000 (Invitrogen), which have been suggested to improve the delivery efficiency without arousing obvious cytotoxicity compared to the conventional delivery formulation [16]. Briefly, both Lipofectamine2000 of 4 μL and 50 μM miRNAs of different volumes (2, 4, 6 and 8 μL) were diluted with Opti-MEM (Gibco) to a final volume of 20 μL, respectively. Then they were mixed together and kept at room temperature for 20 min. Consequently, the oligonucleotide delivery formulations with a given Lipofectamine2000 quantity but different oligonucleotide amounts (50, 100, 150 and 200 nM) were obtained for the cell sheet oligonucleotide delivery. When referring to the oligonucleotide amounts in the delivery formulations, the concentration (nM) of oligonucleotide is used providing that the sample is immersed in 2 mL media. To conduct the cell sheet oligonucleotide delivery, the culture medium was firstly changed into sheet-inducing medium without antibiotics (2 mL/well). Then 24 h later, the oligonucleotide delivery formulations were added to the wells and incubated at 37 °C for 24 h. Finally, the culture medium was changed into sheet-inducing medium for 24 h to accomplish the oligonucleotide delivery.

2.3. Structural observation of the BMSC sheets

Hematoxylin and eosin (H&E) staining and scanning electron microscopy (SEM, Hitachi S-4800) were used to inspect the microstructure of the BMSC sheets. After 10

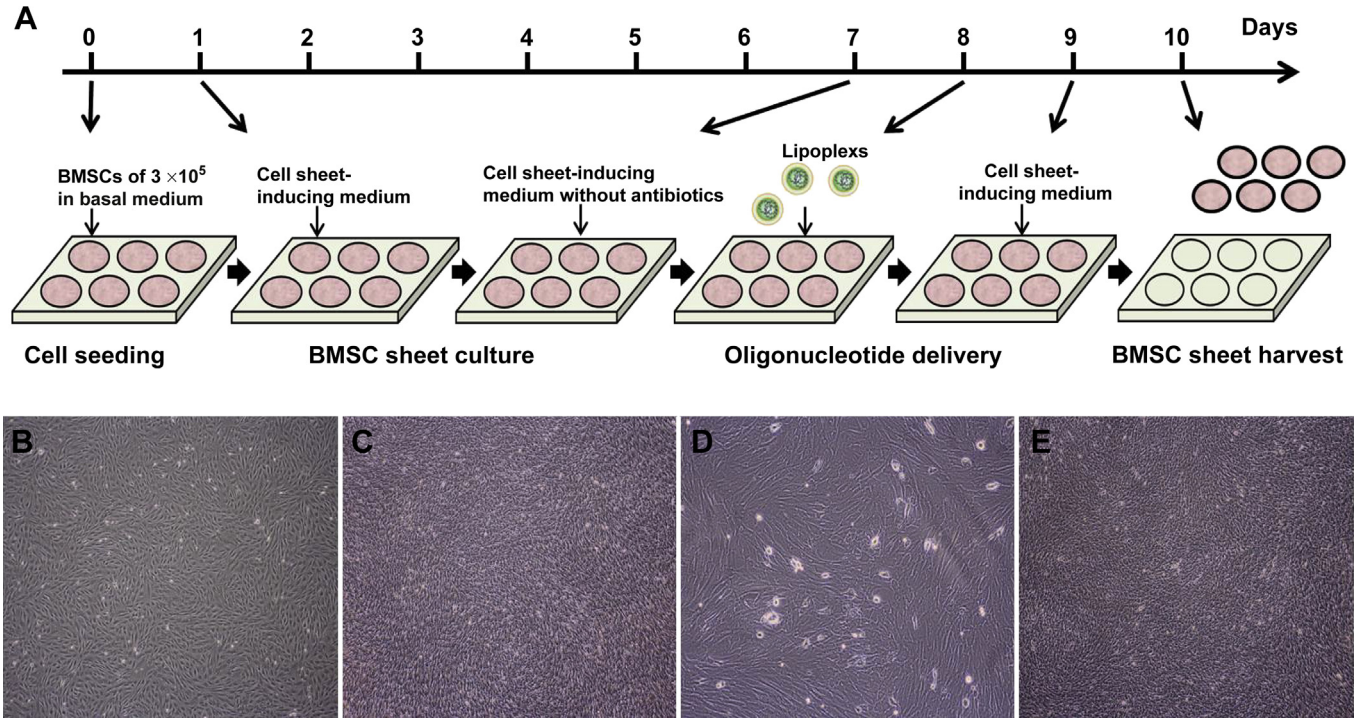

Fig. 1. (A) Procedures for the BMSC sheet fabrication and oligonucleotide delivery; (B) Cell morphology at 24 h post-seeding (40×); (C) Cell sheet morphology after 6 days of cell sheet induction (40×); (D) Cell sheet morphology one day after antimiR-138 delivery (200×); (E) Cell sheet morphology after 9 days of cell sheet induction (40×).