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Reprogramming of mesenchymal stem cells derived from iPSCs seeded on biofunctionalized calcium phosphate scaffold for bone engineering

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ABSTRACT

Human induced pluripotent stem cell-derived mesenchymal stem cells (iPSC-MSCs) are a promising choice of patient-specific stem cells with superior capability of cell expansion. There has been no report on bone morphogenic protein 2 (BMP2) gene modification of iPSC-MSCs for bone tissue engineering. The objectives of this study were to: (1) genetically modify iPSC-MSCs for BMP2 delivery; and (2) to seed BMP2 gene-modified iPSC-MSCs on calcium phosphate cement (CPC) immobilized with RGD for bone tissue engineering. iPSC-MSCs were infected with green fluorescence protein (GFP-iPSC-MSCs), or BMP2 lentivirus (BMP2-iPSC-MSCs). High levels of GFP expression were detected and more than 68% of GFP-iPSC-MSCs were GFP positive. BMP2-iPSC-MSCs expressed higher BMP2 levels than iPSC-MSCs in quantitative RT-PCR and ELISA assays ($p < 0.05$). BMP2-iPSC-MSCs did not compromise growth kinetics and cell cycle stages compared to iPSC-MSCs. After 14 d in osteogenic medium, ALP activity of BMP2-iPSC-MSCs was 1.8 times that of iPSC-MSCs ($p < 0.05$), indicating that BMP2 gene transduction of iPSC-MSCs enhanced osteogenic differentiation. BMP2-iPSC-MSCs were seeded on CPC scaffold bio-functionalized with RGD (RGD-CPC). BMP2-iPSC-MSCs attached well on RGD-CPC. At 14 d, COL1A1 expression of BMP2-iPSC-MSCs was 1.9 times that of iPSC-MSCs. OC expression of BMP2-iPSC-MSCs was 2.3 times that of iPSC-MSCs. Bone matrix mineralization by BMP2-iPSC-MSCs was 1.8 times that of iPSC-MSCs at 21 d. In conclusion, iPSC-MSCs seeded on CPC were suitable for bone tissue engineering. BMP2 gene-modified iPSC-MSCs on RGD-CPC underwent osteogenic differentiation, and the overexpression of BMP2 in iPSC-MSCs enhanced differentiation and bone mineral production on RGD-CPC. BMP2-iPSC-MSC seeding on RGD-CPC scaffold is promising to enhance bone regeneration efficacy.

1. Introduction

Bone defects occur frequently as a result of trauma, tumor ablative surgery, congenital defects, infectious conditions and other causes of loss of skeletal tissue [1,2]. The supply of bone grafts to treat critical-sized bone defects remains a major challenging health issue worldwide [3]. Failure of bone defect repair often leads to severe functional and esthetic deformities. Bone tissue engineering methods using stem cells and scaffolds provide new promising approaches for bone repair [4–11]. Mesenchymal stem cells (MSCs) are progenitor cells for bone regeneration, and bone marrow derived MSCs (BM-MSCs) were extensively investigated [2,12]. However, because of the invasive harvest procedure of BM-MSCs and their decreased potency in senior patients with diseases, alternative sources of MSCs have been investigated including adipose tissue, deciduous tooth, umbilical cord and many other tissues [13].

Recently, a groundbreaking approach by Yamanaka and colleagues generated induced pluripotent stem cells (iPSCs) from adult somatic cells using reprogramming techniques, yielding cell

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proliferation and differentiation potential similar to embryonic stem cells (ESCs) [14–16]. iPSCs provide a promising method for obtaining patient-specific stem cells for tissue regeneration. However, iPSCs have the potential to be expanded indefinitely and may result in tumor formation after transplantation [16]. Therefore, Lian et al. further derived MSCs from human iPSCs, which were referred to as iPSC-MSCs [17]. The biosafety of iPSC-MSCs was improved when compared with the direct use of iPSCs [16]. iPSC-MSCs have superior potential for cell expansion, which is critically important because large numbers of expanded stem cells are needed for cell transplantation and regenerative medicine therapy [18]. Despite of these advantages, only a few investigations have investigated iPSC-MSCs for bone regeneration [19].

Local and sustained delivery of osteoinductive growth factors is an effective approach to promote bone regeneration. Gene therapy strategy has been used for this purpose because it has advantages over direct growth factor delivery, such as low cost and sustained long-term release [20]. Bone morphogenic protein 2 (BMP2), a member of the transforming growth factor β (TGFβ) superfamily, has a remarkable capability of inducing bone formation [21–23]. Preclinical and clinical investigations have successfully applied BMP2 in the therapy of bone defects [24]. Various vectors have been tested to deliver BMP2 by gene therapy, such as liposome-mediated plasmid DNA delivery, adenoviral vectors, and lentiviral vectors [21–23]. Lentiviral vectors with minimal immunogenicity and improved biosafety after a series of modifications could transduce BM-MSCs with high efficiency and long-term stability, which were used to repair bone defect with superior mechanical quality [22,25,26]. However, to date there has been no report on BMP2 gene modification of iPSC-MSCs for bone tissue engineering.

Among various scaffolds for bone regeneration, calcium phosphate (CaP) scaffolds are important for bone repair because they are bioactive, mimic bone minerals and can bond to neighboring bone [27–31]. Calcium phosphate cements (CPCs) can set in situ at the body temperature and conform to complex-shaped bone defects [32–36]. CPC used tetracalcium phosphate and dicalcium phosphate anhydrous, and was approved by the Food and Drug Administration (FDA) of USA in 1996 [32,37–39]. CPC exhibited excellent osteoconductive properties and was resorbed and replaced by new bone *in vivo* [37–40]. Attempts were made to improve the mechanical properties of CPC, for example, by incorporation of chitosan [41]. To enhance the biocompatibility of CPC, immobilization of Arg–Gly–Asp (RGD) on chitosan was recently performed before mixing the chitosan with CPC. This yielded RGD-CPC which significantly improved cell attachment when compared to traditional CPC [41]. However, to date there has been no report on human iPSC-MSC seeding on RGD-CPC scaffold.

The objectives of this study were to genetically modify human iPSC-MSCs for BMP2 delivery, and to seed BMP2 gene-modified iPSC-MSCs on chitosan-CPC scaffold immobilized with RGD for bone tissue engineering. The following hypotheses were tested: (1) BMP2 delivery by gene therapy will promote osteogenic differentiation of iPSC-MSCs while having no significant adverse effect on cell growth; (2) BMP2 gene-modified iPSC-MSCs will attach to chitosan-CPC scaffold with RGD immobilization, survive well and result in enhanced osteogenic differentiation and bone mineral production.

2. Methods and materials

2.1. iPSC culture

The culture of human iPSCs was approved by the University of Maryland Baltimore Institutional Review Board (HP-00046649). Human iPSC BC1 line was derived from adult bone marrow CD34 + cells as described recently, and the cells were kindly provided by Dr. Linzhao Cheng of the Johns Hopkins University [43,44]. Human primary mononuclear cells (MNCs) from a healthy adult marrow donor (code: BM2426) were isolated using a standard gradient protocol by Ficoll-Paque Plus ($p = 1.077$). CD34 + cells were then purified using magnetic-activated cell sorting (MACS) system and cultured with hematopoietic cytokines for 4 days (d) before being reprogrammed by a single episomal vector pEB-C5 [43,44]. iPSCs were expanded on a feeder layer of mitotically-inactivated murine embryonic fibroblasts (MEF) as ESCs [41]. Mitotically-inactivated MEFs as feeder cells were mixed with iPSCs. The iPSC colonies were dissociated into clumps through treatment with 1 mg/mL collagenase type IV for 6 min, followed by mechanical scraping while avoiding the detachment of the MEF layer from the plate surface. Then, the iPSCs were induced to form embryoid bodies (EBs) in suspension culture with ultra-low-attachment plates (Corning, Corning, NY) for 10 d [41]. If residual inactivated MEFs were present, they would attach to the bottom of the flask in the first few days. Once the attached inactivated MEFs were observed, the floating EBs were transferred to a new flask, thereby avoiding the adherent MEFs [42]. In this way, after suspension culture for 10 d, any residual inactivated MEFs had been removed from the EBs. The EB formation medium was composed of Dulbecco's modified Eagle's medium (DMEM)/F12 (Life Technologies, Grand Island, NY), 20% KnockOut Serum Replacement (serum-free; Life Technologies), 1% MEM non-essential amino acids solution (Life Technologies), 1 mM L-glutamine (Sigma–Aldrich, St. Louis, MO), and 0.1 mM 2-mercaptoethanol (Sigma–Aldrich) and changed every 2 d. The EBs were transferred and cultured on 0.1% gelatin-coated plates for 10 more d. The iPSC-MSCs migrated out from the EBs and were then isolated by cell scrapers. iPSC-MSCs were expanded using growth medium composed of low glucose Dulbecco's modified Eagle's medium (DMEM) (Life Technologies), 10% hMSC screened fetal bovine serum (FBS) (Thermo Fisher, Logan, UT) and 1% penicillin/streptomycin/glutamine (Life Technologies). A preliminary investigation characterized the expression of surface markers of iPSC-MSCs using flow cytometry. More than 90% of the cells expressed MSC surface markers CD29, CD44, CD166, CD73. iPSC-MSCs were positive for characteristic MSC markers CD105 and HLA-class I (HLA-ABC). The endothelial marker (CD31), hematopoietic linage marker (CD34), or the iPSC pluripotency markers (TRA-1-81 and Oct3/4) were negative. The preliminary study concluded that iPSC-MSCs expressed surface markers characteristic of MSCs, and were negative for typical hematopoietic and endothelial cell markers. Passage 3 iPSC-MSCs were subjected for lentiviral gene transduction in the present study.

2.2. Lentivirus production and gene transduction

Lentivirus, expressing green fluorescent protein (GFP) or human bone morphogenetic protein 2 (BMP2), was produced commercially (GenTarget Inc., San Diego, CA), as previously described [22,45]. Briefly, GFP or BMP2 gene sequence was sub-cloned into a third generation of human immunodeficiency virus 1 (HIV-1)-based expression lentivector under the transcriptional control of EF-1α promoter. The third generation lentiviral vector system with 3′-LTR self-inactivation mechanism only generates replication-incompetent lentivirus. The inserted sequence was verified by sequencing analysis. Expression lentiviral particles were produced in 293T cells by co-transfection with both the lentivectors and lentiviral packaging plasmids (GenTarget). Virus titers were measured in TH1080 cells via HIV-1 p24 ELISA assay (Advanced BioScience Laboratories, Rockville, MD).

iPSC-MSCs at passage 3 were seeded on a CytoOne 24-well tissue culture polystyrene (TCPS) plate (USA Scientific, Ocala, FL). Upon 50% confluence, iPSC-MSCs were infected with the GFP or BMP2 lentivirus with a multiplicity of infection (MOI) of 15 for 3 d according to the manufacturer's instructions, which were referred to as GFP-iPSC-MSCs or BMP2-iPSC-MSCs, respectively. The MOI of 15 was adopted because a high expression of GFP (more than 68% positive) was observed when iPSC-MSCs were infected with GFP lentivirus using this MOI in preliminary experiment. Then GFP-iPSC-MSCs or BMP2-iPSC-MSCs were expanded in growth medium. Up to passage 8 of gene-modified iPSC-MSCs were examined to determine the stability of target gene transduction. Passage 6 cells were seeded on CPC scaffold.

GFP-iPSC-MSCs were used to evaluate the transduction efficiency and stability. Passage 5 to 8 GFP-iPSC-MSCs were fixed with 4% paraformaldehyde (USB, Cleveland, OH) for 10 min at room temperature and then washed with Dulbecco's phosphate-buffered saline (D-PBS; Life Technologies). Cells were stained with 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI; Roche, Germany; 2 μg/mL) in D-PBS with 0.1% Triton X-100 (Sigma–Aldrich) for 10 min, washed with D-PBS, and immediately subjected for fluorescent assay using epifluorescence microscopy (Eclipse TE2000-S; Nikon, Melville, NY). The transduction efficiency was analyzed by a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) to count the number of GFP-positive cells, using untransducted iPSC-MSCs as negative control.

2.3. BMP2 gene expression and protein secretion

To detect the success and stability of BMP2 gene transduction, passage 5 to 8 BMP2-iPSC-MSCs cultured on 24-well TCPS were harvested. BMP2 gene expression was analyzed by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR, 7900HT, Applied Biosystems, Foster City, CA) using the $2^{-\Delta\Delta Ct}$ method [46]. Briefly, total RNA was extracted using the TRIzol reagent and the PureLink RNA Mini Kit and reverse-transcribed using the High Capacity cDNA