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Osteogenic differentiation of adipose-derived stem cells and calvarial defect repair using baculovirus-mediated co-expression of BMP-2 and miR-148b

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ABSTRACT

Repair of large calvarial bony defect remains a challenge for orthopedic surgeons. Since microRNAs (miRNAs) modulate the osteogenesis of osteoprogenitor cells, we aimed to engineer human adipose-derived stem cells (hASCs), a promising cell source for bone engineering, with miRNA-expressing baculovirus vectors. We constructed 4 baculoviruses each expressing 1 human miRNA (miR-26a, miR-29b, miR-148b, miR-196a) and verified that the miRNA-expressing baculovirus vectors augmented hASCs osteogenesis. Among these 4 miRNAs, miR-148b and miR-196a exerted more potent osteoinductive effects than miR-26a and miR-29b. Furthermore, we unveiled that co-transduction of hASCs with miR-148b-expressing and bone morphogenetic protein 2 (BMP-2)-expressing baculovirus vectors enhanced and prolonged BMP-2 expression, and synergistically promoted the in vitro osteogenic differentiation of hASCs. Implantation of the hASCs co-expressing BMP-2/miR-148b into critical-size (4 mm in diameter) calvarial bone defects in nude mice accelerated and potentiated the bone healing and remodeling, filling ≈94% of defect area and ≈89% of defect volume with native calvaria-like flat bone in 12 weeks, as judged from micro computed tomography, histology and immunohistochemical staining. Altogether, this study confirmed the feasibility of combining miRNA and growth factor expression for synergistic stimulation of in vitro osteogenesis and in vivo calvarial bone healing.

1. Introduction

Large calvarial bone defects may occur due to congenital defects, acquired injuries, neurosurgical procedures or infection, but spontaneous calvarial reossification rarely occurs in adults [1]. Consequently, the repair of massive calvarial bony defects remains challenging in the clinical setting. To facilitate calvarial bone healing, gene therapy and tissue engineering have converged. This approach involves the use of osteoprogenitor cells such as bone marrow-derived mesenchymal stem cells (BMSCs), adipose-derived stem cells (ASCs) or muscle-derived stem cells, which are engineered to overexpress factors promoting osteogenesis (e.g. bone morphogenetic protein 2 (BMP-2)) and angiogenesis (e.g. vascular endothelial growth factor (VEGF)), given the crucial roles of osteogenesis and angiogenesis in bone healing [2–4]. Implantation of the genetically modified cells into critical-size calvarial defects can improve healing, but with limited success [5]. Furthermore, common gene delivery vectors have their respective shortcomings. For instance, nonviral vectors generally suffer from low transfection efficiency while adenovirus mounts potent immune responses. Lentivirus and adeno-associated virus impart

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long-term gene expression, yet concerns regarding random transgene integration and mutagenesis/tumorigenesis impede their applications for bone regeneration [6,7].

Besides these common viral vectors, baculovirus is an insect virus in nature but can transduce ASCs [8] and BMSCs [9] at efficiencies exceeding 95%. Baculovirus is not a human pathogen, nor does it replicate inside mammalian cells, rendering baculovirus a promising gene vector for bone regeneration [5,10]. Implantation of BMSCs engineered with a BMP-2-expressing baculovirus into rat calvarial [11] or rabbit femoral [12] defects substantially improves bone healing. However, transplantation of baculovirus-engineered, BMP-2/VEGF-expressing ASCs into rabbits fails to heal the critical-size calvarial defects [13].

MicroRNAs (miRNAs) are small, ≈22 nucleotide non-coding RNAs that can regulate gene expression at post-transcriptional level by binding to complementary sequences in the 3′-untranslated regions (UTRs) or coding regions of target mRNAs, which leads to translation inhibition/mRNA degradation. MiRNAs are involved in development, cellular differentiation, disease progression, oncogenesis, viral pathogenesis and immunity [14,15]. Furthermore, recent studies have indicated that miRNAs play regulatory roles during osteogenic differentiation [16]. For instance, miR-26a [17], miR-29b [18,19], miR-148b [20], miR-196a [21], miR-322 [22] and miR-218 [23] are reported to enhance osteogenic differentiation. Conversely, miR-23a [24], miR-31 [25], miR-106a [26], miR-17-5p [26], miR-133 [27], miR-135 [27], miR-138 [28] and miR-204 [29] impede in vitro osteogenesis.

Inspired by the promise of miRNA for osteogenesis regulation, in this study we hypothesized that baculovirus-mediated expression of osteogenesis-promoting miRNA (miR-26a, miR-29b, miR-148b or miR-196a) was able to potentiate the osteogenesis of human ASCs (hASCs), and constructed 4 baculovirus vectors (Bac-miR26a, Bac-miR29b, Bac-miR148b and Bac-miR196a), each expressing 1 miRNA. Whether the miRNA-expressing baculovirus improved the hASCs osteogenesis was evaluated. We further assessed whether baculovirus-mediated co-expression of miRNA and BMP-2 synergistically ameliorated hASCs osteogenesis. Whether the hASCs co-expressing miRNA and BMP-2 enabled satisfactory calvarial bone healing was evaluated by implanting the cells into calvarial bone defects in nude mice, followed by micro computed tomography (μCT), histology and immunohistochemical staining analyses.

2. Materials and methods

2.1. Culture of human ASCs (hASCs)

Primary hASCs were obtained from Taiwan Stem Cell Bank (Hsinchu, Taiwan) and propagated in α-minimum essential medium (α-MEM, Sigma) containing 15% fetal bovine serum (FBS, HyClone), 100 U/ml penicillin, 100 mg/ml streptomycin and 4 ng/ml FGF-2 (bFGF, PeproTech). hASCs were cultured in 10-cm dishes at 37 °C with 5% CO_2 and passaged every 3 days. hASCs of passage 11 were used for subsequent experiments.

2.2. Recombinant baculovirus preparation and transduction

In nature, miRNAs are transcribed by RNA polymerase II as primary miRNAs which are then cleaved into hairpin intermediate pre-miRNAs. The pre-miRNAs are exported from nucleus to cytoplasm and processed into the ≈22 nt RNA duplex [30]. Because pre-miRNA flanked by splicing donor (SD) and splicing acceptor (SA) sequences take advantage of natural RNA splicing mechanisms and are more efficiently processed into mature miRNA [31,32], we designed the pre-miRNA flanked by SD and SA sequences in attempt to promote the baculovirus-mediated miRNA expression and processing. The pre-miRNA sequences encoding human miR-26a, miR-29b, miR-148b and miR-196a were designed (see Table S1 for sequences), synthesized together with flanking SD and SA sequences as well as splicing branch sites, and cloned into the pUC57 plasmid (GeneDireX Inc). The SD-pre-miRNA-SA sequences were further subcloned into the pBac-CMV-WPRE plasmid [9] wherein the SD-pre-miRNA-SA sequences were placed under the control of cytomegalovirus (CMV) promoter, followed by downstream woodchuck hepatitis post-transcriptional regulatory element (WPRE) and polyadenylation (poly A) sequences (Fig. 1A). Individual baculovirus vectors were constructed using these plasmids, following the instructions of Bac-to-Bac system (Invitrogen). For BMP-2 expression, we employed a dual baculovirus system that consisted of one baculovirus (Bac-FLPo) expressing FLPo recombinase and the other baculovirus (Bac-FCBW) harboring BMP-2 cassette flanked by Frt sequences [33]. Bac-FLPo and Bac-FCBW were constructed previously [33]. The baculoviruses were amplified by infecting Sf-9 cells and titrated by end-point dilution method [9].

hASCs were transduced as described previously [34] with minor modifications. Briefly, hASCs of passage 11 were seeded to 6-well plates (2×10^5 cells/well), 10-cm dishes ($\approx 2 \times 10^6$ cells/dish) or T-75 flasks (5×10^6 cells/flask), cultured overnight and washed twice with phosphate-buffered saline (PBS, pH 7.4) prior to transduction. Depending on the multiplicity of infection (MOI), a certain volume of virus supernatant was diluted with TNM-FH medium and mixed with $NaHCO_3$-free DMEM at a volumetric ratio of 1:4. The transduction was initiated by directly adding the virus solution to the cells (500 μl/well in 6-well plates, 1 ml per 10-cm dish and 2 ml per T-75 flask) and continued by gentle shaking on a rocking plate at room temperature for 6 h. After the transduction period, the virus mixture was replaced with the osteogenic medium (DMEM containing 10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerol phosphate and 50 μM ascorbic acid 2-phosphate) containing 3 mM sodium butyrate. After 15 h of incubation at 37 °C, the medium was replaced by fresh osteogenic medium and cells were harvested after 9 h (i.e. 1 day post-transduction) for implantation. Alternatively, the cells continued to be cultured with medium exchange every 2–3 days for analyses.

2.3. Detection of transgene expression and hASCs differentiation

To quantify the BMP-2 expression, the medium was sampled at different days post-transduction (dpt) for ELISA analysis using the Human BMP-2 DuoSet ELISA kit (R&D Systems). At different dpt, total RNA was extracted from hASCs for quantitative reverse transcription real-time PCR (qRT-PCR), using primers specific for osteopontin, osteocalcin or alkaline phosphatase genes (Table S2). The miRNA expression was analyzed using the TaqMan MicroRNA Reverse Transcription Kit (Cat. #000405 for miR-26a, #000413 for miR-29b, #000471 for miR-148b, and #000495 for miR-196a) that quantifies only mature miRNA but not precursors. The gene expression levels were normalized to that of U6 (for miRNAs) or *gapdh* (for marker genes) and referenced to that of mock-transduced cells at day 0.

To detect mineralized matrix, hASCs were subject to Alizarin red staining at 14 dpt. After removing the medium, cells were rinsed with PBS 3 times and fixed with fixative solution (4% paraformaldehyde) overnight. The cells were washed with deionized water 3 times, stained with 40 mM Alizarin Red (pH 4.2) for 10 min, followed by 3 PBS washes and microscopic observation. To quantify the calcium deposition, at 14 dpt the cells were rinsed with PBS 3 times, followed by incubation with 0.6 N HCl overnight. The supernatant was collected the next day for calcium phosphate deposition analysis (CALCIUM liquicolor, Human Inc.). The cells were collected and the total protein was quantified using the protein assay kit (Bio-Rad). The calcium deposition is expressed as nmol/μg protein.

2.4. Preparation of hASCs/scaffold constructs

Disk-shaped poly (*L-lactide-co-glycolide*) (PLGA) scaffolds (diameter≈4 mm, thickness≈1 mm, porosity≈92%) were fabricated by solvent casting and a particulate leaching technique [35]. The PLGA scaffolds were sterilized with 70% alcohol and pre-treated with fresh medium that contained 1% gelatin overnight. The transduced hASCs were trypsinized at 1 day post-transduction, resuspended into fresh medium, seeded onto the gelatin-coated PLGA scaffold (5×10^5 cells/scaffold) and allowed to adhere for 2 h. The constructs were cultured overnight prior to implantation.

2.5. Surgical procedures

The 8-week-old immunocompromised nude mice (female BALB/cAnN.Cg-Foxnlnu/CrlNarl, Animal Health Research Institute) were anesthetized by intramuscular injection of Zoletil 50 (25 mg/kg body weight) and 2% Rompun® (0.15 ml/kg body weight). Under sterile conditions, a midline sagittal incision in the scalp was made to expose the parietal bone and the pericranium was removed by blunt scraping. A critical-size (4 mm in diameter) defect was created in the middle of parietal bone using a sterile drill. The constructs were implanted into the defects and the incision was closed.

2.6. μCT

The mice were scanned with a μCT imaging system (BioScan) at different time points. At 12 weeks post-transplantation, the mice were sacrificed and calvarial bones were also removed for μCT scanning. The three-dimensional images were reconstructed using the Amira software (Visualization Science Group). From the μCT images, disk-shaped volume of interest (VOI, diameter = 4 mm, height = 1 mm) was drawn within the parietal defects and analyzed using the PMOD software (PMOD Technologies) to calculate the new bone area, volume and density (expressed as average Hounsfield unit (HU)) in the VOI.