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Enhanced critical-size calvarial bone healing by ASCs engineered with Cre/loxP-based hybrid baculovirus

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ABSTRACT

Calvarial bone repair remains challenging for adults. Although adipose-derived stem cells (ASCs) hold promise to heal bone defects, use of ASCs for critical-size calvarial bone repair is ineffective. Stromal cell-derived factor 1 (SDF-1) is a chemokine capable of triggering stem cell migration. Although recombinant SDF-1 protein is co-delivered with other molecules including BMP-2 to facilitate calvarial bone repair, these approaches did not yield satisfactory healing. This study aimed to exploit a newly developed Cre/loxP-based hybrid baculovirus for efficient gene delivery and prolonged transgene expression in ASCs. We demonstrated that transduction of rat ASCs with the hybrid Cre/loxP-based baculovirus enabled robust and sustained expression of functional BMP-2 and SDF-1. Expression of BMP-2 or SDF-1 alone failed to effectively induce rat ASCs osteogenesis and healing of critical-size calvarial bone defects. Nonetheless, prolonged BMP-2/SDF-1 co-expression in ASCs synergistically activated both Smad and ERK1/2 pathways and hence potentiated the osteogenesis. Consequently, transplantation of the hybrid baculovirus-engineered, BMP-2/SDF-1-expressing ASCs/scaffold constructs potently healed the critical-size (6 mm) calvarial bone defects (filling ≈70% of defect volume), which considerably outperformed the calvarial bone repair using BMP-2/SDF-1 delivered with biomaterial-based scaffolds. These data implicated the potential of Cre/loxP-based hybrid baculovirus vector for ASCs engineering and calvarial bone healing.

1. Introduction

Repair of large calvarial bone defects remains challenging in the clinical setting due to poor spontaneous calvarial re-ossification in adults [1]. To facilitate calvarial bone healing, gene therapy and regenerative medicine have converged by which vectors are used to deliver various genes into osteoprogenitor cells such as bone marrow-derived mesenchymal stem cells (BMSCs) or adipose-derived stem cells (ASCs) [2,3]. Given the crucial roles of osteogenesis and angiogenesis in bone healing, potent osteoinductive BMP-2 (bone morphogenetic protein 2) and angiogenic factor VEGF (vascular endothelial growth factor) are often used to stimulate calvarial bone healing [4]. However, implantation of genetically modified cells expressing BMP-2/VEGF into critical-size calvarial defects usually improves healing with limited success [5–7].

Stromal cell-derived factor 1 (SDF-1) is a chemokine capable of binding CXCR-4 receptor on the cells and activating downstream MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) signaling pathway. SDF-1 can trigger the migration of cells such as BMSCs and plays a crucial role in the BMSCs homing to a fracture site [8]. Furthermore, SDF-1 can promote the secretion of VEGF [9], ameliorate the myocardial repair [10] and potentiate BMP-2-induced bone formation [11]. Inspired by these findings, recombinant SDF-1 protein has been co-delivered with simvastatin [12], VEGF [13], BMP-7 [13] or BMP-2 [14–16] to facilitate calvarial bone repair in recent years. However, bone healing using these approaches did not yield satisfactory outcome, and use of genetically modified stem cells expressing BMP-2 and SDF-1 has yet to be reported.

Baculovirus is an insect virus but can transduce rabbit ASCs and BMSCs at efficiencies exceeding 95% with low cytotoxicity [17].

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Baculovirus is non-pathogenic to humans and does not replicate inside mammalian cells, rendering baculovirus a promising gene vector for tissue engineering [17,18]. Recently, we have developed a hybrid baculovirus system based on codon-optimized FLP recombinase (FLPo) and Frt to prolong and enhance transgene expression [19]. The FLPo/Frt-based hybrid vector system consists of 2 baculoviruses: one expressing FLPo and the other substrate baculovirus accommodating a transgene cassette flanked by two Frt sequences. Co-transduction of ASCs enables FLPo/Frt-mediated transgene cassette excision from the baculovirus genome and re-circularization into episomal DNA minicircles in ≈90%–96% of rabbit ASCs [19]. This hybrid baculovirus system was employed to express transgenes in ASCs to facilitate the repair of cartilage [20], bone [21,22] and heart [23]. Furthermore, we have developed another Cre/loxP-based baculovirus system [19] which, similarly, comprises two baculoviruses: one expressing Cre recombinase and the other harboring the transgene cassette flanked by loxP sites. Co-transduction of human and rabbit ASCs confers more robust DNA minicircle formation/transgene expression and lower cytotoxicity than the FLPo/Frt-based baculovirus system [19]. The Cre/loxP baculovirus system was recently exploited to transduce rat BMSCs for prolonged BMP-2 and microRNA sponge expression and femoral bone repair in osteoporotic rat models [24].

In this study, we hypothesized that the new Cre/loxP-based baculovirus can confer prolonged and enhanced BMP-2 and SDF-1 co-expression in rat ASCs. Since ASCs are less amenable to BMP-2 stimulation towards osteogenic differentiation than BMSCs [25,26], we first examined whether the hybrid baculovirus-mediated BMP-2/SDF-1 expression was able to synergistically improve the ASCs osteogenesis *in vitro*. Whether the engineered ASCs expressing BMP-2/SDF-1 could improve the critical-size calvarial bone healing in rats was evaluated by micro computed topography (μCT), histology, histochemical and immunohistochemical staining.

2. Materials and methods

2.1. Isolation and culture of rat ASCs

All animal experiments were approved by the National Tsing Hua University Institutional Animal Care and Use Committee. Rat BMSCs were isolated and cultured as described [24]. Rat ASCs were isolated from 4-week old female Sprague-Dawley rats (BioLasco, Taiwan). Briefly, an abdomen incision (3–5 cm) was made between the caudal edge of the rib cage and the base of the tail. The fat pads were removed with tweezers, washed with phosphate-buffered saline (PBS, pH 7.4) 3 times, minced, immersed in 0.1% Collagenase I (Sigma) and incubated at 37 °C for 30 min. The cells were centrifuged (1700×g) and the red blood cells were removed by RBC lysis buffer (BioLegend). The isolated rat ASCs were cultured in 10-cm dish at 37 °C using α-MEM containing 10% fetal bovine serum (FBS, Hyclone), 100 IU/ml penicillin and 100 IU/ml streptomycin. After confluence, the cells were passaged twice. Passage 3 cells were used for subsequent experiments.

2.2. Baculovirus preparation and ASCs transduction

All transgenes were expressed under the control of rat EF-1α promoter. Hybrid baculovirus vectors Bac-Cre expressing Cre recombinase and Bac-LEBW expressing BMP-2 were constructed previously [24]. The rat EF-1α promoter was PCR-amplified from pVITRO1-neo-mcs (Invivogen) and cloned into pBacLCW [27] using *XhoI/BamHI* to replace the CMV promoter and yield pBacLEW. The gene encoding rat SDF-1α (referred to SDF-1 thereafter) was PCR-amplified from rat BMSCs using specific primers (Table S1) and subcloned into pBac-LEW in between loxP sites by *EcoRI/StuI* to yield pBac-LESW, which contained the WPRE sequence to enhance the mRNA stability. The donor plasmid pBac-LESW was used to generate baculovirus Bac-LESW using the Bac-To-Bac® system (Invitrogen) as described [18]. Virus titers were determined by end-point dilution method and are expressed as plaque forming units per milliliter (pfu/ml) [18].

Baculovirus transduction of rat ASCs was performed as described [18]. Briefly, rat ASCs were seeded to 6-well plates (2×10^5 cells/well) or 10-cm dishes (1×10^6 cells/dish), cultured overnight and washed twice with PBS prior to co-transduction with Bac-Cre/Bac-LEBW, Bac-Cre/Bac-LESW or Bac-Cre/Bac-LEBW/Bac-LESW. Depending on the multiplicity of infection (MOI, pfu/cell), a certain volume of virus supernatant was mixed with $NaHCO_3$-free DMEM at a volumetric ratio of 1:4 (total volume was 0.5 and 2 ml in 6-well plates and 10-cm dishes, respectively) and added to the cells. For mock transduction, virus-free TNM-FH medium was mixed with $NaHCO_3$-free DMEM at a volumetric ratio of 1:4 and added to the cells. The cells were gently shaken on a rocking plate at room temperature for 6 h, after which the virus mixture was replaced with osteoinduction medium (α-MEM containing 10% FBS, 100 IU/ml penicillin, 100 IU/ml streptomycin, 0.1 μM dexamethasone, 10 μM β-glycerol phosphate and 50 μM ascorbic acid 2-phosphate) containing 3 μM sodium butyrate (all from Sigma). After 15 h of incubation at 37 °C, the medium was replaced by fresh osteoinduction medium and continued to be cultured using osteoinduction medium with medium replacement every 2 days. The cells were trypsinized at different day post-transduction (dpt) for assays.

2.3. Cell migration assay

Rat BMSCs were cultured in T-75 flasks to 80% confluency, trypsinized and seeded into the transwell (Corning Costar) membrane inserts (pore size = 8 μm, 1×10^4 cells/insert) and cultured using α-MEM containing 2% FBS and 1 ng/ml TNF-α (R&D system). In parallel, the transduced ASCs were seeded to the transwell plate (1.5×10^4 cells/well) and cultured using α-MEM containing 10% FBS and 5 mM sodium butyrate. After 1 day, we replaced the medium in the transwell plate and the membrane inserts with fresh α-MEM containing 2% FBS, and placed inserts to the transwell plate. Two days later, the BMSCs remaining on the upper membrane were removed with cotton wool and the cells that have migrated through the membrane were stained with 0.1% crystal violet (Sigma) for 5 min, washed with PBS twice, imaged with optical microscope and counted using Image Pro software.

2.4. ELISA and quantitative real-time reverse-transcription PCR (qRT-PCR)

The supernatant from the ASCs cultured in osteoinduction medium was sampled at different dpt and analyzed using the Human BMP-2 DuoSet ELISA kit (R&D Systems) or Human CXCL12/SDF-1 DuoSet ELISA kit (R&D Systems). Alternatively, total RNA was isolated from ASCs using the NucleoSpin RNA II kit (Machereye-Nagel) and reverse transcribed to cDNA using the MMLV Reverse Transcription 1st-strand cDNA Synthesis Kit (Epicentre Biotechnologies). The genes encoding Runt-related transcription factor 2 (Runx2), osterix (OSX) and osteopontin (OPN) were analyzed by qPCR using StepOnePlus Real-Time PCR Systems (Applied Biosystems) and gene-specific primers (Table S1). The *gapdh* expression served as an internal control. The gene expression levels were normalized to those of mock-transduced cells.