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Adipose-derived stem cell sheets functionalized by hybrid baculovirus for prolonged GDNF expression and improved nerve regeneration

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ABSTRACT

Peripheral nerve regeneration requires coordinated functions of supporting cells (e.g. Schwann cells) and neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF), but nerve regeneration is usually far from complete. Here we constructed a Cre/loxP-based hybrid baculovirus (BV) vector which enabled intracellular formation of episomal DNA minicircle for effective transduction of rat adipose-derived stem cells (ASCs) and prolonged expression of functional GDNF capable of recruiting Schwann cells. The GDNF expression persisted for >20 days with the peak level (≈128 ng/ml) tremendously exceeding the picogram levels of GDNF secreted by neuroprogenitor cells. We further developed a facile method to fabricate and transduce cell sheets composed of undifferentiated ASCs in 2 days, without the need of thermo-responsive polymer commonly used for cell sheet fabrication. Implantation of the hybrid BV-engineered, GDNF-expressing ASCs sheets into sciatic nerve transection site in rats significantly improved the nerve repair, as judged from the enhanced functional recovery, nerve reinnervation, electrophysiological functionality, Schwann cells proliferation/infiltration, axon regeneration, myelination and angiogenesis. The hybrid BV is able to functionalize ASCs sheets by intracellular episomal DNA minicircle formation that circumvents undesired gene integration, and the ASCs sheets fabrication is rapid and simple. These data and features implicate the potentials of ASCs sheets functionalized by the hybrid BV for peripheral nerve regeneration.

1. Introduction

Peripheral nerve injuries lead to pain and significant disability, affecting 20 million Americans and costing ≈150 billion in annual health care expenditure in the United States [1]. Following traumatic injury, the distal stump of peripheral nerve is disconnected from the proximal stump and undergoes Wallerian degeneration [2]. Macrophages and monocytes migrate into the nerve stumps to remove resulting myelin and axon debris, while Schwann cells (SCs) dedifferentiate and proliferate to form bands of Bungner, and produce neurotrophic factors and extracellular matrix (ECM) molecules to stimulate axon regeneration. The regenerating axons extend until reaching their synaptic target to achieve functional reinnervation (for review see Ref. [2]). In the clinical setting, end-to-end suturing is common for injuries caused by transection, but nerves are only capable of limited regeneration and the clinical outcomes are often unsatisfactory especially when nerve repairs are delayed beyond 1 month [3]. Conversely, autologous nerve grafting is the gold standard for treatment of severe injuries with a gap [4], but it is associated with donor site morbidity. More critically, regeneration is usually far from complete and <50% of patients regain full function after treatment [2].

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SCs are the principle supporting cells that mediate peripheral nerve regeneration thanks to their ability to produce ECM and various neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) [5]. SCs also participate in the critical remyelination process by migrating towards axons, elongating and ensheathing axons [6]. These properties render SCs the top choice as a cell source for peripheral nerve regeneration. However, the donor nerve morbidity associated with SCs harvesting limit their use in nerve repair [7]. Although neural stem cells (NSCs) can be used as an alternative, isolation of NSCs remains a hurdle [8]. Additionally, embryonic stem cells, induced pluripotent stem cells, mesenchymal stem cells (MSCs) and adipose-derived stem cells (ASCs) are potential cell sources for nerve regeneration [7,9]. Among these cell types, ASCs are particularly promising as ASCs can be isolated in large quantities during liposuction and be cultured easily [10]. ASCs are often induced to differentiate into SCs-like cells, which can enhance neuron survival, stimulate neurite outgrowth, improve myelin formation and promote nerve regeneration (for review see Ref. [11]). Under appropriate culture conditions, ASCs are able to form cell sheets composed of cells, cell-to-cell junction molecules and abundant ECM, which can be rolled into rods and form engineered neural tissue to promote peripheral nerve repair [12].

Aside from cells, neurotrophic factors such as GDNF [13], NGF [14] and BDNF [15] have been assessed for nerve regeneration, among which GDNF triggers migration and proliferation of SCs [13], enhances functional recovery [3] and results in better motor neuron survival than BDNF [3]. Recombinant GDNF can be delivered into the nerve injury site using the drug delivery system [3,13,16] or nerve conduit [4] for local and sustained GDNF release. However, a large amount of recombinant GDNF may be required [17]. This problem may be tackled by cell-based GDNF delivery by which genetically engineered SCs overexpressing GDNF are implanted to stimulate the nerve repair [18,19].

For cell-based gene delivery, baculovirus (BV) is a promising vector capable of transducing various stem cells including ASCs at efficiencies exceeding 95% [20]. We previously developed an FLPo/Frt-based hybrid BV vector system which enables intracellular recombination and DNA minicircle formation [21]. This system has been explored for transducing rabbit ASCs sheets, conferring 97% transduction efficiency and recombination/minicircle formation in 78% of cells in ASCs sheets [22]. Similarly, we developed a Cre/loxP-based hybrid BV system comprising two vectors: one expressing Cre recombinase (BacCre) and the other substrate BV harboring the transgene cassette flanked by loxP sites [21]. Co-transduction of ASCs results in Cre-mediated excision of the transgene cassette off the substrate BV genome, thereby enabling intracellular formation of episomal DNA minicircle that encompasses the transgene. The hybrid Cre/loxP-based BV system confers similar transduction efficiency and recombination efficiency when compared with the FLPo/Frt-based system [21], allows for sustained expression of various transgenes such as bone morphogenetic protein-2 [23], miR-214 sponge [24] or stromal cell-derived factor 1 [25], and improves ASCs differentiation and bone healing *in vivo* [24,25].

Given the potentials of GDNF for nerve regeneration, ASCs sheets for tissue engineering and the hybrid BV for cell-based gene delivery, here we aimed to exploit the new Cre/loxP-based BV vector for genetic engineering of undifferentiated ASCs sheets for GDNF overexpression and sciatic nerve regeneration in rats. We first evaluated whether the new hybrid BV vector enabled robust and persistent expression of functional GDNF in rat ASCs and next examined whether the hybrid BV transduction affected the rat ASCs sheets formation. Whether the hybrid BV-engineered, GDNF-expressing ASCs sheets improved nerve repair was further assessed.

2. Materials and methods

2.1. Recombinant BV preparation

The recombinant BV (BacCre) expressing the Cre recombinase was constructed previously [21] while the substrate BV (BacLEGW) was constructed in this study following the procedure described earlier [24,25]. BacLEGW harbored the human *gdnf1* gene under the transcriptional control of rat EF-1α promoter and contained the WPRE (Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element) sequence to enhance the mRNA stability. The expression cassette was flanked by loxP sites for Cre recognition and excision. The BV stocks were produced by infecting insect cell Sf-9 and titered by end-point dilution method as described [23].

2.2. ASCs isolation, ASCs sheets preparation and transduction

All animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology, Taiwan). The experimental protocols were approved by the Institutional Animal Care and Use Committee of National Tsing Hua University. Rat ASCs were isolated from the inguinal fat pads of 4-week old male Sprague-Dawley (SD) rats (BioLasco, Taiwan) as described [25]. Briefly, the fat pads were removed with tweezers, minced, immersed in 0.1% collagenase I (Sigma) and incubated at 37 °C for 30 min. The digested tissues were centrifuged (1700 ×*g*), filtered and red blood cells were removed by using the RBC lysis buffer (BioLegend). After centrifugation (1700 ×*g*), the pellets were resuspended in α-MEM containing 10% fetal bovine serum (FBS, Hyclone), 100 IU/ml penicillin and 100 IU/ml streptomycin, and the cells were plated into T-75 flasks ($\approx 1 \times 10^5$ cells/cm^2) and incubated at 37 °C (5% CO_2). After 3 days, non-adherent cells were removed, and adherent cells continued to be cultured in α-MEM containing 10% FBS and antibiotics for 2–4 days until subconfluence. The ASCs were passaged 3 to 5 times for experiments.

For cell sheets fabrication, rat ASCs were seeded to polystyrene 6-well plates (Corning Costar) at 2×10^6 cells/well and cultured in α-MEM containing 20% FBS and antibiotics at 37 °C (5% CO_2). After 24 h, ASCs sheets were visible and BV transduction was performed as described previously [22]. Briefly, ASCs sheets were gently washed with phosphate-buffered saline (PBS), and a certain amount of virus was diluted with TNM-FH medium (the medium for insect cell culture and BV vector production, Sigma) depending on the multiplicity of infection (MOI) to be used. The virus solution was mixed with $NaHCO_3$-free α-MEM at a volumetric ratio of 1:4 (total volume = 500 μl per well) and added to the wells. For mock-transduction control, fresh TNM-FH medium was mixed with virus-free, $NaHCO_3$-free α-MEM at a volumetric ratio of 1:4 and added to the cells. Cell sheets were gently shaken on a rocking plate at room temperature for 6 h, and the transduction mixture was removed, followed by culture at 37 °C in α-MEM medium containing 3 mM sodium butyrate and 20% FBS. At 48 h post-seeding, the transduced ASCs sheets were washed with PBS twice and briefly trypsinized using 0.05% trypsin-EDTA (0.5 ml/well, Gibco) for 10 s. After trypsin removal, ASCs sheets were washed twice, incubated in PBS and gently shaken. The transparent ASCs sheets spontaneously detached from the well and shrank, which were handled with a forceps for ensuing implantation.

2.3. Expression level and functional assay of GDNF

To confirm prolonged GDNF expression, rat ASCs were seeded to