A One-step Method to Fabricate BMP-2 Gene Activated Porous PLLA Scaffold for Bone Formation

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Introduction: Enhancement of bone regeneration is needed to heal large bone defects and to treat fracture-delayed unions or nonunions. In addition, osteoporotic fractures are common in the elderly population. Therefore, developing an effective technology for large bone repair is critical in improving the nation’s health. Rapid advances in regenerative medicine have raised the hope of repairing bone defects with the combination of biomaterials, cells and growth factors [1]. A major challenge in cell-based bone tissue engineering is to promote efficient osteogenic differentiation of cells seeded within three-dimensional (3D) biomaterial scaffolds, which requires the sustained introduction of soluble osteoinductive biofactors [2], such as bone morphogenetic protein-2 (BMP-2). However, their short half-lives and rapid clearance by the bloodstream as seen in experimental animals limit the ability of these biofactors. Recombinant adeno-associated viral (rAAV) vector-based genetic engineering of stem cells is a promising therapeutic approach to promote bone tissue healing [3]. However, traditional ex vivo gene transfer of BMP-2 to stem cells, prior to their seeding onto biomaterial scaffolds for tissue engineering and implantation, is a time consuming process and requires complicated cell culture in vitro [4]. The current study builds on the recent technological advancements in our labs in the design and fabrication of a novel 3D porous scaffold for bone tissue engineering. The ice-based microparticle porogenization method [5] enables the single-step fabrication of regenerative bone scaffolds encapsulated with human bone marrow mesenchymal stem cells (hBMSCs) and viral vector encoding BMP-2. We report here the efficient expression of BMP-2 in vitro and osteoinductive effects on naïve hBMSCs implanted in the hindlimb muscle of SCID mice.

Methods: Both human BMP-2 and the green fluorescent protein (GFP) reporter genes under control of the CMV promoter were constructed in a self-complementary AAV vector (scAAV), respectively. Serotypes 2, 6 and 8 of the scAAV-CMV-GFP vectors were produced according to our published protocol [6]. hBMSCs were isolated with IRB approval from the femoral heads of patients undergoing total hip arthroplasty. Poly-L-lactide (Sigma, Lot#SLBD6608V) was dissolved in chloroform (10 wt %) and cooled to -20 °C overnight. Ice-based microparticles were generated by injecting AAV solution diluted in deionized water through specialized nozzles (World Precision Instruments, Inc., Cat: TIPOSTW1F-L) into liquid nitrogen (Figure 1A). PLLA solution and the AAV ice micro-particles were mixed evenly in pre-cold stainless steel container and shaped by pre-cold stainless steel molds on dry ice. Finally, the 4x5x5 mm³ scaffold samples were kept in liquid nitrogen overnight and freeze-dried in lyophilizer for 4 hours. For structural observation, samples without AAV were bisected just before freeze-drying. After fabrication, scaffolds were sterilized by 70% ethanol for 10 minutes and rinsed once with phosphate-buffered saline (PBS). 20 ul (about 1.6x10^5 cell) of hBMSC cell suspension were then seeded evenly into the AAV-activated PLLA scaffolds. Cell viability was assessed via Calcein acetoxymethyl ester (calcein-AM) and ethidium homodimer-1 (EthD-1) staining (Live/Dead Kit, Invitrogen) before 24 hrs and 7 days. After the entire fabrication process, the activity of the AAV vector was also tested by release kinetics analysis. BMP-2 concentration in the medium after 7 days was measured by BMP-2 ELISA kit (R&D). To test the efficacy of the AAV6-CMV-BMP-2 activated porous scaffold in enhancing bone formation in vivo, we implanted the hBMSCs-seeded BMP-2 gene-activated porous scaffolds within the hindlimb muscle in immune-deficient (SCID) mice. Bone formation was vitally monitored with micro-CT using Scanco VivaCT40 system.

Results: 1. Fabrication of AAV gene-activated PLLA porous scaffold: Ice-based micro-particles were generated with high efficiency with a median diameter of 250 µm, ranging from 100 to 500 µm, as shown in Figure 1B. As shown in Figure 1 C & D, the PLLA scaffold was highly porous, with good pore interconnectivity. The diameter of the holes ranged from 100 to 500 µm, consistent with the size of the ice-based micro-particles. The interconnectivity is important to allow the passage of cells, nutrients, and waste products in and out of the scaffold. 2. Cell viability and AAV vector activity after fabrication: Live/Dead staining results showed high cell viability (>95%) 24hrs (Figure 2A) and 7 days (Figure 2B) after fabrication, but cell distribution was not uniform. The surface of the scaffold had more cells than the interior, limited by the cell seeding method and scaffold property. Compared to other serotypes of AAV vectors, serotype 6 of AAV showed high efficiency of GFP reporter gene transduction in hBMSCs (Figure 2 C). The AAV6 viral particles remained active after the entire fabrication process and continued to be released during 100 hours of culture, and infected cells outside the scaffold (Figure 2D).
3. Functionality of AAV-mediated BMP-2 gene-activated constructs in vitro and in vivo: As shown in Figure 3A, three experimental groups were examined: (A) scaffold + ex-vivo transferred BMP-2 hBMSCs (AAV-BMP-2-hBMSCs); (B) AAV6-BMP-2 activated scaffold + hBMSCs; and (C) scaffold + hBMSCs. After 7 days culture, cell culture medium was collected for ELISA assay of BMP-2 protein activities. Results showed that the rAAV6-BMP-2 gene activated 3D PLLA scaffold (group B) could release functional rAAV-BMP-2 to efficiently transduce seeded hBMSCs within 3D scaffold, with no significant difference as compared to group A of scaffold that was loaded with prior traditional ex-vivo rAAV-BMP-2 transduced hBMSCs (p > 0.05). As expected, both groups A and B had significantly higher activities of BMP-2 protein as compared to the scaffold seeded with hBMSCs alone (group C) (p <0.05). Four weeks post-implantation in vivo, the presence of mineralized bone was observed (Figure 3B & C). We found that the embedded matrix components and AAV vectors could deliver transduction-competent osteoinductive rAAV-BMP-2 to enhance hBMSCs osteogenic differentiation in vivo, resulting in improved bone formation in muscle in SCID mice as compared to naïve/BMP-2 protein-laden scaffolds (1ug of BMP-2 protein per scaffold).

Discussion: These findings indicate that combining 3-D PLLA scaffolds and rAAV gene transfer to target hBMSC osteogenic differentiation is an exciting new candidate technology to optimize the osteoinductive activity of soluble growth factors and tethering capabilities of extracellular matrix for bone tissue engineering.

Significance: The ice-based porogenized scaffolds are well-suited for the repair of simple and uncomplicated bone defect, and present potential technology readiness towards clinical applications, involving a single-step fabrication method for gene-activated biomaterial scaffold, targeted to promote hBMSC osteogenesis to enhance bone formation.

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Figure 1. Schematic of AAV activated PLLA porous scaffold fabrication. (A) AAV solution was injected through a needle with specialized nozzles into liquid nitrogen to generate ice-based micro-particle; (B) diameter of ice-based micro-particle ranged from 100 to 300 μm; (C) PLLA porous scaffold fabricated with ice-based micro-particles; (D) SEM cross-section of porous PLLA scaffold fabricated using ice-based micro-particles.

Figure 2. Cell viability and AAV vector activity after fabrication: (A) cell attachment to the scaffold 2hrs after seeding; a and b show Live/Dead staining of scaffold surface respectively; c and d show Live/Dead staining of scaffold cross section respectively (40x); (B) cell proliferation on the scaffold 7 days after seeding: a and b show Live/Dead staining of scaffold surface respectively; c and d show Live/Dead staining of scaffold cross section respectively (40x). (C) Efficiency of different vectors of AAV vectors in 2D hBMSCs; (D) Release kinetics of AAV6-D-ChimericCMV-GFP from gene activated constructs; viral particles per scaffold (3x10⁶, 1x10⁷). Figure 3. BMP-2 gene expression and ectopic bone formation in muscle (A) BMP-2 activity assessed by ELISA assay; (B) implantation of construct into muscle; (C) ectopic bone formation in tAAV-BMP-2 activated scaffolds seeded with hBMSCs (circle), but similar areas were not seen with BMP-2-protein-induced scaffold (circle).

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