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Dual chemotactic factors-secreting human amniotic mesenchymal stem cells *via* TALEN-mediated gene editing enhanced angiogenesis

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ABSTRACT

Background: Even though mesenchymal stem cells (MSCs) have angiogenic property, their cytokine secretory capacity is limited to treat ischemic vascular disorders. In present study, we produced genome-edited MSCs that secreted dual chemokine granulocyte chemotactic protein-2 (GCP-2) and stromal-derived factor-1α (SDF-1α) and determined their therapeutic potential in the context of experimental ischemia.
Methods: GCP-2 and SDF-1α genes were integrated into safe harbor site at the safe harbor genomic locus of amniotic mesenchymal stem cells (AMM) *via* transcription activator-like effector nucleases (TALEN). GCP-2 and SDF-1α gene-edited AMM (AMM/GS) were used for quantitative (q)-PCR, Matrigel tube formation, cell migration, Matrigel plug assays and *in vivo* therapeutic assays using hindlimb ischemia mouse model.
Results: AMM/GS-derived culture media (CM) induced significantly higher tube lengths and branching points as compared to AMM/S CM and AMM CM. Interestingly, Matrigel plug assays revealed that significantly higher levels of red blood cells were found in AMM/GS than AMM/S and AMM Matigel plugs and exhibited microvascular like formation. Cells was transplanted into ischemic mouse hindlimbs and compared with control groups. AMM/GS injection prevented limb loss and augmented blood perfusion, suggesting that enhances neovascularization in hindlimb ischemia. In addition, transplanted AMM/GS revealed high vasculogenic potential *in vivo* compared with transplanted AMM/S.
Conclusion: Taken together, genome-edited MSCs that express dual chemokine GCP-2 and SDF-1α might be alternative therapeutic options for the treatment of ischemic vascular disease.

1. Introduction

Critical limb ischemia (CLI) is a peripheral arterial disease caused by arterial obstruction/stenosis in the limb. CLI can be treated with surgical bypass or endovascular therapy. Approximately 30% of CLI patients are not eligible for conventional therapies; 20% die within 6 months; and 40% undergo leg amputation [1]. Therefore, a novel therapeutic modality is needed to improve the poor prognosis of patients with untreatable CLI.

Recently, stem cells have shown promise in regenerative medicine for the treatment of ischemic vascular diseases. However, randomized placebo-controlled clinical trials revealed that stem cell therapy did not significantly increase amputation-free survival [2] and clinical trials results of meta-analyses showed marginal heart function following stem cell therapy [3]. Thus, functional improvements in stem cells are necessary to improve the therapeutic results in the treatment of ischemic (cardio) vascular diseases.

Chemotactic cytokines (chemokines), known for their inflammation-mediating and immune surveillance properties, play a pivotal role in angiogenesis by recruiting pro-angiogenic or endothelial progenitor cells [4–6]. In addition, chemokines regulate endothelial function *via* downstream signaling pathways. Recently, it was reported that the overexpression of granulocyte chemotactic protein-2 (GCP-2/CXCL6) and stromal-derived factor-1α (SDF-1α/CXCL12) enhanced the angiogenic potential of mesenchymal stem cells (MSCs) [7,8].

In this study, we developed a dual chemokine-expression system at the safe harbor genomic locus of amniotic mesenchymal stem cells (AMM) *via* transcription activator-like effector nucleases (TALEN)-mediated editing of their genome to allow for long-term chemokine secretion. We characterized the angiogenic properties of dual GCP-2 and SDF-1α gene-edited AMM (AMM/GS) and their therapeutic effects on a hindlimb ischemia model.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) and human dermal fibroblast (HDF) were purchased from ATCC (Manassas, VA, USA). Human amniotic mesenchymal stem

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cells (AMM) were purchased from Thermo Scientific, Inc. (Watlham, MA, USA). HDF and AMM were cultured in low-glucose Dulbecco's Modified Eagle Medium (DMEM; GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco) [9,10]. HUVECs were cultured in endothelial growth complete media (EGM-2) (Lonza, Walkersville, MD, USA).

2.2. Donor construction

GCP-2-T2A-SDF-1α or SDF-1α was synthesized and inserted into the AAVS1 safe harbor site targeting donor vector (System Biosciences, Palo Alto, CA, USA) at the NdeI and SalI restriction sites.

2.3. Transfection and selection

Human AMM cells were maintained in DMEM supplemented with 10% FBS. For electroporation, human AMM cells were harvested and counted; 1×10^5 cells were resuspended with 0.6 μg of AAVS1 left TALE-Nuclease vector (System Biosciences), AAVS1 right TALE-Nuclease vector (System Biosciences), and AAVS1 HR Donor (System Biosciences) in 10 μL electroporation buffer; and the cells were electroporated using a Neon Transfection System (Thermo Fisher Scientific). After 5 days of transfection, GCP-2/SDF-1α or SDF-1α knock-in cells were selected by incubating with 5 μg/mL puromycin for 11 days.

2.4. Fluorescence-activated cell sorting

After puromycin selection, the cells were harvested on day 50. Cells were washed once with phosphate-buffered saline (PBS) following the 0.05% Trypsin/EDTA treatment for cell detachment. The cells were resuspended in PBS for sorting. The cells were sorted on a S3e Cell Sorter (Bio-Rad, Hercules, CA, USA).

2.5. Genomic DNA extraction and junction PCR

Genomic DNA from cultured cells was extracted using a G-spin™ Total DNA Extraction Mini Kit (Intron Biotechnology) according to the manufacturer's instructions. Next, 120 ng of genomic DNA was amplified by touch-down PCR (36 cycles). Touch-down PCR conditions were as follows: 1 cycle at 98 °C for 30 s, followed by 22 cycles of 98 °C for 30 s, 72–60 °C for 30 s (a decrease of 1 °C every two cycles) and 72 °C for 1 min, followed by 14 additional cycles at 98 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, and the final extension step at 72 °C for 10 min. For the second PCR, 0.5 μL of the touch-down PCR product was used. The second PCR conditions were as follows: 1 cycle at 98 °C for 30 s, followed by 35 cycles at 98 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min, with a final extension step at 72 °C for 10 min.

2.6. Quantitative-reverse-transcriptase PCR and reverse-transcriptase PCR analyses

Quantitative (q) reverse-transcriptase (RT) PCR analysis was conducted as previously described [11]. Briefly, total RNA was isolated from cells using RNA-stat (Iso-Tex Diagnostics, Friendswood, TX, USA). Extracted RNA was reverse-transcribed using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The synthesized cDNA was subjected to qRT-PCR or RT-PCR using human-specific primers and probes. RNA levels were quantitatively assessed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Relative mRNA expression normalized to GAPDH expression was calculated as described previously [12].

2.7. RT-PCR and qPCR primers

The primers used in qRT-PCR were for human GCP-2 (Hs00237017_m1), SDF-1α (Hs00171022_m1) and GAPDH (Hs99999905_m1) and for mouse (Angiopoietin) Ang-1 (Mm00456498_m1), epidermal growth factor (EGF) (Mm00438696_m1), fibroblast growth factor (FGF)-2 (Mm00433287_m1), vascular endothelial growth factor (VEGF)-A (Mm00437306_m1), and GAPDH (Mm99999915_g1). The following paired RT-PCR primers were used: 5′-cctgttttgggggcagttat/gacagttgctggtctgggtg-3′ for kinase insert domain receptor (KDR) (266 bp), 5′-ccaaagagtctccatgccct/ttaaagttgccacacagccc-3′ for von Willebrand factor (189 bp), 5′-ttatttctgtgaagggcgagttcg/aatatcaggtacttcatgccggg-3′ for Tie-2 (223 bp), 5′-gaaggtggagaagctccctg/ttctttagccatgtggcctg-3′ for CCR2 (206 bp), 5′-atgactgtgagcggagcaag/ggaatgggatgtatctgccc-3′ for CCR3 (192 bp), 5′ gcaacatgctggtcatcctc/caaacacagcatggacgaca-3′ for CCR5 (279 bp), 5′-aaggcagaagcaacccaaat/tcaaggttcgtccgtgttgt-3′ for CXCR2 (145 bp) and 5′-tggggcagcccagaacatca/gccgcctgcttcacacctt-3′ for GAPDH (198 bp). All primer/probe sets were purchased from Applied Biosystems.

2.8. Culture medium preparation

Culture media (CM) were prepared as described previously [13]. Briefly, (10^6 cells) were seeded into T-75 cell culture flasks and grown in low-glucose DMEM (Gibco) containing 2% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco) for 48 h until the cells reached approximately 90% confluence. CM were then centrifuged and the supernatants were collected and used for analysis.

2.9. Scratch migration assay

Scratch wound assays were also conducted as described previously [13]. HDFs were seeded to a final density of 10^5 cells/well in 24-well culture and incubated at 37 °C in 5% CO_2 for 27 h to produce confluent monolayers. Monolayers were scratched using a sterile pipette tip and incubated with each of the CM, AMM, AMM/S, and AMM/GS; each of these media had been previously cultured for 5 days. To evaluate cell mobility, images were obtained at 5 random fields in the scratched areas. Scratch areas were examined using the NIH Image program (http://rsb.info.nih.gov/nih-image/).

2.10. Matrigel tube formation assay

To evaluate the tube formation potential, HUVECs were seeded with each CM (AMM, AMM/S, and AMM/GS) at a concentration of 1×10^4 cells/well on Matrigel (BD Biosciences, San Jose, CA, USA)-coated 2-well glass slides (Nunc, Roskilde, Denmark). After 24 h of incubation, fields were randomly photographed and the number of branching points and tube length of each sample were measured.

2.11. Matrigel plug in vivo assay

To examine the *in vivo* vasculogenic potential, a Matrigel plug assay was conducted as previously described [14]. First, 2×10^5 cells with 500 μL Matrigel were transplanted subcutaneously into nude mice. After 14 days, the Matrigel plugs were collected and hemoglobin levels were evaluated using Drabkin's Reagent Kit (Sigma, St. Louis, MO, USA).

2.12. Ischemic hindlimb mice model and cell injection

Ischemic hindlimb was induced as previously described [11]. Briefly, 7–10-week-old male nude mice (Joongang Laboratory Animal Inc., Seoul, Korea) weighing 17–23 g each were used. Mice were anesthetized with isoflurane (induction: 450 mL air with 4.5% isoflurane, maintenance: 200 mL air with 2.0% isoflurane, Baxter International, Inc., Deerfield, IL, USA) and the right femoral artery was surgically ligated. Next, 1×10^6 cells were intramuscularly injected into the ischemic hindlimb area after surgery ($n = 8$ for each group). The animals were sacrificed by intravenous injection of thiopental sodium (40 mg/kg). Blood flow in the hindlimb was analyzed by laser Doppler perfusion imaging (Moor Instruments, Axminster, UK).

2.13. Histological analysis

Histological analysis was performed as previously described [11]. Briefly, the adductor muscles were harvested, fixed in paraformaldehyde for 4 h, and incubated overnight in 15% sucrose solution. The tissues were embedded in OCT compound (Sakura Finetek USA, Torrance, CA, USA) and sectioned into 10-μm-thick sections. Five frozen sections from each group of ischemic tissues were stained with biotinylated isolectin B4 (ILB4, 1:250; Vector Laboratories, Inc., Burlingame, CA, USA) primary antibodies, followed by streptavidin Alexa Fluor 555 (1:400; Invitrogen, Carlsbad, CA, USA) secondary antibodies for capillary density measurement. Next, 5 fields from 6 tissue sections were randomly selected, and the number of capillaries was examined.

2.14. Statistical analysis

All data are presented as the mean ± standard deviation. Statistical analyses were conducted using Student's *t*-test to compare 2 groups [15] and analysis of variance with Bonferroni's multiple comparison test using SPSS v11.0 (SPSS, Inc., Chicago, IL, USA). Data with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Targeted knock-in of GCP-2/SDF-1α into the AAVS1 safe harbor locus

To generate a stable stem cell line expressing the dual chemokine GCP-2/SDF-1α or single SDF-1α using transcription activator-like effector nuclease (TALEN)-mediated integration, we chose the adeno-associated virus integration site 1 (AAVS1) on chromosome 19 as the target integration site. AAVS1 is known as a safe harbor locus and transgene expression is stable [16,17]. The targeting donor plasmid DNA was designed to carry PGK promoter-driven GCP-2-P2A-SDF-1α and EF1α promoter-driven GFP-T2A-Puromycin (Fig. 1A). The donor plasmid was transfected into human AMM with a pair of TALENs, leading to cleavage of the AAVS1 locus. Although <4% of cells were GFP-positive cells after transfection, we improved the GFP-positive cell proportion to 99.78% by fluorescence-activated cell sorting followed by puromycin drug selection (42.12%) (Fig. 1C). To confirm genomic integration of the donor plasmid into AAVS1, we performed genomic DNA PCR followed touch-down PCR, a method in which primers avoid amplifying

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