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Minicircle microporation-based non-viral gene delivery improved the targeting of mesenchymal stem cells to an injury site

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ABSTRACT

Genetic engineering approaches to improve the therapeutic potential of mesenchymal stem cells (MSCs) have been made by viral and non-viral gene delivery methods. Viral methods have severe limitations in clinical application because of potential oncogenic, pathogenic, and immunogenic risks, while non-viral methods have suffered from low transfection efficiency and transient weak expression as MSCs are hard-to-transfect cells. In this study, minicircle, which is a minimal expression vector free of bacterial sequences, was employed for MSC transfection as a non-viral gene delivery method. The conventional cationic liposome method was not effective for MSC transfection as it resulted in very low transfection efficiency (less than 5%). Microporation, a new electroporation method, greatly improved the transfection efficiency of minicircles by up to 66% in MSCs without any significant loss of cell viability. Furthermore, minicircle microporation generated much stronger and prolonged transgene expression compared with plasmid microporation. When MSCs microporated with minicircle harboring firefly luciferase gene were subcutaneously injected to mice, the bioluminescence continued for more than a week, whereas the bioluminescence of the MSCs induced by plasmid microporation rapidly decreased and disappeared in mice within three days. By minicircle microporation as a non-viral gene delivery, MSCs engineered to overexpress CXCR4 showed greatly increased homing ability toward an injury site as confirmed through *in vivo* bioluminescence imaging in mice. In summary, the engineering of MSCs through minicircle microporation is expected to enhance the therapeutic potential of MSCs in clinical applications.

1. Introduction

Mesenchymal stem cells (MSCs) are adult multipotent stem cells possessing the capabilities of self-renewal and differentiation into mesodermal lineages. MSCs have been highlighted as attractive cell sources for stem cell therapy because they can be isolated from various tissues without raising ethical issues and expanded through *in vitro* culture [1–3]. Through many investigations, MSCs have been shown to enhance tissue repair, which was achieved either by direct cell engraftment to the injury site or by indirect paracrine secretion of soluble factors and extracellular vesicles (exosomes and microvesicles) including mRNA and miRNA [1–3]. Currently, it is believed that the therapeutic effects of MSCs depend mainly on paracrine activity because engrafted MSCs are short-lived, which does not correlate with the success of MSC therapy [1,3]. To enhance the therapeutic efficacy of MSCs, many genetic engineering approaches have been explored, with the purpose to enhance homing ability toward injury sites, survivability in hostile *in vivo* conditions, and differentiation to the targeted lineages [2,4]. Viral vectors such as retrovirus, lentivirus, and adeno-associated virus have been widely employed for the efficient introduction of transgenes to MSCs, which provided a long-term and stable expression. Despite these obvious advantages, the clinical applications of viral vectors are severely limited because of the potential problems such as oncogenic transformation by unpredicted

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chromosomal integration, pathogenic risks, and the induction of immune responses [4].

In order to avoid the potential safety problems of viral vectors in clinical application, many efforts have been made to develop alternative non-viral gene delivery systems for MSCs. Although many non-viral systems using cationic liposomes and nanoparticles have been developed, most of them have suffered severely from very low transfection efficiency and short sustainability, especially in MSCs, which are known as "hard-to-transfect" cells. Recently, lipid-based nanoparticles containing MSC-targeting and nuclear localization signaling peptides were shown to achieve very high transfection efficiency (~52%) with long-lasting gene expression (longer than 170 h) [5–7]. However, this system employed sleeping beauty transposon accompanying the process of insertion into host genome, which might cause the potential problems such as oncogenic transformation. Therefore, non-viral gene delivery system using plasmid vectors without the concern of host genome integration need to be developed. Several engineering strategies to modulate the elements of plasmid vectors have been shown to improve transfection efficiency and sustainability [4,8–12]. Modification of the conventional polyadenylation signals resulted in the increase of transgene expression [8]. The plasmids devoid of CpG dinucleotide sequences have been shown to generate higher and more sustainable expression of transgenes [9,10]. Among such efforts, minicircles have been highlighted as minimized eukaryotic expression cassettes that are devoid of bacterial plasmid backbone containing many CpG motifs [11,12]. They are usually generated by site-specific recombination to remove bacterial backbone sequences after the propagation of parental plasmid [13–16]. Especially, genetically engineered *Escherichia coli* capable of inducible *in vivo* recombination and following the enzymatic degradation of bacterial backbone made a great improvement in minicircle production by reducing time and labor to similar levels as those of routine plasmid DNA preparation [16]. Minicircles have been employed for various purposes including transgene expression, gene therapy, DNA vaccine, and the generation of induced pluripotent stem cells [11,12,17]; however, there have been only a few studies that used minicircles for transfection of MSCs [18,19]. In these studies, minicircles were delivered to MSCs by the conventional cationic liposome method or nucleofection technology, but the resulting transfection efficiencies were low (less than 35%).

In this study, we delivered minicircles to MSCs with a very high transfection efficiency by microporation, which is a newly developed electroporation technology using a pipette tip as an electroporation space and a capillary type of electric chamber [20–23]. By minicircle microporation, the transfected MSCs with overexpression of C-X-C chemokine receptor type 4 (CXCR4) were shown to migrate efficiently to the injury site in mice, which is a practical genetic engineering application for enhancing the therapeutic potential of MSCs by a non-viral gene delivery method.

2. Materials and methods

2.1. Construction of parental plasmids

The parental plasmids of minicircles containing CMV (pMC.CMV-MCS-SV40polyA) or EF1α promoter (pMC.EF1-MCS-SV40polyA) were purchased from System Biosciences, Inc. (Mountain View, CA, USA). The gene encoding enhanced green fluorescent protein (EGFP) was amplified from pIRES2-EGFP (Clontech, Palo Alto, CA, USA) by polymerase chain reaction (PCR) using a pair of primers, McEGFP-fwd and McEGFP-rev (Supplementary Table S1). The resulting PCR product was cloned into pMC.CMV-MCS-SV40polyA and pMC.EF1-MCS-SV40polyA using *Eco*RI and *Xba*I restriction enzymes, which generated pP-McCMV-EGFP and pP-McEF1-EGFP, respectively. For the generation of control vector pcDNA3.1-EGFP, EGFP gene amplified by PCR using p3.1EGFP-fwd and p3.1EGFP-rev primers (Supplementary Table S1) was also inserted to pcDNA3.1 using *Bam*HI and *Nhe*I restriction enzymes. The gene encoding firefly luciferase linked with 2A sequence (fLuc-2A), synthesized by Bioneer (Daejeon, Republic of Korea), was amplified by PCR using McfLuc-fwd and McfLuc-rev primers (Supplementary Table S1). The resulting PCR product was cloned into pP-McCMV-EGFP using EZ-Fusion cloning kit (Enzynomics, Daejeon, Republic of Korea), which generated pP-McCMV-fLuc-2A-EGFP. The amplified fLuc-2A DNA using p3.1fLuc-fwd and p3.1fLuc-rev primers (Supplementary Table S1) was cloned to pcDNA3.1-EGFP using EZ-Fusion cloning kit, which resulted in pcDNA3.1-fLuc-2A-EGFP. The gene encoding mouse CXCR4 (mCXCR4) was amplified from the clone pCMV-SPORT6-CXCR4 (Korea Human Gene Bank, Medical Genomics Research Center, KRIBB, Korea, http://genbank.kribb.re.kr/) using two pairs of primers CMV-mCXCR4-fwd/rev and EF1-mCXCR4-fwd/rev primers (Supplementary Table S1), respectively. Two amplified products were inserted into pMC.CMV-MCS-SV40polyA and pMC.EF1-MCS-SV40polyA using *Sal*I/*Eco*RI and *Xba*I/*Ecor*I restriction enzymes, which generated pP-McCMV-CXCR4 and pP-McEF1a-CXCR4, respectively.

2.2. Preparation of minicircles

For the production of minicircles, the parental plasmids were transformed to ZYCY10P3S2T *E. coli* strain (System Biosciences) [16]. Growth and induction of the minicircles were performed according to the manufacturer's instructions and the protocol in Kay et al. [16]. Briefly, a single colony was pre-cultured at 37 °C for 2 h in Luria-Bertani broth (LB) containing kanamycin (50 μg/ml), followed by seed culture in Terrific broth (TB) containing kanamycin (50 μg/ml) at 30 °C for overnight. The next day, induction medium supplemented with 0.01% L-arabinose was added to the overnight culture, and the mixed culture was further incubated at 32 °C with shaking at 250 rpm for 7 h. From the harvested *E. coli* cells, minicircle DNA was extracted by using NucleoBond Xtra Maxi Plus plasmid purification kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. In order to improve the purity, the minicircle was further purified from the corresponding band (negatively supercoiled circular minicircle DNA) excised from agarose gel by using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions.

2.3. Cells and mice

Human bone marrow (hBM)-MSCs purchased from Lonza (Basel, Switzerland) and adipose tissue (hAT)-MSCs purchased from Life Technologies (Carlsbad, CA, USA) were cultured in MSC basal medium (MSCBM; Lonza) and MesenPRO RS medium (Life Technologies), respectively, with supplementations of growth supplement and L-glutamine according to the manufacturer's instructions. Human Embryonic Kidney (HEK) 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose (4.5 g/l) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin in a 5% CO_2 incubator at 37 °C.

Five to six weeks old male Balb/C and nude mice were purchased from (Orient Bio Inc., Seongnam, Republic of Korea). All experiments employing mice were performed in accordance with protocols approved by the Animal Care and Use Committee of Korean Research Institute of Bioscience and Biotechnology (KRIBB). The mouse BM (mBM)-MSCs were isolated and cultured as