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In vivo gene correction with targeted sequence substitution through microhomology-mediated end joining

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ABSTRACT

Genome editing technology using programmable nucleases has rapidly evolved in recent years. The primary mechanism to achieve precise integration of a transgene is mainly based on homology-directed repair (HDR). However, an HDR-based genome-editing approach is less efficient than non-homologous end-joining (NHEJ). Recently, a microhomology-mediated end-joining (MMEJ)-based transgene integration approach was developed, showing feasibility both *in vitro* and *in vivo*. We expanded this method to achieve targeted sequence substitution (TSS) of mutated sequences with normal sequences using double-guide RNAs (gRNAs), and a donor template flanking the microhomologies and target sequence of the gRNAs *in vitro* and *in vivo*. Our method could realize more efficient sequence substitution than the HDR-based method *in vitro* using a reporter cell line, and led to the survival of a hereditary tyrosinemia mouse model *in vivo*. The proposed MMEJ-based TSS approach could provide a novel therapeutic strategy, in addition to HDR, to achieve gene correction from a mutated sequence to a normal sequence.

1. Introduction

DNA double-strand breaks (DSBs) are induced by a variety of endogenous and exogenous types of damage [1,2]. However, cells have unique mechanisms to repair DSBs such as homology-directed repair (HDR), non-homologous end-joining (NHEJ), and microhomology-mediated end-joining (MMEJ) [3–6]. Genome editing technology with artificial site-specific nucleases such as zinc finger nucleases, transcription activator-like effector nucleases, and RNA-guided nucleases has rapidly advanced, which enables site-specific gene engineering such as gene knockout and knock-in through exploiting these natural DNA repair mechanisms [4,7–13].

Although gene knock-in has been mainly achieved by the HDR method, this approach usually requires the use of a donor template containing long homologous sequences, requiring a laborious construction protocol. Furthermore, the efficiency of transgene knock-in through HDR is low and varies among cell types [14]. Moreover, since HDR can only occur in the late G/S2 phases, it cannot be applied in post-mitotic cells [15]. Recently, an MMEJ-based approach was introduced as a potential substitute to the conventional HDR method for inserting genes into the target site [12,13]. The MMEJ mechanism is considered to occur from G1 to

Abbreviations: HDR, Homology-directed repair; NHEJ, Non-homologous end-joining; MMEJ, Microhomology-mediated end-joining; DSB, Double-strand break; gRNA, Guide RNA; TSS, Targeted sequence substitution; NTBC, 2-(2-Nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione; ssODN, Single-strand oligodeoxynucleotide; RFP, Red fluorescent protein; PBS, Phosphate-buffered saline; qRT-PCR, Quantitative real-time polymerase chain reaction; FAH, Fumarylacetoacetate hydrolase.

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early S phase and is thus a cell cycle-independent process [11]. Since MMEJ occurs in a phase in which HDR cannot happen, MMEJ has come into the spotlight as an alternative gene correction method.

In this study, we developed an MMEJ-based targeted sequence substitution (TSS) method using double-guide RNAs (gRNAs) with a donor sequence flanking short microhomologies and the target sequences of gRNAs both *in vitro* and *in vivo*. The feasibility of the method and efficiency of sequence substitution was tested *in vitro* and *in vivo*. For the *in vitro* experiment, we developed a fluorescence-reporter cell line derived from human embryonic kidney 293T (HEK293T) cells, and compared the gene correction efficiency between the MMEJ-based and HDR-based methods. For the *in vivo* experiment, we applied the MMEJ-based TSS approach in the $Fah^{mut/mut}$ mouse line, a model of human familial tyrosinemia, to express the fumarylacetoacetate hydrolase (FAH) protein [16].

2. Materials and methods

2.1. Cell culture, transfection, and lentivirus production

HEK293T cells were purchased from American Type Culture Collection (Manassas, VA, USA) and used to establish reporter cell lines. The HEK293T cells and reporter cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 μg/ml ciprofloxacin (Sigma, St. Louis, MO, USA) and 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA).

The reporter cells were transfected with mixtures of plasmids encoding a Cas9-red fluorescent protein (RFP)-puromycin expression plasmid, gRNA-expressing plasmid(s) (pRG2, Addgene: 104174), and donor constructs at a 1:1:10 M ratio using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The efficiencies of gene correction were analyzed at 72 h after transfection.

For lentivirus production to make reporter cell line, transfer lentiviral vector, psPAX2, and pMD2. G were mixed at a weight ratio of 4:3:1, and transected into HEK293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The supernatant containing the virus was collected at 48 h after the initial transfection, and then filtered through a Millex-HV 0.45-μm low-protein-binding membrane (Millipore, Darmstadt, Germany).

2.2. Production of the mVenus-off reporter cell line

For generating the 2X stop codon-mVenus reporter cell line, we constructed a lentiviral vector encoding the cytomegalovirus promoter, an mVenus fluorescence coding sequence with 2X stop codon sequences, and a puromycin resistance sequence. We introduced two continuous stop codons into wild-type *mVenus* coding sequences by site-directed mutation to achieve complete knock-out. Due to the presence of the 2X stop codons, translation of the *mVenus* gene is prematurely terminated so that the mVenus protein cannot be expressed (designated mVenus-off). The mutated sequence was integrated into HEK293T cells by lentivirus transduction. After 24 h, the cells were treated with 2 μg/ml puromycin (Invitrogen) for the following 7 days to eliminate any untransduced cells, and the *in vitro* experiment was performed with the cells selected on a puromycin-containing medium (Fig. 1A, Supplementary Fig. 1).

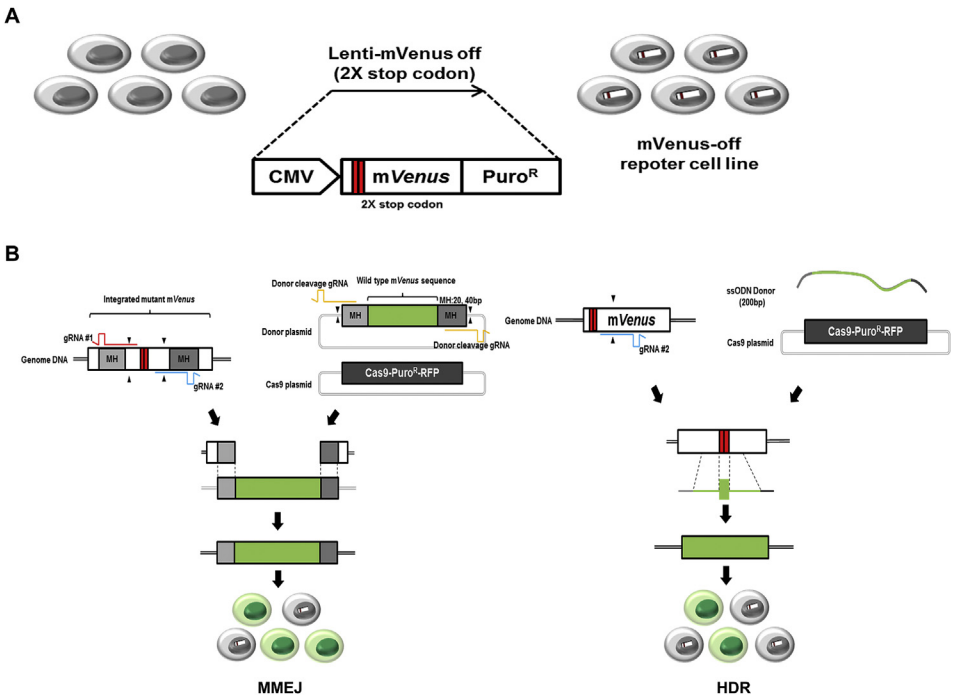

Fig. 1. Schematic diagram of the *in vitro* mVenus-off reporter assay.
(A) HEK293T cells were transduced by a lentivirus having an mVenus-off lentivirus construct generated through site-directed mutagenesis with two stop codons introduced into the m*Venus* wild-type sequence; the reporter cell line was established through puromycin selection. (B) The left panel shows the microhomology-mediated end joining (MMEJ)-based strategy and the right panel shows the homology-directed repair (HDR)-based strategy. In the case of MMEJ, two guide RNAs (gRNAs) were used to cut inside of the microhomologies (MH) on the genome, and donor cleavage gRNA, which cuts outside of the microhomologies on the donor plasmid, was used for linearization of the donor template. Through the common microhomology sequences, the mVenus-off sequence is substituted with the wild-type m*Venus* sequence. In the case of HDR, a single-strand oligodeoxynucleotide (ssODN) and one gRNA were used. When the mutated sequences are corrected, mVenus would be normally expressed.

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