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Targeted introduction of tissue plasminogen activator (TPA) at the AAVS1 locus in mesenchymal stem cells (MSCs) and its stable and effective expression

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ABSTRACT

Thrombolytic therapy using tissue plasminogen activator (TPA) is an effective method for treating acute myocardial infarction. However, the systemic administration of TPA is associated with the risk of hemorrhage. Mesenchymal stem cells (MSCs) from bone marrow are characterized by low immunogenicity and homing toward damaged tissues and are therefore ideal cell carriers to achieve lesion-targeting medication. In this article, TPA gene was integrated into the AAVS1 of mesenchymal stem cells, which has been confirmed to be a safe chromosomal locus. The targeting efficiency was 83%. The clones with the site-specific integration retained the stem cell traits of MSCs, displayed a normal karyotype and could persistently and effectively express TPA, as demonstrated by an average expression activity of 1.5 units/mL (3.4-fold that of the control group). After subculture and subsequent growth for two weeks, the clones showed an average TPA activity of 1.43 units/mL and exhibited no significant differences among the individual clones. In summary, the foreign TPA gene can be specifically introduced to the AAVS1 locus, whereby it can be stably and effectively expressed. MSCs can serve as cell carriers for the targeted treatment of a thrombus using TPA.

1. Introduction

Clinical studies have revealed that thrombolytic therapy can increase the survival rate of those patients suffering from acute myocardial infarction or cerebrovascular accidents. The thrombolytic approach involving tissue plasminogen activator (TPA), which has become a clinical method for treating acute myocardial infarction, has the ability of reducing the infarct size, protecting ventricular function and curbing the mortality rate [1,2]. Additionally, in the treatment of acute ischemic stroke, TPA has been shown to display the effects of improving the nerve function of the patients [3,4]. However, TPA is currently administered to patients systemically, which risks hemorrhage, including pulmonary or cerebral hemorrhage, which can be lethal. Hence, an ideal administration strategy of TPA is to express it locally in the lesions at a high level [5].

Mesenchymal stem cells (MSCs), a type of adult stem cell, can differentiate into cells of mesoderm lineage [6,7]. Because MSCs have the crucial features of homing toward damaged tissues and modulating inflammation, such cells with a genetic modification can aggregate in and around the infarcted blood vessels and secrete therapeutic factors, thereby subjecting the injured tissues and organs to a targeted cure [8,9]. Currently, the MSC-based cell therapy for ischemic stroke has entered the stage of clinical trials and has achieved satisfactory outcomes [10]. As such, MSCs are likely to be the ideal cell carrier to accomplish TPA expression targeting infarcted areas [11–13].

So far, numerous methods have been employed to modify MSCs originating from various tissues in which the gene therapy for tumor and hereditary diseases is attempted. However, most of these modification methods are based on random integration, which, due to the uncertainty of the insertion sites, can cause various issues. For example, random integration may occur in heterochromatin regions, leading to gene silencing [14]. Alternatively, it may occur in a coding region of an intrinsic gene that destroys its function, affects the transcription and expression of neighboring genes or, in the worst case, results in the malignant transformation of cells and tumorigenesis [15]. Because of the genetic risks associated with random integration-based gene modification, the targeted introduction of a foreign gene into a "safe site" on the chromosome has become a more appealing option. AAVS1 has been

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demonstrated to be a safe transgenic locus, and the concurrent expression of zinc-finger nuclease (ZFN) enables foreign genes to be introduced into the AAVS1 site of multiple cell lines with high efficiency [16]. This study attempts to introduce the TPA gene into the AAVS1 locus of MSCs, thereby exploring the feasibility of using MSCs as the cell carrier for targeted gene therapy.

2. Materials and methods

2.1. Separation and culture of MSCs

Signed informed consent was obtained from volunteers and approved by the Ethics Committee of Central South University before bone marrow was acquired from their iliacs. The bone marrow was subject to separation with a Histopaque-1077 (Sigma–Aldrich, St. Louis, MO) density gradient, as described previously [7,17]. The cells were cultured in MSC medium (DMEN low glucose + 10% FBS + 10 ng/ml bFGF).

2.2. Construction of vectors

AAVS-SA-2A-PURO was acquired through Addgene (Cambridge, MA). The full-length cDNA of TPA, a generous gift from Dr. Xiang Wang at Central South University, was inserted into the *Not*I site of pcDNA3.1 to generate the expression cassette of CMV-TPA-BGH-polyA. Then, the CMV-TPA-BGHpolyA expression cassette was inserted into the *Sal*I site of the AAVS-SA-2A-PURO plasmid to generate the donor vector AAVS-PURO-TPA. The construction of the ZFN-expression vector was based on a previous study [18]. The amino acid sequences of the four ZFN-L zinc-fingers, which determine the relevant recognition sites, were YNWHLQR, RSDHLTT, HNYARDC and QNSTRIG; the crucial amino acids of the four ZFN-R zinc-fingers were QSSNLAR, RTDYLVD, YNTHLTR and QGYNLAG.

2.3. Gene targeting

MSCs were separated and propagated until passage 3 and were subjected to co-transfection of AAVS-PURO-TPA along with ZFN-L and ZFN-R using the nuclear transfection method. The amounts of the donor vector and ZFN-expression vector were 5 and 1 μg, respectively, which were used to transfect 3×10^5 MSCs. The transfected cells were grown in DMEM + 10% FBS for 48 h, followed by puromycin supplementation at 0.5 μg/ml for selection. The antibiotic was removed after approximately 15 days. After culturing for another two weeks, single clones were selected and propagated. Meanwhile, some clones were trypsinized to prepare the mixed clonal culture. The remaining clones were stained with crystal violet and enumerated.

2.4. Validation of the site-specific integration clones

To confirm the site-specific integration clones, the gDNA was extracted using a routine method and examined with PCR using the primers of screen up (5'-AACTACCTAGACTGGATTCGTGAC-3') and screen dn (5'-TGAGAAGCTGATGCAAGTTATGAG-3').

2.5. Southern blot

A total of 5 μg of gDNA was digested with *Ahd*I overnight before being electrophoresed in 0.8% agarose and transferred to a positively charged nylon membrane. The probes, labeled with DIG-dUTP, were used to hybridize the gDNA fragments on the nylon membrane, which was then incubated at 42 °C overnight. The hybridization signals were detected using CDP-Star. Details of the probes are illustrated in Fig. 1: P1 corresponds to the 6443–6885 region of the PPP1R12C gene, whereas P2 is the PCR product amplified using AAVS-PURO-TPA as the template and ATTACGGGGTCATTAGTTCA and AATGGGGCGGAGTTGTTACGA as the primers.

2.6. Determination of multiple differentiation potential

To assess the multiple differentiation potential of the MSCs, we studied their differentiation into osteoblasts, adipose cells and chondrocytes using the StemPro® Osteogenesis Differentiation Kit, StemPro® Chondrogenesis Differentiation Kit and StemPro® Adipogenesis Differentiation Kit (Invitrogen, Carlsbad, CA), respectively. The experimental procedures were performed according to the corresponding instructions. The osteoblasts were stained with Alizarin red, the adipose cells with Oil Red O, and the chondrocytes with Alcian blue.

2.7. Assessment of TPA activity

The MSCs with site-specific integration were inoculated into a 6-well plate at 2×10^5 cells/well, with each clone in triplicate wells. After 24 h of growth, the medium was harvested and centrifuged; the resulting supernatant was ready for detection. The cells in each well were trypsinized and enumerated. The determination of the TPA activity was accomplished using the Tissue type Plasminogen Activator human Chromogenic Activity Assay Kit (ab108905; Abcam, Cambridge, UK) following the manufacturer's instructions. A standard curve was generated using specimens with known TPA activity values, which was then used to assess the TPA activity of the test samples.

3. Results

3.1. Construction of the targeting system

We constructed the AAVS1-targeting vector AAVS1-puro-TPA, which contains a promoterless expression cassette of puromycin.

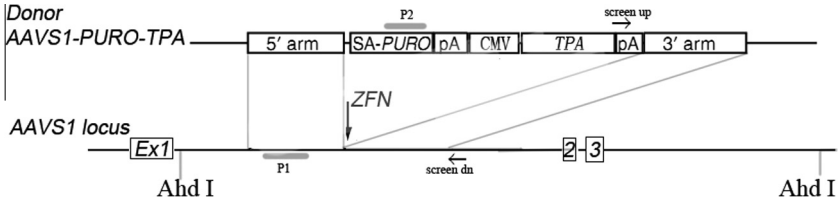

Fig. 1. Schematic organization of the targeting vector AAVS1-puro-TPA. The targeting vector AAVS1-puro-TPA comprises 5' and 3' homologous arms, the promoterless puromycin expression cassette SA-PURO-pA and the TPA expression cassette CMV-TPA-pA under the control of the CMV promoter. The ZFN recognition site is located in the first intron of the PPP1R12C gene in the AAVS1 locus. 'screen up' and 'screen dn' are the primers used to validate the site-specific integration clones and are at the 3' ends of pA and the 3' homologous arm, respectively. P1 and P2 are the Southern blot probes used to ascertain the site-specific integration clones.

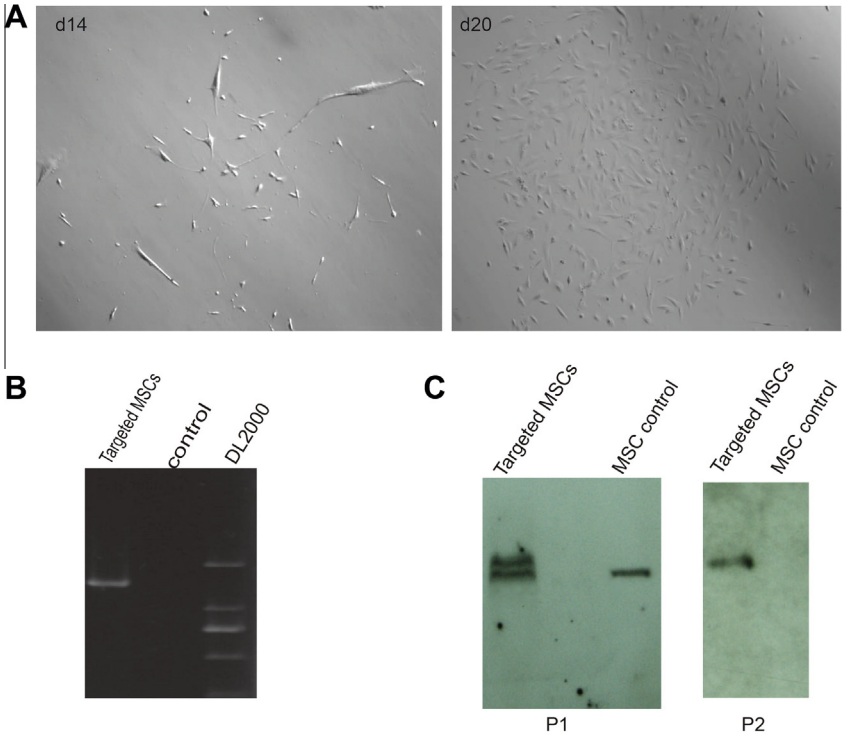

Fig. 2. Gene targeting at the AAVS1 locus of MSCs. (A) The resistant clones of MSCs, showing the morphology of resistant clones after 14 and 20 days of selection; (B) Representative result of site-specific integration clones based on Junction PCR; (C) Representative result of site-specific integration clones based on Southern blot. P1 and P2 are the corresponding probes illustrated in Fig. 1.

Table 1

Test	Transfected cells	Examined clones	Positive clones	Targeting efficiency (%)
1st	2×10^5	50	42	84
2nd	2×10^5	50	41	82

Upon being integrated at the AAVS1 locus, the antibiotic gene can be transcribed due to the $P_{PPP1R12C}$ promoter. In addition, the vector also contains the P_{CMV}-controlled TPA expression cassette (Fig. 1).

3.2. Gene targeted MSCs

MSCs were co-transfected with AAVS1-puro-TPA and the ZFN-expressing vector. Forty-eight hours later, puromycin was added to screen for positive clones. Two weeks later, many single clones were visible (Fig. 2a). After further growth for two weeks, single clones were picked and propagated. gDNA samples were extracted from 50 randomly chosen clones and analyzed with junction PCR, which revealed 42 clones harboring the expected 1.6-kb band (Fig. 2b). Subsequently, the Southern blot showed that the expected 18-kb band was detected in all PCR-positive clones (Fig. 2c). The gene targeting experiment was performed twice, yielding targeting efficiencies of 84% and 82% (Table 1).

3.3. Trait examination for the MSC clones with site-specific integration

To investigate the stem cell traits of the derived MSCs, we determined their phenotypes and multipotency. The results showed that the GFP-positive cells retained the expression profile of classical surface markers associated with MSCs, namely CD34−, CD45−, CD44+, CD73+, CD90+ and CD109+ (Fig. 3a). Meanwhile, these cells maintained the multipotency of differentiation toward osteoblasts, adipose cells and chondrocytes (Fig. 3b). Karyotype identification indicated that the site-specific integrated MSCs retained the normal karyotype of 46, XY (Fig. 3c).

3.4. TPA expression in the clones with site-specific integration

To determine whether TPA is effectively expressed at the AAVS1 locus in the MSCs, we randomly collected supernatant samples from MSCs with site-specific integration, modification and examined their TPA activities. The results revealed that after 24 h of growth, the average TPA activity of these MSCs in the supernatant reached 1.5 units/mL, which was 3.4-fold that of the relevant value of the control MSCs. In addition, the 6 clones exhibited comparable TPA activities, with no significant differences among them. The cells of these 6 clones were subcultured and grown for 2 weeks before their TPA activities were examined. The results showed that the TPA activity was 1.43 units/mL and that there was no significant difference among the individual clones (Fig. 4).

4. Discussion

MSCs are an ideal cell carrier for gene therapy, and the advancement of gene targeting technologies has promoted the establishment of MSC site-specific modification. This study used a ZFN to introduce TPA into a safe docking site for foreign genes in human MSCs, the AAVS1 locus, and achieved an effective expression.

Many methods have been reported to facilitate gene modification in MSCs, with most integrating the therapeutic genes randomly into the genome. Because the random integration of a foreign expression cassette harbors the risk of insertional mutation and often results in silencing of the foreign gene, the genetically modified cells have tremendous differences in the expression

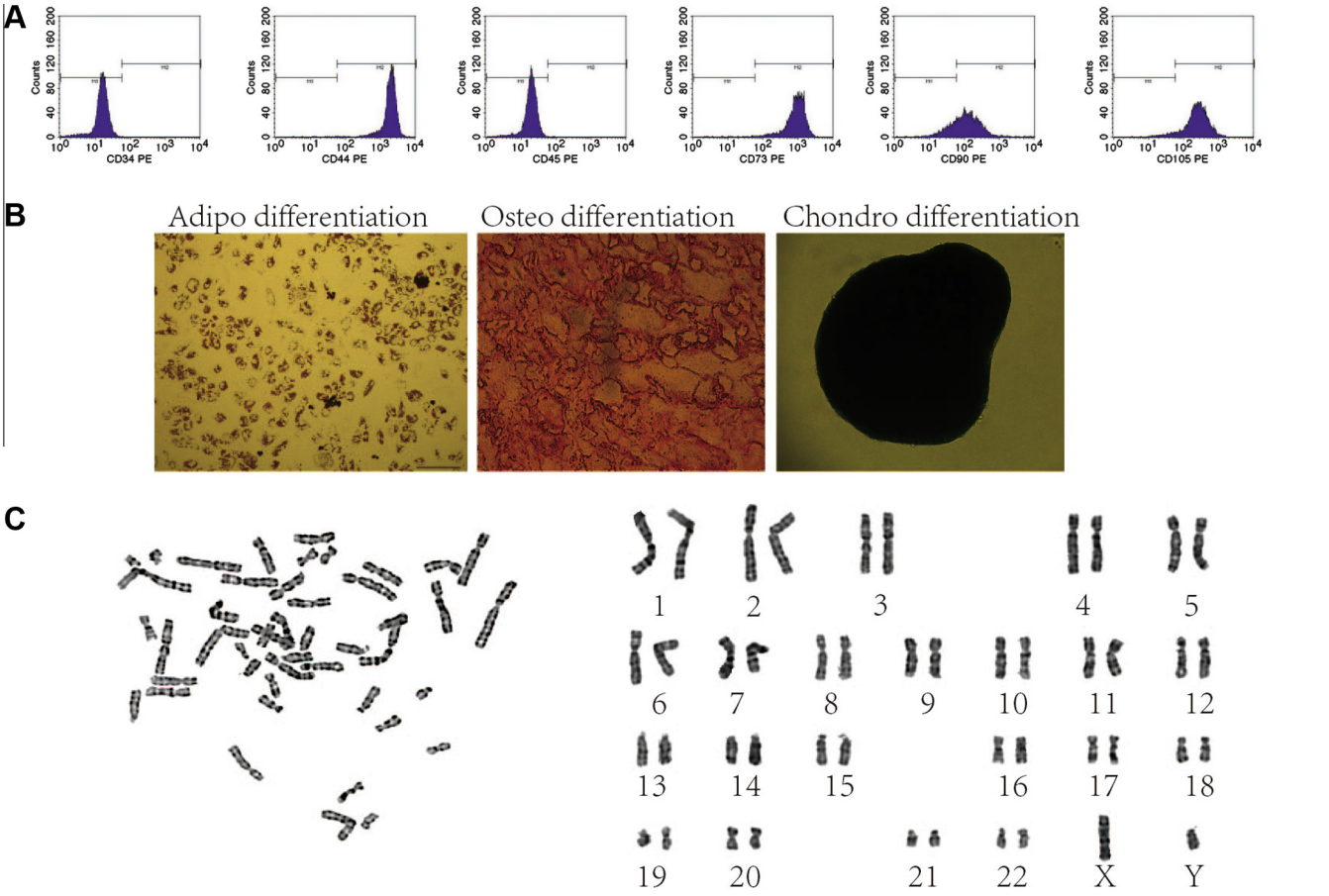

Fig. 3. Examination of the stem cell traits for the MSC clones with site-specific integration. (A) Expression analysis of cell surface markers: PE-conjugated antibodies (CD34, CD44, CD45, CD73, CD90 and CD105) were incubated with MSCs and then analyzed with flow cytometry. (B) Differentiation of the MSCs with site-specific integration toward adipose cells, osteoblasts and chondrocytes. The differentiated adipose cells, osteoblasts and chondrocytes were stained with Oil Red O, Alizarin red and Alcian blue, respectively. (C) Karyotype identification showed that the MSCs with site-specific integration retained the normal 46, XY karyotype. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

levels [14,19]. Such gene silencing has been reported to be mediated by methylation-dependent or -independent mechanisms [20]. In contrast to the previously reported modification methods, this study introduced the gene of interest, TPA, specifically into the AAVS1 locus to achieve its expression. None of the isolated clones were shown to exhibit gene silencing, which may be due to the presence of natural insulators in the locus that protect genes from being silenced [21]. Previously, a foreign gene was introduced into the AAVS1 locus in human or mouse embryonic stem cells and was reported to be effectively expressed [22]. Based on this finding, we reported herein for the first time that a foreign gene that is integrated into the AAVS1 locus of MSCs can be effectively expressed. This finding is significant for using MSCs to perform gene therapy. Importantly, TPA expression exhibits no apparent differences among the individual MSC clones, which is crucial for achieving repeatability in gene therapy research and stable clinical efficacy.

Thus far, four chromosomal loci in MSCs have accommodated successful targeting, namely A1AT, COL1A1, COL1A2 and CCR5, all of which encode proteins of pivotal functionality. Mutations in the A1AT gene may lead to a common genetic metabolic disease in liver, α-1-antitrypsin deficiency. Defects in COL1A1 or COL1A2, encoding Type I or II collagen, may cause osteogenesis imperfecta. Because the integration of foreign genes into these loci can result in known diseases, they are not suitable sites for docking foreign genes. Because individuals with a homozygous CCR5 deletion (δ32) can resist the infection of human immunodeficiency virus (HIV), CCR5 is considered a safe place for the incorporation of foreign genes and has been reported to accommodate gene

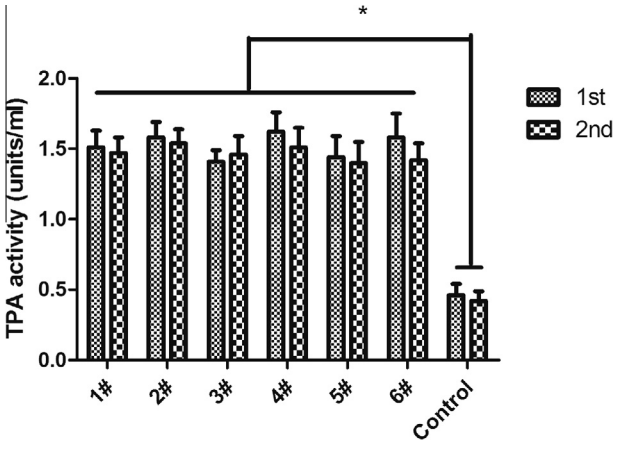

Fig. 4. Determination of TPA activity. Six MSC clones with site-specific integration were randomly selected for harvesting the cell culture supernatant and examining the corresponding TPA activity. 1#, 2#, 3#, 4#, 5# and 6# are the IDs of the individual clones, and the control is the MSC without site-specific integration. The experiment was repeated twice. The first test was performed for newly isolated clones, which were subcultured and grown for 2 weeks before the second test was conducted.*$p < 0.05$.

knock-in mediated by an integration-defective lentivirus vector [23]. Nevertheless, the insertion of a foreign gene into the CCR5 locus can interfere the expression of flanking genes [16], which raises questions for its suitability as a transgenic docking site. Importantly, AAVS1 is considered to be a safe docking site for foreign genes [16,24,25]. In this study, we showed that the introduction of the TPA gene at AAVS1 does not alter the stem cell traits of the MSCs and that the gene is stably expressed. Therefore, this locus is an excellent foreign gene docking site in MSCs.

Currently, the clinical application of TPA to treat artery embolization is accomplished by systemic administration. Although this approach has many advantages compared with other therapeutic methods, it harbors a high risk of complications. The most significant consequence is hemorrhage, especially pulmonary and cerebral hemorrhage, which can be life-threatening [1,5]. Our preliminary study revealed that in addition to avoiding systemic side effects, the local, restricted overexpression of TPA can prevent or successfully dissolve blood clots [5]. Because they have the feature of homing toward lesions and damaged sites, MSCs have been widely used to perform ischemic treatment studies, with several that have entered the stage of clinical trials. This report established a method to utilize MSCs for expressing TPA, which has the prospect of achieving TPA high-expression targeting embolization tissues. Furthermore, we succeeded in integrating the TPA gene into a safe chromosomal site, which is not lost due to cell division and has exhibited long-term, stable expression. As such, this technique may prevent the recurrence of thrombus after its ablation.

Acknowledgments

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