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Reduction of MBS85 gene expression after the targeted integration of a transgene into the AAVS1 site using adeno-associated virus integration machinery

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ABSTRACT

Introduction: The adeno-associated virus (AAV) integrates into a particular location on human chromosome 19 (19q13.4), which is known as AAVS1, through the binding of a replication protein, Rep, to the viral inverted terminal repeat (ITR). Aims: Using the AAV integration machinery, we performed the AAVS1-targeted insertion of a reporter gene into KM-102 cells, a stromal cell line established from human bone marrow cells, and examined the impact of the AAVS1-targeted insertion of the transgene on the host cells. Methods: We co-transfected KM-102 cells with a Rep plasmid and a plasmid containing GFP and a blasticidin resistance gene flanked by ITR sequences. After culturing the cells with blasticidin S, thirty clones were obtained and analyzed for AAVS1 - specific integration and then had their myosin binding subunit 85 (MBS85) mRNA levels measured. Results: Out of 30 selected clones, three clones containing the GFP gene at AAVS1 were obtained. These three clones grew well, similar to the wild-type KM-102 cells, but showed a decreased level of MBS85 mRNA expression. These results indicated that although the insertion of the transgene at AAVS1 affected MBS85 expression, it did not appear to cause phenotypic changes in the KM-102 cells. Conclusions: The AAVS1 site is a safe harbor for transgene insertion although it results in impaired MBS85 expression in KM-102 cells.

Keywords: Site-specific transgene integration; Adeno-associated virus; Rep protein; AAVS1 locus; MBS85

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INTRODUCTION

Retroviral vectors are capable of integrating transgenes into host chromosomes. However, the safety of retroviral vectors is a major issue since they integrate into the human genome at random locations and thus can potentially activate proto-oncogenes, triggering the development of cancer [1]. Thus, to aid the emergence of gene therapy it is necessary to develop safe strategies that allow therapeutic genes to be inserted into predetermined sites in the cellular genome.

The adeno-associated virus (AAV) genome is a 4.8 kb (kilobase) linear, single-stranded DNA, which contains

a 145 nucleotide (nt) inverted terminal repeat (ITR) sequence at both ends (figure 1A). The AAV preferentially integrates into the AAVS1 site on chromosome 19 (19q13.4). The AAVRep78 or Rep68 required for AAVS1-specific integration [2]. Rep78/68 specifically binds to a sequence motif within the ITR, the Rep binding site (RBS), and cleaves it in a site- and strand-specific manner at the terminal resolution site (trs) located 16 nt upstream of the RBS. A sequence homologous to the RBS/trs is present in the AAVS1 locus (figure 1). The AAVS1 RBS motif is located 14 nt upstream of the translation initiation codon for the protein phosphatase 1 regulatory inhibitor subunit 12C gene (PPP1R12C), which is also called MBS85(myosin binding subunit 85). MBS85 is essential for actin depolymerization and cell motility. Site-specific integration using the AAV machinery enables the targeting of a therapeutic gene to a predetermined site, thereby minimizing the risk caused by insertional mutagenesis. Preferential integration, using an AAV plasmid-based system, has been demonstrated in cell lines derived from human embryonic kidney cells, HEK293 cells, [2] a human erythroid leukemia cell line, K562 [3], a human cervical carcinoma cell line [4] and human embryonic stem cells [5] (see Urabe et al., 2007 [6] for review).Rep-mediated integration system can efficiently insert ~200 kb foreign DNA into AAVS [7].

Marrow stromal cells are the cellular constituent of the marrow microenvironment and are essential for supporting hematopoiesis. In addition they are easy to obtain and rapidly expand in vitro. They are one of ideal targets for site-specific insertion of transgene since they can be vehicles to deliver therapeutic proteins [8].

AIMS: We performed the AAVS1-targeted integration of a gene of interest into a bone marrow-derived stromal cell line, KM-102, as a model and examined the impact of the AAVS1-targeted insertion of the transgene on the host cells.

MATERIALS AND METHODS

Plasmid construction: pRVK contains a 3.4kb AAVS1 sequence. pSVR68 expressing Rep68 under the control of the SV40 promoter (figure 2), was derived from pCMVR68 [2]. The SV40 promoter was PCR amplified

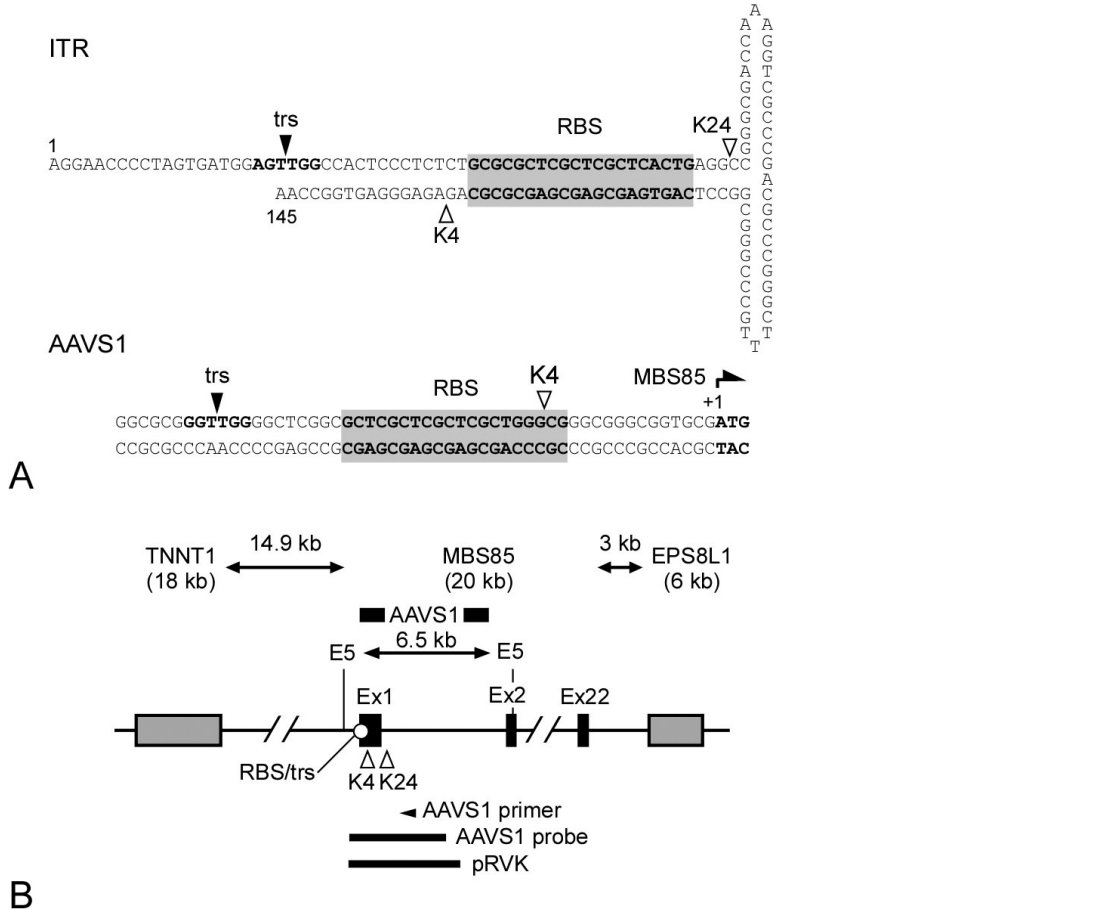

Figure 1: A) Sequence alignment of the ITR sequence and the AAVS1 site. The Rep binding site (RBS) is shown in gray, and the terminal resolution site (trs) is indicated by an arrowhead. The initiation codon for MBS85 is indicated by an arrow. The breakpoints in the K4 and K24 KM-102 clones are indicated by white arrowheads. B) Genetic organization of the AAVS1 site. The AAVS1 site crosses the first exon and the first intron of the MBS85 genome, which is flanked by slow skeletal troponin T1 (TNNT1) and epidermal growth factor receptor pathway substrate 8-like protein 1 (EPS8L1) genes. The RBS/trs site is indicated by a white circle. The breakpoints in K4 and K24 cells are indicated by white arrowheads. The EcoRV sites (E5), the AAVS1-specific probe for Southern blot and the PCR primer for amplification of plasmid-AAVS1 junction and the sequence cloned into pRVK are also shown.

from pCMV-Tag2B (Stratagene, Pallo Alto, CA, USA) and inserted into pCMVR68 upstream of the Rep68 gene. pEB2, which harbors the CMV promoter, IRES (internal ribosome entry site), and a blasticidin S resistance gene (bsr) was described previously [9]. A humanized green fluorescent protein (hrGFP) gene was excised from phrGFPII-1 (Stratagene) and inserted into pEB2 upstream of the IRES. The hrGFP/bsr cassette was then inserted between the ITR sequences (pWGFPEB).

Cell culture and transfection: The KM-102 stromal cells [10] were maintained in McCoy's 5A medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO, USA). The transfection of the KM-102 cells was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly a mixture of 2 μg of pSVR68 and an equal quantity of pWGFPEB was transfected into 3.5x105 KM-102 cells per well in a six-well plate. One day after transfection the cells were reseeded at an appropriate ratio. KM-102 cell clones were collected after being cultured for 14 days in the presence of 5 μg/ml of blasticidin S.

Southern blot analysis: Southern blot analysis was performed as described previously [11]. A 3.0-kb AccI fragment derived from pRVK was used as an AAVS1-specific probe (figure 1B), and a 3.0-kb NotI fragment from pWGFPEB was employed as a transgene probe (figure 2).

Quantitative real-time PCR for MBS85 mRNA: Total RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany). One microgram of total RNA was subjected to reverse transcription (SuperScript One-Step RT-PCR, Invitrogen) and qPCR (QuantiTect SYBR Green PCR Kit, Qiagen) in triplicate with primers for MBS85 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (table 1).

RESULTS

Site-specific integration in KM-102 cells: We analyzed 30 KM-102 clones by Southern blotting after digestion with EcoRV. Figure 3A shows a representative blot. The left panel is a blot that was hybridized with the AAVS1 probe and shows a 6.5-kb basal band in each lane (arrow). In some lanes, additional bands with slower mobility were detected, indicating the rearrangement of AAVS1. The right panel shows the same blot re-probed with a transgene-specific probe. Bands that hybridized with both the AAVS1 and transgene probes are indicated by arrowheads. Clones K4, K24, and K25 were considered to harbor the transgene at the AAVS1 locus and also confirmed to express GFP (figure 4A).

To verify that the GFP transgene had been inserted into the AAVS1 locus in these clones, we tried to PCR-amplify the junction sequence with primers specific to the CMV promoter or the bsr gene and an AAVS1-specific primer (figures 1B, 2, table 1). In the K4 cells, the GFP/bsr transgene cassette was inserted upstream of the initiation codon for MBS85 in reverse orientation (figures 1 and 3B). In the K24 cells, the transgene cassette was inserted in the same direction as the MBS85 gene and it appeared that the first exon had been deleted. The breakpoints detected in the K4 and K24 cells are consistent with those described in previous reports [4, 12, 13]. No sequence was amplified from the K25 cells.

Impact of AAVS1 integration on the expression of MBS85: Since the AAVS1 site overlaps with the MBS85 gene, the insertion of foreign DNA into AAVS1 disrupts the MBS85 gene. Integration at AAVS1 also results in the deletion of the AAVS1 locus [4, 12, 13]. We quantified the expression of MBS85 mRNA relative to GAPDH mRNA using real-time RT-qPCR. The clones

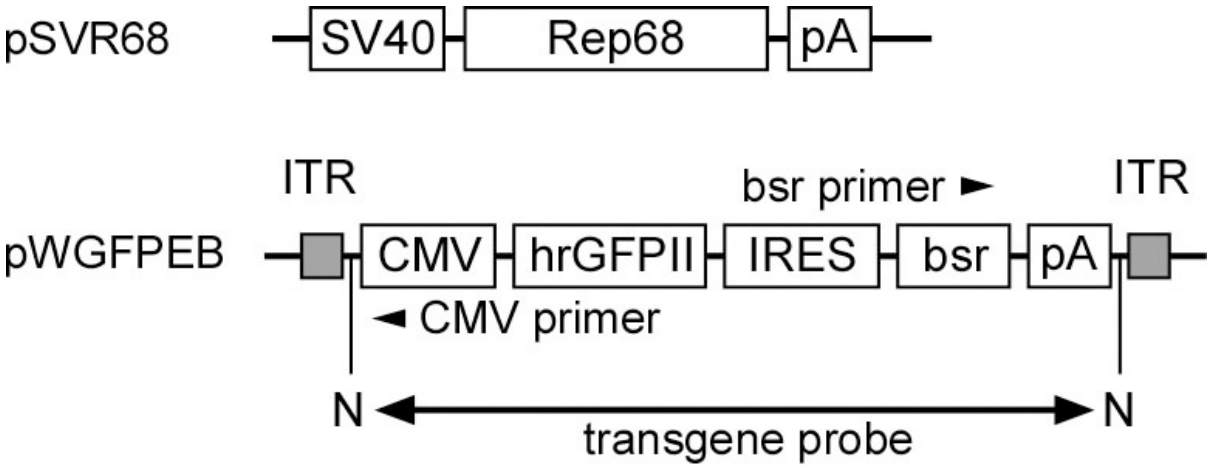

Figure 2: Plasmid DNA used to transfect the KM-102 cells. pSVR68 expresses Rep68 under the control of the SV40 promoter. pWGFPEB harbors a humanized GFP gene (hrGFP) driven by the CMV promoter and a bsr gene under the translational control of the IRES. The expression cassette is flanked by the ITR. Positions of CMV and bsr primers for the amplification of plasmid-AAVS1 junction are indicated by arrowheads.

Table 1: Primers used in the study.

CMV	5'-GGGTCGTTGGGCGGTCAGC-3'
bsr	5'-TCGAGTGGTAAGTCCTTGTGGTATGT-3'
AAVS1	5'-CGGGGAGGATCCGCTCAGAGGACA-3'
MBS85	5'-CTAGCAGCAAACACAGAAGGA-3'
	5'-CGGGGAGGAGACGCCATTGA-3'
GAPDH	5'-TGGCAAAGTGGAGATTGTTGCC-3'
	5'-CTAAAGATGGTGATGGGCTTCCCG-3'

that harbored the transgene at the AAVS1 site (K4, K24, K25 cells) showed decreased MBS85 mRNA levels compared to the parental KM-102 cells and the K23 cells, which contained the transgene at a non-AAVS1 site (figure 4B).

G-CSF levels in the culture supernatants: KM-102 cells produce G-CSF upon stimulation with IL-1 [14]. To examine the phenotypic changes induced in KM-102 cells after their transfection with a GFP plasmid and clonal expansion, we investigated the responsiveness of

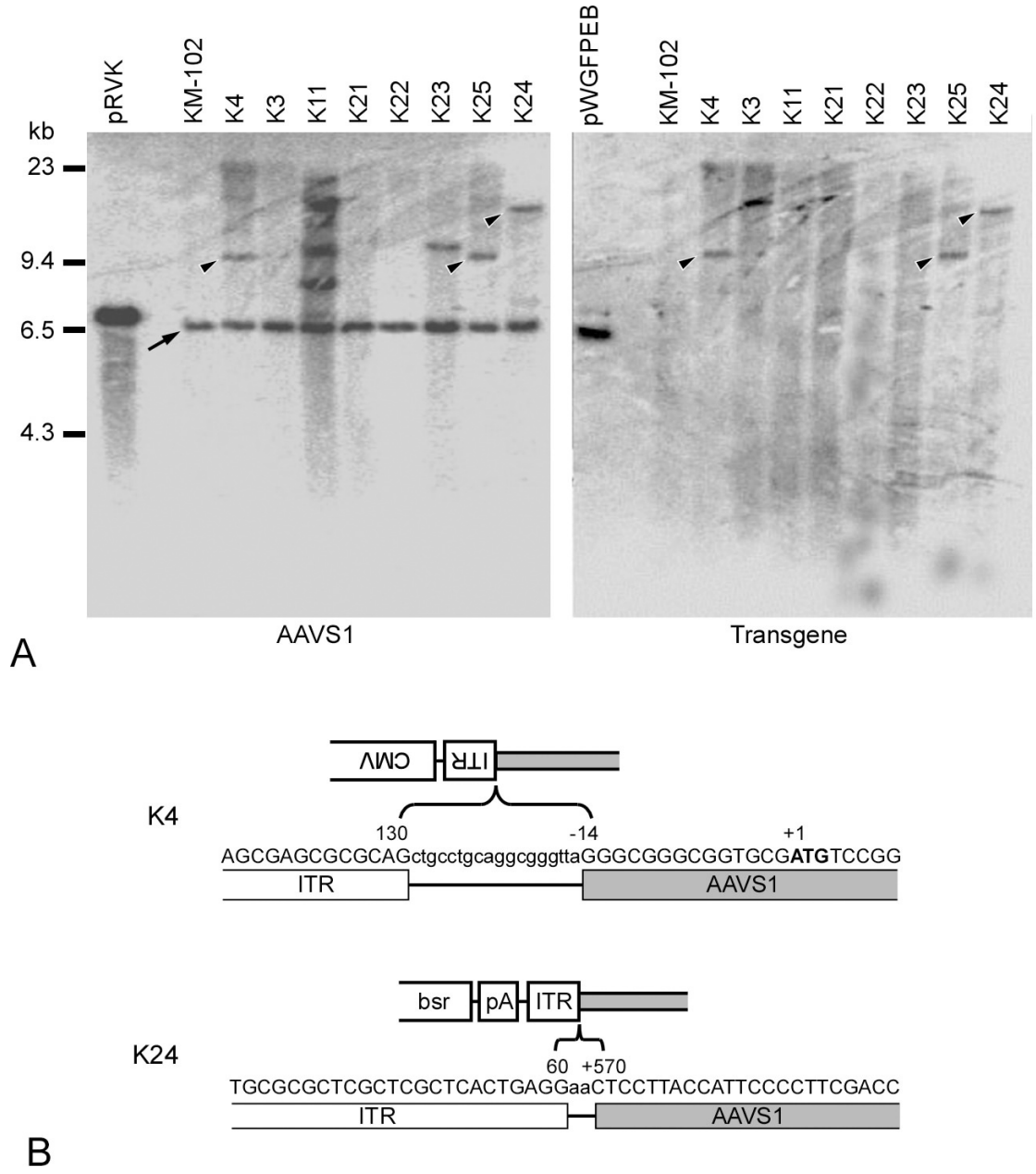

Figure 3: A) Representative Southern blot analysis of cell clones obtained after co-transfection of the KM-102 cells with pWGFPEB and pSVR68. The EcoRV-digest was probed with an AAVS1-specific probe or a transgene probe. The arrowhead indicates a rearranged band that also hybridized with the transgene probe. The arrow in the left panel indicates the 6.5-kb AAVS1 basal band. Linearized pRVK or pWGFPEB was used as positive control for Southern analysis. B) Junction sequence between the GFP plasmid and the AAVS1 locus in the AAVS1-integrants. The junction sequence was successfully PCR-amplified from the K4 and K24 cells. The positions of the residues in the AAVS1 sequence were numbered from the initiation codon for MBS85, which was defined as +1 (see also Figure 1). Unidentified nucleotides in the ITR-AAVS1 junction are indicated by small letters.

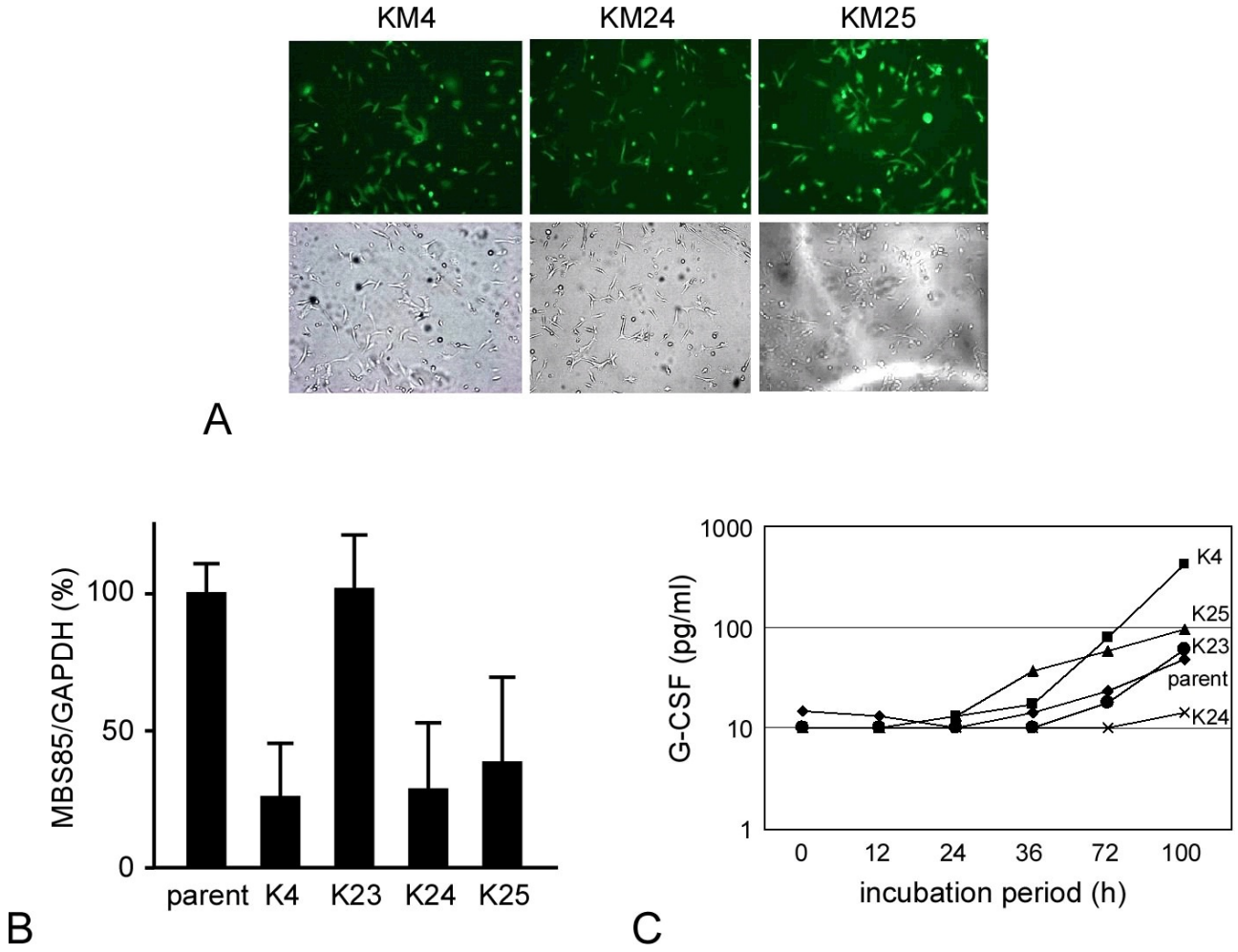

Figure 4: (A) –Photographs of KM-102 clones. Isolated clones express GFP. (B) – Quantitative real-time RT-PCR for MBS85 mRNA expression. The expression of MBS85 was normalized to that of GAPDH (M±S.D.). In the K4, K24, and K25 clones, the GFP/bsr gene is inserted into the AAVS1 locus. In the K23 cells, the GFP/bsr gene was inserted into a site other than AAVS1. (C) –Secretion of G-CSFby KM102 clones upon stimulation with IL-1β.

the clones to IL-1. Parental KM-102 cells and the transgenic clones were cultured in the presence of IL-1 (10 ng/ml), and then the G-CSF levels in the culture supernatants were measured. The clones secreted G-CSF after exposure to IL-1(like the KM-102 cells (figure 4C). This result suggested that the incorporation of foreign DNA into the AAVS1 site did not affect the ability of the cells to respond to IL-1.

DISCUSSION

In the present study, we tested AAVS1-targeted insertion of transgene in KM-102 cells, a cell line derived from marrow stromal cells, and were able to specifically insert a GFP gene into the AAVS1 locus. Southern blot analysis demonstrated the AAVS1-specific integration of the transgene into KM-102 cells in three out of thirty clones (10%), which was confirmed by PCR analysis of the junction sequences between the transgene plasmid and the AAVS1 sequences in two clones. We failed to amplify the junction in K25 cells, which may be due to more extensive deletion of the MBS85 genome in K25 cells beyond the AAVS1 primer annealing site (figure 3A). Recently integration of AAV into AAVS1 has been reported to accompany partial duplication of the MBS85 gene and can preserve the normal expression of its mRNA [15]. They employed wild-type AAV and an AAV vector expressing GFP for targeted insertion in HeLa cells and mouse ES cells, whereas we used plasmid DNA in KM-102 cells. The different AAVS1 targeting strategy may affect the restoration of the MBS85 genome. In addition the same group had reported previously that the integration of AAV caused extensive deletion over the adjacent gene TNNT1 [16]. Thorough understanding of Rep-mediated integration will be required for safe gene insertion technology based on AAV.

The insertion of foreign DNA into the AAVS1 site inevitably edits one allele of the MBS85 gene. However,

KM-102 cells containing the transgene at AAVS1 did not appear to show phenotypic changes although their MBS85 mRNA level decreased (Figure 4B). Therefore, the disruption of one of the MBS85 gene's alleles might not have serious effects in KM-102 cells although the impact of such MBS85 gene disruption should be thoroughly investigated. The integration of a transgene into a predetermined site in the human genome, which minimizes the risk caused by insertion mutagenesis, is particularly important for the application of gene transfer to proliferating cells such as stem cells. Our strategy for delivering a therapeutic gene into the AAVS1 locus using the AAV components ITR and Rep is an attractive approach for gene therapy and regenerative medicine.

CONCLUSION

The AAVS1 site is a safe harbor for transgene insertion although it results in impaired MBS85 expression under some condition.

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Author Contributions

Ajalli Rahim – Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting the article, Final approval of the version to be published
Masashi Urabe – Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting and critical revision of the article, Final approval of the version to be published
Hiroaki Mizukami – Conception and design, Analysis and interpretation of data, Critical revision of the article, Final approval of the version to be published
Akihiro Kume – Conception and design, Analysis and interpretation of data, Critical revision of the article, Final approval of the version to be published
Keiichi Ichimura – Conception and design, Critical revision of the article, Final approval of the version to be published
Keiya Ozawa – Conception and design, Analysis and interpretation of data, Critical revision of the article, Final approval of the version to be published

Guarantor

The corresponding author is the guarantor of submission.

Conflict of Interest

The authors declare that they have no competing interests.

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