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Transgene Integration into the Human AAVS1 Locus Enhances Myosin II-Dependent Contractile Force by Reducing Expression of Myosin Binding Subunit 85

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Abstract

The adeno-associated virus site 1 (AAVS1) locus in the human genome is a strong candidate for gene therapy by insertion of an exogenous gene into the locus. The AAVS1 locus includes the coding region for myosin binding subunit 85 (MBS85). Although the function of MBS85 is not well understood, myosin II-dependent contractile force may be affected by altered expression of MBS85. The effect of altered expression of MBS85 on cellular contractile force should be examined prior to the application of gene therapy. In this study, we show that transgene integration into AAVS1 and consequent reduction of MBS85 expression changes myosin II-dependent cellular contractile force. We established a human fibroblast cell line with exogenous DNA knocked-in to AAVS1 (KI cells) using the CRISPR/Cas9 genome editing system. Western blotting analysis showed that KI cells had significantly reduced MBS85 expression. KI cells also showed greater cellular contractile force than control cells. The increased contractile force was associated with phosphorylation of the myosin II regulatory light chain (MRLC). Transfection of KI cells with an MBS85 expression plasmid restored cellular contractile force and phosphorylation of MRLC to the levels in control cells. These data suggest that transgene integration into the human AAVS1 locus induces an increase in cellular contractile force and thus should be considered as a gene therapy to effect changes in cellular contractile force.
Highlights

· Transgene integration to human AAVS1 locus decreases MBS85 expression.

· Decrease in MBS85 expression increases cellular contractile force.

· MBS85 is involved in myosin regulatory light chain phosphorylation.

Keywords

myosin binding subunit 85; adeno-associated virus site 1; cellular contractile force; myosin regulatory light chain phosphorylation; genome editing
1. **Introduction**

Recently developed genome editing techniques have opened the door for gene therapy. One of the anticipated types of gene therapy is gene replacement therapy, in which exogenous DNA (a transgene) is transferred to the human genome to replace a defective gene with a functional gene. Transgene delivery has traditionally been accomplished using viral vectors. However, a potential problem with this method is random insertion of the exogenous DNA into the host cell genome. In the worst case, transgene insertion into an inappropriate genomic site could result in malignant transformation. Therefore, genome editing techniques have been used to insert transgenes into defined genomic sites. Although no genomic site has been fully validated as a genomic “safe harbor”, some candidates from the human genome have been proposed, including the adeno-associated virus site 1 (AAVS1) locus, the chemokine (CC motif) receptor 5 (CCR5) locus, and the human orthologue of the mouse ROSA26 locus [1]. Among these candidates, the AAVS1 locus stands out for the ubiquitous, constitutive, and high-level expression of the integrated transgene [2]. Mali et al. proposed a revolutionary method for the insertion of transgenes into the AAVS1 locus with the development of CRISPR/Cas system [3]. The next step is to carefully consider the effect of transgene insertion into AAVS1 locus as well as the availability of the locus.

The safety of transgene insertion into the AAVS1 locus is still being investigated. The
locus encodes a protein named myosin binding subunit 85 (MBS85). Transgene integration into the AAVSI locus reduces MBS85 expression at the mRNA level, but this reduction can be avoided by modifying the transgene [4]. Pluripotent stem cells with exogenous DNA inserted into the AAVSI locus show normal phenotype and differentiation potential [5, 6]. However, misgivings have arisen because the function of MBS85 is not yet completely understood [1]. MBS85 is a member of the myosin phosphatase targeting protein (MYPT) family (see the review by Grassie et al. [7] and the references therein). One well-investigated protein in the MYPT family is MYPT1. Some of the domains of MBS85 are similar to those of MYPT1. MYPT1 forms a protein complex that dephosphorylates the myosin regulatory light chain (MRLC). Since phosphorylation of MRLC enhances cellular contractile force [8], reduction of MBS85 expression may increase cellular contractile force by increasing MRLC phosphorylation. Cellular contractile force is the sole contributor to the mechanical function of organs and tissues such as the heart and skeletal muscle. The development of therapies to regenerate and replace these organs and tissues would be facilitated by investigating how cellular contractile force is affected by transgene integration into AAVSI.

The purpose of this study was to determine whether transgene integration into the AAVSI locus and the consequent reduction of MBS85 expression would increase cellular contractile force. We inserted the green fluorescent protein (GFP) gene into the AAVSI locus in
human fibroblasts (knock-in cells). Cellular contractile force was compared between the knock-in cells and control cells. The relationship between MBS85 expression and MRLC phosphorylation was examined by western blotting. We discuss the use of gene editing from the viewpoint of cellular contractile force.
2. Materials and methods

2.1. Cells culture and genome editing

The human fibroblast cell line (MRC-5 SV1 TG1) was purchased from the RIKEN Cell Bank (Tsukuba, Japan) and cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM; D6046, Sigma, St. Louis, MO, USA) supplemented with 10% bovine serum and 1% antibiotics (A5955, Life Technologies Corporation, Carlsbad, CA, USA) in 5% CO₂ at 37 °C. Transfection of plasmids was performed using Lipofectamine 2000 (Life Technologies Corporation).

Insertion of GFP gene into the AAVSI locus (i.e., knock-in) was performed as previously described [3]. Three plasmids were obtained from Addgene (Cambridge, MA, USA): a Cas9 expression vector, pSpCas9(BB)-2A-Puro (PX459); a guide RNA expression vector, phH1-gRNA; and a donor plasmid, AAV-CAGGS-EGFP. To create double-strand breaks at the AAVSI locus, we chose two target sequences (T1 and T2) as previously described [3]. Two weeks after transfection, cells were subcloned in a 96-well plate. To confirm that the intended gene editing had been accomplished, we subjected genomic DNA from GFP-positive clones to PCR using primers HR_AAVSI-F and HR_Puro-R [3]. Representative results are shown in Supplemental Figure 1. To determine whether the transgene integrations were mono- or biallelic, we subjected genomic DNA from knock-in clones and wild-type cells to PCR using primers
HR_AAVS1-F and AAVS1-R. The PCR was repeatedly successful for the wild-type cells. In contrast, we failed to obtain a PCR product from the knock-in subclone used in this research. Therefore we concluded that the knock-in cells used in this research harbored a biallelic integration.

MBS85 knock-out cells were established similarly to the knock-in cells. A 23 base pair target sequence (5’– GGCGGCTGGCCGGGGGCGGG – 3’) was chosen from the first exon of MBS85, and guide RNA for the target sequence was inserted into phH1-gRNA. The guide RNA and Cas9 expression plasmids were cotransfected. Two weeks after transfection, cells were subcloned in a 96-well plate. MBS85-knock-out clones were confirmed by DNA sequencing (Supplemental Figure 2) and western blotting (Figure 1).

An MBS85 expression plasmid (pECE-M2-PPP1R12C wt, Addgene) was used for rescue experiments with the knock-in cells.

2.2. Western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were performed as previously described [8]. Information on the primary antibodies used in this study is presented in Supplemental Table 2.
2.3. Collagen gel compaction assay and widefield microscopy

To compare cellular contractile force between test and control cells, we used a collagen gel compaction assay [9]. In this assay, cells are cultured in a free-floating collagen gel and the gel is compacted by cellular contractile force. This method is simple and enables evaluation of cellular force.

We used porcine-derived type I collagen (Cellmatrix-IP, Nitta Gelatin Inc., Osaka, Japan) and carried out collagen gelation according to the manufacturer’s instructions. The final concentrations of collagen and cells were 1.6 mg/mL and $10^6$ cells/mL, respectively. One hundred microliters of collagen cell mixture was transferred to a well in a 96-well plate and then gelation was performed for 30 min at 37 °C. After gelation, each gel was transferred to a dish filled with DMEM. The bottom plane of the gel was observed using widefield light microscopy with a 2× objective lens (Ti-E and CFI-Plan Apo λ series, NIKON, Tokyo, Japan), and images were captured with an sCMOS camera (Zyla 4.2, Andor Technology Ltd., UK). The bottom area of the gel was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The change in gel diameter during 24 h of cell culture was calculated as follows:

$$Normalized\ diameter = \sqrt{\frac{S_{\text{compacted}}}{S_{\text{initial}}}}$$

where $S_{\text{initial}}$ denotes the bottom area of the gel immediately after gelation and $S_{\text{compacted}}$ denotes the bottom area of the gel after 24 h of cultivation.
To determine whether collagen gel compaction was related to phosphorylation of MRLC [10], we treated cells with 20 μM Y-27632 (Y0503, Sigma), a selective inhibitor of Rho-associated coiled-coil forming kinase, and measured gel relaxation.

2.4. Cell growth

To evaluate cell growth, we measured the absorbance of cell suspensions at 600 nm (OD$_{600}$). Cells were cultured on a plastic dish at a concentration of $5 \times 10^2 \pm 2 \times 10^1$ cells/mm$^2$. After cultivation, trypsinized cells were washed with phosphate-buffered saline and the OD$_{600}$ of the cell suspension was measured with an absorption spectrometer (SmartSpec Plus, Bio-Rad, Hercules, CA, USA).

2.5. Statistical analysis

Statistical analysis was carried out using Microsoft Excel. In comparisons of two sets of data, equality of the variances was tested using the $F$-test. The $\alpha$ level was set at 0.05. Since the variances were found to be equal, Student’s $t$-test was carried out. Confidence intervals are stated at the 95% confidence level.

Cell growth analysis, western blotting, and the collagen gel compaction assay were performed at least four times to assess repeatability.
3. Results and discussion

3.1. Deletion of the MBS85 gene and knock-in to AAVS1 do not alter the phenotype of human fibroblasts

First, we examined whether the cellular features of MBS85 knock-out cells and GFP knock-in cells differed from those of wild-type cells. Figure 1(A) shows typical cell morphologies. MBS85 knock-out cells and GFP knock-in cells were cultured for 6 months, and GFP knock-in cells continued to express GFP. There were no noticeable differences in cellular shape among MBS85 knock-out, GFP knock-in, and wild-type cells. Differences in cell growth rate among the three cell lines were evaluated by measuring the OD_{600} of cell suspensions (Fig. 1(B)). The OD_{600} values were normalized to the absorbance at day zero. No clear differences in growth rate were observed among MBS85 knock-out, GFP knock-in and wild-type cells. These data suggest that MBS85 gene knock-out and GFP knock-in to the AAVS1 locus do not alter the cellular fundamental cellular features of human fibroblasts.

3.2. Deletion of the MBS85 gene and knock-in to AAVS1 enhance cellular contractile force

Next, we assessed whether deletion of the MBS85 gene and knock-in to AAVS1 affected cellular contractile force. Prior to evaluating cellular contractile force, we assessed MBS85 protein expression by western blotting (Fig. 1(C)). As expected, no visible signal was detected
from MBS85 knock-out cells. However, a faint band was observed in the lane corresponding to GFP knock-in cells. Densitometrical analysis showed that MBS85 expression in GFP knock-in cells less than half that in wild-type cells. The cellular contractile force was then compared among the three cell lines (Fig. 2). Cells were embedded in the free-floating collagen gel, and the degree of gel compaction due to cellular contractile force was measured. Representative images of cells embedded in the collagen gel after 24 h of cultivation are shown in Figure 2(A). The bottom area of the gels was measured manually, and changes in the diameter were compared (Fig. 2(B, C)). The degree of gel compaction was significantly greater with MBS85 knock-out and GFP knock-in cells than with wild type cells. To determine whether collagen gel compaction was due to cellular contractile force, we treated the compacted gels with Y-27632, which inhibits myosin II-dependent contractile force. After 1 h of incubation, the bottom area of each gel was measured. The diameter increased by 5–10% for each cell line. These data suggest that deletion of the MBS85 gene or reduction of MBS85 expression by knock-in of exogenous DNA to AAVS1 increases cellular contractile force.

3.3. Reduction of MBS85 expression is associated with myosin regulatory light chain phosphorylation

To explore the molecular mechanism by which cellular contractile force was altered, we
assessed the amount of phosphorylated MRLC by western blotting (Fig. 3(A)). MRLC exists in de-phosphorylated, mono-phosphorylated, and diphosphorylated states [11]. Anti-phospho-MRLC (Ser19) recognizes both mono- and diphosphorylated MRLC, while anti-phospho-MRLC (Thr18/Ser19) recognizes only diphosphorylated MRLC. The amount of diphosphorylated MRLC in GFP knock-in cells was significantly greater than that in wild-type cells, although there were no differences in total MRLC or the sum of mono- and diphosphorylated MRLCs.

To confirm the effect of reduced MBS85 expression on the MRLC phosphorylation and cellular contractile force, we transfected GFP knock-in cells with an MBS85 expression plasmid. Genome editing techniques can cause unwanted off-target effects [12]. Such effects could alter MRLC phosphorylation and cellular contractile force in an MBS85-independent manner. To exclude this possibility, we introduced MBS85 into GFP knock-in cells and evaluated the amount of phosphorylated MRLC and the cellular contractile force (Fig. 3). The introduction of MBS85 clearly rescued the changes in MRLC phosphorylation and cellular contractile force. These results suggest that reduction of MBS85 expression induces phosphorylation of MRLC and enhances cellular contractile force.

In this study, we showed that MBS85 knock-out and reduction of MBS85 expression by transgene knock-in to the human AAVS1 locus increased cellular contractile force by increasing
phosphorylation of MRLC (Fig. 4). We generated GFP knock-in cells and used PCR to confirm biallelic integration of the GFP gene into the AAVSI locus. Western blotting analysis showed that MBS85 protein expression was significantly reduced by insertion of the transgene into the AAVSI locus. The reduction of MBS85 expression induced an increase in MRLC phosphorylation and cellular contractile force. Thus, insertion of exogenous DNA into the human AAVSI locus by genome editing increases cellular contractile force even though fundamental cellular features such as cell morphology and cell growth are maintained. Previous research showed that transgene integration into the human AAVSI locus did not change cell growth rate [13] or pluripotency [2]. Thus, the AAVSI locus is thought to be a strong candidate for gene therapy. However, we should take into account increase in cellular contractile force when we apply a technique or therapy involving in AAVSI locus to the cellular contractile force-related organs such as heart and skeletal muscle. Of course, enhancement of cellular contractile force is not always a priority in the development of gene therapies. Compared with modification of T cells to treat cancer [14], the relevance of cellular contractile force diminishes. Nevertheless, since gene therapy may be applied to organs where cellular contractile force plays important role, other loci that can be targeted without affecting this cellular function should be investigated. The chemokine (CC motif) receptor 5 (CCR5) gene [15] and the human ROSA26 locus [16] are likely candidates. The next step in our research is to elucidate the effect of
knock-in to these loci on cellular contractile force.
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References


Figure legends

Figure 1.

Effect of MBS85 knock-out and knock-in to AAVS1 on the cellular morphology and growth rate of human fibroblasts. (A) Phase contrast and fluorescent images of wild-type cells (WT-phase), MBS85 knock-out cells (KO-phase), and green fluorescent protein (GFP) knock-in cells (KI-phase and KI-GFP). (B) Cellular growth rates were analyzed. Error bars denote standard error. (C) MBS85 expression was assessed by western blotting.

Figure 2.

Differences in cellular contractile force among cells lines were evaluated using a collagen gel compaction assay. Wild-type cells (WT), MBS85 knock-out cells (KO), and GFP knock-in cells (KI) were embedded in free-floating collagen gels. (A) Representative morphologies of cells embedded in collagen gel after 24 h of cultivation. (B) Changes in the collagen gel were evaluated by measuring each gel area. (C) Changes in gel diameter were statistically analyzed. Error bars denote standard error. An asterisk indicates a significant difference ($p < 0.05$).
Figure 3.

Reduction of MBS85 expression is associated with myosin regulatory light chain (MRLC) phosphorylation. (A) Western blotting analysis of wild-type cells (WT), GFP knock-in cells overexpressing MBS85 (KI + OE), and GFP knock-in cells (KI). (B) The change in diameter of the collagen gel embedded with GFP knock-in cells overexpressing MBS85 was determined. The data for wild-type and GFP knock-in cells are the same as shown in Figure 2. Error bars denote standard error.

Figure 4.

Summary of this research. Reduction of MBS85 expression by integration of exogenous DNA into the human AAAS1 locus increases cellular contractile force by increasing phosphorylation of MRLC.
Figure 1.
Figure 2.
Figure 3.
Figure 4.