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Evaluation of the correlation between nuclear localization levels and genome editing efficiencies of Cas12a fused with nuclear localization signals

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ABSTRACT

Genome editing technology using the CRISPR-Cas system is attracting much attention not only as a promising experimental tool for analysis of genome functions, but also as a novel therapeutic approach for genetic disorders. Among the various types of Cas proteins, Cas12a is expected to be a promising gene editing tool due to its unique properties, including low off-target effects. As Cas proteins are of prokaryotic origin, they need to be fused with appropriate localization signals to perform their function in eukaryotic cells. Cas12a proteins fused with a nuclear localization signal (NLS) have been developed so far, but the relation between the nuclear localization activity and the genome editing efficiency has not been fully elucidated. Here, utilizing two Cas12a orthologs, AsCas12a and LbCas12a, with various number of NLSs derived from various origins, we revealed that the improved nuclear localization resulted in increased genome editing efficiencies when expressed using adenovirus (Ad) vector in cultured cells. However, when they were expressed in mouse liver, the improvement of the nuclear localization activity was not necessarily required to achieve the maximum genome editing efficiency four weeks after Ad vector administration. These data indicated that the optimized NLS modification of Cas12a proteins *in vitro* situations differed from that *in vivo*.

Introduction

The clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated 9 (Cas9) system is a genome editing technology based on the acquired immunity mechanism of bacteria and archaea.[1,2] Cas9 induces double-strand breaks (DSBs) in target genes, enabling knockout and knock-in of targeted genes in the genome. Gene therapy using the CRISPR-Cas9 system is expected to become a fundamental treatment for congenital genetic disorders. Clinical trials using CRISPR-Cas9 system-mediated genome editing have already been conducted for several diseases. For example, several clinical trials using genome editing technology have been performed for the treatment of Leber congenital amaurosis (LCA) and sickle cell disease (SCD).[3,4] Moreover, medicines for SCD utilizing CRISPR technology have already been approved for use in the UK and the US.[5] However, the CRISPR-Cas9 system also has several problems, including insufficient genome editing efficiencies and off-target effects.

Cas12a (also known as Cpf1) is derived from prokaryotes, has attracted much attention as an alternative Cas protein because it possesses several properties distinct from those of Cas9.[6] First, Cas12a recognizes T-rich PAMs (protospacer adjacent motif), whereas Cas9 recognizes the G-rich PAMs. Therefore, Cas12a is able to target genome regions which Cas9 is not able to cleave.[6,7] Second, when Cas12 cleaves a target DNA it leaves a 5′-overhang at the cleavage site, while Cas9 leaves a blunt end at the cleavage site.[6] Third, Cas12a exhibits lower off-target effects than Cas9.[8,9] Fourth, Cas12a cuts pre-

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mature long precursor RNAs, producing crisprRNA (crRNAs).[10–12] These properties of Cas12a led us to consider that Cas12a might serve as an alternative tool for gene therapy of genetic disorders.

Since the CRISPR-Cas system is based on prokaryotic immune mechanisms, the original form of Cas proteins is not actively transported to the nucleus. In addition, the molecular weights of Cas9 and Cas12a are approximately 150 kDa, making these proteins too large to easily pass through the nuclear pore. Cas12a possesses nuclear export signal (NES)-like amino acid sequences.[13] Efficient and active transport of Cas proteins to the nucleus is essential to achieve efficient genome editing in eukaryotic cells. For this purpose, the Cas proteins used in eukaryotic cells are usually endowed with nuclear localization signals (NLSs). Several types of NLS-containing Cas9 proteins have already been developed in previous studies.[14,15] The most commonly used Cas9 protein is fused with the SV40 NLS at the N-terminal and the nucleoplasmin NLS at the C-terminal.[16] However, NLS modification of Cas12a and the correlation between the nuclear localization levels and genome editing efficiencies of Cas12a have not been fully evaluated. Optimization of the NLS-mediated active transport of Cas12a will thus be highly important for efficient genome editing.

In this study, we first examined the nuclear localization levels and genome editing efficiencies of Cas12a fusion proteins containing different types and numbers of NLSs in cultured cells. Next, we produced Cas12a-expressing adenovirus (Ad) vectors and evaluated the nuclear localization levels and genome editing efficiencies of Cas12a in the mouse liver after intravenous administration. This study contributes to genome editing therapeutics by providing important information for efficient Cas12a-mediated genome editing.

Materials and methods

Ad vectors

Ad vectors plasmids were prepared by an improved *in vitro* ligation method.[17,18] Briefly, shuttle plasmids containing gRNA and Cas12a proteins expression cassettes flanked with I-*Ceu*I and PI-*Sce*I sites were digested with I-*Ceu*I and PI-*Sce*I and cloned into pAdHM4-E4-122aT.[19] Ad vector plasmids were linearized by digestion with *Pac*I, and then were transfected into HEK293 cells using Lipofectamine 2000 (Thermo Fisher Scientific, San Jose, CA). The Ad vectors were then propagated in HEK293 cells, purified by two rounds of cesium chloride gradient ultracentrifugation, dialyzed, and stored at −80 °C. The virus particle (VP) titers were determined by spectrophotometric method.[20] The infectious units (IFU) were determined using an Adeno-X Rapid Titer Kit (Clontech Laboratories; Mountain view, CA).

Mouse

C57BL/6 J mice (4 weeks old) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Ad vectors were intravenously administered via tail vein at the indicated doses. All animal experimental procedures used in this study were performed in accordance with the institutional guidelines for animal experiments at Osaka University.

Immunostaining of Cas12a proteins

Huh-7 cells were transduced with Ad vectors as described above. Cells were fixed with 4 % paraformaldehyde after a 48-h incubation. HA-tagged Cas12a proteins were detected with mouse anti-HA.11 epitope tag antibody and Alexa Fluor 488-labeled donkey anti-mouse IgG (Thermo Fisher Scientific). For immunostaining of mouse liver sections, mouse livers were harvested at the indicated day after Ad vector administration and were fixed with 4 % paraformaldehyde. Subsequently, the organs were embedded in optimal cutting temperature (OCT) compound (Sakura Finetech Japan, Tokyo, Japan) and frozen at a temperature of −80℃. Frozen samples were sectioned at a thickness of 10 μm and incubated in PBS containing 5 % Immuno-Block (KAC Co., Ltd, Kyoto, Japan) and 0.1 % Triton X-100 for 1hr. Cas12a proteins were detected with anti-HA rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA) and Alexa Fluor 488-labeled donkey anti-rabbit IgG (Thermo Fisher Scientific). The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence images were captured using a BZ-X800 microscope (Keyence, Osaka, Japan).

T7 endonuclease I (T7E1) assay

Huh-7 cells were transduced with Ad vectors as described above. Total cellular DNA was recovered from the cells after a 48hr incubation. Indels were assessed by a T7E1 assay as previously described.[21,22] Briefly, target region of the AAVS1 region in the genome was amplified by PCR using the primer set for AAVS1 (Table 1). PCR was performed using PrimeSTAR Max DNA polymerase (TaKaRa Bio, Otsu, Japan). The resulting PCR amplicons (100 ng) were subjected to heat-denature and re-annealing followed by digestion with 50–80 units of T7E1 enzyme (New England Biolabs, Ipswich, MA) for 30 min at 37 °C. The cleaved fragments derived from mismatch-pairings were resolved in 10 % PAGE. Images were taken using FAS5 (NIPPON Genetics, Tokyo, Japan). The signal intensity of each band was quantitated using ImageJ software. Estimation of indels was calculated following the formula: % indels = 100x(1-(1-cleaved band intensity/total band intensities)1/2).

Statistical analysis

Statistical significance was determined using one-way ANOVA analysis followed by Bonferroni's multiple comparison test. Data are presented as means ± standard deviations (S.D) or standard errors (S.E).

Results

Cas12a fusion proteins containing different types and numbers of NLSs and their nuclear localization levels in cultured cells

In order to evaluate the effects of addition of NLSs on the nuclear translocation and genome editing efficiencies of Cas12a, three types of Cas12a fusion proteins composed of AsCas12a (derived from Acidaminococcus BV3L6) and LbCas12a (derived from Lachnospiraceae bacterium ND2006) fused with different types and numbers of NLSs were overexpressed in cultured human cells by plasmid transfection. The type and number of NLSs was determined on the basis of previous reports. AsCas12a- and LbCas12a-1xNLS possessed one copy of nucleoplasmin NLS at the C-terminus.[6] AsCas12a- and LbCas12a-2xNLS possessed one copy of nucleoplasmin NLS and the SV40 NLS at the C-terminus.[23] AsCas12a- and LbCas12a-6xNLS possessed six copies of c-Myc NLS at the C-terminus (Fig. 1A).[13] Each plasmid contained the expression unit for gRNA targeting the AAVS1 locus, a human safe harbor region. Western blot analysis demonstrated that AsCas12-1xNLS, -2xNLS, and -6xNLS were efficiently expressed at similar levels following transfection. LbCas12a-1xNLS, -2xNLS, and -6xNLS showed similar results (Fig. 2A). When the cellular localization was examined, both AsCas12a-1xNLS and LbCas12a-1xNLS were mainly localized in the cytoplasm, with a small amount also present in the nucleus. In contrast, AsCas12a or LbCas12a either with -2xNLS or -6xNLS were mainly localized in the nucleus, with AsCas12a-6xNLS and LbCas12a-6xNLS showing particularly strong nuclear

Table 1
PCR Primer List.

No.	Oligo DNA and Primer	Sequence(5′→3′)
1	AAVS1 gRNA Fw	AGAT**GCCACCTCTCCATCCTCTTGCTTT**
2	AAVS1 gRNA Rv	AAAAAAAGCAAGAGGATGGAGAGGTGGC
3	Rosa26 gRNA Fw	AGAT**GCCAGCCAGTGGTGGTGCTTGCCT**
4	Rosa26 gRNA Rv	AAAAAGGCAAGCACCACCACTGGCTGGC
5	Cas12a Fw	GTGGCACCTTCCAGGGTCAA
6	Cas12a Rv	CTGTGCCTTCTAGTTGCCAGC
7	human GAPDH Fw	GGTGGTCTCCTCTGACTTCAAC
8	human GAPDH Rv	GTGGTCGTTGAGGGCAATG
9	mouse GAPDH Fw	CAATGTGTCCGTCGTGGATCT
10	mouse GAPDH Rv	GTCCTCAGTGTAGCCCAAGATG
11	AAVS1 T7E1 primer Fw	GGACAGATAAAAGTACCCAGAACC
12	AAVS1 T7E1 primer Rv	CTGTGCCATCTCTCGTTTCTTAG
13	Rosa26 T7E1 primer Fw	CAGAAAGGTAGACGGATTTAGCC
14	Rosa26 T7E1 primer Rv	CATAACTGCAGACTTGTGGGATAC

Sequences in bold are the Cas12a target sequences of AAVS1 and Rosa26.

localization (Fig. 2B). These data indicated that nuclear localization of Cas12a was significantly enhanced by addition of multiple NLSs.

The genome editing abilities of various NLS-containing Cas12a proteins co-relates to the nuclear localization activities

Next, we evaluated the genome editing efficiencies of the Cas12a fusion proteins containing the NLSs. In order to efficiently overexpress Cas12a, especially *in vivo*, Ad vectors expressing Cas12a were produced. Since Cas12a expression in the Ad vector-packaging cells often inhibited the amplification of Ad vectors,[22] a liver-specific AHA promoter that, does not work in HEK293 cells but efficiently drives transcription in the hepatocytes was used for Cas12a expression.[24] All the Ad vectors were recovered in high titers (Fig. 1B) (Table 2).

In order to examine the genome editing efficiencies of the Cas12a-expressing Ad vectors, the Ad vectors were administrated

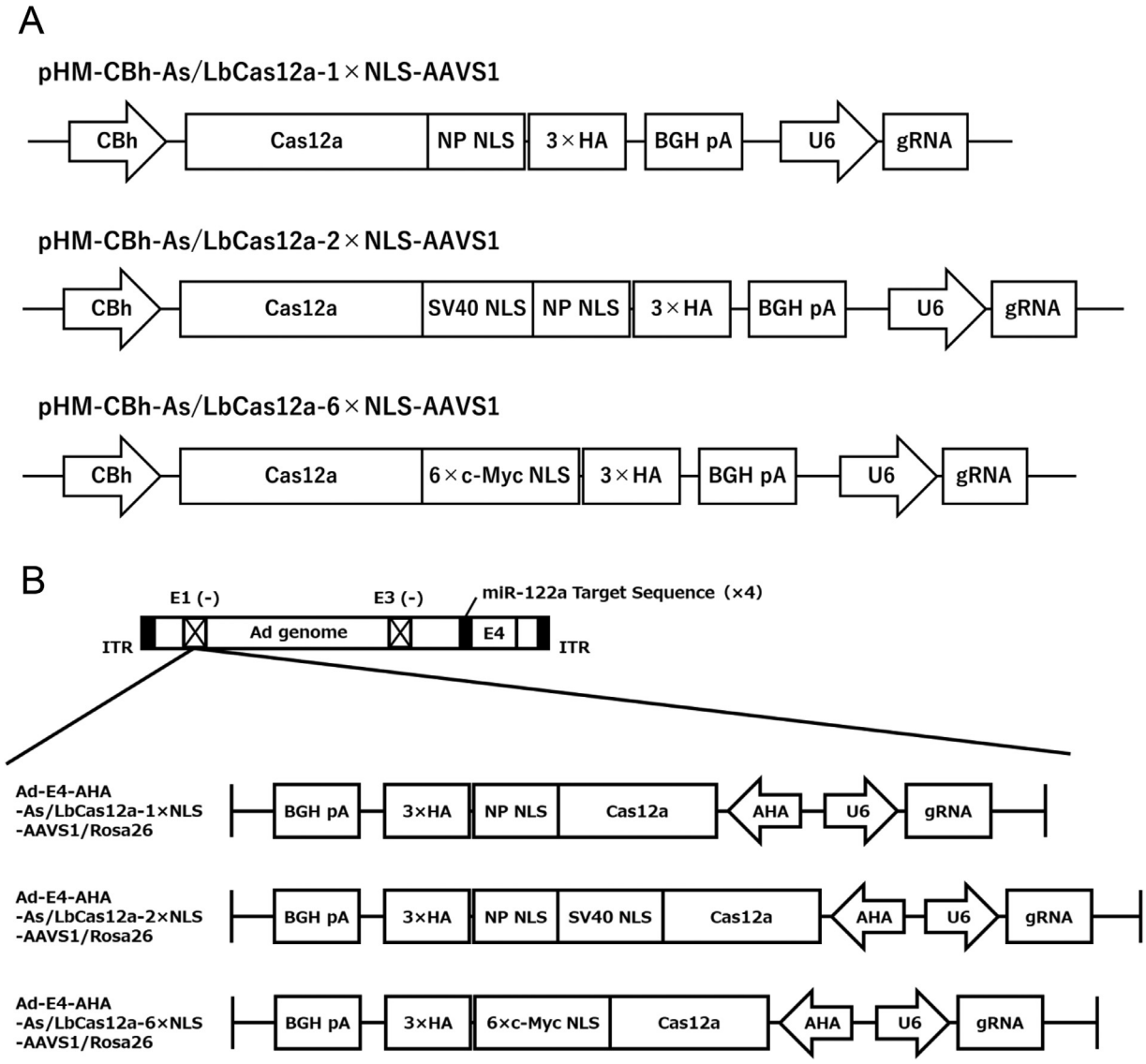

Fig. 1. A schematic structure of plasmids and Ad vectors co-expressing Cas12a fused with various NLSs and gRNA.(A and B) Transcription of Cas12a and gRNA targeting the AAVS1 locus were driven by the CBh promoter and U6 promoter, respectively. CBh, chicken β-actin hybrid promoter; NP NLS, nucleoplasmin NLS; SV40 NLS, simian virus 40 large T antigen NLS; 6x c-Myc NLS, 6 copies of c-Myc NLS; 3xHA, 3 copies of HA-tag; BGH pA, bovine growth hormone (BGH) poly A signal; AHA, a liver-specific apolipoprotein E enhancer-hepatocyte control region- and a human alpha1-antitrypsin promoter; ITR, inverted terminal repeat; gRNA, a guide RNA sequence targeting for AAVS1 or Rosa26 locus.

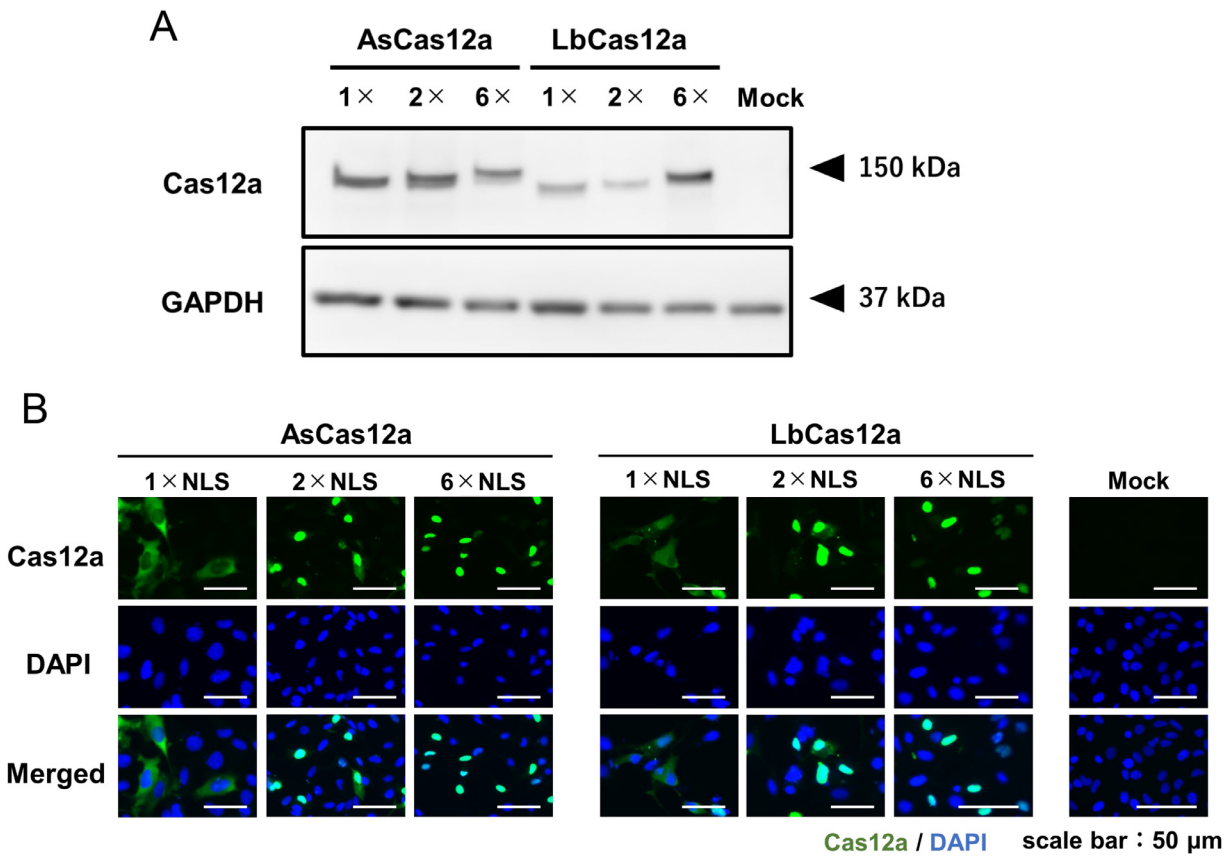

Fig. 2. Nuclear localization of AsCas12a and LbCas12 fused with different types and numbers of NLSs in cultured cells.(A) Western blotting images of AsCas12a and LbCas12a expression in HeLa cells following transfection. HeLa cells were transfected with the plasmids shown in Fig. 1A, followed by western blotting analysis at 48 h after transfection. HA-tagged AsCas12a and LbCas12a were detected by using anti-HA antibody. Representative images were shown from three independent experiments. Immunofluorescent images of AsCas12 and LbCas12a following plasmid transfection in HeLa cells. (B) HeLa cells were stained with anti-HA antibody and Alexa fluor 488-labeled secondary antibody at 48 h after plasmid transfection. Cell nuclei were stained with DAPI. Representative images were shown from three independent experiments. Scale bars indicate 50 μm. Mock; non-transfected group.

to Huh-7 cells, a human hepatocellular carcinoma cell line. No statistically significant difference of the mRNA levels was observed in the cells transduced by each Ad vector expressing either AsCas12a or LbCas12a, even when the number of NLSs was different (Fig. 3A). However, the Cas12a protein expression levels were slightly different (Fig. 3B). These results indicated that fusion with NLSs affected the translation and/or stabilities of Cas12a proteins in the cells.

Immunostaining analysis of Cas12a proteins in the cells demonstrated that both AsCas12a-1xNLS and LbCas12a-1xNLS were mainly localized to the cytoplasm, with little nuclear localization observed (Fig. 3C). On the other hand, AsCas12a-2xNLS and LbCas12a-2xNLS were mainly localized to the nucleus, although small amounts of Cas12a proteins were found in the cytoplasm. AsCas12a-6xNLS and LbCas12a-6xNLS were hardly found in the cytoplasm and almost entirely localized in the nucleus. These data indicated that nuclear localization of Cas12a was significantly enhanced by addition of multiple NLSs, when Cas12a proteins were expressed by an Ad vector, as was the case with Cas12a-expressing plasmids.

Table 2
List of Ad vector titers.

Ad vector	VP/ml($\times 10^{12}$)	IFU/ml($\times 10^{11}$)	ratio
Ad-E4-AHA-AsCas12a-1xNLS-AAVS1	0.349	0.304	11.491
Ad-E4-AHA-AsCas12a-2xNLS-AAVS1	0.600	0.388	15.471
Ad-E4-AHA-AsCas12a-6xNLS-AAVS1	1.172	1.346	8.701
Ad-E4-AHA-LbCas12a-1xNLS-AAVS1	0.536	0.580	9.246
Ad-E4-AHA-LbCas12a-2xNLS-AAVS1	0.941	1.327	7.090
Ad-E4-AHA-LbCas12a-6xNLS-AAVS1	0.569	0.458	12.443
Ad-E4-AHA-AsCas12a-1xNLS-Rosa26	1.001	1.426	7.022
Ad-E4-AHA-AsCas12a-2xNLS-Rosa26	3.058	5.125	5.967
Ad-E4-AHA-AsCas12a-6xNLS-Rosa26	1.906	1.663	11.458
Ad-E4-AHA-LbCas12a-1xNLS-Rosa26	1.128	1.337	8.436
Ad-E4-AHA-LbCas12a-2xNLS-Rosa26	1.392	1.297	10.729
Ad-E4-AHA-LbCas12a-6xNLS-Rosa26	1.551	1.921	8.076

Next, the effects of addition of NLSs on the Cas12a-mediated genome editing efficiencies were evaluated in Huh-7 cells by the estimation of indel frequencies by T7E1 assay. AsCas12a-1xNLS and LbCas12a-1xNLS mediated genome edition at indel frequencies of 2.0 % and 4.4 %, whereas AsCas12a-2xNLS and LbCas12a-2xNLS upregulated genome edition to 29.4 % and 26.6 %, respectively (Fig. 3D). Furthermore, LbCas12a-6xNLS mediated an indel frequency of 51.5 %. On the other hand, despite its efficient nuclear localization, AsCas12a-6xNLS mediated an indel frequency of 14.6 %, which was lower than that for AsCas12a-2xNLS. These data indicated that although addition of multiple NLSs to Cas12a proteins significantly improved the nuclear localization, the nuclear localization levels of Cas12a were not always correlated with the genome editing efficiencies of Cas12a in cultured cells.

In vivo *genome editing efficiencies of Ad vectors expressing Cas12a proteins fused with NLSs*

In order to evaluate the *in vivo* genome editing efficiencies by Ad vector-mediated overexpression of Cas12a proteins, Cas12a-expressing Ad vectors and a Rosa26 locus-targeted gRNA-expressing Ad vector were intravenously co-administered to mice (Fig. 4A). It is well

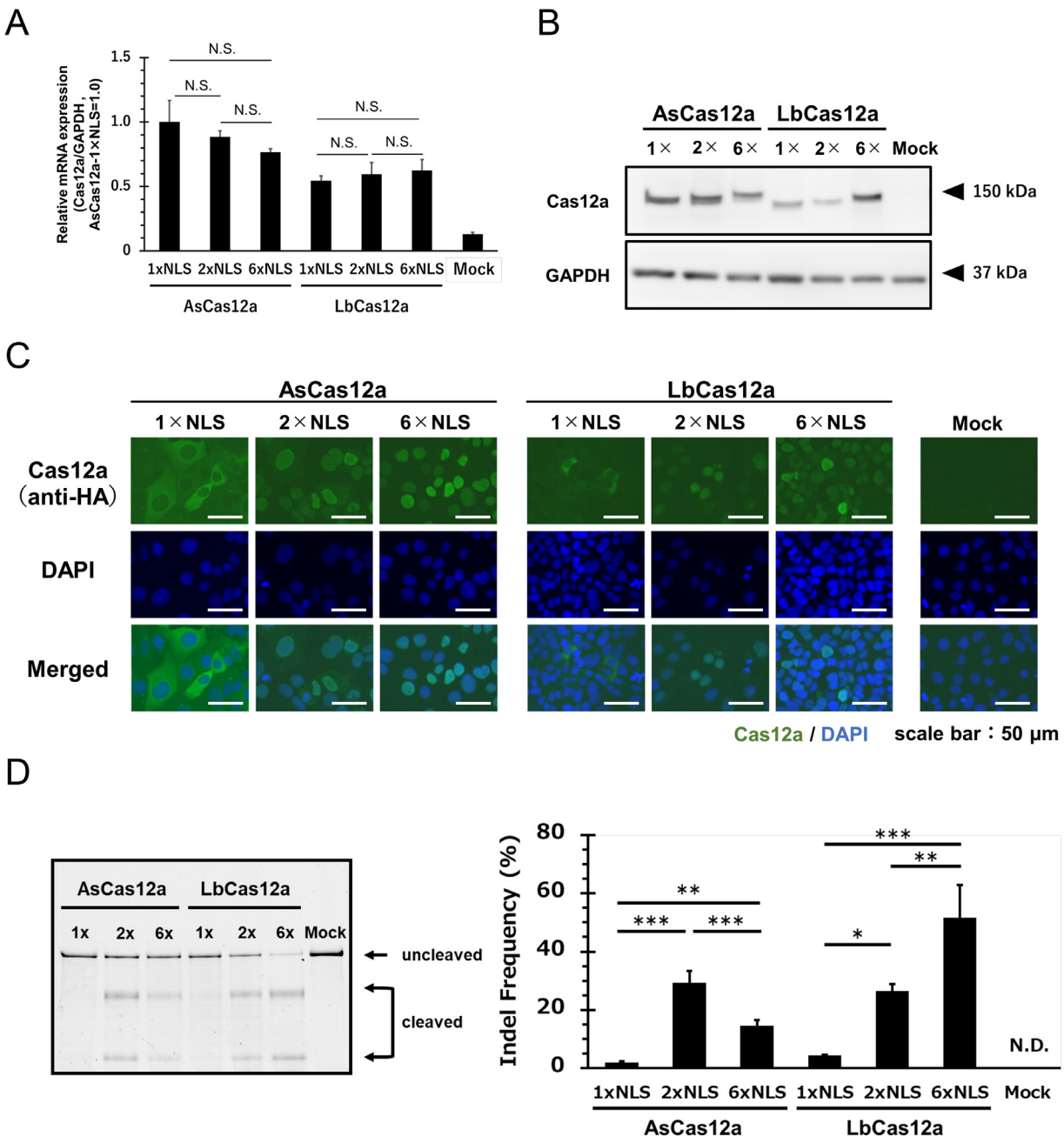

Fig. 3. Functional evaluation of Ad vectors carrying CRISPR-Cas12a with various NLSs.(A) Real-time RT-PCR analysis of mRNA levels of Cas12a genes containing various NLSs in Huh-7 cells at 48 h after transduction with Ad vectors. The data are normalized with GAPDH expression and presented as mean ±S.D. (n = 3 of biological replicates). The value of AsCas12a-1xNLS is set to 1. (B) Western blot analysis of Cas12a-1xNLS, -2xNLS and -6xNLS protein expressions in Huh-7 cells at 48 h after transduction with Ad vectors. (C) Immunostaining of Cas12a-1xNLS, -2xNLS and -6xNLS proteins in Huh-7 cells at 48 h after transduction with Ad vectors. Cell nuclei were stained with DAPI. Scale bars indicate 50 μm. (D) Genome editing activities of Cas12a-1xNLS, -2xNLS and -6xNLS expressing Ad vectors in Huh-7 cells. Genomic DNAs were isolated at 48 h after transduction. indels were analyzed by a T7E1 assay. Cleaved and uncleaved PCR products were quantified using Image J software. The data are presented as mean ±S.D. (n = 3 of biological replicates) One-way ANOVA followed by Bonferroni's post-hoc comparisons tests were performed for statistical analyses. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; N.S., not significant; N.D., not detected. Mock; Ad vector untreated group.

known that intravenous administration of an Ad vector results in efficient transgene expression in the liver.[25] The mouse Rosa26 locus is a safe harbor region in the mouse genome. The expression levels of AsCas12a and LbCas12a fused with different types and numbers of NLSs in the liver were slightly different (Fig. 4B), although the mRNA levels of AsCas12a-1xNLS, -2xNLS, and -6xNLS and LbCas12-1xNLS, -2xNLS, and -6xNLS were similar, both at 1 week and at 4 weeks after administration (Fig. 4C).

In order to examine the intracellular localization of the Cas12a proteins containing the NLSs in the liver, immunofluorescence staining of Cas12a was performed (Figs. 4D and 4E). Both AsCas12a-1xNLS and LbCas12a-1xNLS were localized mainly in the cytoplasm at both 1 and 4 weeks after administration, whereas AsCas12a-2xNLS and LbCas12a-2xNLS were significantly migrated in the nucleus. Furthermore, AsCas12a-6xNLS and LbCas12a-6xNLS accumulated in the nucleus. These results indicated that the nuclear localization of Cas12a proteins was significantly improved in the tissues as well as in cultured cells, as the numbers of NLSs were increased.

Next, in order to assess the correlation between the nuclear localization levels of Cas12a proteins and *in vivo* genome editing

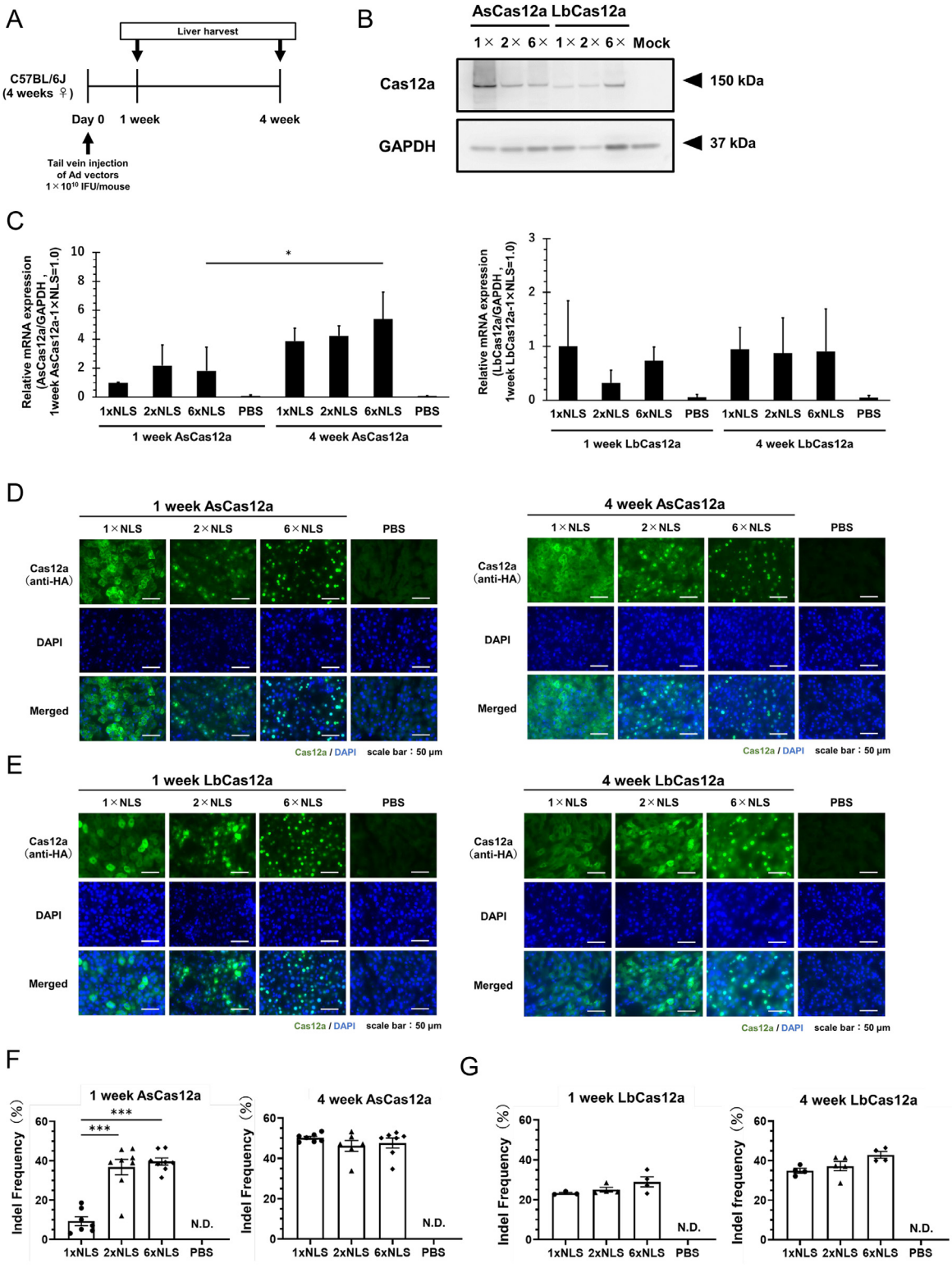

Fig. 4. *in vivo* genome editing by Ad vectors expressing CRISPR-Cas12a fused with various NLSs in the mouse liver.(A) Experimental schedule. Ad vectors were intravenously administered at a dose of 1×10^{10} IFU/mouse and were sacrificed 1 and 4 weeks post injection. (B) Western blot analysis of Cas12a-1xNLS, -2xNLS and -6xNLS protein expression in the livers 1 week post administration of Ad vectors. Mock; Ad vector untreated group. (C) The Cas12a mRNA levels in the livers following intravenous administration of Ad vectors expressing Cas12a. The data are normalized with the data of GAPDH and presented as mean ±S.E. (n = 3 of biological replicates). The value of AsCas12a-1xNLS or LbCas12a-1xNLS is set to 1 in each panel. One-way ANOVA followed by Bonferroni's post-hoc comparisons tests were performed for statistical analysis (*$p < 0.05$.). (D and E) Immunostaining of AsCas12a (D) and LbCas12a (E) protein in the liver following intravenous administration of Ad vectors. Cell nuclei were stained with DAPI. Scale bars indicate 50 μm. (F and G) Genome editing activities of AsCas12a-1xNLS, -2xNLS and -6xNLS expressing Ad vectors in (F) and LbCas12a-1xNLS-, -2xNL- and -6xNLS- expressing Ad vectors in (G) in mouse livers. Genomic DNAs were prepared one and four weeks after transduction were analyzed for mutations by T7E1 assay. Cleaved and uncleaved PCR products were quantified using Image J software. The data are presented as mean ±S.E. (AsCas12a; n = 7 or 8, LbCas12a; n = 3 or 4 of biological replicates) One-way ANOVA followed by Bonferroni's post-hoc comparisons tests were performed for statistical analyses. ***$p < 0.001$; N.D., not detected.

efficiencies, the indel frequencies at the Rosa26 locus were evaluated by a T7E1 assay. The indel frequencies of AsCas12a-1-NLS in the liver were 9.2 % at 1 week post-administration, while those of AsCas12a-2-NLS and AsCas12a-6-NLS were 36.7 % and 39.6 %, respectively (Fig. 4F and Fig. S1A). These data suggested that improvement of the nuclear localization of AsCas12a increased the indel frequencies in the liver, at least at 1 week after administration. On the other hand, all of the Ad vectors expressing AsCas12a proteins showed similar indel frequencies of approximately 50 % at 4 weeks after administration, regardless of the numbers of NLSs attached to Cas12a. These data indicated that at 1 week after administration, the nuclear localization levels of AsCas12a were correlated with the genome editing efficiencies, although comparable indel frequencies were achieved for all the fusion protein of AsCas12a and NLSs at 4 weeks after administration. LbCas12a-1xNLS, -2xNLS, and -6xNLS showed comparable indel frequencies of approximately 20 % at 1 week and 40 % at 4 weeks after administration (Fig. 4G and Fig. S1B), although LbCas12a-2xNLS and -6xNLS were more efficiently accumulated in the nucleus than LbCas12a-1xNLS. These data showed that addition of more than two copies of NLSs to LbCas12a did not appear to improve the genome editing efficiencies in the liver.

Discussion

In this study, we examined the correlation between the nuclear localization and genome editing efficiencies of AsCas12a and LbCas12a containing NLSs in cultured cells and mouse liver. This study demonstrated that the nuclear localization and genome editing efficiencies of LbCas12a increased along with the enhancement of LbCas12a nuclear-localization in the cultured cells. On the other hand, although nuclear localization of AsCas12a-6xNLS was superior to that of AsCas12a-2xNLS, the genome editing efficiencies of AsCas12a-2xNLS were higher than those of AsCas12a-6xNLS. These data suggested that six copies of c-Myc NLS inhibited the cleavage activity of AsCas12a (Fig. 3C and 3D). Since c-Myc NLS has 9 amino acids, a total of 54 amino acids were included at the C-terminus of AsCas12a-6xNLS. Cas12a has a nuclease-active region mainly at the C-terminus.[26] Six copies of c-Myc NLS might cause steric hindrance in AsCas12a-mediated genome cleavage. Furthermore, NLSs are cationic because they contain many basic amino acids such as lysine and arginine.[27,28] Six copies of c-Myc NLS might interact with genomic DNA, resulting in inhibition of the proper interaction between AsCas12a and genomic DNA.

In vivo nuclear localization of Cas12a, similar to the *in vitro* localization, was significantly different depending on the number of NLSs added to Cas12a (Fig. 4D and 4E). However, the genome editing efficiencies in the livers were comparable for AsCas12a-1xNLS, -2xNLS, and -6xNLS at 4 weeks after administration and for LbCas12a-1xNLS, -2xNLS, and -6xNLS at both 1 and 4 weeks after treatment (Fig. 4F and 4G). Since there were approximately $3–4 \times 10^8$ hepatocytes in the mouse liver and >90 % of the injected doses of an Ad vector accumulated in the liver in this study, the hepatocytes were transduced with the Ad vectors at an MOI of approximately 30 when 1 x 10^{10} IFU of Ad vectors were intravenously administered, suggesting that Cas12a was expressed in almost all of the hepatocytes. Actually, immunostaining analysis of liver sections demonstrated that almost all the hepatocytes expressed Cas12a after intravenous administration of Cas12a-expressing Ad vectors (Fig. 4D and 4E). It is considered that sufficient amounts of LbCas12a were expressed and transported into the nucleus for efficient genome editing even when one copy of NLS was attached to LbCas12a. As for AsCas12a, the genome editing efficiencies of AsCas12a-1xNLS were lower than those of AsCas12a-2xNLS and AsCas12a-6xNLS at 1 week after administration, while all the AsCas12a containing the NLSs exhibited comparable levels of genome editing efficiencies at 4 weeks after administration. Probably, AsCas12a-1xNLS gradually cleaved the target genomic region in the nucleus over the 4 weeks post-administration to mediate efficient genome editing, although the nuclear localization translocation efficiencies of AsCas12a-1xNLS were low.

AsCas12a-2xNLS and AsCas12a-6xNLS showed higher levels of genome editing efficiencies in the liver than AsCas12a-1xNLS at 1 week after administration, indicating that enhancement of nuclear translocation of AsCas12a by addition of NLSs resulted in efficient genome editing in the liver in the early post-administration period (Fig. 4F). Long-term overexpression of Cas protein occasionally exhibited cytotoxic effects. Previous reports have shown that overexpression of SpCas9 at high doses inhibited cell growth and metabolic activity.[29] Several approaches have been developed to control Cas protein expression by drugs or light to actively eliminate or stop the activity of Cas proteins after genome editing.[30–32] This study demonstrated that AsCas12a-2xNLS and -6xNLS showed efficient genome editing in the liver at 1 week after administration, suggesting that efficient genome editing at an early time point was achieved by efficient nuclear localization of AsCas12a. Efficient nuclear localization of Cas12a allows reduction or cessation of the Cas12a activity at an early time point after treatment, leading to a reduction in the risk of Cas12a-mediated off-target effects and cytotoxicity.

This study demonstrated that the effects of nuclear localization levels of Cas12a proteins on genome editing efficiencies were different between cultured cells and livers. Enhancement of the nuclear localization of Cas12a resulted in significant elevation in the genome editing efficiencies in the cultured cells, while there was no apparent correlation between the nuclear localization levels and genome editing efficiencies in the liver at 4 weeks after administration. This might be partly due to the difference in cell division rates between cultured cells and tissues. Generally, the amounts of proteins in the cells decreased during mitosis due to repression of transcription and translation.[33,34] Proteins that had once migrated to the nucleus returned to the cytoplasm as the nuclear membrane disassembled.[35] These previous findings suggested that enhanced nuclear migration of Cas12a has clearly observable effects on the genome editing efficiencies in actively dividing cultured cells. Further information might be obtained by evaluating the effects of NLS attached to Cas12a in tumor cells in tumor-bearing animal model. Since this study demonstrated that the effects of NLSs on *in vitro* genome editing efficiencies did not always correlate with those on *in vivo* genome editing efficiencies, we should pay attention to modification of Cas12a with NLSs when Cas12a-mediated *in vivo* genome editing is performed.

In summary, this study demonstrated that nuclear translocation of Cas12a proteins and the corresponding genome editing efficiencies were improved by addition of multiple NLSs *in vitro* and *in vivo*, although the correlation between the nuclear localization levels of Cas12a proteins and the genome editing efficiencies was slightly different between cultured cells and tissues. This information provides important clues for the development of not only Cas12a-mediated genome editing gene therapy but also basic research into the functions of genomes.

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Author contribution

Tomohito Tsukamoto: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Haruna Mizuta:** Validation, Methodology, Formal analysis, Data curation. **Eiko Sakai:** Supervision, Writing – review & editing. **Fuminori Sakurai:** Writing – review & editing, Writing – original draft, Supervision. **Hiroyuki Mizuguchi:** Conceptualization, Supervision, Writing - review and editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.xphs.2024.10.029.

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