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Efficient generation of adenovirus vectors carrying the Clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR associated proteins (Cas)12a system by suppressing Cas12a expression in packaging cells.

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ABSTRACT

Clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR associated proteins (Cas) 9 system is a powerful tool for genome editing and still being aggressively improved. Cas12a, a recently discovered Cas9 ortholog, is expected to become complementary to Cas9 due to its unique characteristics. Previously we attempted to establish an adenovirus (Ad) vector-mediated delivery of CRISPR-Cas12a system since Ad vector is widely used for gene transfer in basic researches and medical applications. However, we found difficulties preparing of Ad vectors at an adequate titer. In this study, we have developed Ad vectors that conditionally express Cas12a either by a tetracycline-controlled promoter or a hepatocyte specific promoter to avoid putative inhibitory effects of Cas12a. These vectors successfully proliferated in packaging cells, HEK293 cells, and were recovered at high titers. We have also developed packaging cells that express shRNA for Cas12a to suppress expression of Cas12a. Using the cells, the Ad vector directing constitutive expression of Cas12a proliferated efficiently and was successfully recovered at a high titer. Overall, we improved recovery of Ad vectors carrying CRISPR-Cas12a system, thus provided them as a tool in genome editing researches.

Key words: Clustered regularly interspaced short palindromic repeat, Cas12a, Adenovirus vector

INTRODUCTION

Clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR associated proteins (Cas) system is now in a widespread use both in basic research and medical applications as a programmable genome editing tool via generating site - specific double-strand breaks (DSBs) in the genome. DSBs in eukaryotic genomes are repaired by endogenous DNA repair-machineries, resulting in insertions and deletions (indels) at the breaks. In addition to the prevailing Cas nuclease, Cas9, recently discovered Cas12a (also known as Cpf1) can be also programmed with guide RNAs (gRNAs) complementary to the target DNA sequence to induce DSBs under the guidance of a CRISPR RNA (crRNA)[1]. In contrast to Cas9, Cas12a recognizes a T-rich protospacer adjacent motif (PAM) different from the G-rich PAM recognized by Cas9, which increases the range of targetable genomic sites[1]; cleaves DNA leaving 5'-overhang at the break[1], which might facilitate the introduction of DNA fragments into the break site via compatible ends[2]; excises crRNA from long precursor RNAs[3], which allows multiplex genome engineering using a single crRNA[2]; induces fewer off-target events, which makes Cas12a safer for genome editing[4][5]. Due to these unique properties, the CRISPR-Cas12a system together with Cas9 is being considered as a versatile tool for the genome editing technology. *Acidaminococcus*-derived Cas12a (AsCas12a) and *Lachnospiraceae*-derived Cas12a (LbCas12a) were so far reported to be functional in human cells[1]. Each ortholog has different properties in genome editing, such that LbCas12a shows stronger genome cleavage activity compared to AsCas12a[6][7], rendering LbCas12a more promising for genome editing technology.

Adenovirus (Ad) vectors have been widely used as gene transfer tools both *in vitro* and *in vivo*, including delivery of CRISPR-Cas9 system[8][9][10], due to their advantages such as a non-integrating nature, a large loading capacity and ease in preparation at high titers. Previously we have developed Ad vectors carrying AsCas12a and LbCas12a expression systems[11]. However, the Ad vector could be prepared at a low titer for AsCas12a only after repeated failures and, moreover, could not be prepared for LbCas12a. These observations raised

the suspicion that Cas12a proteins might be inhibitory to Ad vector production. In this study, we have developed Ad vectors that conditionally express Cas12a proteins under a hepatocyte-specific promoter or a tetracycline-inducible promoter to suppress their expression in packaging cells. We have also developed packaging cells expressing shRNA for Cas12a to see whether Ad vectors directing constitutive expression of Cas12a could be prepared.

MATERIALS AND METHODS

Packaging cells

Lentivirus vectors were prepared as previously described[12]. Briefly, each lentivirus vector plasmid was co-transfected with pCMV-VSV-G-RSV-Rev[12] and pCAG-HIVgp[12] into HEK293T cells (human transformed embryonic kidney cell lines) using PEI polyethylenimine (Polysciences; Warrington PA). The supernatants over the next 72 hours were collected and virus particles were purified by ultracentrifugation. Transduction units (TU) were determined based on virus genomes integrated in the host cell genomes detected by quantitative polymerase chain reaction (qPCR) with predetermined standard curves for venus-positive cells transduced with CS-CDF-RVLuP[13]. HEK293 cells (human transformed embryonic kidney cell lines) were transduced with the lentivirus vectors at 20 TU/cells and cultured at least for 10 days in the presence of puromycin (1.5 μg /ml; InvivoGen; San Diego, CA), and used as packaging cells.

Quantitative analysis of Ad vector proliferation

Packaging cells seeded on a 6-cm culture dish at $2.5x10^6$ cells/well were transfected with Ad vector plasmids (7 μg) linearized by digestion with PacI using Lipofectamine2000 (Invitrogen Life Technologies; Carlsbad CA). Cell extracts were prepared 10 days after transfection by repeated freezing and thawing. DNA was isolated from the cell extracts and subjected to quantitative polymerase chain reaction (qPCR) analysis with the primer sets for the conserved vector region (Table S1). Ad vector plasmids were used for creating standard curves to determine the Ad genome copy number. The cell extracts were applied to the fresh packaging cells at one Ad genome copy/cell, genomic DNA was collected 48 hrs later and Ad copy number was determined by qPCR.

Alternatively, packaging cells seeded on 24-well plates at $2.5x10^5$ cells/well were transduced with purified Ad vectors at 1 MOI (multiplicity of infection) and genomic DNA was collected 4 days after transduction for the analysis of Ad vector copy number.

T7 endonuclease I (T7E1) assay

Cells were seeded at $3.5x10^4$ cells/well in 24-well plates and transduced with Ad vectors at various MOIs. Indels were assessed by T7E1 assay 2 days later as previously described[14][11]. Briefly, the target region of adeno-associated virus integration site 1 (*AAVS1*) region was amplified from genomic DNA by PCR using the primer set for *AAVS1* (Table S1). PCRs were performed using PrimeSTAR Max DNA polymerase (TaKaRa Biomedicals; Otsu, Japan). The resulting PCR amplicons (100 ng) were subjected to heat-denature and re-annealing followed by digestion with 30-50 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 (National Institute of Health). Estimation of indels was calculated following the formula: % indels = $100x(1-(1-\text{cleaved band intensity/total band intensities})^{1/2})$

Cell viability assay

Cells seeded on 96-well plates at $5x10^4$ cells/well for HEK293 cells and $1.0x10^4$ cells/well for the other cell lines were transduced with Ad vectors in the presence or absence of doxycycline (DOX) (1 μg/ml) and cell viability was measured 4 days after transduction using Cell Counting Kit-8 (DOJINDO; Kumamoto, Japan) according to the manufacturer's instructions.

Statistical analysis

Statistical significance was determined using one-way ANOVA analysis followed by Bonferroni's or Dunnett's, or two-way ANOVA analysis followed by Bonferroni's Multiple comparison Test or Student's t-test. Data are presented as means ± standard deviations (SD) or standard errors (SE).

RESULTS

Generation of Ad vectors conditionally expressing Cas12a proteins.

To avoid the putative inhibitory effects of Cas12a expression on Ad vector production, we have developed Ad vectors conditionally expressing Cas12a proteins under the tetracycline-responsive promoter or a synthetic liver-specific promoter composed of apolipoprotein E enhancer, the hepatocyte control region, and human α1-antitrypsin (AHA) promoter, which enables suppression of Cas12a expressions in packaging cells. All the vectors were generated based on the previously developed Ad vector where the hepatocyte-enriched microRNA miR-122a target motifs were integrated downstream the Ad E4 gene to suppress the leaky expression of viral genes[15] (Figure.1A). A gRNA sequence targeting *AAVS1* region was newly selected using CHOPCHOP v2[16](Table S1). After validation by T7E1 assay, its expression cassette was co-integrated with the Cas12a expression cassette into the E1-deleted region of each Ad vector (Figure 1A). According to the standard protocol, Ad vector plasmids were transfected into HEK293 cells and the cell lysates containing virus particles were recovered when a substantial cytopathic effect (CPE) was observed. Then the lysates were added to fresh packaging cells for the next round of amplification. After serial scaling-up, Ad vectors were purified from cell lysates. In consistent with our previous observations, Ad vectors constitutively expressing LbCas12a under the chicken β-actin hybrid (CBh) promoter without gRNA expression cassettes (Ad-CBh-LbCas12a(g-)) could not be recovered, while Ad-CBh-AsCas12a(g-) was recovered but at a low titer (Table 1). Furthermore, both Ad vectors with gRNA expression cassettes (Ad-CBh-AsCas12a and Ad-CBh-LbCas12a) could not be recovered. Contrary to these, Ad vectors conditionally expressing LbCas12a (Ad-teton-LbCas12a and Ad-AHA-LbCas12a) were successfully recovered (Table 1). In addition, Ad vectors conditionally expressing AsCas12a (Ad-teton-AsCas12a and Ad-AHA-AsCas12a) were recovered at higher titers compared to Ad-CBh-AsCas12a(g-) by 5- and 2-fold, respectively (Table 1). These observations strongly suggested that expression of Cas12a in packaging cells

inhibited virus production.

Quantitative analysis of Ad genome copy number revealed inhibitory effects of Cas12a expression on Ad vector production.

To explore the cause of the differences in vector titers, viral genome copy number was examined in an initial stage of vector production. The cell lysates containing virus particles were recovered from the cells transfected with each Ad vector plasmid 10 days after transfection, and genome copy number was determined by qPCR. The collected cell lysates were added to fresh HEK293 cells at one genome copy/cell, and then Ad genome copy number was quantitated 48 hours after transduction (Figure 1B). In the cases of Ad vectors constitutively expressing Cas12a (Ad-CBh-AsCas12a and Ad-CBh-LbCas12a), genome copy number was reduced by ~100 and ~1000 fold, respectively, compared to control GFP-expressing Ad vector (Ad-CBh-GFP). On the other hand, the genome copy number of conditional Ad vectors (Ad-AHA-AsCas12a, Ad-teton-AsCas12a and Ad-tenon-LbCas12a) was up-regulated to ~10-fold reduction compared to Ad-CBh-GFP. Furthermore, production of Ad-AHA-LbCas12a was increased to a level comparable to Ad-CBh-GFP. These observations suggested that the inhibitory effects of Cas12a expression on Ad vector recovery were derived from inhibited vector proliferation by Cas12a expression.

Functional evaluation of Ad vectors conditionally expressing AsCas12a or LbCas12a.

To confirm the conditional expression of Cas12a proteins, human hepatocarcinoma cells, Huh-7 cells, and human non-small cell lung carcinoma cells, H1299 cells, were transduced with the Ad vectors. AsCas12a protein expression in Huh-7 cells transduced with Ad-AHA-AsCas12a was detected at a comparable level to the control Ad-CBh-AsCas12a(g-). LbCas12a protein in Huh-7 cells transduced with Ad-AHA-LbCas12a was also detected but at much lower levels compared to the control (Figure 1C). In H1299 cells, however, both AHA promoter-driven Ad vectors revealed very low protein expression compared to the control, verifying the hepatocyte specific expression of Cas12a proteins

(Figure 1D). Doxycycline-dependent expression of Cas12a from Ad-teton-AsCas12a or Ad-teton-LbCas12a was also confirmed (Figure 1C and 1D). Notably, LbCas12a protein revealed lower expression again than AsCas12a protein.

Next, the genome editing activities of these Ad vectors were evaluated in Huh-7 cells (Figure 1E). Ad vectors with the AHA promoter (Ad-AHA-AsCas12a and Ad-AHA-LbCas12a) revealed genome editing activity at high doses (600 MOI) resulting in 3.6 % and 8.8 % indels, respectively. Contrary to this, Ad vectors with the tetracycline-regulatory system (Ad-teton-AsCas12a and Ad-teton-LbCas12a) revealed higher genome editing activities than Ad vectors with AHA promoters as 30 % and 50 % indels at 600 MOI, respectively. These results indicated that the obtained Ad vectors were functional for genome edition and the genome editing efficiency was dependent on the promoter driving Cas12a expression. Also it was noticeable that LbCas12a, which showed lower expression compared to AsCas12a in either promoter systems (Figure 1C), had comparable to or even higher genome editing activity than AsCas12a, suggesting that LbCas12a protein have higher genome editing potentials than AsCas12a as reported in previous studies[6][7].

Taken together, Ad vectors expressing functional Cas12a could be prepared at practical titers by utilizing conditional expression systems. Moreover, Ad vector for LbCas12a expression was prepared for the first time.

Generation of packaging cells suppressing Cas12a expression.

Our findings strongly suggested Cas12a expression in packaging cells was harmful to Ad vector production. Therefore, we tested whether Ad vectors constitutively expressing Cas12a proteins could be recovered at higher titer by using packaging cells where Cas12a expression was suppressed. The commonly used Ad vectors are replication-defective due to deletion of the virus E1 region and thus produced in packaging cells like HEK293 cells where the E1 gene is expressed. We have developed packaging cells designed to suppress Cas12a expression by modifying HEK293 cells. HEK293 cells were transduced

with lentivirus vectors expressing shRNAs for AsCas12a and LbCas12a, generating shAsCas12a-HEK293 and shLbCas12a-HEK293, respectively (Figure 2A). These packaging cells were transfected with plasmids directing AsCas12a or LbCas12a expression under the CBh promoters (pHM-CBh-AsCas12a and pHM-CBh-LbCas12a). It was confirmed that the expression level of AsCas12a and LbCas12a in the corresponding shRNA-expressing cells was decreased (Figure 2B).

shAsCas12a-HEK293 cells rescued Ad-CBh-AsCas12a production.

Next, shAsCas12a-HEK293 and shLbCas12a-HEK293 cells were tested for production of Ad vectors constitutively expressing AsCas12a and LbCas12a under the CBh promoters, respectively (Figure 2C and 2D). When Ad genome copy number was assessed as in Figure 1B, Ad-CBh-LbCas12a remained to be suppressed in the shAsCas12a-HEK293 cells as expected, while Ad-CBh-AsCas12a was detected at a comparable level to the control Ad-CBh-GFP (Figure 2C), which showed a sharp contrast to ~100-fold suppression in parental HEK293 cells (Figure 1B). Consistent with these results, Ad-CBh-AsCas12a could be prepared using shAsCas12a-HEK293 cells at a comparable titer to control Ad-CBh-GFP (Table 2). On the other hand, Ad-CBh-LbCas12a in shLbCas12a-HEK293 was improved but still at much lower level compared to the control (~1/1000 in HEK293 vs. ~1/100 in shLbCas12a-HEK293) (Figure 1B and 2D). Consistently, Ad-CBh-LbCas12a could not be recovered using shLbCas12a-HEK293 cells (Table 2). These results revealed that suppressing AsCas12a expression, but not LbCas12a, by shRNA was effective for rescuing Ad vector production from inhibitory effects caused by Cas12a. Finally, the obtained Ad vector was functionally verified (Figure 2E and 2F). Protein expression of AsCas12a from Ad-CBh-AsCas12a prepared in shAsCas12a-HEK293 cells was detected at a comparable level to the control Ad-CBh-AsCas12a(g-). The genome editing activity was also detected similarly to the control (25% vs 28% at 400 MOI of AsCas12a-expressing Ad vectors) (Figure 1E and 2F). These results indicated that suppressing Cas12a in packaging cells by shRNA was effective for recovery

of functional Ad vectors constitutively expressing Cas12a, at least in case of AsCas12a.

Taken together, we demonstrated that inhibitory effects of Cas12a expression on Ad vector production could be rescued by suppressing Cas12a expression either by utilizing conditional promoter or shCas12a-expressing packaging cells.

Cas12a-expression suppressed Ad vector proliferation.

In Ad genome analysis starting from plasmid transfection shown in Figure 1B, a majority of the Ad genomes recovered 10 days after transfection was assumed to be non-replicated and/or non-packaged. Therefore, the amount of infectious Ad vectors used for the next transduction step was not estimated, leaving the question whether expression of Cas12a inhibited the Ad vector proliferation or the initial replication and/or packaging from the transfected Ad plasmids. In order to precisely evaluate the effects of Cas12a expression, we examined the Ad vector proliferation starting from purified Ad vectors including Ad-CBh-AsCas12a newly prepared in shAsCas12a-HEK293 cells (Figure 3A). HEK293 cells were transduced with Ad vectors and the Ad genome copy number was quantitated 4 days later. The genome copy numbers of Ad vectors conditionally expressing AsCas12a (Ad-AHA- and Ad-teton-AsCas12a) and LbCas12a (Ad-AHA- and Ad-teton-LbCas12a) were comparable to Ad-CBh-GFP, while a striking decrease was observed in Ad-CBh-AsCas12a, indicating AsCas12a expression was inhibitory to Ad vector proliferation. Furthermore, induced LbCas12a expression from Ad-teton-LbCas12a revealed a significant decrease in the genome copy number compared to the non-induced vector, indicating LbCas12a expression was also inhibitory to Ad vector proliferation (Figure 3B). Noticeably, the decease in the genome copy number by induced LbCas12a expression from Ad-teton-LbCas12a was greater than that by induced AsCas12a expression from Ad-teton-AsCas12a, suggesting LbCas12a suppressed Ad vector proliferation more severely than AsCas12a.

Taken together, these observations clearly demonstrated that Cas12a expression suppressed Ad vector proliferation, which would lead to the difficulties in preparation of Ad vectors in packaging cells.

Cytotoxic effects of Cas12a expression in various cell lines.

Since the Ad proliferation relies on host cell metabolisms, cell viability was examined to explore the cause of inhibitory effects of Cas12a on Ad vector proliferation. HEK293 cells were transduced with Ad-CBh-AsCas12a and Ad-CBh-GFP at increasing MOIs and cell viabilities were determined 48 hours after transduction (Figure 4A). A prominent decrease in cell viabilities, which was presumably due to the CPE and subsequent cell death caused by Ad vector proliferation, was observed in Ad-CBh-GFP, while significantly higher viabilities were observed at most MOIs in Ad-CBh-AsCas12a compared to Ad-CBh-GFP, suggesting that Cas12a expression did not induce massive cell death over the naturally occurring CPE and cell death by Ad vector proliferation in HEK293 cells. However, the cytotoxic effects caused by Cas12a expression might have been overwhelmed by cell death caused by Ad vector proliferation, or underestimated due to the inhibitory effects of Cas12a on Ad vector proliferations. Therefore, cell lines, which do not support proliferation of E1-deleted Ad vectors, were examined instead of HEK293 cells. Cell viabilities in H1299 and HeLa cells were clearly decreased in Ad-CBh-AsCas12a compared to Ad-CBh-GFP (Figure 4A). Similarly, cell viabilities in these cell lines were decreased in Ad-teton-AsCas12a in the presence compared to the absence of DOX (Supplementary Figure S1). Meanwhile, no or mild effects of AsCas12a expression on cell viabilities were observed in Huh-7 cells (Figure 4A and S1). Induced expression of LbCas12a also suppressed the cell viability moderately in H1299 cells and mildly in HeLa cells, but not in Huh-7 cells (Figure 4B).

Overall, Cas12a expression caused cytotoxic effects at various extents depending on cell lines. Furthermore, the toxicities were not parallel to the inhibitory effects on Ad vector proliferation, such that induced expression of LbCas12a suppressed Ad vector proliferation more severely but caused less toxicities compared to AsCas12a.

DISCUSSION

Our observations that Ad vectors directing conditional expression of Cas12a could avoid the inhibitory effects on Ad vector production suggested that Cas12a expression was the causative factor for the inhibition. This is reminiscent to the previous reports: the Ad vector carrying CRISPR-Cas9 systems was prepared at high titer ($1.3x10^{11}$/ml) when Cas9 expression was directed by a weak promoter, a mouse phosphoglycerate kinase (PGK) promoter[17], while it was prepared at lower titers (up to $1x10^{10}$/ml) when Cas9 was expressed under a relatively strong promoter, a human elongation factor-1 alpha (EF-1α) promoter[13]. Although details of virus preparation were not described in these studies, considering our observations, it is highly possible that higher expression of Cas nucleases, Cas9 and Cas12a, inhibited Ad vector production.

The molecular basis of the inhibitory effects caused by Cas12a is currently unknown. The introduced DNA sequence of Cas12a itself could not be the case because Ad vectors with conditionally regulated Cas12a expression cassettes did not show any inhibitory effects. In addition, post-transcriptional suppression of AsCas12a by shRNA in packaging cells canceled the inhibition. These results suggested expression products were the ones inducing the inhibitory effects. In previous studies, a growth defect in stably Cas9-expressing human parasites, *T. vaginalis* and T. cruzi, and a microalga, *C. reinhardtii*, resulting in failure in isolating those cells, was observed[18][19]. Also DSBs induced by Cas9 were reported to be toxic in human pluripotent stem cells in a P53-dependent fashion[20]. Furthermore, the prolonged expression of CRISPR-Cas9 system by helper-dependent adenovirus vectors revealed toxicity on human hematopoietic stem cells (HSCs)[21]. These results strongly suggested that the expression of Cas nuclease systems or Cas protein itself is harmful to host eukaryotic cells. As multitudes of viruses proliferate in a single host cell when Ad vectors were prepared, extremely high amount of Cas12a was accumulated in the cells, which might lead to cell toxicity resulting in defective host cell functions and, in turn, decrease in host cell-dependent virus production. In fact, we have shown in this study that Cas12a expression caused cell death in some cell lines.

However, we observed that Ad-CBh-AsCas12a caused less cell death compared to Ad-CBh-GFP in HEK293 cells, suggesting that any cell toxicities, which could be detected by the cell viability assay, were not the direct causes of inhibitory effects on Ad vector proliferations. Supporting this notion, the detected cytotoxic effects were not correlated to the inhibitory effects on Ad vector proliferations: induced expression of LbCas12a revealed less toxicity but inhibited Ad vector proliferation more severely compared to AsCas12a. Therefore, Cas12a might have altered cellular functions in HEK293 cells, which is, for example, activation of cellular immunity leading to exclusion of invaders, or digestion of single-stranded (ss) replication intermediates of Ad genome by the non-specific nuclease activity of Cas12 targeting ssDNAs[22][23]. Detailed mechanisms require to be elucidated in the future studies using HEK293 cells stably expressing Cas12a proteins.

We have shown in this study that shAsCas12a-expressing packaging cells successfully produced Ad-CBh-AsCas12a at the comparable level to Ad-CBh-GFP. We also observed that the titers of Ad-CBh-GFP prepared in shAsCas12a-HEK293 or shLb-Cas12a-HEK293 were lower compared to parental HEK293 cells (Table1 and 2). The precise reason for these observations is unknown. However, since the Ad vector production was largely influenced by cell-culturing conditions, it is possible that the cell metabolisms of shRNA-expressing HEK293 cells might not be fully recovered from suppressive effects by drug selection, leading to decreases in Ad vector production. Further studies are required to elucidate the basis of this phenomena, for example, by using clonally isolated packaging cells where the drug could be withdrawn from the maintenance culture media.

The observation that expression of shRNA for LbCas12a in packaging cells could not rescue Ad vector production might be because the expression level of shRNA was not sufficient for suppressing LbCas12a mRNA. Alternatively, as LbCas12a has different properties from AsCas12a, such as stronger genome editing activity than AsCas12a[6][7], escaped expression of LbCas12a might be

sufficient to show its inhibitory effects. If the latter was the case, one could imagine the DSBs in the host genomes might be the cause for cell toxicities as previously observed for Cas9[20]. Supporting this notion, functional inhibition of Cas9 by anti-CRISPR proteins, AcrII4 and AcrII2, has shown to overcome the negative effects of CRISPR-Cas9 system in HSCs[21]. However, in our previous study[11] as well as in this study, Ad vectors carrying the Cas12a expression cassette without gRNA also showed difficulties to prepare (Table 1), suggesting that inhibitory effects might be caused mainly by Cas12a protein itself rather than its enzymatic activities which would be exhibited in the presence of gRNA. Enzymatic activities, however, might be a part of the inhibitory effects because Ad virus expressing AsCas12a with gRNA was more difficult to prepare than that without gRNA.

In conclusions, we have improved preparation of Ad vectors carrying functional CRISPR-AsCas12a and -LbCas12a systems either by selecting promoter for Cas12a expression or utilizing appropriate packaging cells. These obtained vectors would facilitate biological and medical researches in future studies.

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Figure legends

Figure 1 Generation and functional evaluation of Ad vectors expressing Cas12a under the conditional promoters.

(A) Schematic structures of Ad vectors are shown. Cas12a expression is regulated by AHA promoter, tetracycline-regulated promoter and CBh promoter in Ad-AHA-Cas12a, Ad-teton-Cas12a and Ad-CBh-Cas12a, respectively. AHA, apolipoprotein E enhancer and human alpha1-antitrypsin promoter hybrid promoter; BGH pA, bovine growth hormone (BGH) poly A signal; SV40 pA, SV40 poly A signal; hPGK, human phosphoglycerate kinase promoter; Tet-on 3G, modified rtTA; TRE 3G, 3rd-generation of Tet-responsive promoter; U6, U6 promoter; ITR, inverted terminal repeat; CBh, chicken β-actin hybrid promoter. (B) Quantitative analysis of Ad genome copy number. HEK293 cells were transfected with Ad vector plasmids and cell lysates prepared 10 days later were used for transducing fresh HEK293 cells. Ad genome was prepared 48 hours after transduction from HEK293 cells and copy number was quantified by qPCR. Results were presented as mean ± SE (n=3); **p<0.01, ***p<0.001 compared to Ad-CBh-GFP. Statistical significance was determined using one-way ANOVA analysis followed by Bonferroni's Multiple comparison Test. (C and D) Western blot analysis of AsCas12a and LbCas12a expression. AsCas12a and LbCas12a protein expression was analyzed in Huh-7 cells (C) and H1299 cells (D) transduced with Ad-AHA-Cas12a, Ad-teton-Cas12a or Ad-CBh-AsCas12a(g-) at 200 MOI in the presence or absence of DOX (1 μg/ml). Ctrl indicates Ad-CBh-AsCas12a(g-) as a control. Cell lysates prepared 48 hours after transduction were subjected to western blot analysis. Cas12a expression levels were quantified using Image J software. Results were presented as mean ± SE (n=3). (E) Genome editing activities of Cas12a-expressing Ad vectors. Huh-7 cells were transduced with Ad-AHA-Cas12a (lanes 4-9) or Ad-teton-Cas12a (lanes 11-22) at 200, 400 and 600 MOI in the presence or absence of DOX (1μg/ml). The control Ad-CBh-AsCas12a(g-) (Ctrl) was used with proportional MOI of Ad-gRNA (200, 400 and 600 MOI) (lanes 1-3). Genomic DNAs prepared 48 hours after transduction were analyzed by T7E1 assay. Cleaved and uncleaved PCR products were quantified using Image J software and shown in the lower panel. Results were presented as mean ± SE (n=3); N.D., not

detected.

Figure 2 Generation of Ad vectors expressing Cas12a under the CBh promoter utilizing shCas12a-expressing packaging cells.

(A) Schematic representation of generation of shCas12a-expressing HEK293 cells. HEK293 cells were transduced with shCas12a-expressing lentiviral vector and used for Ad vector preparation. PuroR; Puromycin Resistance, LTR; long terminal repeat. (B) AsCas12 (left) or LbCas12a (right) expression was analyzed by qRT-PCR in shAsCas12a- or shLbCas12a-HEK293 cells transfected with AsCas12a- or LbCas12a-expression plasmids, respectively. Expression in HEK293 cells was taken as 1.0. Results were presented as mean ± SD (n=3); *p<0.05. Statistical significance was determined using Student's t-test. (C and D) Quantitative analysis of Ad genome copy number. Ad genome was prepared from shAsCas12a- (C) or shLbCas12a-HEK293 cells (D) transduced with Ad-CBh-GFP, -AsCas12a and -LbCas12a, respectively, as in the materials and methods section and copy number was quantified by qPCR. Results were presented as mean ± SE (n=3); *p<0.05, **p<0.01. Statistical significance was determined using one-way ANOVA analysis followed by Dunnett's Multiple comparison Test. (E) Western blot analysis of AsCas12a expression. Huh-7 cells were transduced with Ad-CBh-AsCas12a or Ad-CBh-AsCas12a(g-) at 200 MOI and cell lysates prepared 48 hours after transduction were subjected to western blot analysis. Cas12a expression levels were quantified using Image J software. Results were presented as mean ± SE (n=3). (F) Genome editing activities of Ad-CBh-AsCas12a. Huh-7 cells were transduced with Ad-CBh-AsCas12a at 200 and 400 MOI. Genomic DNAs prepared 48 hours after transduction were analyzed by T7E1 assay. Cleaved and uncleaved PCR products were quantified using Image J software and shown in the right panel. Results were presented as mean ± SD (n=3); N.D., not detected.

Figure 3 Quantitative analysis of Ad genome copy number in HEK293 cells transduced with purified Ad vectors.

(A) HEK293 cells were transduced with purified Ad vectors at 1 MOI and Ad genome was prepared 4 days after transduction. Ad genome copy number was

quantified by qPCR. Results were presented as mean ± SD (n=3); **p<0.01, ***p<0.001, ****p<0.0001 compared to Ad-CBh-AsCas12a. Statistical significance was determined using one-way ANOVA analysis followed by Bonferroni's Multiple comparison Test. (B) HEK293 cells were transduced with purified Ad-teton-AsCas12a (left) and Ad-teton-LbCas12a (right) at 1 MOI and cultured in the presence or absence of DOX (1 μg/ml). Ad genome was prepared 4 days after transduction and copy number was quantified by qPCR. Results were presented as mean ± SD (n=3); **p<0.01, N.S., not significant. Statistical significance was determined using Student's t-test.

Figure 4 Cell viability analysis of various cell lines transduced with Cas12a-expressing Ad vectors.

(A) HEK293, Huh-7, H1299 and HeLa cells were transduced with Ad-CBh-GFP and Ad-CBh-AsCas12a at the indicated MOIs. (B) Huh-7, H1299 and HeLa cells were transduced with Ad-teton-LbCas12a at the indicated MOIs and cultured in the presence or absence of DOX (1μg/ml). Cell viability was determined 48 hours after transduction. Mock-transduced cells in (A) and without DOX in (B) were set to 100%. Results were presented as mean±SD (n=3); *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, N.S., not significant compared to Ad-CBh-GFP in (A) and DOX (-) in (B). Statistical significance was determined using two-way ANOVA analysis followed by Bonferroni's Multiple comparison Test.