Generation of the Adenovirus Vector-Mediated CRISPR/Cpf1 System and the Application for Primary Human Hepatocytes Prepared from Humanized Mice with Chimeric Liver

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The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) 9 system is now widely used as a genome editing tool. CRISPR-associated endonuclease in Prevotella and Francisella 1 (Cpf1) is a recently discovered Cas endonuclease that is designable and highly specific with efficiencies comparable to those of Cas9. Here we generated the adenovirus (Ad) vector carrying an Acidaminococcus sp. Cpf1 (AsCpf1) expression cassette (Ad-AsCpf1) for the first time. Ad-AsCpf1 was applied to primary human hepatocytes prepared from humanized mice with chimeric liver in combination with the Ad vector expressing the guide RNA (gRNA) directed to the Adeno-associated virus integration site 1 (AAVS1) region. The mutation rates were estimated by T7 endonuclease I assay around 12% of insertion/deletion (indel). Furthermore, the transduced human hepatocytes were viable (ca. 60%) at two weeks post transduction. These observations suggest that the Ad vector-mediated delivery of the CRISPR/AsCpf1 system provides a useful tool for genome manipulation of human hepatocytes.

Key words adeno virus vector; genome editing; human hepatocyte; humanized mouse liver cell; clustered regularly interspaced short palindromic repeat-associated endonuclease in Prevotella and Francisella (Cpf1)

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) 9 system, a powerful tool for genome editing, is now being used for a wide variety of applications in cultured cells and living organisms. 1–3 Therefore, its potential is being explored in the research for treatment of human diseases such as sickle cell anemia, 4 hemophilia, 5,6 and human immunodeficiency virus (HIV) infection. 7 Liver diseases have been also regarded as targets of genome editing applications because of the variety of serious inherited and acquired genetic disorders caused by mutations in single genes. 8 Primary human hepatocytes are preferably used for investigating human liver disorders because of species differences in many aspects of biological features that typically appear in enzymes responsible for drug metabolisms. However, primary human hepatocytes do not proliferate in culture. To overcome the problem, chimeric mice with humanized livers, 9–11 in which the liver has been repopulated with functional human hepatocytes, have been developed and could serve as a source of human liver cells.

Recently, CRISPR-associated endonuclease in Prevotella and Francisella 1 (Cpf1) was identified as another CRISPR/Cas DNA endonuclease. 12 Cpf1 proteins have characteristics different from Cas9, which might provide advantages for genome editing: T-rich protospacer adjacent motif (PAM) sequences (5’TGTN or TTN) that increase the range of targetable genomic sites in addition to the T-rich sequences recognized by Cas9 proteins; 5’-overhang at the double strand break that potentially facilitates knock-in of donor fragments via annealing with target sites using sticky ends; shorter CRISPR RNA (crRNA) without trans-activating CRISPR RNA (tracrRNA) sequences that is easy to synthesize. Among so far identified Cpf1s, Acidaminococcus-derived Cpf1 (AsCpf1) and Lachnospiraceae-derived Cpf1 (LbCpf1) have been shown to be effective and widely used for genome manipulation in human cells. 12 Viral vectors including retrovirus, lentivirus, adeno-associated virus (AAV) and adeno virus (Ad) vectors have been used as gene delivery tools of CRISPR. 13 AAV and Ad vectors have been preferably utilized because of their advantages such as their episomal nature, and efficient introduction of DNA into non-transformed, either dividing or quiescent post-mitotic cells such as adult hepatocytes. The effectiveness of AAV vectors carrying AsCpf1 has been demonstrated on targeting to primary neuronal cells as well as to brain in mouse model. 14 In contrast, Ad vectors have not been tested for delivery of Cpf1 in any host system. Here we report, for the first time, generation of Ad vectors for expression of CRISPR/AsCpf1 and investigated its efficacy of manipulating human AAV integration site 1 (AAVS1) region, as a model locus, in primary human hepatocytes prepared from humanized mice with chimeric liver.

MATERIALS AND METHODS

Cell Cultures HEK293 cells (a human transformed embryonic kidney cell line) were cultured in Dulbecco’s modified Eagle’s medium (WAKO, Osaka, Japan) supplemented with 10% fetal bovine serum, streptomycin (100 μg/mL; Nacalai Tesque, Kyoto, Japan) and penicillin (100 U/mL; Nacalai

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H1299 cells (a human non-small cell lung carcinoma cell line) were cultured in RPMI 1640 (Sigma-Aldrich, St.
Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum, streptomycin (100μg/mL) and penicillin (100 U/mL).
Primary human hepatocytes prepared from humanized mice with chimeric liver (hereinafter referred to as PHHs) were purchased
from PhoeniXBio (Higashi-Hiroshima, Japan) and cultured in type I-collagen-coated plates (Corning, Corning, NY,
U.S.A.) with dHCGM supplied by the manufacturers.

**Plasmids**
The plasmids expressing hemagglutinin (HA)-tagged AsCpf1 (pCDNA3.1-hAsCpf1, #69982) and HA-tagged LbCpf1 (pCDNA3.1-hLbCpf1, #69988) were purchased from Addgene (Cambridge, MA, U.S.A.). The plasmids for expression of guide RNA (gRNA), BPK3079 (#78742) and BPK3082 (#78742), which carry the upstream crRNA spacer sequences for AsCpf1 and LbCpf1, respectively, were also purchased from Addgene. To obtain the shuttle plasmids, the elongation factor 1 alpha (EF1α) promoter of pMEF5 was replaced with a hybrid chicken beta-actin (CBh) promoter by ligating pMEF5 digested with NheI and NotI from pHM-CBh-hSpCas9 (Addgene, #42230) digested with XbaI and NotI to produce pHM-CBh-hSpCas9. Cas9-coding region was replaced with Cpf1-coding region by ligating pHM-CBh-hSpCas9 digested with AgeI and NotI and pHM-CBh-LbCpf1, respectively. The plasmids expressing gRNAs for AsCpf1 and LbCpf1 were constructed by inserting double-stranded oligonucleotides (Table S1) into the BsmBI sites of BPK3079 and BPK3082, producing pU6-AsgRNA-AAVSI and pU6-lbgRNA-AAVSI, respectively. pHM-U6-AsgRNA-AAVSI, pHM-U6-lbgRNA-AAVSI and pHM-U6-cas9gRNA-AAVSI were generated by insertion of U6-asgRNA-AAVSI, U6-lbgRNA-AAVSI and U6-cas9gRNA-AAVSI fragments from pU6-AsgRNA-AAVSI, pU6-lbgRNA-AAVSI and pX330-lbgRNA-AAVSI into pHM5 shuttle vector. The reporter plasmid pCAG-EGxxFP-AAVSI containing approximately 500 bp long genomic sequence of AAVSI locus and the control plasmid pMEF5-mCherry were previously described.

**Viruses**
The Ad vectors for expression of AsCpf1 (Ad-AsCpf1), LbCpf1 (Ad-LbCpf1) and gRNA (Ad-asgRNA-AAVSI) were prepared by an improved in vitro ligation method. Briefly, the fragments of expression cassettes from pHM-CBh-AsCpf1, pHM-CBh-LbCpf1, pHM-U6-asgRNA-AAVSI and pHM-U6-lbgRNA-AAVSI were individually integrated into the I-CeuI sites of pAdHM4. The Ad vector expressing the green fluorescent protein (GFP) (AdCA-GFP) was previously described. These Ad vectors were amplified in HEK293 cells and purified by two rounds of cesium-chloride-gradient ultracentrifugation. The virus particle (VP) titers were determined by spectrophotometric method. The infectious units (IFU) were determined using an Adeno-X Rapid Titer Kit (Clontech Laboratories, Mountain View, CA, U.S.A.). For transducing cells, cells suspended in the culture media were mixed with Ad vectors at the indicated multiplicity of infection (MOI) and were subsequently seeded.

**Western Blot Analysis**
HEK293 cells (2×10^5 cells/well, 24-well plates) were transfected with 400 ng of pHM-CBh-AsCpf1 or pHM-CBh-LbCpf1 using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, U.S.A.) according to the manufacturer’s instructions. Total cell lysates were prepared 48 h after transfection. H1299 cells (1×10^5 cells/well, 12-well plates) or PHHs (4×10^5 cells/well, 24-well plates) were transduced with Ad vectors. Cell lysates were prepared from H1299 cells 48 h after transduction and from PHHs 5 d after transduction. Western blot analysis was performed as previously described. Briefly, cell lysates were electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels (PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Darmstadt, Germany). The HA-tagged Cpf1 proteins were detected using mouse Anti-HA antibody (Covance, Princeton, NJ, U.S.A.). Mouse anti-β-actin (Sigma-Aldrich) was used for loading controls.

**Validation of gRNAs by EGxxFP Assay**
EGxxFP system was used for gRNA validation as previously described with some modifications. HEK293 cells were seeded on poly-L-lysine-coated 24-well plates (1×10^5 cells/well) and co-transfected with 400 ng of Cpf1-expressing plasmids. 400 ng of reporter pCAG-EGxxFP-AAVSI and 200 ng of pHM5-mCherry as a transfection control using Lipofectamine 2000. Forty-eight hours after transfection, images were captured and processed using BIOREVO digital camera (BZ-9000, Keyence Japan, Osaka, Japan).

**T7 Endonuclease I (T7EI) Assay**
The cleavage activity of Cpf1 at endogenous loci was quantified based on the mutation rates resulting from non-homologous end joining (NHEJ) repair. H1299 cells (7×10^5 cells/well, 24-well plates) were co-transfected with 400 ng of pHM-CBh-AsCpf1 or pHM-CBh-LbCpf1, and 400 ng of pHM-U6-asgRNA-AAVSI or pHM-U6-lbgRNA-AAVSI using Lipofectamine 2000. For transduction experiments, H1299 cells and PHHs were suspended in culture medium, mixed with Ad-AsCpf1 and Ad-asgRNA-AAVSI at various MOIs and seeded onto 24-well plates at 7×10^4 and 4×10^5 cells/well, respectively. The insertion/deletion (indel) mutations were assessed by T7EI assay 2 and 14 d after transduction in H1299 cells and PHHs, respectively, as previously described. Briefly, genomic DNA was prepared from the cells and the target region of human AAVSI region was amplified by PCR using the primer set for AAVSI (Table S1). PCRs were performed using PrimeSTAR Max DNA polymerase (TaKaRa Biomedicals, Otsu, Japan) following the manufacturer’s instructions. The resulting PCR amplicons (100 ng) were denatured and re-annealed by heating and gradual cooling followed by digestion with 30–50 units of T7EI enzyme (New England Biolabs, Ipswich, MA, U.S.A.) for 30 min at 37°C. The cleaved fragments derived from mismatch-parings were resolved by 10% PAGE followed by staining with Midori Green Advance (NIPPON Genetics, Tokyo, Japan). Images were taken using FAS5 (NIPPON Genetics, Tokyo, Japan). Estimation of indels was determined following the formula: % indels=100×(1–(1–cleaved band intensity/total band intensities)^1/2)

**Cell Viability Assay**
PHHs were seeded on collagen-coated 96-well plate (7×10^5 cells/well). Cell viability was assayed 14 d after transduction using an alamarBlue Cell Viability Reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer’s instructions.

**Statistical Analysis**
Statistical significance was determined using one way ANOVA analysis followed by Bonferroni’s multiple comparison test. Data are presented as
RESULTS

Validation of gRNA of AAVSI Region. We used the AAVSI locus as the target region because it is a well-characterized model locus for genome manipulation.\textsuperscript{23–25} For initial validation of gRNAs designed for Cpf1, we used the reporter plasmid pCAG-EGxxFP-AAVS1, which was previously constructed to contain an approximately 500 bp genomic region of AAVSI\textsuperscript{14} integrated into the enhanced green fluorescent protein (EGFP) coding region (Supplementary Fig. S1A). Two targeting sites (gRNA1 and gRNA2) with PAM (TTTN) at the 5’ end of the sequence were selected using CHOPCHOP v2,\textsuperscript{14} an improved CRISPR/Cas9 target prediction tool, and gRNAs were designed for each target sequence (Fig. S1). The pCAG-EGxxFP-AAVS1 reporter plasmids were co-transfected into HEK293 cells with AsCpf1- or LbCpf1-expressing plasmids (pcDNA3.1-hAsCpf1 and pcDNA3.1-hLbCpf1) along with the gRNA-expressing plasmids (pU6-asgRNA-AAVS1 and pU6-lbgRNA-AAVS1). Cells were assayed for the double strand break (DSB) repair of target AAVSI region using reconstituted EGFP fluorescence as a marker (Figs. S1B, S1C). The gRNA1 showed better activity both for AsCpf1 and LbCpf1. Also, we conducted off-target predic-

Fig. 1. Genome Editing via Plasmid-Mediated Expression of CRISPR/Cpf1 in Human Cell Lines

(A) Western blot analysis of AsCpf1 (151kD) and LbCpf1 (143kD) expression in HEK293 cells. Cell lysates were prepared from cells 48h after transfection and subjected to Western blot analysis. The HA-tagged Cpf1 proteins were detected using anti-HA antibody. (B) Analysis of homology-directed repair (HDR)-mediated gene editing by EGxxFP assay. HEK293 cells were transfected with the reporter plasmid, Cpf1- and gRNA-expressing plasmids as well as the control plasmid for transfection efficiencies, pHMEF5-mCherry. Images were captured 48h after transfection and signal intensities were quantitated based on the images and normalized by mCherry expressions. Results were presented as mean±S.D. (n=3); *p<0.05. ****p<0.0001 compared with Cas9. Scale bar, 200µm. (C) Genome editing activities in H1299 cells. Genomic DNAs were harvested 72h after transfection and subjected to T7E1 assay. Cleaved and uncleaved PCR products were quantified using Image J software and the calculated % indels are shown below each lane. N.D., not detected. (Color figure can be accessed in the online version.)
tion for gRNA1 sequence using Cas-OFFinder (http://www. rgenome.net/cas-offinder/) and found sequences mismatched to gRNA1 at least by three nucleotides in the human genome (Table S2). Kim et al. reported by systemic analyses using Next Generation Sequencing (NGS) analysis that Cpf1 barely caused off-target mutations with gRNAs having doubly mismatched sequences within the 19 nucleotide-region adjacent to PAM sequences.27) Thus, we reanalyzed gRNA1 regarding the 19 nucleotides adjacent to PAM and confirmed that all the off-target candidates had at least two mismatches within the region (Table S3). Therefore, it was selected for further analysis. Next we evaluated the activity of AsCpf1, LbCpf1 and Cas9 in parallel by EGxxFP assay in HEK293 cells, where all the enzymes were expressed under the same promoter (pHM-CBh-AsCpf1, pHM-CBh-LbCpf1 and pHM-CBh-hSpCas9) for comparison. pHM-U6-cas9gRNA-AAVS1 contains a previously validated sequence of gRNA for AAVSI.16) Western blot analysis confirmed the expression of AsCpf1 and LbCpf1 proteins (Fig. 1A). Both Cpf1s revealed reconstitution activity comparable to Cas9 with LbCpf1 having a higher activity than AsCpf1 (Fig. 1B). This plasmid-mediated expression of Cpf1s in combination with the gRNA was further validated for editing the endogenous AAVSI region by T7E1 assay in H1299 cells resulting in clear detection of mutations in the endogenous target region (Fig. 1C).

**Generation of Cpf1-Expressing Ad Vectors** Aiming to target primary cells, we next sought to generate Ad vectors carrying AsCpf1 (Ad-AsCpf1) and LbCpf1 (Ad-LbCpf1) expression cassette in the E1-deleted region of Ad vectors20) (Fig. 2A). The gRNA-expressing Ad vectors (Ad-asgRNA-AAVS1 and Ad-lbgRNA-AAVS1) were independently generated. Ad-AsCpf1 and the gRNA-expressing Ad vectors were successfully obtained at reasonably high titers (Fig. 2B). However, Ad-LbCpf1 could not be prepared in repeated trials probably due to the cytotoxicity of LbCpf1 during viral amplification. The expression of AsCpf1 was confirmed in H1299 cells transduced with Ad-AsCpf1 (Fig. 2C). We focused on Ad-AsCpf1 hereafter.

**Genome Editing Activity of Ad Vector-Mediated CRISPR/AsCpf1 Expression in H1299 Cells** We next evaluated the genome processing activity of Ad vector-mediated CRISPR/AsCpf1 systems in a cell line. H1299 cells were co-transduced with increasing MOIs of Ad-AsCpf1 and Ad-asgRNA-AAVS1, and the mutations introduced into the target region in the AAVSI region were analyzed by T7E1 assay (Fig. 2D).
The increasing amount of cleaved fragments was detected in a dose-dependent manner on Ad-asgRNA-AAVS1 and Ad-AsCpf1. The maximum indel rates were estimated around 16% when Ad-AsCpf1 and Ad-asgRNA-AAVS1 were used at 100 MOI and 50 MOI, respectively. This was comparable to the reported efficiency (13–40%) with AAV vector-mediated delivery of AsCpf1 targeting the neuronal genes,[14] which was estimated by SURVEYOR nuclease assay in primary neuron cells. These results clearly indicated that Ad vector-mediated expression of AsCpf1 works for genome edition of endogenous locus.

**Genome Editing Activity of Ad Vector-Mediated Cpf1 Expression in PHHs**

Next, we applied the Ad vector-mediated CRISPR/Cpf1 system to PHHs. PHHs were transduced with various Ad vectors followed by the analysis of protein expressions, genome editions and cell viabilities (Fig. 3A). To examine the transduction efficiency of Ad vectors, PHHs were transduced with the GFP-expressing Ad vector (Ad-CA-GFP) at various MOI and cell viabilities were compared using the AlamarBlue assay (Fig. 3E). Ubiquitous expression of GFP was determined, indicating highly efficient gene expression by Ad vector-mediated delivery in PHHs. To test the genome editing activity, PHHs were co-transduced with Ad-AsCpf1 (2.5 MOI) and Ad-asgRNA-AAVS1 (15 MOI). Ad-CA-GFP (15 MOI) was used as the control for cytotoxic effects caused by transduction with Ad vectors. Genomic DNAs were prepared 14d after transduction and mutations were analyzed by T7E1 assay. Cleaved and uncleaved PCR products were quantified using Image J software and the calculated % indels are shown below each lane and in the bar graph. Results were presented as mean±S.D. (n=3); *p<0.05; N.S., not significant; N.D., not detected. (E) Cell viability analysis by AlamarBlue assay. PHHs transduced with Ad vectors as in (D) were subjected to AlamarBlue assay 14d after transduction. Results were presented as mean±S.D. (n=3); N.S., not significant. (Color figure can be accessed in the online version.)
The ratio of editing was quantitatively estimated as ca. 12% of indels. The cell viability analysis revealed that some decreases (ca. 60%) in the cell viability were observed at day 14 in the cells transduced by Ad-AsCpf1 compared to mock cells but no differences were observed between Ad-asgRNA-AAVSI- and the control Ad vector-transduced cells (Fig. 3E).

DISCUSSION

In the present study, the Ad vector-mediated CRISPR/Cpf1 system was generated for the first time and was shown effective in genome edition in primary human hepatocytes from humanized mice with chimeric liver. Although the cell viability was impaired to some extent, the majority of the transduced cells were viable at least for 2 weeks. Therefore, the Ad vector-mediated delivery of CRISPR/Cpf1 system could be a useful tool for ex vivo genome manipulation of primary human hepatocytes, potentially to be transplanted for both research and therapeutic purposes in future studies. The observed impairment of cell viability might be because of inhibitory effects of AsCpf1 protein itself or leaky expression of Ad proteins.35 The former case seems less likely since any impairment of cell viability was not reported in the previous studies where Cpf1 proteins were expressed using plasmids4,29 or AAV vectors.45 In either case, controlling the Cpf1 expression, for example, by using the tetracycline-regulation system, or suppressing the leaky expression of Ad proteins by improved Ad vectors30 could be the solution for this newly uncovered problem.

In general, AAV vectors seem to be safer than Ad vectors for therapeutic applications in humans because of their low immunogenicity. On the other hand, AAV vectors have smaller packaging capacity (ca. 4.5 kb) than Ad vectors (ca. 8.1 kb), which restricts the cargo size. Besides having larger capacity of cargos, Ad vectors have advantages over AAV vectors when applied in humanized chimeric mouse-derived hepatocytes as it efficiently targets human hepatocytes45 while AAV vectors has been reported to preferably accumulate in the residual murine hepatocytes at various extents depending on serotypes, that could cause decrease in gene editing efficiency in human hepatocytes.35 In addition, Ad vectors generally showed more rapid expression of cargo genes than AAV vectors, that would be critical for genome manipulation of hepatocytes since cultured primary human hepatocytes rapidly lose hepatocytic functions such as cytochrome P-450 activity25 and expression of hepatocyte nuclear factor 4α.35 Moreover, several types of improved Ad vectors with reduced immunogenicity have been developed.30,34,35 Therefore, generation of Ad vector-mediated CRISPR/Cpf1 system could provide more options for delivery tools of genome editing in primary cells.

Since the final goal of the genome editing technology in primary cells would be gene corrections, the first generation of cleavages and subsequent replacement of the target regions are required. In this regard, Suzuki et al. have recently reported that CRISPR/Cas9 systems achieved knock-in into the target Tubb3 locus in non-dividing mouse primary neuronal cells by homology-independent targeted integration (HITI) using plasmid-mediated and AAV vector-mediated delivery.36 However, the efficiency was not high possibly because of the nature of Cas9 that it generates DSB with blunt ends. DSB with blunt ends mainly allows homology-directed repairs (HDRs) which barely occur in non-dividing cells,37 leading to the observed low efficiency. Conversely, in those cells, non-homologous end joining (NHEJ) is the main homology-independent repair pathway where the alignment of only one to a few complementary bases at most are required for the re-ligation of two ends.37 Therefore, the characteristics of Cpf1, that it cleaves genome with 5’-staggered ends, might facilitate HITI of donor fragments through sticky-ends. In fact, AsCpf1 and LbCpf1 have been shown recently to successfully perform correction of disease-related mutations in patient-derived induced pluripotent stem (iPS) cells via plasmid-electroporation as well as germline correction in the model mouse via mRNA- and gRNA-injection.29 Taken these potential benefits, viral vector-mediated delivery of CRISPR/Cpf1 would become a promising tool for knock-in approaches in primary cells, such as hepatocytes. Our study revealed that Ad vector-mediated CRISPR/Cpf1 system could generate genome cleavages in PHHs. The genome replacement with the donor fragment is required to be challenged as the next step in line of this study.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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