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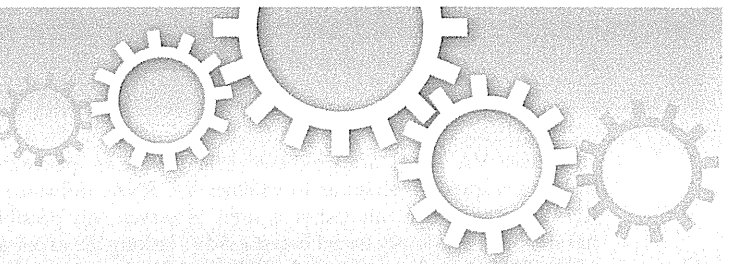

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Adenovirus vectors lacking virus-associated RNA expression enhance shRNA activity to suppress hepatitis C virus replication

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First-generation adenovirus vectors (FG AdVs) expressing short-hairpin RNA (shRNA) effectively downregulate the expressions of target genes. However, this vector, in fact, expresses not only the transgene product, but also virus-associated RNAs (VA RNAs) that disturb cellular RNAi machinery. We have established a production method for VA-deleted AdVs lacking expression of VA RNAs. Here, we showed that the highest shRNA activity was obtained when the shRNA was inserted not at the popularly used E1 site, but at the E4 site. We then compared the activities of shRNAs against hepatitis C virus (HCV) expressed from VA-deleted AdVs or conventional AdVs. The VA-deleted AdVs inhibited HCV production much more efficiently. Therefore, VA-deleted AdVs were more effective than the currently used AdVs for shRNA downregulation, probably because of the lack of competition between VA RNAs and the shRNAs. These VA-deleted AdVs might enable more effective gene therapies for chronic hepatitis C.

RNA interference (RNAi) technology is a versatile tool for analyzing the function of genes *in vitro* and *in vivo* in various research fields. It also presents a therapeutic approach for the treatment of human diseases and for the selection of effective drugs. Two types of small non-coding RNAs function as RNAi, small interfering RNAs (siRNAs) and microRNAs (miRNAs). Short-hairpin RNA (shRNA) is artificially produced RNAi that downregulates the expression of the target gene.

First-generation adenovirus vectors (FG AdVs), which lack the E1 and E3 regions, have been widely used not only for basic studies of various gene functions *in vitro* and *in vivo*, but also for preclinical and clinical gene therapy. The transduction efficiency of this vector is very high, and viral stocks with a high titer are easily obtained. FG AdVs are often used to deliver shRNA or miRNA expression cassettes into target cells *in vitro*[1,2]. They are also used in *in vivo* studies, particularly in gene therapy fields[3–5]. FG AdVs are usually considered not to express any viral gene products because they lack the E1A gene, which is essential for the expression of all viral genes driven by polymerase II. In fact, however, FG AdVs express viral-associated RNAs (VA RNAs), which are vector-encoded small RNAs that are always expressed together with the transgene product both *in vitro* and *in vivo* since they are transcribed by polymerase III.

The VA RNAs, known as VAI and VAII, consist of 157–160 nucleotides (nt) and are encoded at about 30 map units on the genome of adenoviruses. In the normal life cycle of adenoviruses possessing the E1 genes, these VA RNAs are abundantly present during the late phase of infection and inhibit cellular RNAi pathways by saturating Exportin 5, RISC, and Dicer[6]. They are also processed and generate miRNAs[7,8], known as mivaRNAI and mivaRNAII, that disturb the expression of many cellular genes, with the probable result of blocking cellular antiviral machinery. VA RNAs are also expressed during the early phase of viral infection, though their functions during this phase remain unknown and the target genes of mivaRNAs have not been adequately studied. VA RNAs are expressed in AdV-transduced target cells at a level similar to that during the early phase and are considered to be a cause of severe immune responses[9,10], which are a major drawback of this vector. Therefore, AdVs lacking the expression of VA RNAs (VA-deleted AdVs) are desired for both basic and clinical studies and may enable safer gene therapy.

Because VA RNAs are processed using the same pathway as shRNAs, a question arises as to whether VA RNAs influence the RNAi strategy when this vector is used. However, this possibility has not been previously tested because AdVs lacking the expression of VA RNAs have been extremely difficult to develop[11], though low titers of VA-deleted, E1-containing adenoviruses have been obtained. Recently, however, we have established a method for the very efficient production of VA-deleted AdVs[12] that is sufficient for practical use *in vitro* and *in vivo*. The titers of the VA-deleted AdVs are comparable to those of the currently used FG AdVs. Also, we established 293 cell lines that constitutively express VA RNAs and support the growth of shRNA-expressing VA-deleted AdVs (unpublished data). These progresses have enabled us to examine whether VA RNAs actually influence the shRNA strategy.

Hepatitis C virus (HCV) infects 2%–3% of the world's population and is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma[13–15]. The most abundant genotype of HCV worldwide is genotype 1, which has two prevalent subtypes (1a and 1b). Genotype 2 is the second most common genotype. In addition to standard care combined with interferon (IFN) and rivavirin, the emergence of direct-acting antivirals, such as HCV protease inhibitors, has enabled advances in treatment. However, considering the development of drug-resistant viruses and side effects induced by drug–drug interactions, additional options for anti-HCV therapy are inevitably needed. HCV is considered to be an attractive target for shRNA-based therapy[16–19] because the viral genome is a single-stranded RNA of positive polarity and all the steps of viral replication occur in the cytoplasm.

shRNA-expressing cassettes are usually inserted at the E1 cloning site. However, the suitability of this site has not been examined. Here, we report that the shRNA activity was influenced by the position and orientation of the shRNA in the vector genome, and the most effective position/orientation the E4 position. We also show that shRNAs expressed by VA-deleted AdVs inhibited HCV replication more efficiently than those expressed by FG AdVs. The present report is the first to demonstrate that VA RNAs expressed from FG AdVs do indeed reduce the shRNA activity and that VA-deleted AdVs are useful for shRNA strategies.

Results

shRNA-expressing unit inserted at the E4 position worked more efficiently than that at the E1 position in the AdV genome. We constructed four VA-deleted AdVs containing shRNA that suppresses GFP expression (Fig. 1a). Cassettes containing anti-GFP shRNA under the control of the human U6 promoter were inserted at the E1 and E4 insertion positions in left (L) and right (R) orientations (AxdV-aGFP-E1L, -E1R, -E4L, and -E4R, respectively). To assay shRNA suppression we used FC-18 cells that constitutively express GFP under the control of the EF1α promoter from its gene integrated in the chromosome[20]. The cells were infected with these AdVs at a multiplicity of infection (MOI) of 50, and the copy numbers of the GFP mRNA present in the cells were measured using quantitative PCR (qPCR). The suppression efficiency of the E4L vector (Fig. 1b, left, bar 3) was significantly higher than those of the E1L and E1R vectors (bars 1and 2). Therefore, the E4L position/orientation was more effective than the E1L or E1R position/orientation. This finding is notable because the E1 position is popularly used for the insertion of shRNA cassettes. The present results were confirmed using a FACS analysis (Supplementary Fig. S1). We also examined the suppression efficiency of mRNA transiently expressed from a transgene located on the AdV genome: a Cre-expressing AdV was co-infected together with each of the four VA-deleted AdVs expressing anti-Cre shRNA instead of anti-GFP shRNA (AxdV-aCre-E1L, -E1R, -E4L, and -E4R, respectively). The suppression efficiencies of the E4L and E4R anti-Cre vectors were higher than those of the E1L and E1R vectors at MOI 50 (Fig. 1b, right; compare bars 3 and 4 with bars 1 and 2, respectively) and at MOI 200 (Supplementary Fig. S2), while the efficiencies of the E4L and E4R anti-Cre vectors were not statistically different. Based on these data, the E4L position/orientation was adopted for further experiments examining HCV replication.

Anti-HCV shRNA activity in cells replicating HCV subgenome RNA was enhanced using VA-deleted AdV. The anti-HCV activity of AdVs carrying shRNA was assessed in Huh-7-derived cells carrying the viral subgenomic replicon RNA (viral structural genes

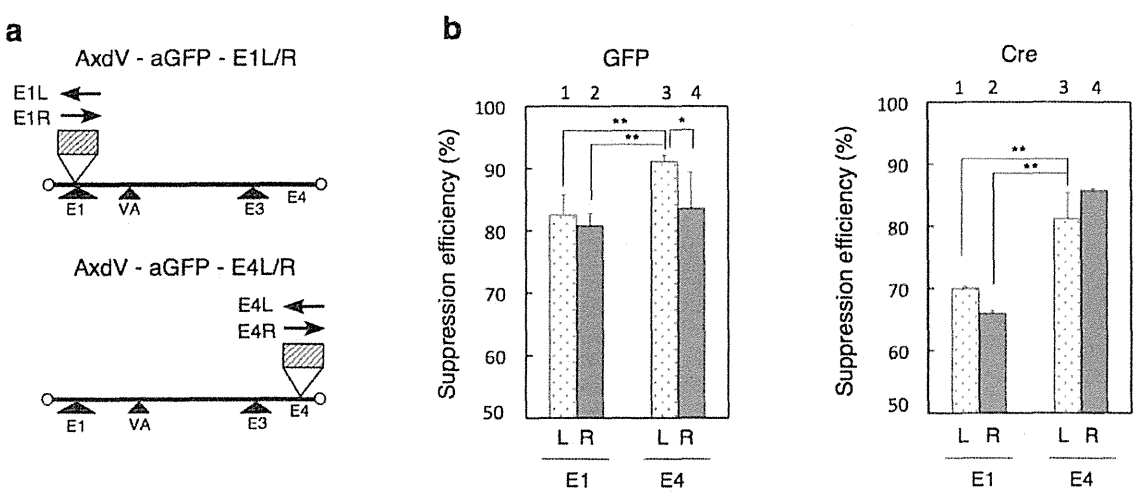

Figure 1 | Structures of vectors containing shRNA cassettes and suppression efficiencies. (a) Structures of AdV-aGFP vectors. The arrow shows the orientation of transcription. Hatched box, shRNA cassette including the human U6 promoter. (b) (Left) Suppression efficiency of GFP RNA expressed from the cell line using anti-GFP vectors. FC-18 cells that constitutively express GFP RNA were infected with the vectors at MOI 50. (Right) Suppression efficiency of Cre RNA expressed from the AdV genome using anti-Cre vectors. FC-18 cells were doubly infected with AdV expressing Cre under the control of the CAG promoter at MOI 10 and anti-Cre vectors at MOI 50. Three days after infection, the amount of cytoplasmic RNA of GFP and Cre were measured using qPCR. The suppression efficiency for vector-infected FC-18 cells was calculated using copy numbers per cell, where uninfected FC-18 cells were denoted as 0% suppression of GFP RNA, while that of CV1 cells, the parent cells of FC-18 that do not contain the GFP gene, is denoted as the control of 100% suppression. Copy number, n = 6. $*P < 0.05$, $**P < 0.01$ compared with the E4L vector (unpaired Student's *t*-test).

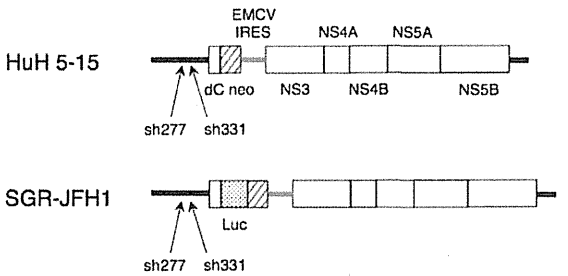

Figure 2 | Schematic representation of the HCV replicons. The coding regions in the HCV polyproteins are indicated by the open boxes. The bold lines indicate the HCV 5′-untranslated region (UTR), which is the target of sh277 and sh331 (arrows), and the 3′-UTR. Gray bars, EMCV internal ribosome entry site; dC, 5′-region of Core gene; neo, neomycin-resistance gene; Luc-neo, firefly luciferase gene fused with neo gene.

are replaced by either the neomycin-resistant [neo] gene or the luciferase-neo fusion gene, and all the nonstructural genes sufficient for replication of the viral genome are retained; Fig. 2). The HCV RNA is constitutively maintained in these cells. Two kinds of shRNAs were used (Fig. 2, arrows). The shRNA331 (called sh331 in this paper) targeting nt 322–342 at the 5′ end of the HCV genome has been reported to inhibit HCV replication efficiently[16]. The shRNA277 (called sh277 in this paper) targeting nt 279–297 is well conserved among genotypes and was newly constructed in this study. HuH 5–15 cells, which are genotype 1b-replicon cells[21], were infected with FG AdVs expressing either sh277 or sh331 or VA-deleted AdVs (VA-del AdVs in this figure) that were identical to AxdV-aGFP-E4L but used sh277 and sh331 as the shRNA (Fig. 3a). These AdVs contained cassettes expressing shRNAs under the control of the human U6 promoter at the E4L position/orientation. HCV RNA copies in the cells were measured using qPCR, and the suppression efficiency was calculated. VA-deleted AdVs expressing sh277 suppressed the viral RNA replication more efficiently than the corresponding FG AdV at MOI 2 (Fig. 3a, bars 3 and 4). A higher anti-HCV activity was also observed for the VA-deleted AdV compared with the FG AdV at MOI 10 (bars 5 and 6). Similarly, VA-deleted AdV expressing sh331 suppressed HCV RNA replication more efficiently than the corresponding FG AdV at both MOI 2 and MOI 10 (bars 7 to 10). Among these settings, VA-deleted AdV expressing sh277 at MOI 10 yielded the highest suppression efficiency (bar 6, 71%).

SGR-JFH1/LucNeo cells (called SGR-JFH1), which harbor a genotype 2a subgenomic replicon carrying a luciferase-neo fusion gene (Fig. 2, lower), were infected with a series of AdVs carrying shRNAs as described above and the numbers of HCV RNA copies in the cells were determined. We obtained results that were very similar to those obtained using HuH 5–15: the viral RNA level in cells infected with VA-deleted AdVs expressing sh277 or sh331 at MOI 2 or MOI 10 was significantly lower than that in cells infected with the corresponding FG AdVs, respectively (Fig. 3b, bars 3 to 10). Thus, these results, together with those obtained in HuH5–15 cells, demonstrated that VA-deleted AdV expressing shRNA was more effective than FG AdV for the suppression of HCV-RNA replication.

To evaluate whether VA-deleted AdV is advantageous to the anti-HCV effect of IFN, VA-deleted AdV and FG AdV expressing human α2-IFN were also constructed. As shown in Fig. 3, both the VA-deleted AdVs and the FG AdVs enabled the efficient suppression of HCV RNA replication in HuH 5–15 and SGR-JFH1 cells (bars 11 and 12). However, in contrast to the results obtained with shRNAs, no significant difference in the suppression efficiency was observed between these two AdVs, supporting the notion that the antiviral mechanisms of IFN are distinct from those of shRNAs.

Coinfection of a VA-deleted AdV expressing sh277 and a VA-deleted AdV expressing sh331 enabled more efficient suppression than single infection alone. Because the sequences of sh277 and sh331 do not overlap, they may work independently and suppress more efficiently when doubly introduced to cells. Therefore, HuH 5–15 cells were coinfected with a VA-deleted AdV expressing sh277 and a VA-deleted AdV expressing sh331, and the resulting suppression efficiency was compared with that in cells infected with either the sh277 AdV or the sh331 AdV alone (Fig. 4a). As a result, the suppression efficiency of the coinfected AdVs was higher than those of the singly infected AdVs at MOI 5 (compare bar 4 with bars 2, 3, 5 and 6) and at MOI 10 (compare bar 4 with bars 7 and 8).

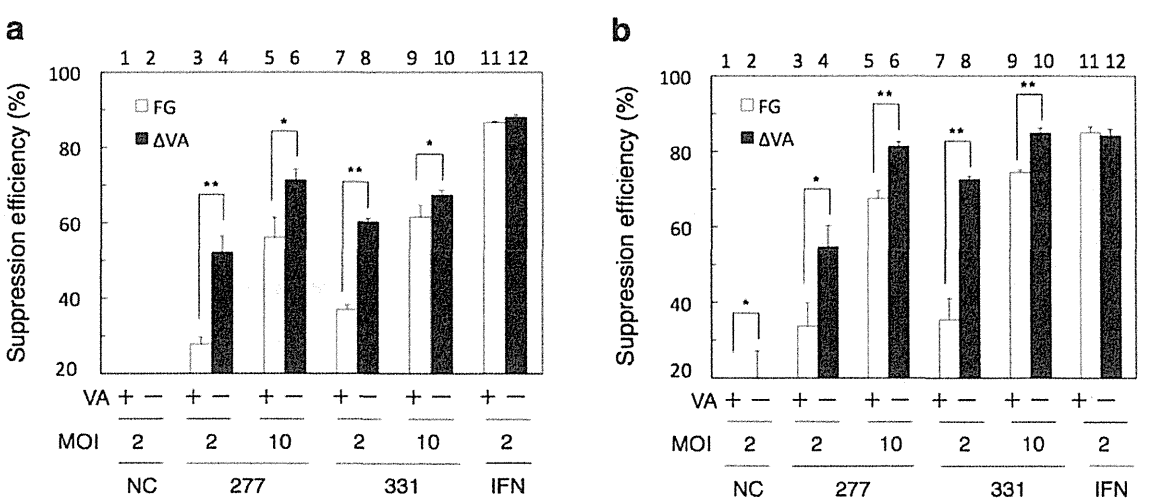

Figure 3 | Suppression of HCV RC RNA by the AdVs expressing sh277 and sh331. Effects on HCV RNA replication in HuH 5–15 cells (a) and SGR-JFH1 cells (b). The cells were infected with FG AdVs (FG, white bars) and VA-deleted AdVs (VA-del, black bars). NC, AdVs expressing negative-control shRNA. The copy numbers of intracellular HCV RNA were measured at 72 h after infection. The suppression efficiency was calculated relative to the copy numbers in uninfected cells as 0%; the copy numbers of HCV RNA in the control, uninfected cells were 4.0×10^4 copies/cell and 1.1×10^4 copies/cell in HuH 5–15 cells (a) and SGR-JFH1 cells (b), respectively. The suppression efficiencies of NC FG AdV and NC VA-deleted AdV were (a) 3.8 (±6.7) and 15.1 (±4.4), while those were (b) −4.1 (±3.4) and 18.2 (±9.0), respectively. Each data point represents an average of n = 3, mean ± S.D. (error bars). $^*P < 0.05$, $^{**}P < 0.01$.

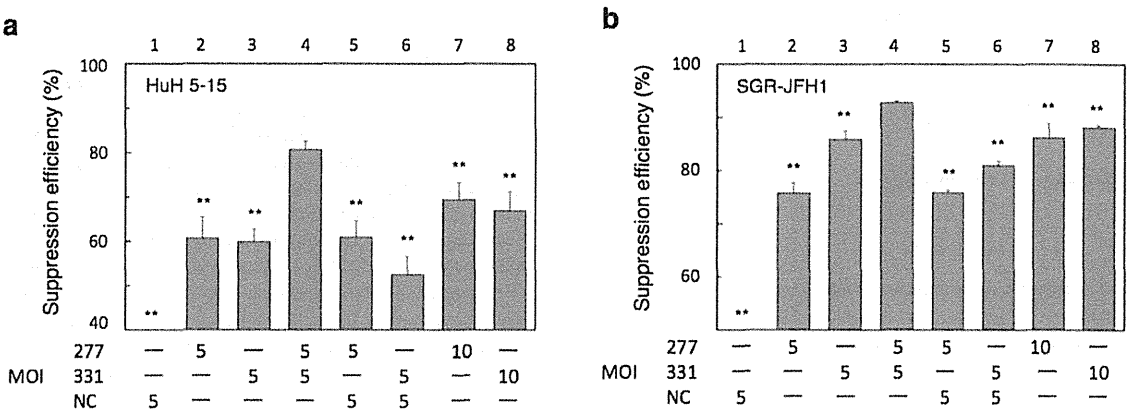

Figure 4 | Suppression of HCV RC RNA by double infection of the shRNA-expressing AdVs. HuH 5–15 cells (a) and SGR-JFH1 cells (b) were infected with the VA-deleted AdVs expressing shRNAs and, three days later, the intracellular RNA levels of HCV RC were measured. The copy numbers of HCV RC were 5.7×10^4 copies/cell and 1.4×10^4 copies/cell in the HuH 5–15 cells (a) and the SGR-JFH1 cells (b), respectively. The suppression efficiencies of NC FG AdV and NC VA-deleted AdV were (a) 8.7 (±13.6) and (b) 18.7 (±2.0), respectively. $^{**}P < 0.01$ against the value of the coinfection (bar 6) (a), (b). The other presentations are the same as in Fig. 3.

These results were confirmed using SGR-JFH1 (Fig. 4b; compare bar 4 with bars 7 and 8). Interestingly, the coinfection of AdV expressing commercially available control shRNA appeared to decrease the activity of sh331 (Fig. 4a and 4b, bars 3 and 6) but not that of sh277 (Figs. 4a and 4b, bars 2 and 5) in both cell lines. These results might suggest that the control shRNA might compete with sh331, but not with sh277, at some step in siRNA processing.

VA-deleted AdVs expressing shRNA or IFN efficiently suppressed HCV replication in HCV-infected cells. The anti-HCV activity of AdVs expressing shRNA or IFN was further investigated in cells infected with the HCV JFH-1 strain[22]. The cells were infected with VA-deleted AdVs and FG AdVs expressing sh331, sh277, or IFN. Three days later, the HCV RNA copy number was measured (Fig. 5a). The VA-deleted AdVs expressing sh277 or sh331 exhibited higher anti-HCV activities than the FG AdVs with only one exception: for sh277-expressing AdVs at MOI 10, suppression efficiencies were similar between VA-deleted AdVs and FG AdVs (bars 5 and 6), though at MOI 2 VA-deleted AdV was significantly more efficient than that of FG AdV (bars 3 and 4). The results might be explained that the effect of sh277 may be saturated at MOI 10, because the copy numbers of HCV genome were very low (figure legends of Fig. 5).

The expression of the HCV protein NS5A in the cells was determined using immunoblotting (Fig. 5b). The level of NS5A protein in the cells infected with the VA-deleted AdV expressing sh331 was nearly undetectable and was comparable with those for the cells infected with FG AdV and VA-deleted AdV expressing IFN (lanes 3, 7 and 8). Meanwhile, as for sh277 NS5A protein was significantly less when using VA-deleted AdV than that using FG AdV (lanes 5 and 6). However, the difference was not evident compared with sh331 correlating with the suppression efficiency of sh277 at MOI 2 and MOI 10 (Fig. 5a, bars 3 to 6). Altogether, these results demonstrated that, although a limited anti-HCV activity was obtained using

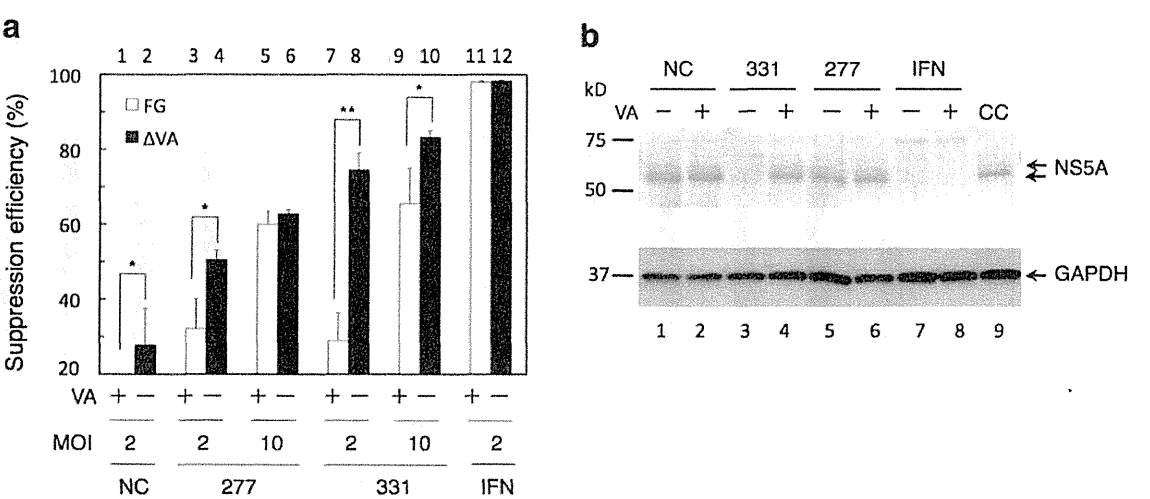

Figure 5 | Suppression of replicating HCV genome and the expressed proteins in the HCV-infected HuH cells. (a) Suppression levels of HCV genomes. HCV-infected HuH-7 cells were infected with shRNA-expressing AdVs and interferon-expressing AdVs at MOI 2 (bars 1 to 4, 7, 8, 11 and 12) or at MOI 10 (bars 5, 6, 9 and 10). The copy numbers of HCV RNA were 1.0×10^3 copies/cell. The suppression efficiencies of NC FG AdV and NC VA-deleted AdV were 3.7 (±5.5) and 27.8 (±9.7), respectively. The other presentations are the same as in Fig. 3. (b) Western blot analysis of HCV NS5A protein expressed in the viral-infected cells. Three days after the AdV infection at MOI 10, HCV NS5A and GAPDH proteins were detected using specific antibodies. CC, Cells infected with HCV but not with AdVs expressing the shRNAs. Arrows for NS5A indicate its hyper- (upper) and hypo- (lower) phosphorylated forms.

the FG AdV, a more efficient shRNA-mediated inhibition of HCV replication was achieved using the VA-deleted AdV.

Discussion

We showed here that the highest shRNA activity was obtained when the shRNA-expressing cassette was Inserted at the E4-insertion site in the leftward orientation. This site is located upstream of the E4 promoter and is very close to the right terminal of the vector genome[23]. The above results appear to be true for the FG AdVs that were used in our preliminary experiments. Although the E4 site offered the best shRNA activity, the E1 insertion site is commonly used for the insertion of shRNA cassettes. In contrast to the above-mentioned findings for shRNAs, the expressions of transgenes under the control of the CMV, CAG, and EF1α promoters at the E4 site were lower than those of transgenes under the control of these promoters at the E1 site (MS and YK, manuscript in preparation). In the present study, we used a polymerase III promoter of the human U6 promoter. A specific sequence that enhances this particular promoter might exist near the E4 site. Alternatively, differences in the position effect between polymerases II and III might explain this difference, at least in part. In either case, the above information is valuable for designing more effective shRNA strategies using AdVs both *in vitro* and *in vivo*, including strategies for gene therapy. Notably, the present results suggest that, when one wants to express a transgene product and an shRNA simultaneously, the best AdV is likely to contain the transgene at the E1 position and an shRNA cassette at the E4L position/orientation.

We showed that VA RNAs expressed from currently used FG AdVs decreased the activity of shRNA in the assay system for HCV replication. Because VA RNAs are processed and produce miRNAs, this result can be explained by competition with the shRNAs that are processed using the same pathway. The results also indicated that VA-deleted AdVs are a more efficient vehicle for shRNA strategies than FG AdVs, at least for the suppression of HCV replication. The advantage of using VA-deleted AdVs may not be restricted to the HCV system. Our preliminary experiment showed that, when using a commercially available anti-GFP shRNA, the suppression efficiency of the VA-deleted AdV was slightly higher than that of the FG AdV (85% ± 1.1% and 77% ± 4.1%, respectively) at MOI 200. This level was similar when using SGR-JFH1cells and shRNAs of sh277 and sh331 at MOI 10 (for example, Figs. 3b and 5a, bars 5, 6, 9 and 10). The difference in the suppression efficiency was much more evident when measuring HCV subgenomic and genomic RNAs at MOI 2 (Figs. 3 and 5a, bars 3, 4, 7 and 8). The reason for this finding may be that HCV RNAs are self-replicating and possibly amplified differences in the suppression efficiency, especially in the early step of replication. Alternatively, VA RNAs might increase HCV replication by interacting with some regulation factors, such as mir122. In any case, we demonstrated, for the first time, that VA RNAs expressed in FG AdV did reduce the shRNA activity, at least for HCV RNA.

VA-deleted AdVs are useful not only for shRNA strategies, but also for other purposes, since VA RNAs disturb the RNAi machinery. VA-deleted AdVs with a high titer nearly comparable to FG AdVs can now be obtained using the production method reported by Maekawa *et al.*[12]. This production efficiency is sufficient for practical use, even for large-scale preparations, and the production protocol is almost the same as that currently used for FG AdVs. FLP-expressing 293 cells (293hde12) are used in the last two passages to remove the VA RNA genes on the "pre-vector" genome. We expect that VA-deleted AdVs may become as prevalent as FG AdVs. In fact, we no longer use FG AdVs in our laboratory, having replaced them with VA-deleted AdVs instead.

The efficiency at which the HCV RNA genome is inhibited using sh277 and sh331 is almost comparable with that in cells infected with AxEF-IFN, an AdV expressing interferon under the control of the potent EF1α promoter (AM and YK, unpublished results). Because the inhibition mechanisms of shRNA and interferon differ, they are likely to work independently. Therefore, as noted above, VA-deleted AdVs containing both an IFN-expressing unit located at the E1 position and an anti-HCV shRNA at the E4L position/orientation might serve as powerful vectors for gene therapy for hepatitis C. Although high immune responses are a major concern with AdVs, we have reported that this problem can be overcome, at least in part, by avoiding the aberrant expression of the viral pIX gene arising from the use of the CAG or CMV promoter. We have called this type of AdV a "low inflammatory AdV"[24]. Thus, the combination of these improvements, i.e. VA-deleted, low-inflammatory AdVs expressing IFN and shRNA at the position/orientation described above, may yield an effective vector for gene therapy for chronic hepatitis C. The examination of this possibility is underway.

Methods

Cells and vector titration. FG AdVs were prepared using 293 cells[25], which are derived from human embryonic kidney and constitutively express adenoviral E1 genes and support the replication of E1-substituted AdVs. VA-deleted AdVs were prepared according to the method using 293hde12 cells described by Maekawa *et al.*[12] or using 293U6VA-1 cells that constitutively express both VAI and VAII. The VA-deleted AdVs in this study grew efficiently in this cell line. To establish 293U6VA-51 cell line we constructed a plasmid pU6VA-SVPur. This plasmid contains VAI and VAII genes located downstream of human U6 promoter and puromycin gene under the control of SV40 early promoter. 293 cells were transfected with pU6VA-SVPur and puromycin-resistant cell clones were isolated. Among these clones 293U6VA-51 expressed highest level of VA RNAs measured using qPCR primers described in Maekawa *et al.*[12] FC-18 cells[20] are derived from the monkey kidney cell line CV1 and conditionally express GFP: GFP expression can be turned off by infection with AxCAhFLPe, which expresses a codon-humanized FLPe.[26].

The human hepatocarcinoma cell line HuH-7 and its derived-HCV replicon cells were cultured in DMEM supplemented with nonessential amino acids and 10% fetal calf serum. HuH 5–15 cells support the replication of the subgenomic HCV replicon derived from the genotype 1b, Con 1 strain in HuH-7 cells[21]. pSGR-LucNeo-JFH1 plasmid was constructed by inserting the firefly luciferase gene into the 5′ end of the neomycin phosphotransferase gene of pSGR-JFH1 carrying subgenomic RNA from the genotype 2a, JFH-1 strain[27] and was introduced into HuH-7 cells. SGR-JFH1/LucNeo cells (simply called SGR-JFH1 in this paper) were developed after 1 month of culture with 0.5 mg/mL of G418-containing medium. The VA-deleted AdVs and FG AdVs were titrated using the methods described by Pei *et al.*[28]. Briefly, the copy numbers of a viral genome that was successfully transduced into infected target cells, (HeLa cells in this study) were measured using real-time PCR (relative virus titer: rVT). The titer of the standard virus was determined using the copy number of serially diluted plasmid DNA. When FG AdVs are used, the rVT (copies/mL) normally corresponds to about one fifth of the $TCID_{50}$ titer, when the gene product is not deleterious; the reason for this difference is probably that the transduction efficiency of 293 cells is exceptionally higher than that of the other cells.

Quantitative real-time PCR. The copy numbers of the expressed Cre RNA were quantified using the primers and the probe: forward primer, 5′-ATCCAGCAACAT-TTGGGCC-3′; probe, 5′-CGACAAGCAGAAGAACGGCATCAAGG-3′; reverse primer 5′-ACGACCAAGTGACAGC-3′. HCV RNA copies in the replicon cells and HCV-infected cells were determined using the primers and the probe: forward primer, 5′-GAGTGTCGTGCAGCCTCCA -3′; probe, 5′-CTGCTAGCCGAGT-AGTGTTGG -3′; reverse primer 5′-CACTCGCAAGCACCCTATCA -3′. The sequences of the primers and the probe for quantifying GFP RNA have been reported[12]. The total RNA of the infected cells was extracted, and the amounts of the expressed target RNA and 18S-rRNA (correction standard) were quantified using reverse-transcription and real-time PCR (Applied Biosystems Prism 7000), and the ratio of the target RNA to 18S-rRNA was then calculated. The qPCR reaction was performed according to the manufacturer's protocol: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min (Applied BioSystems).

Immunoblotting. Cells were lysed with the lysis buffer (1% Triton X-100, 25 mM Tris [pH7.5], 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktail and phosphatase inhibitor cocktail [Roche, Basel, Switzerland]) and were diluted 1 : 2 (v/v) with 3 × sampling buffer after removing the cell debris by centrifugation. The cell lysates were separated using SDS-PAGE and were transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA, USA). After blocking in 4% BlockAce (DS Pharma Biomedical, Osaka, Japan), the blots were incubated with the respective primary antibodies, followed by the secondary antibody in TBST (25 mM Tris, [pH7.5], 150 mM NaCl, and 0.1% Tween 20). The primary antibodies that were used were anti-NS5A antibody[29] and anti-GAPDH antibody (clone 6C5; Santa Cruz, CA, USA). Anti-rabbit IgG, HRP-linked antibody and anti-mouse IgG, HRP-linked antibody (Cell Signaling Technology, Danvers, MA, USA) were used as a secondary antibodies. Finally, the proteins were visualized using an

enhanced chemiluminescence (ECL) reagent (ECL Select Western Blotting Detection Reagent;, GE Healthcare, Little Chalfont, UK).

Construction of vector cosmids. All the AdVs described here lacked the expression of VAI and VAII and were constructed using a cosmid cassette, pAxdVw4c, derived from pAxcw containing the full-length AdV genome[30] except for the VAI and VAII genes, which were disrupted because of 15-nt and 17-nt deletions in their B-box sequences, respectively[12]. This cassette contains cloning sites for *Swa*I in the E1 region and for *Cla*I upstream of the E4 region[23]. A cassette expressing shRNA under the control of the human U6 promoter was purchased from Takara Bio. The AdV genome was excised with *Pac*I and was transfected into 293U6VA-51 cells. The cassette cosmids for construction of VA-deleted AdVs are described in Maekawa *et al*[12].

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Author contributions

Z.P. performed most of the experiments. G.S. and M.I. designed the experiments for evaluation of anti-HCV activity of AdV. S.K. discussed the experimental data and helped with troubleshooting. A.M. and M.S. constructed AdV-generating cosmids and continuously encouraged Z.P. I.S. and T.S. advised Z.P., G.S. and M.I. and wrote the manuscript. Y.K. organized this study and performed virological work. All authors read and approved the manuscript.

Additional information

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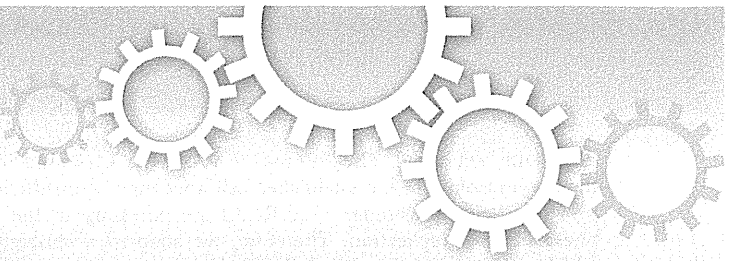

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Efficient production of adenovirus vector lacking genes of virus-associated RNAs that disturb cellular RNAi machinery

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First-generation adenovirus vectors (FG AdVs) are widely used in basic studies and gene therapy. However, virus-associated (VA) RNAs that act as small-interference RNAs are indeed transcribed from the vector genome. These VA RNAs can trigger the innate immune response. Moreover, VA RNAs are processed to functional viral miRNAs and disturb the expressions of numerous cellular genes. Therefore, VA-deleted AdVs lacking VA RNA genes would be advantageous for basic studies, both *in vitro* and *in vivo*. Here, we describe an efficient method of producing VA-deleted AdVs. First, a VA RNA-substituted "pre-vector" lacking the original VA RNA genes but alternatively possessing an intact VA RNA region flanked by a pair of FRTs was constructed. VA-deleted AdVs were efficiently obtained by infecting 293hde12 cells, which highly express FLP, with the pre-vector. The resulting transduction titers of VA-deleted AdVs were sufficient for practical use. Therefore, VA-deleted AdVs may be substitute for current FG AdV.

E1- and E3-deleted adenovirus vectors (AdVs) developed in the middle of 1990's[1,2] are commonly known as first-generation (FG) AdVs, and have been extensively used not only for the basic studies of various gene functions *in vitro* and *in vivo* but also for preclinical and clinical gene therapy. Because FG AdVs lack E1A gene, which is an essential transactivator in all other viral promoters driven by RNA polymerase II. Therefore, FG AdVs were usually considered that they do not express any viral gene products. However, FG AdVs, in fact, express VA RNAs that are transcribed by RNA polymerase III when using these vectors both *in vitro* and *in vivo*, because its activity is probably independent of RNA polymerase II.

VA RNAs, VAI and VAII, located at about 30 map units on adenovirus 5 (Ad5), are non-coding RNAs consisting of 157–160 nucleotides (nt). These VA RNAs are extremely abundant during the late phase of infection and inhibit cellular RNA-interference pathways by saturating Exportin 5 and Dicer[3]. Also, VAI inhibits protein kinase R (PKR) activity and, consequently, eliminates the block of the cellular translation machinery to allow the efficient production of viral proteins. Moreover, VA RNAs were processed and generate miRNAs[4,5], known as mivaRNAI and mivaRNAII that disturb the expression of many cellular genes, with the probable result of blocking cellular antiviral machinery. Therefore, there is no doubt that the AdVs lacking VA RNA genes are superior to current FG AdVs.

VAI and VAII are also transcribed not only during the early phase of Ad5 but also in E1A-deleted FG AdV. An E1-containing Ad5 mutant virus is reported which lack the expressions of both VAI and VAII and can proliferate in human cells, but its titers are approximately 60-fold lower than that of wild-type Ad5[6]. Therefore, VA RNAs are not essential, but play an important role in efficient viral growth by overcoming cellular antiviral machinery. Although the E1-containing mutant virus lacking expression of VA RNAs can slightly grow, efficient systems for producing E1-, E3- and VA-deleted AdVs (simply denoted VA-deleted AdVs) are extremely difficult to develop. One system for generating VA-deleted AdV expressing GFP using a 293 cell line that inducibly expresses the VAI gene has been reported[7]. However, the transduction titer produced using this system is approximately 1,000-fold lower than that of current FG AdVs. Furthermore, 18 days (including a secondary passage) are required before VA-deleted AdV are first observed, and the aid of expression markers, such as GFP or luciferase fluorescence, may be necessary to isolate the VA-deleted AdV. Therefore, this production system using a VA-expressing cell line is impractical for general use.

We hypothesized that, although VA RNAs are not essential for viral growth, VA-deleted AdV cannot grow during the initial step of vector generation, where only a few copies of the viral genome are present per cell, possibly because viral genes other than VA RNAs that block the cellular antiviral machinery may not be

sufficiently expressed. The amount of VA RNAs expressed from the chromosomes of the established cell lines may be insufficient, probably because abundant VA RNAs are necessary in the late phase of viral replication. Therefore, we adopted a strategy in which a VA-containing FG AdV (denoted pre-vector) is extensively amplified using 293 cells and the pre-vector is then converted to VA-deleted AdV through the removal of the VA genes using recombinase-expressing 293 cells to obtain a large amount of VA-deleted AdVs sufficient for the simultaneous introduction of numerous viral copies to the cells. However, this strategy appeared unrealizable because it requires the 293 cell line highly expressing the recombinase sufficient to remove VA RNA genes completely from rapidly replicating pre-vector genomes. We have established the 293 cell line, 293hde12[8], that contains the codon-humanized FLPe (hFLPe) that highly expresses thermo-stable FLPe recombinase[9]. We here showed that the VA genes present in the pre-vector genome were virtually completely removed using this cell line and high-titer VA-deleted AdVs were efficiently obtained.

Results

Efficient production of VA-deleted AdVs. We first constructed a VA-expressing DNA fragment, FVF, that contains intact VAI and VAII genes (Fig. 1a) flanked with a couple of FRTs (F refers to FRT in this study). The splicing acceptor site present between the initiation codon of the terminal protein precursor (pTP) and the 5′ end of VAI was disrupted by replacing T with C to prevent possible aberrant splicing. Then, we constructed two structurally different EF-AdV pre-vectors expressing GFP under the control of the EF1α promoter, AxdV-4FVF-GFP and AxdV-FVF-GFP, in which the original VAI and VAII genes were disrupted and, instead, the functional FVF fragment was inserted at different positions. The former pre-vector lacks both B-box sequences of the VAI and VAII that are essential for the activity of the internal polymerase-III promoter because of the deletions of 15 nt and 17 nt, respectively, (Fig. 1b), and instead bears the FVF fragment at the E4 insertion site near the right end of the viral genome[10] (Fig. 1a, upper left). The latter pre-vector lacks most of the VAI and VAII regions because of a deletion of 381 nt (Fig. 1c) and contains the FVF fragment within this region (Fig. 2a, lower left). Both pre-vectors grew well in the 293 cells, and their transduction titers were only slightly lower than that of the control FG AdV (Table 1, 37×10^7 and 60×10^7 versus 83×10^7 copies/mL).

Then, 293hde12 cells that constitutively and highly express codon-humanized FLPe recombinase[8,11] were infected with pre-vectors to obtain the first stock of VA-deleted AdV. The vector lacking VA RNA genes grew efficiently in the 293hde12 cells, similar to the pre-vector genome in 293 cells, because the VA RNAs were supplied from the excised circular DNAs consisting of VA RNA genes and one copy of FRT (Fig. 2a, middle) and from the pre-vector genome prior to the FLP-mediated excision. Then, five times more volume of the first stock than usual was used to infect the 293hde12 cells to create the second stock. The vector replicated in the 293hde12 cells, probably because a large amount of VA-deleted AdV were infected: the titer of the vector in the first stock was very high, and a much larger volume of stock was used for infection. The resulting vectors were named AxdV-4F-GFP and AxdV-F-GFP, respectively (Fig. 2a, upper and lower right). When the vectors in the second stock were used to infect 293hde12 cells, a minimal degree of replications was observed. Using a high multiplicity of infection (MOI), however, the slight replication was observed though the replication progressed slowly. Vectors from the second stock were used for further characterization.

The transduction titers of VA-deleted AdVs were sufficient for practical use. Because VA-deleted AdV is difficult to grow in 293 cells, the conventional titration method for measuring viral growth cannot be used. Therefore, the transduction titers[12] of the VA-deleted AdV and the pre-vector were measured; these titers show the copy number of viral genomes successfully transduced into infected target cells as evaluated using real-time PCR. The titers of VA-deleted AdV, pre-vector, and commonly used FG AdV can be compared using this method. The transduction titers of both AxdV-4F-GFP and AxdV-F-GFP were only one order lower than those of their pre-vectors (14% and 12%, respectively, Table 1), indicating that the titers of the VA-deleted vectors were sufficient for practical use as an alternative to FG AdVs. Moreover, using the same strategy, we produced other VA-deleted AdVs, such as AxdV-4F-NCre expressing Cre recombinase under the control of EF1α promoter and AxdV-F-SRChe expressing the Cherry marker under the control of the SRα promoter, and the resulting transduction titers were similar to those for AxdV-4F-GFP and AxdV-F-GFP (Table 1). Of note, the titer of Cre-expressing VA-deleted AdV was successfully obtained without trouble and was also quantitatively sufficient for general use, since Cre-expressing FG AdVs often exhibit a low titer and vector expansion is sometimes difficult.

Viral stocks of VA-deleted AdV likely contain a small amount of pre-virus that escaped the removal of the FVF fragment. Hence, HuH-7 cells were infected with AxdV-F-GFP and three days later, the transduced AdV DNA was examined using a Southern blot technique (Fig. 2b). The pre-vector DNA (2.7 kb) containing the VA genes clearly shifted to VA-deleted AdV DNA (2.2 kb), and no 2.7-kb band was detected in this assay. The result indicates that the VA RNAs in the pre-vector were efficiently removed during the replication of the pre-vector genome in 293hde12 cells. The same results were obtained when AxdV-4F-GFP was used (data not shown). The copy numbers of these viral genomes were examined using quantitative real-time PCR (qPCR) and VAI- and VAII-specific probes (Fig. 1a). Although the secondary structures of VAI and VAII are very similar, these probes were highly specific for one to the other (Supplementary Fig. S1). The pre-vector genome was present at approximately 1/90 or less, indicating that the purity of the VA-deleted AdV was about 99% or more.

Almost complete removal of contaminated pre-vector. To examine the level of VA RNAs expressed from contaminated pre-vector, the total cellular RNA of HuH-7 cells infected with AxdV-F-GFP was extracted and the expressed VA RNAs were analyzed using a northern blot technique (Fig. 2c). Neither VAI nor VAII were detected. In contrast, the pre-vector AxdV-FVF-GFP expressed considerable amounts of VAI and VAII. The same results were obtained when AxdV-4F-GFP was used (data not shown). The FG AdV AxCAGFP expressing GFP under control of CAG promoter does express similar amount of VA RNAs (data not shown). Expressed VAI and VAII RNA in cells infected with AxdV-4F-GFP or AxdV-F-GFP were also examined using qPCR after reverse transcription (Table 2). Both VAI and VAII were hardly detected, compared with the pre-vector infected in parallel, especially when using AxdV-4F-GFP, though 1% to 3% of the VA RNAs were detected when infected with AxdV-F-GFP. The Table 2 also showed that AxdV-4F-GFP and AxdV-F-GFP preparations expressed VA RNAI only about 1/300 and 1/120 less than FG AdV (0.02/5.97 and 0.05/5.97), respectively. These results correlated well with those of the Southern blot and qPCR analyses described above. Because contamination by the pre-vector of AxdV-4F-GFP was hardly detected using qPCR, an extremely sensitive bioassay was performed; 293 cells were infected with the VA-deleted AdV stock and the possible presences of pre-vector genome and VA RNAs expressed from the pre-vector were examined using Southern and northern blot techniques, respectively. Only the pre-vector genome must replicate efficiently in 293 cells. The amplified pre-vector genome was still not detected in a Southern blot analysis (Fig. 3a),

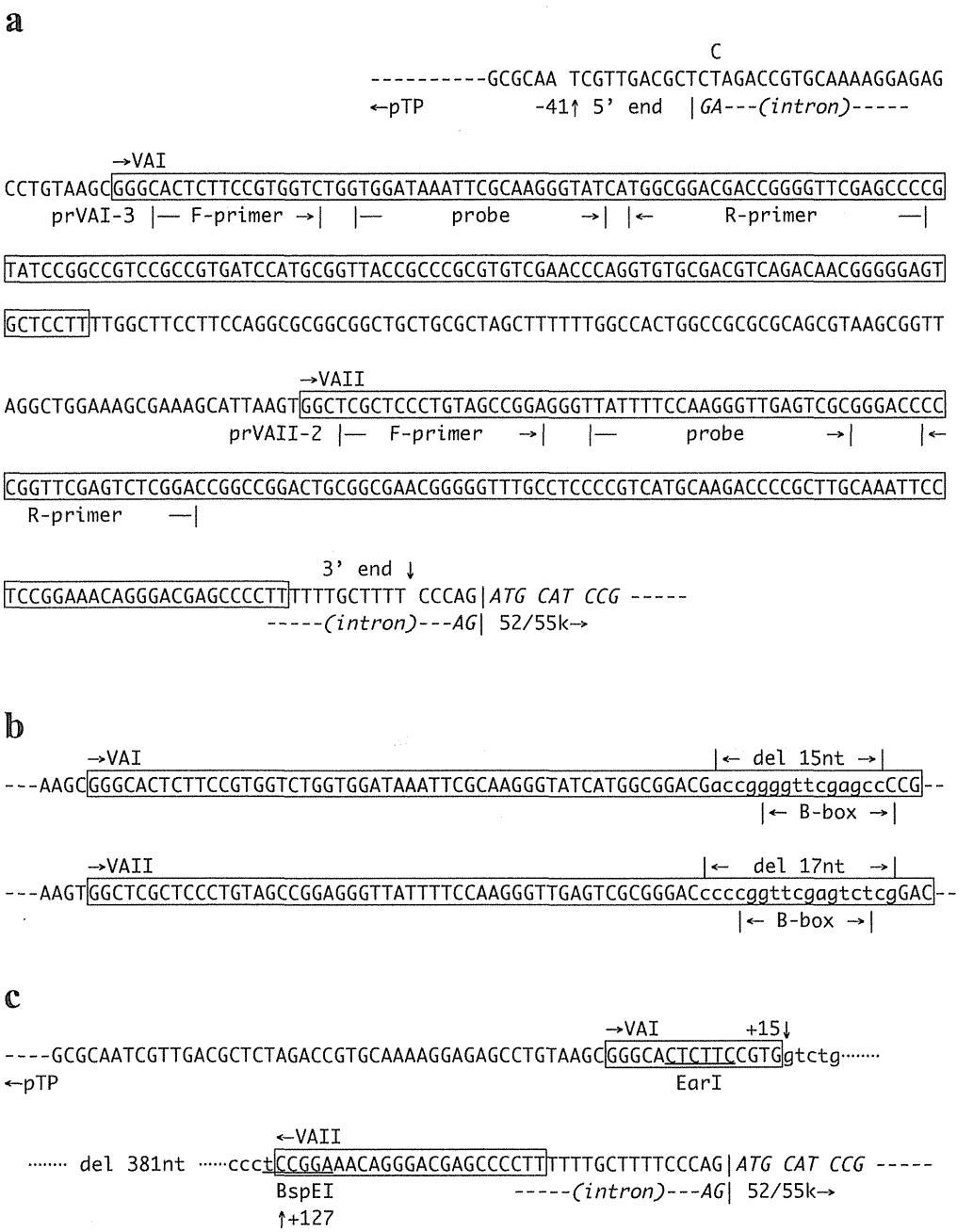

Figure 1 | Nucleotide sequences for VAI-VAII region. (a) VAI and VAII genes in the FVF fragment. VAI and VAII are boxed. prVAI-3 and prVAII-2 are the primers used for qPCR for the detection and quantification of VAI and VAII, respectively. Note that the actual R-primers are complementary to the sequences shown in this figure. pTP, terminal protein precursor; 52/55 k, 52 k and 55 k proteins. (b) B-box deletions of internal promoter in AxdV-4FVF-GFP and AxdV-4F-GFP. The deleted nucleotides are shown in small letters. (c) VAI-VAII deletion in AxdV-FVF-GFP and AxdV-F-GFP. The deleted region is shown in small letters. The deletion of 381 nt starts at 15 nt and 127 nt downstream from the 5′ end of VAI and VAII, respectively.

whereas the VA RNAs were slightly detected using a northern blot technique (Fig. 3b). Although the experiments using 293 cells were not quantitative, these results suggested that the amount of VA RNAs expressed from contaminated pre-vector in target cells other than 293 cells was minimal when the second stock was used. These results indicated that the VA-deleted AdVs produced using this production system were almost pure and may be used without consideration of the effect of VA RNAs in most studies. The contaminated circular DNA containing the VA-RNA genes was completely removed by purification of the vector.

To examine whether the VA-deleted AdV expresses a gene product as efficiently as FG AdV, HeLa cells were infected with VA-deleted AdVs and pre-vectors expressing GFP at the same transduction unit value and the resulting fluorescence was measured (Table 3). The VA-deleted AdVs showed similar expression levels to those of their pre-vectors.

Discussion

The efficient removal of VA RNA genes from the pre-virus genomes is crucial for this strategy. We here showed that 293hde12 cell line

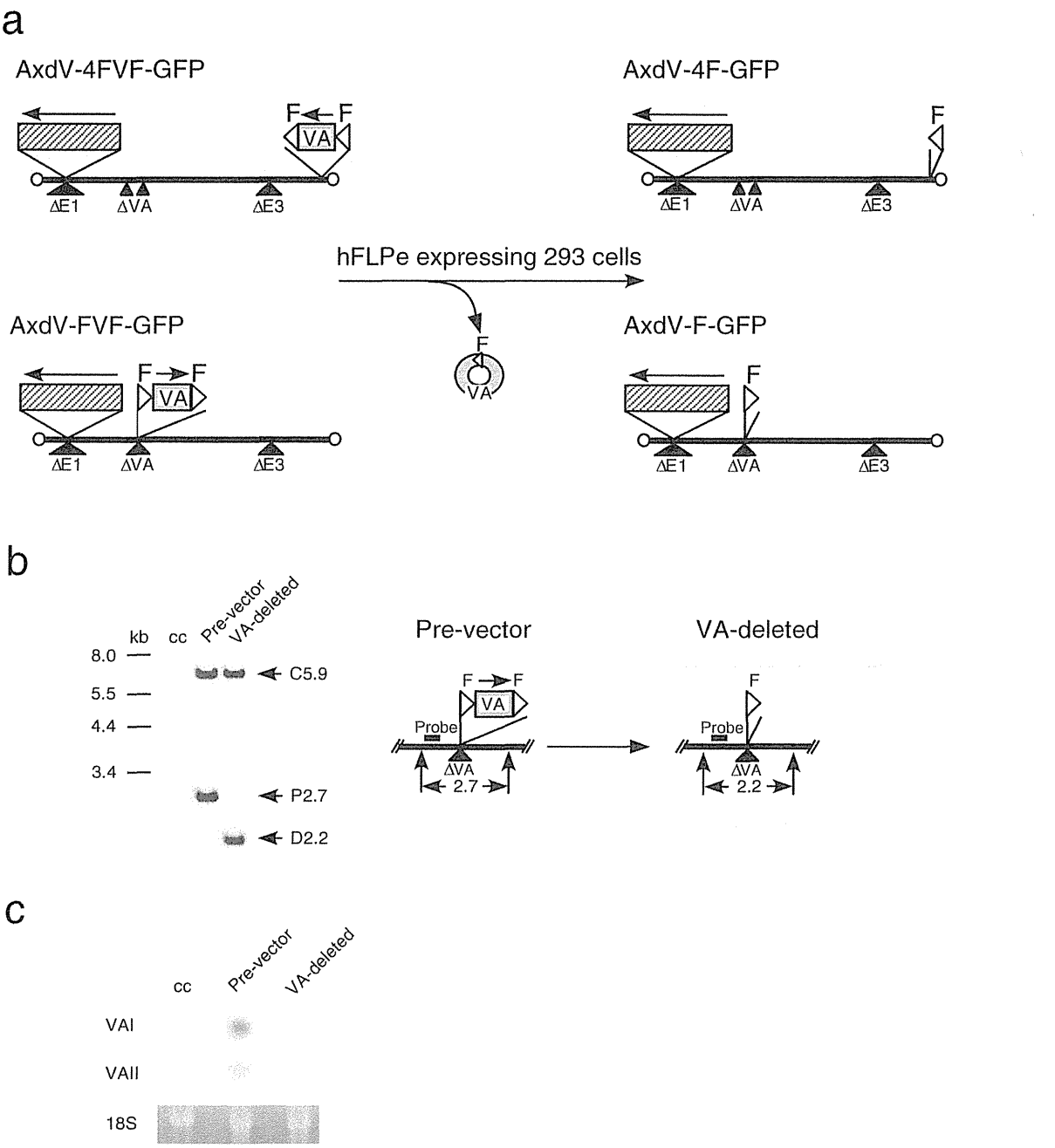

Figure 2 | Structures of pre-vectors and VA-deleted AdVs. (a) Strategy for the generation of VA-deleted vectors. F, FRT. Hatched box, GFP expression unit including EF1α promoter. An arrow shows the orientation of the transcription. (b) Removal of FVF fragment from the pre-vector AxdV-FVF-GFP genome. Total DNAs of infected HuH-7 cells were digested with *Kpn*I, and the DNA fragment containing FVF and F was detected using a Southern blot technique using the probe shown in the figure (enzyme, positions). C5.9 refers to the 5.9-kb fragment derived from outside of this region (1.7-kb *Bgl*II fragment, nt 30823-82495), showing that the amounts of the vector genomes were almost the same. cc, uninfected control cells; P, pre-vector; D, VA-deleted AdV. (c) Detection of expressed VA RNAs. Total RNAs of the same infected HuH-7 cells were analyzed using a northern blot technique. 18 S, 18 S ribosomal RNA.

that highly and constitutively express FLP recombinase was sufficient for this purpose. The cell lines constitutively expressing Cre have been used for production of helper-dependent AdVs, which contain a large DNA up to about 30kb. The viral packaging region of the helper virus must be excised out from the replicating viral genome but the excision efficiency is less than that described here[13,14] and hence the contaminated helper virus must be removed by the buoyant centrifugation using cesium chloride. Probably such incomplete excision might result from the toxicity of highly expressed Cre in 293 cells[15–18], while no toxicity of FLP has so far been reported. Therefore, 293hde12 cells played the most important role in this production system.

Furthermore, we demonstrated in this work that the AdVs containing two FRT sequences at the VA RNA region in the viral

Table 1 | Transduction titers of VA-deleted AdVs

AdV	Type	Transduction titer copies/mL ($\times 10^7$)	Ratio (%)
AxdV-4FVF-GFP	pre-vector	37	100
AxdV-4F-GFP	VA-deleted	5	14
AxdV-FVF-GFP	pre-vector	60	100
AxdV-F-GFP	VA-deleted	7	12
AxdV-4FVF-NCre	pre-vector	47	100
AxdV-4F-NCre	VA-deleted	4	8
AxdV-FVF-SRChe	pre-vector	20	100
AxdV-F-SRChe	VA-deleted	2	12

The transduction titer of the control FG AdV, AxEFGFP, was 83×10^7 copies/mL. The transduction titer was called the relative vector titer (rVT)[12], which is about five-times lower than the conventional $TCID_{50}$ titer of the pre-virus measured using 293 cells (see Methods).

genome, AxdV-F-GFP etc., were viable and their titers were comparable to FG AdV. When a foreign DNA sequence is inserted into the AdV genome, even when the sequence is short such as recombination targets, the AdVs are often not viable or their titers are very low, probably because the small insertion influences on the replication of the AdV genome. For this reason only three positions, i.e. as the substitutions of E1 and E3 genes and the insertion upstream of E4 genes, are generally used. Hence, we first constructed AxdV-4F-GFP because we knew that the AdVs containing foreign DNA at the E4 insertion position was very stable shown by our early works[1,19]. Then, we tried the original site of VA RNA genes and demonstrated that the FRTs can successfully be inserted at the VA RNA region causing only slight reduction of the AdV titers.

This production system described here may allow the practical application of VA-deleted AdVs as a possible alternative to current FG AdVs, since the transduction titer was only one order lower than that of FG AdVs and, in fact, we have already produced more than twenty VA-deleted AdVs expressing various genes without any difficulty. Because VA RNAs may disturb experimental results that are obtained using FG AdVs to some extent, VA-deleted AdVs are likely to be preferable for many types of basic research using adenovirus vectors. When examining the influences of VA RNAs on previously obtained results, the pre-vector can be used as an ideal control for VA-deleted AdVs. Although the target genes of VA RNAs are not clear at present, Aparicio *et al.*[5] reported that the expressions of many genes including TIA-1, a splicing and translation regulator, are downregulated by mivaRNAs, which are the processed products of VA RNAs, and they proposed that TIA-1 may be one such target of mivaRNAs. We compared the expression of TIA-1 using a VA-deleted AdV and its pre-vector and observed that TIA-1 expression was slightly downregulated but was not statistically significant in our assay system. Because they did not use a VA-deleted vector but rather E1-containing viruses that efficiently replicate in infected cells and probably express much more VA RNAs. Therefore, their results are not necessarily contradictory and may be explained by the quantitative differences of expressed VA RNAs. We found other genes that were downregulated by VA RNAs (YK, unpublished results). Studies on the influences of VA RNAs on immune responses *in vivo* are also underway. So far, we found that the immune response caused by VA RNA was lower than that by aberrantly expressed viral pIX protein[20] but quantitative analyses are needed to clarify this issue. Notably, VA-deleted AdVs are probably superior to the current FG AdV for researches involving AdVs expressing siRNA and miRNA, since VA RNAs expressed by FG AdVs may compete with them and disturb the effects of these RNAs[3]. In fact, an shRNA expressed using VA-deleted AdV was more effective than that using current FG AdV (manuscript in preparation). These small-RNA technologies are extensively used in various research fields including signal transduction, cell differentiation and iPS study. Because more than three hundred papers have been published so far using FG AdVs expressing siRNA or miRNA based on our PubMed searching, VA-deleted AdV may be valuable in these fields.

The production level may be sufficient to apply VA-deleted AdVs to gene therapy as a safer alternative to existing practices. To what extent VA-deleted AdVs reduce immune responses in gene therapy of humans remain to be elucidated. Another possible contribution to gene therapy using AdVs is that, VA-deleted AdVs may considerably reduce contamination with replication-competent AdVs (RCA), one of the problems in the production of gene-therapy grade AdVs, because even if VA-deleted RCA is generated, only very low levels of replication would occur in human cells. Moreover, as proposed by Carnero *et al.*[4], a VA-deleted replication-competent adenovirus produced using this method could be used as an oncolytic virus that specifically replicate in cells with inactive PKR, such as tumor cells with activated ras or Epstein-Barr virus.

The same strategy described here could also be applied to studies of other DNA viruses, such as herpes viruses, because drug-resistant cell lines that highly express viral genes essential for the viral life cycle are often difficult to obtain because of their slight toxicity to cell growth. Moreover, using this strategy and recombinase-expressing cells, two or more genes could be deleted simultaneously using mutant *lox*P 2272[21] and FRT mutants[22,23] that exclusively recombine two identical mutant recombinase-targets. In conclusion, this strategy may accelerate studies using adenovirus vectors and may contribute to gene therapy.

Methods

Cells and virus titration. Human cell lines of 293[24], HeLa and HuH-7 are derived from the embryonic kidney, the cervical carcinoma and the hepatocellular carcinoma, respectively. These cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS). The 293 cells constitutively express adenoviral E1 genes and support the replication of E1-substituted AdVs. 293hde12 is a 293 cell line possessing the hFLPe gene[8], an improved version of the FLPe gene[9], in which the codon usage has been changed to that used in humans and which produces more FLPe enzyme. 293hde12 cells were cultured in DMEM supplemented with 10% FCS plus geneticin (0.75 mg/mL). After infection with AdVs, the cells were maintained in DMEM supplemented with 5% FCS without geneticin.

All the AdVs were titrated using the method of transduction titer known as the relative vector titer (copies/mL)[12]. In this titration method, the copy number of viral genomes successfully transduced into the infected target cells, such as HeLa or HuH-7 cells, are measured using real-time PCR. The transduction titer method can be used not only for VA-deleted AdV, but also for FG AdVs, including the pre-vectors. This method enabled us to compare the various titers, since the transduction titer is not influenced by the growth rate of 293 cells, even if an expressed gene product (such as Cre or dsRed) is slightly deleterious to 293 cells[12]. When the gene product is not deleterious, the titer obtained using this method corresponds to about one fifth of that using either a 50% tissue culture infectious dose ($TCID_{50}$/mL)[25,26] or a plaque assay; the reason for this difference is probably because the transduction efficiency of 293 cells is much higher than that of other cells. The sequences of TaqMan probes for the titration (named AdV-1[10,12]) are derived from Ad5 pIX gene: forward primer, 5′-TGTGATGGGCTCCAGCATT-3′; probe, 5′-ATGGTCGCCCCGTCCTGCC-3′; reverse primer, 5′-TCGTAGGTCAAGGTAGTAGAGTTTGC-3′. A recommended protocol of the titration is available as Supplementary data of reference 12.

Table 2 | Expression levels of VAI and VAII RNAs measured using qPCR

		VA RNAs			
		I		II	
AdVs	Type	Copies $\times 10^8$	Ratio (%)	Copies $\times 10^8$	Ratio (%)
AxdV-4FVF-GFP	pre-vector	4.47 ± 0.81	100	1.36 ± 0.15	100
AxdV-4F-GFP	VA-deleted	0.02 ± 0.00	<1	ND	<1
AxdV-FVF–GFP	pre-vector	1.81 ± 0.06	100	1.83 ± 0.32	100
AxdV-F-GFP	VA-deleted	0.05 ± 0.00	2.9	0.03 ± 0.00	1.4
AxCAGFP	control FG AdV	5.97 ± 0.83		4.17 ± 0.16	

HuH-7 cells were infected with 50 μL of conventional stock of the pre-vector and the second stock of VA-deleted AdV. The method for preparation of the second stock is described in the Method section.

Plasmids. The pVA41da plasmid contains a DNA fragment covering the entire VAI and VAII from nt position 10576–11034 (Fig. 1a) of adenovirus type 5. A splicing acceptor site upstream of the pTp gene was disrupted, as described in the text. The region of functional VAI and VAII was excised as a *Hin*dIII-*Xba*I fragment and inserted at a *Swa*I site of pUFwF[27]; the resulting plasmid and DNA fragment containing intact VAI and VAII flanked two FRTs were named pUFVA41daF and FVF fragment, respectively.

Vector construction. All the AdVs described here were constructed using the cosmid cassette pAxcwit2 containing the full-length AdV genome[28]. The pre-vector cassette pAxdV-4FVF-w (w refers to the SwaI cloning site at the E1 region, see below) possesses the AdV genome in which VAI and VAII genes are disrupted by 15-nt and 17-nt deletions in their B-box sequences, respectively (Fig. 1b). The AdV genome contains the FVF fragment at the *Sna*BI site (nt position 35770) located in the E4 region at 165-nt downstream from the right end of the Ad5 genome[10]. A GFP-expressing unit under the control of the EF1α promoter[29] was inserted into the *Swa*I cloning site at the authentic E1 substitution region to obtain the pre-vector cosmid pAxdV-4FVF-GFP. The EF1α promoter in the left orientation was adopted to express the GFP because the use of the promoter in this manner greatly reduces the immune response of AdV, compared with the CAG and CMV promoters[20,30]. The pre-vector AxdV-4FVF-NCre and VA-deleted AdV AxdV-4F-NCre possess identical structures to AxdV-4FVF-GFP and AxdV-4F-GFP, respectively, except that the GFP gene is replaced by a Cre recombinase gene tagged with a nuclear localization signal[31]. The other pre-vector cosmid cassette pAxdV-FVF-4c lacks the VAI and VAII genes because of a large deletion from nt 10635 to nt 11012 and instead carries the FVF fragment. Note that this cassette contains two cloning sites, the *Swa*I site in the E1 region and the *Cla*I site in the E4 region (as described above), and is useful for the simultaneous expression of two genes. The pre-vector cosmid pAxdV-FVF-GFP was obtained by inserting the GFP expression unit described above at the *Swa*I site. Also, another pre-vector cosmid pAxdV-FVF-4Che contains the Cherry marker gene (Clontech) under the control of an SRα promoter[32] at this *Cla*I site. Then, the pre-vector AxdV-FVF-GFP, the VA-deleted vector AxdV-F-GFP, the pre-vector AxdV-FVF-4SRChe, and the VA-deleted AdV, AxdV-F-4SRChe were obtained using these pre-vector cosmid cassettes. The procedure to produce VA-deleted AdVs was as follows: the pre-vector genome in the cosmid cassette described above was excised with *Pac*I and transfected into 293 cells. The obtained pre-vector was then amplified twice using 293 cells and was used to infect 293hde12 cells at 10 copies/cell of transduction multiplicity of infection (MOI) to obtain VA-deleted AdVs. Then, This VA-deleted AdVs were used to infect 293hde12 cells at 10 copies/cell. The resultant viral stock is called the second stock in the Result section.

Quantitative Real-time PCR. The RNA expressions of VAI and VAII were quantified using the primers and probes described in Fig. 1a. The sequences of the GFP primers were as follows:
forward primer, CTACAACAGCCACAACGTCTATATCA;
probe, CGACAAGCAGAAGAACGGCATCAAGG;
reverse primer, ATGTTGTTGCGGATCTTGAAG.
The total RNA of the infected cells was extracted, and the amounts of the expressed

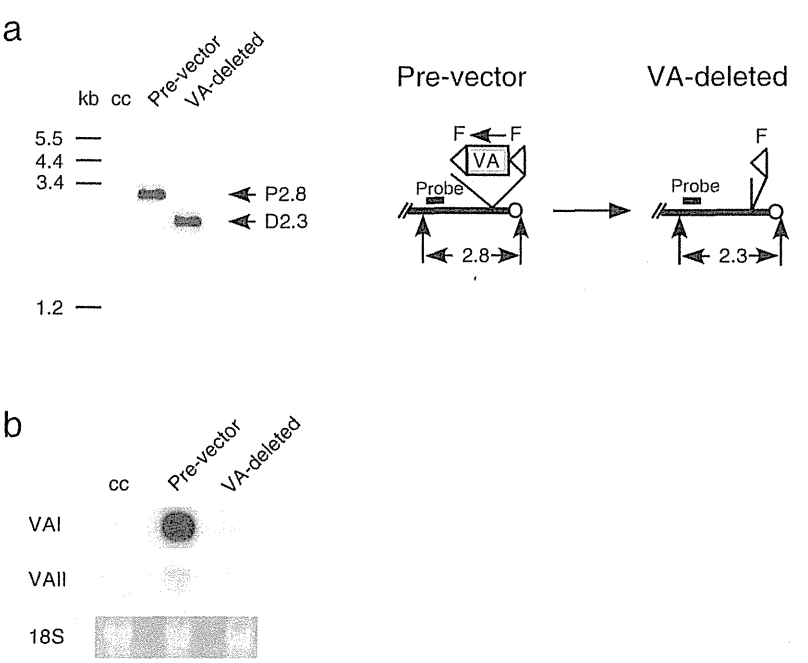

Figure 3 | Viral genome and expressed VA-RNA of contaminated pre-vector replicating in 293 cells. (a) Removal of the FVF fragment from the genome of the replicating pre-vector. Total DNAs of 293 cells infected with 15 μL of AxdV-4F-GFP stock were digested with *Eco*RV and the DNA fragment containing FVF and F was detected using a Southern blot technique. Other presentations are the same as in Fig. 1. (b) Detection of the expressed VA RNAs. Total RNAs of the same infected 293 cells were analyzed using a northern blot technique. 18 S, 18 S ribosomal RNA.

Table 3 | GFP expression in target cells infected with VA-deleted AdVs

AdV	Type	Ratio	
		fluorescence	expressed mRNA
AxdV-4FVF-GFP	pre-vector	1	1
AxdV-4F-GFP	VA-deleted	0.97 ± 0.15	0.81 ± 0.16
AxdV-FVF-GFP	pre-vector	1	1
AxdV-F-GFP	VA-deleted	1.14 ± 0.31	1.18 ± 0.20

HeLa cells were infected with pre-vector and VA-deleted AdV at a transduction MOI of 10. Three days later, GFP fluorescence was measured using a fluorometer and the GFP mRNA was quantified using reverse transcriptase and qPCR.

target RNA and 18S-rRNA (correction standard) were quantified using reverse-transcription and real-time PCR (Applied Biosystems Prism 7000); the ratio of the target RNA to 18S-rRNA was then calculated. The linear correlation between the amount of infected vector and the Ct values was confirmed (Supplementary Fig. S2). To quantify the DNA amount of VA RNAs and the AdV genome, the infected total cell DNA was prepared from cells using a previously described method[33,34] or a DNA preparation kit (Macherey-Nagel, through TaKaRa Bio). Quantitative PCR was performed to detect the AdV genome using a probe for the pIX gene described above[10,12]. The amount of chromosomal DNA was simultaneously measured to correct the Ct values of the viral genome per cell, and the corrected Ct was shown throughout. The probes were derived from the sequence of the human β-actin gene for HeLa and HuH7[10]. The qPCR reaction was performed according to the manufacturer's protocol: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min (Applied BioSystems).

Southern blotting analysis. HuH-7 cells in a 6-cm dish were infected with VA-deleted AdV and its pre-vector, and three days later the total DNA was prepared from the dish. Before alkaline treatment, the agarose gel was exposed to 0.1-N HCl for partial depurination, causing the DNA fragmentation of several hundred base pairs to obtain the complete transfer to the membrane[33]. The DNA was then transferred to the nylon membrane Hybond-N (Amersham GE) using the capillary-transfer method. Specific DNA was detected using a DIG DNA Labeling and Detection Kit (Roche Diagnostics). The probe DNA fragment derived from the viral genome was labeled with digoxigenin-UTP, and specific DNA was detected using the chemiluminescence of CDP-Star (Roche Diagnostics); the bands were visualized using LAS-4000 (Fuji Film).

Northern blotting analysis. The cells indicated in the figure legends were infected with pre-vector and VA-deleted AdV, respectively, at a transduction MOI of 10 copies/mL. The total RNA of the infected cells was extracted, and 30 μg per lane was electrophoresed in the agarose gel with tris-acetate-EDTA buffer. The RNA was transferred to the nylon membrane Hybond-N$^+$ (Amersham GE) using the capillary-transfer method. Specific RNA was detected using a DIGDNA Labeling and Detection Kit (Roche Diagnostics).

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Author contributions

A.M. performed the experiments and contributed to the writing of the manuscript. Z.P., M.S., H.F., Y.O. and S.K. performed the experiments. I.S. discussed the data and wrote the manuscript. Y.K. designed the strategies and performed the experiments. All the authors discussed the results and commented on the manuscript.

Additional information

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