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

Aya Maekawa, Zheng Pei, Mariko Suzuki, Hiromitsu Fukuda*, Yohei Ono, Saki Kondo, Izumu Saito & Yumi Kanegae

Laboratory of Molecular Genetics, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo, Japan.

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.

Received 17 November 2012
Accepted 27 December 2012
Published 25 January 2013

Correspondence and requests for materials should be addressed to Y.K. (kanegae@ims.u-tokyo.ac.jp)

* Current address: Japan Animal Referral Medical Center, 2-5-8 Kuij, Takatsu-ku, Kawasaki-shi, Kanagawa 213-0032, Japan.
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 unrealistic 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, that contains the codon-humanized FLPe (hFLPe) that highly expresses thermo-stable FLPe recombinase. 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 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 versus 83 × 10^7 copies/mL).

Then, 293hde12 cells that constitutively and highly express codon-humanized FLPe recombinase 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 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-SRCh 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 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),
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 AdVs 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
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\(^1\)\(^3\)\(^4\) 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\(^1\)\(^5\)\(^6\)\(^7\)\(^8\), 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

---

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 EF1\(\alpha\) 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 \(KpnI\), 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 \(BglII\) 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.
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\textsuperscript{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.\textsuperscript{5} reported that the expressions of many genes including TIA-1, a splicing and translation regulator, are down-regulated 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\textsuperscript{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 or miRNA, since VA RNAs expressed by FG AdVs may compete with them and disturb the effects of these RNAs. 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.\textsuperscript{5}, 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 loxP 2272 and FRT mutants\textsuperscript{20,21} that exclusively recombine two identical mutant recombine-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\textsuperscript{\textregistered}, HeLa and HuH-7 are derived from the embryonic kidney, the cervical carcinoma and the hepatocellular carcinoma, respectively. These cells were cultivated 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\textsuperscript{8}, an improved version of the FLPe gene, 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 genetin (0.75 mg/mL). After infection with AdVs, the cells were maintained in DMEM supplemented with 5% FCS without genetin.

All the AdVs were titrated using the method of transduction titer known as the relative vector titer (copies/mL)\textsuperscript{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 dRed) is slightly deleterious to 293 cells\textsuperscript{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\textsubscript{50}/mL)\textsuperscript{12,26} 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\textsuperscript{+}F\textsuperscript{+}F\textsuperscript{+})\textsuperscript{12,26} are derived from Ad5 pIX gene forward primer, reverse primer, 5'-TGTGATGGGCTCCAGCATT-3'; probe, 5'-ATGTGCGCCGCTTCTGCC-3'; reverse primer, 5'-TCGATGTCAGTGAGATGTTGC-3'. A recommended protocol of the titration is available as Supplementary data of reference 12.

| AdV               | Type         | Transduction titer copies/mL (×10\textsuperscript{3}) | Ratio (%) |
|-------------------|--------------|------------------------------------------------------|-----------|
| AxdV-4FV-GFP      | pre-vector   | 37                                                   | 100       |
| AxdV-4F-GFP       | VA-deleted   | 7                                                    | 12        |
| AxdV-FVR-GFP      | pre-vector   | 47                                                   | 100       |
| AxdV-4FNCre       | VA-deleted   | 2                                                    | 8         |
| AxdV-FVF-SRChe    | pre-vector   | 20                                                   | 100       |
| AxdV-5FR-SRChe    | VA-deleted   | 7                                                    | 12        |

The transduction titer of the control FG AdV, AxEFGFP, was 83 × 10\textsuperscript{3} copies/mL. The transduction titer was called the relative vector titer (VVT)\textsuperscript{14}, which is about five-times lower than the conventional TCID\textsubscript{50} titer of the pre-virus measured using 293 cells (see Methods).
**Table 2 | Expression levels of VAI and VAII RNAs measured using qPCR**

| AdVs Type          | VA RNAs | 
|--------------------|---------| 
|                    | Copies × 10^6 | Ratio (%) | Copies × 10^6 | Ratio (%) |
| AxdV-4FVF-GFP      | 4.47 ± 0.81  | 100       | 1.36 ± 0.15  | 100       |
| AxdV-4F-GFP        | 0.02 ± 0.00  | <1        | ND           | <1        |
| AxdV-FVF–GFP       | 1.81 ± 0.06  | 100       | 1.83 ± 0.32  | 100       |
| AxdVF-GFP          | 0.05 ± 0.00  | 2.9       | 0.03 ± 0.00  | 1.4       |
| AxCAGFP            | 3.97 ± 0.83  |           | 4.17 ± 0.16  |           |

HuH7 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 HindIII-XbaI fragment and inserted at a SwaI site of pUFwF27; the resulting plasmid and DNA fragment containing intact VAI and VAII flanked two FRTs were named pUFVA41daF 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 HindIII-XbaI fragment and inserted at a SwaI site of pUFwF27; the resulting plasmid and DNA fragment containing intact VAI and VAII flanked two FRTs were named pUFVA41daF and pUFVA41daI.

**Vector construction.** All the AdVs described here were constructed using the cosmid cassette pAxcwit2 containing the full-length AdV genome. 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 SnaBI site of pUFwF27; the resulting plasmid and DNA fragment containing intact VAI and VAII flanked two FRTs were named pUFVA41daF and pUFVA41daI.

**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, CTACAACAGCCAACGCTCTATATCA; reverse primer, ATGGTGTGCGGATCCTTGAAG. The total RNA of the infected cells was extracted, and the amounts of the expressed VA RNAs were measured using qPCR.

**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 EcoRV 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. 18S, 18S ribosomal RNA.
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 method33,34 or a DNA preparation kit (Macherey-Nagel, through genome, the infected total cell DNA was prepared from cells using a previously described method). From the sequence of the human β-actin gene for HeLa and HuH7. 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.** HuH7 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 30 min to obtain the complete transfer to the membrane33. The DNA was then transferred to the nylon membrane Hybond-N (Amersham GE) using the capillary-transfer method. Specific DNA was detected using 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 DIG DNA Labeling and Detection Kit (Roche Diagnostics). The probe DNA fragment derived from the viral genome was labeled with digoxigenin-UTP, and specific RNA was detected using the chemiluminescence of CDP-Star (Roche Diagnostics); the bands were visualized using LAS-4000 (Fuji Film).

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

| AdV | Type | fluorescence | expressed mRNA |
|-----|------|--------------|---------------|
| AxdV4PFV-GFP | pre-vector | 1 | 1 |
| AxdV4PFV-GFP | VA-deleted | 0.97 ± 0.15 | 0.81 ± 0.16 |
| AxdVFVF-GFP | pre-vector | 1 | 1 |
| AxdVFVF-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.

1. Miyake, S. et al. Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. *Proc. Natl. Acad. Sci. U S A* 93, 1320–1324 (1996).
2. Anton, M. & Graham, F. L. Site-specific recombination mediated by an adenovirus vector expressing the Cre recombinase protein: a molecular switch for control of gene expression. *J. Virol.* 69, 4600–4606 (1995).
3. Lu, S. & Cullen, B. R. Adenovirus VAI noncoding RNA can inhibit small RNA interference and MicroRNA biogenesis. *J. Virol.* 78, 1268–12678 (2004).
4. Carrero, E., Sutherland, J. D. & Fortes, P. Adenovirus and miRNAs. *Biochim. Biophys. Acta* 1809, 660–667 (2011).
5. Aparicio, O. et al. Adenovirus VA RNA-derived miRNAs target cellular genes involved in cell growth, gene expression and DNA repair. *Nucleic Acids Res* 38, 750–763 (2010).
6. Bhat, R. A., Domer, P. H. & Thimmappaya, B. Structural requirements of adenovirus VAI RNA for its translation enhancement function. *Mol. Cell Biol.* 5, 187–196 (1985).
7. Machitani, M. et al. Development of an adenovirus vector lacking the expression of virus-associated RNAs. *J. Control Release* 154, 285–289 (2011).
8. Takata, Y., Kondo, S., Goda, N., Kanegae, Y. & Saito, I. Comparison of efficiency between FLPs and Cre for recombinase-mediated cassette exchange in vitro and in adenovirus vector production. *Genes Cells* 16, 765–777 (2011).
9. Buchholz, F., Angrand, P. O. & Fortes, P. Efficient properties of FLp recombinase evolved by cycling mutagenesis. *Nat. Biotechnol.* 16, 657–662 (1998).
10. Kanegae, Y. et al. High-level expression by tissue/cancer-specific promoter with strict specificity using a single-adenoviral vector. *Nucleic Acids Res.* 39, 67 (2011).
11. Kondo, S., Takata, Y., Nakano, M., Saito, I. & Kanegae, Y. Activities of various FLP recombinases expressed by adenovirus vectors in mammalian cells. *J. Mol. Biol.* 390, 221–230 (2009).
34. Nakano, M. et al. Production of viral vectors using recombinase-mediated cassette exchange. *Nucleic Acids Res.* 33, e76 (2005).

**Acknowledgements**

We thank Ms M. Terashima for excellent technical support and Ms E. Kondo for secretarial assistance. This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology to Y.K. and S.K. and the Ministry of Health, Labour and welfare ; by Research on the innovative development and the practical application of new drugs for hepatitis B to I.S.

**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**

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

**Competing financial interests:** The authors declare no competing financial interests.

**License:** This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/3.0/

**How to cite this article:** Maekawa, A. et al. Efficient production of adenovirus vector lacking genes of virus-associated RNAs that disturb cellular RNAi machinery. *Sci. Rep.* 3, 1136; DOI:10.1038/srep01136 (2013).