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Quantitative Analysis of Virus-associated RNAI Expression following Transduction with a Replication-incompetent Adenovirus Vector *In Vitro* and *In Vivo*

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Abstract

The adenovirus (Ad) genome encodes one or two non-coding small RNAs called virus-associated (VA)-RNAs, that are transcribed by polymerase III and support Ad replication. As previously reported, a replication-incompetent Ad vector, which is widely used in not only gene therapy studies, including clinical trials, but also basic researches as a gene delivery vehicle, as well as wild-type Ad (WT-Ad) express VA-RNAs, and VA-RNAs activate innate immunity, including the production of type I interferons. In addition, VA-RNAs perturb cellular microRNA (miRNA) expression profiles via competitive inhibition of key components involved in the miRNA maturation pathway. Although these characteristics of VA-RNAs might negatively affect the application of Ad vectors, VA-RNA expression profiles following transduction with an Ad vector have been not fully examined. In this study, we quantitatively analyzed the expression profiles of VA-RNAI, which is a major species of VA-RNAs, following transduction with Ad vectors *in vitro* and *in vivo* using real-time RT-PCR. The VA-RNAI expression levels in the cells transduced with a conventional Ad vector expressing luciferase (Ad-CAL2) at a multiplicity of infection (MOI) of 100 were approximately 2000- to 3000-fold lower than those infected with WT-Ad at the same MOI at 48 h after treatment. The expression levels of VA-RNAI in the mouse liver following administration with Ad-CAL2 were approximately 600-fold lower than those following administration with WT-Ad at 48 h post-administration. miRNA-mediated suppression of leaky expression of the Ad E4 genes resulted in about five-fold reduction in the VA-RNAI copy numbers in the liver following systemic administration in mice. These data provide informative clues for the development of novel safer Ad vectors.

Keywords: Adenovirus vector; VA-RNA; Real-time RT-PCR; Gene therapy

Introduction

A replication-incompetent adenovirus (Ad) vector is one of the most promising vectors for gene therapy as well as basic research thanks to several advantages as a gene delivery vehicle, such as high-titer production and highly efficient transduction into a variety of dividing and non-dividing cells *in vitro* and in vivo. However, it is well known that systemic administration of Ad vectors causes innate and adaptive immune responses, leading to multi-tissue damage, including hepatotoxicity [1,2]. Although adaptive immune responses following administration with Ad vectors has been relatively well studied [3], the mechanisms underlying Ad-induced innate immune responses have remained to be clarified. Several groups, including ours, have reported that Ad components, including capsid proteins, Ad genome DNA, and transcripts, are recognized by cellular receptors and sensors, leading to the induction of innate immune responses [4-9].

Recently, we and Minamitani et al. [10] demonstrated that a conventional replication-incompetent Ad vector as well as wild-type Ad (WT-Ad) each express two small RNAs (virus-associated RNAs: VA-RNAI and II), which are approximately 160nt-long non-coding RNAs transcribed by RNA polymerase III, and that VA-RNAs induce innate immune responses via the retinoic acid-inducible gene I (RIG-I) and/or interferon (IFN)-beta promoter stimulator-1 (IPS-1) pathway [8,10]. In addition, VA-RNAs inhibit microRNA (miRNA) production by competitive inhibition of key components involved in the miRNA processing pathway, such as exportin-5, dicer, and argonaute 2 [11,12]. These characteristics of VA-RNAs might contribute to Ad vector-induced hepatotoxicities, which are main side effects of systemic administration of an Ad vector, because inhibition of miRNA production in the liver leads to severe hepatotoxicities [13].

Expression of VA-RNAs would interfere with the transduction efficiencies and safety profiles of an Ad vector; however, expression profiles of VA-RNAs following transduction with an Ad vector have remained to be examined.

In this study, expression profiles of VA-RNAI following transduction with a conventional replication-incompetent Ad vector *in vitro* and *in vivo* were quantitatively examined by real-time RT-PCR analysis, which allows quantitative analysis of VA-RNA copy numbers, even when expression levels of VA-RNAI are quite low. Detectable levels of VA-RNAI were expressed from a replication-incompetent Ad vector expressing luciferase (Ad-CAL2), although the expression levels of VA-RNAI from Ad-CAL2 were much lower than those from WT-Ad *in vitro* and in vivo. VA-RNAI copy numbers remained almost constant or declined gradually following transduction. In addition, Ad-E4-122aT, a modified Ad vector that can suppress the leaky expression of not only the E4 gene but also other Ad genes in an miR-122a–dependent manner [14], showed reductions in the expression levels of VA-RNAI as well as of the E4 genes in the liver, suggesting that leaky expression of other Ad genes is involved in VA-RNAI expression.

Materials and Methods

Cells and viruses

HUVEC (normal human umbilical vein endothelial cells) were cultured in the medium recommended by the manufacturer (Lonza, Basel, Switzerland). SK HEP-1 (a human hepatoma cell line) and HEK293 (a transformed embryonic kidney cell line) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/ml), and penicillin (100 U/ml).

We used a previously prepared E1- and E3-deleted first-generation replication-incompetent Ad vector possessing a CA (chicken beta-actin promoter with cytomegalovirus, CMV, enhancer) promoter-driven firefly luciferase expression cassette in the E1-deleted region in the reverse orientation (Ad-CAL2) [15]. Ad-E4-122aT-CAL, which contains four tandem copies of sequences with perfect complementarity to miR-122a in the 3'-untranslated region (UTR) of the E4 gene, was prepared in a manner similar to that described using pAd-E4-122aT [14] and pCAL3-1 [16]. We confirmed by real-time PCR analysis that none of the viral stocks used in this study contained a detectable replication-competent virus [17]. WT-Ad (human type 5) was obtained from American Type Culture Collection (ATCC). These Ads were amplified in HEK293 cells and purified by two rounds of cesium chloride-gradient ultracentrifugation, dialyzed, and stored at -80°C [18]. Determination of infectious units (IFU) was accomplished using an Adeno-X Rapid Titer Kit (Clontech, Mountain View, CA).

Mice

Female C57BL/6 mice aged 5 weeks were obtained from Nippon SLC (Hamamatsu, Japan). All animal experimental procedures used in this study were performed in accordance with the institutional guidelines for animal experiments at Osaka University.

Analysis of VA-RNAI expression in cultured cells

Ads were added to the cells at multiplicities of infection (MOIs) of 10 and 100. After 1-h incubation, the medium containing Ads was replaced with fresh medium. Cells were harvested at 6, 12, 24, 48, 72, and 96 h after transduction, and total RNA was extracted from the cells. Complementary DNA was synthesized with a Superscript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA). VA-RNAI copy numbers were determined using the StepOnePlus real-time PCR systems (Applied Biosystems, Foster City, CA) [19] and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. The sequences of the primers and probes used in this study are given in Table 1.

No.	Name	Sequence (5' – 3')
1	hGAPDH-F	ggtggtctcctctgacttcaaca
2	hGAPDH-R	Gtggtcgttgagggcaatg
3	hGAPDH-Probe	FAM-cactcctccacctttgacgctggg-TAMRA
4	mGAPDH-F	Caatgtgtccgtcgtggatct
5	mGAPDH-R	Gtcctcagtgtagcccaagatg
6	mGAPDH-Probe	FAM-cgtgccgcctggagaaacctgcc-TAMRA
7	VA-RNAI-F	gggcactcttccgtggtctg
8	VA-RNAI-R	aggagcactcccccgttgtc
9	E2A-F	cactacggtgcgagtgcaa
10	E2A-R	ggtagctgccttcccaaaaag
11	E2A-Probe	FAM-tcaaagcctgggcacgcgc-TAMRA
12	E4-F	gggatcgtctacctcctttga
13	E4-R	gggcagcagcggatgat

14	E4-Probe	FAM-acagaaacccgcgctaccatactggag-TAMRA
15	Hexon-F	Aacgatgacaacgaagacgaagtag
16	Hexon-R	ggcgcctgcccaaatac
17	Hexon-Probe	FAM-cgagcaagctgagcagcaaaaaactca-TAMRA
18	Fiber-F	gcgcctatccgaacctctagt
19	Fiber-R	agaggccgttgcccattt
20	Fiber-Probe	FAM-acctccaatggcatgcttgcgc-TAMRA
21	Luc-F	tcctatgattatgtccggttatgtaaa
22	Luc-R	tgtagccatccatccttgtcaa
23	Luc-Probe	FAM-aatccggaagcgaccaacgcc-TAMRA

Table 1: The primers and probes used in the study.

Analysis of VA-RNAI and other Ad gene expression in mouse liver

Ads were intravenously administered to mice at a dose of 1010 IFU/mouse via the tail vein. Total RNA was extracted from each liver at the indicated days following administration. The expression levels of VA-RNAI and the Ad genes were measured as described above.

Statistical analysis

Statistical significance was determined using Student's t-test.

Results

VA-RNAI expression following transduction with an Ad vector in cultured cells

First, in order to examine the expression profiles of VA-RNAI in cultured cells following transduction with an Ad vector, HUVEC and SK HEP-1 cells were transduced with Ad-CAL2, a replication-incompetent Ad vector expressing luciferase, at MOIs of 10 and 100. Although a replication-incompetent Ad vector genome was amplified independently of the Ad E1 gene following transduction with an Ad vector in several tumor cell lines, SK HEP-1 cells did not support replication of the Ad vector genome [20], which make it possible to circumvent the influences of Ad vector genome replication on VA-RNAI expression levels. In HUVECs, detectable levels of VA-RNAI expression were found from 6 h after transduction with Ad-CAL2 at MOIs of both 10 and 100 (Figure 1A). The VA-RNAI expression levels were slightly elevated 12 h after transduction, and subsequently VA-RNAI expression remained almost constant or decreased gradually. Transduction with Ad-CAL2 at an MOI of 100 resulted in approximately 3- to 30-fold higher levels of VA-RNAI than transduction at an MOI of 10. On the other hand, copy numbers of VA-RNAI continuously increased up to 96 h post-infection with WT-Ad. There were approximately 2000- and 12000-fold differences in VA-RNAI copy numbers following treatment with Ad-CAL2 and WT-Ad at an MOI of 100 at 48 and 96 h post-transduction, respectively (Figure 1B), probably because WT-Ad efficiently replicated in the infected cells while Ad-CAL2 did not.

In SK HEP-1 cells, the expression patterns of VA-RNAI following transduction with Ad-CAL2 were similar to those in HUVEC cells (Figure 1C and 1D). The VA-RNAI expression levels following transduction with Ad-CAL2 at an MOI of 100 were approximately 3000-fold lower than those following infection with WT-Ad at an MOI of 100 at 48, 72, and 96 h post-transduction. The VA-RNAI expression levels reached a plateau at 48 h after infection with WT-Ad and thereafter remained constant in SK HEP-1 cells. These results demonstrated that a replication-incompetent Ad vector as well as WT-Ad expresses VA-RNAI, although Ad-CAL2 mediated much lower levels of VA-RNAI expression than WT-Ad. Ad-CAL2 expressed slightly but significantly higher levels of VA-RNAI than WT-Ad at 6 and 12 h after transduction at an MOI of 100 in HUVEC and at MOIs of 10 and 100 in SK HEP-1 cells; however, it remained unclear why higher copy numbers of VA-RNAI were found for Ad-CAL2 than for WT-Ad at 6 and 12 h after treatment.

Expression profiles of VA-RNAI in mouse liver following administration of an Ad vector

Next, in order to examine the expression profiles of VA-RNAI in mouse organs following systemic administration of an Ad vector, expression levels of the Ad genes, including VA-RNAI, in the liver, in which systemically administered Ad vector is mainly distributed, were determined by real-time RT-PCR analysis following Ad vector administration. VA-RNAI expression in the liver was detected 24 h after injection of Ad-CAL2 and decreased gradually thereafter. Expression profiles of firefly luciferase and the other Ad genes, including the E2A, E4, hexon, and fiber proteins, were similar to those of VA-RNAI. On the other hand, WT-Ad exhibited the highest levels of VA-RNAI expression at 48 h after administration (Figure 2). The VA-RNAI expression levels by WT-Ad were more than 600-fold higher than those by Ad-CAL2 at 48 and 96 h after administration. The other Ad genes exhibited expression patterns similar to those of VA-RNAI following injection of WT-Ad, although their expression levels were individually different. These results demonstrated that a replication-incompetent Ad vector expresses VA-RNAI in the mouse liver following administration, even though VA-RNAI expression by Ad-CAL2 was much lower than that by WT-Ad.

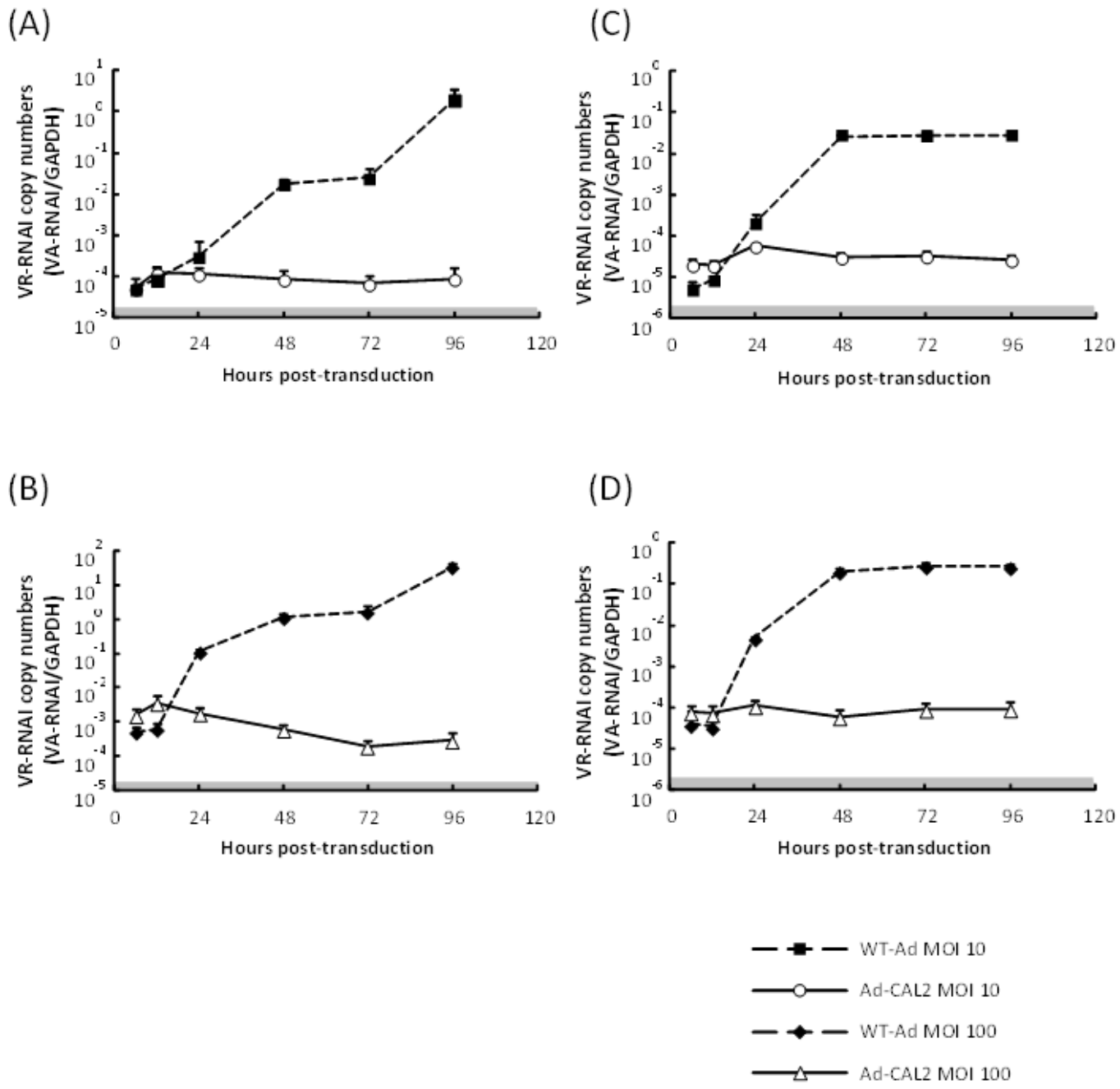

Figure 1: Time course profiles of VA-RNAI expression in HUVEC and SK HEP-1 cells following treatment with an Ad vector and WT-Ad. (A, B) HUVEC and (C, D) SK HEP-1 cells were treated with Ad-CAL2 or WT-Ad at MOIs of 10 (A, C) and 100 (B, D) for 1 h, and the VA-RNAI expression levels were determined 6, 12, 24, 48, 72, and 96 h after treatment by real-time RT-PCR analysis. The gray shaded boxes indicate VA-RNAI levels with no significant differences between VA-RNAI expression in cells transduced with Ad-CAL2 and mock-transduced cells ($p>0.05$). The data are expressed as means ±S.D. (n = 3 or 4).

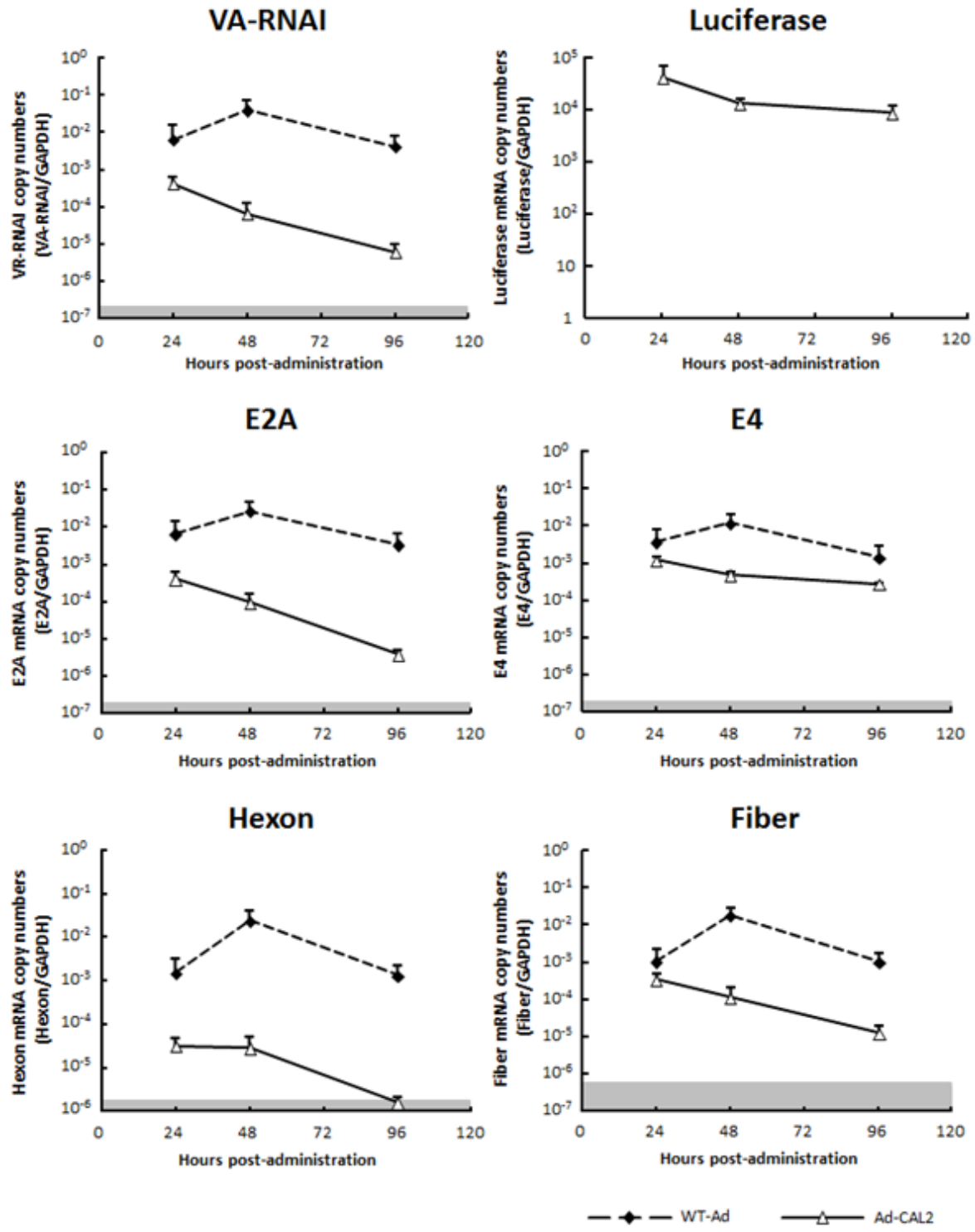

Figure 2: Time course profiles of VA-RNAI expression in mouse liver following administration with an Ad vector and WT-Ad. C57BL/6 mice were intravenously administered with Ad-CAL2 or WT-Ad at a dose of 1 × 1010 IFU/mouse. Livers were harvested 24, 48, and 96 h after administration. The Ad gene expression levels were determined by real-time RT-PCR analysis. The gray shaded boxes indicate RNA levels with no significant differences between gene expression in the liver transduced with Ad-CAL2 and mock-transduced cells ($p>0.05$). The data are expressed as means ± S.D. (n=5-6).

Reduction in VA-RNAI expression in mouse liver by suppression of leaky expression of the Ad genes

In order to examine the relationship between the expression of VA-RNAI and other Ad genes, Ad-E4-122aT-CAL, which carries miR-122a–targeted sequences into the 3'-UTR of the E4 gene and shows significantly low leaky expression levels of not only the E4 gene but also other Ad genes, was administered to mice (Figure 3A) [14]. As previously demonstrated [14], the E4 mRNA level in the liver at 48 h after administration with Ad-E4-122aT-CAL was approximately five-fold lower than those following administration with Ad-CAL2 (Figure 3B). Ad-E4-122aT-CAL exhibited an approximately 70% reduction in VA-RNAI expression levels in the liver following administration, compared with those following administration with Ad-CAL2 (Figure 3C). These results suggest that the expression of other Ad genes, including the E4 gene, would contribute to VA-RNAI expression.

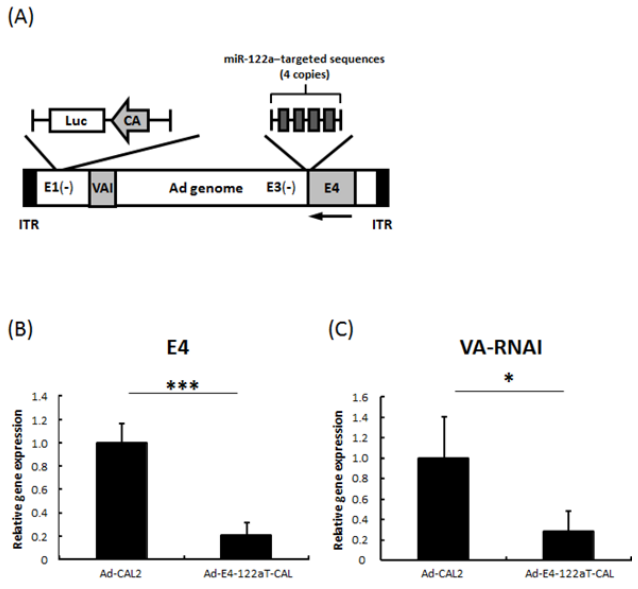

Figure 3: Reduction in VA-RNAI expression in mouse liver following administration with Ad-E4-122aT-CAL. (A) Schematic diagram of Ad-E4-122aT-CAL. A luciferase expression cassette was inserted into the E1-deleted region. CA, chicken beta-actin promoter with cytomegalovirus, CMV, enhancer; ITR, inverted terminal repeat; VAI, virus-associated RNA I; 122aT, miR-122a –targeted sequences. (B, C) C57BL/6 mice were intravenously administered Ad-CAL2 or Ad-Luc-E4-122aT at a dose of 1 × 1010 IFU/mouse. Two days after administration, the livers were harvested. The Ad gene expression levels in the livers were determined by real-time RT-PCR analysis. The data are expressed as means ± S.D. (n=5-6). $^{*}p<0.01$, $^{***}p<0.0001$.

Discussion

Previous studies demonstrated that replication-incompetent E1-deleted Ad vectors express VA-RNAI [8]. However, the expression profiles of VA-RNAI following transduction with an Ad vector have remained to be clarified, although VA-RNAs induce innate immune responses [8,10] and perturb miRNA expression profiles [10,11]. Elucidation of the expression profiles of VA-RNAI contributes to reduction in Ad vector-induced side effects and development of safer Ad vectors. Although VA-RNAI expression levels were often analyzed by northern blotting [12,21,22], northern blotting is semi-quantitative. In addition, real-time RT-PCR analysis shows a lower detection limit of VA-RNAI than northern blotting analysis shows. Thus, in the present study, we determined the VA-RNAI expression profiles following transduction with an Ad vector by real-time RT-PCR analysis. A replication-incompetent Ad vector, Ad-CAL2, mediated VA-RNAI expression in cultured cells and mouse liver, although the VA-RNAI expression levels by Ad-CAL2 were much lower than those by WT-Ad.

Almost constant or gradually declining VA-RNAI expression levels were found in the cultured cells following transduction with an Ad vector. On the other hand, VA-RNAI expression levels in the liver following systemic administration of an Ad vector declined more rapidly than those in the cultured cells. This is probably because transduced cells in the liver were damaged via innate immune responses following systemic administration of an Ad vector. Systemic administration of an Ad vector results in the rapid induction of inflammatory cytokines, which induce tissue damage, within 12 h after injection [7,23-27].

Previous studies reported that VA-RNAI was detected 1-3 h after infection with WT-Ad [28]. Similar results were obtained in the present study. In addition, VA-RNAI expression from an Ad vector was found as early as 6 h after transduction. We previously demonstrated that type I IFN expression, which is induced by VA-RNAs via an IPS-1–dependent pathway, was detected at 12 h after transduction [8]. These results suggest that VA-RNAI was rapidly transcribed from the Ad vector genome, resulting in the induction of type I IFN expression. It remains to be clarified why the VA-RNAI expression levels by Ad-CAL2 were higher than those by WT-Ad at 6 h after infection; however, in this experiment, Ad-CAL2 contains the CA promoter for expression of the luciferase gene. The CA promoter exhibits the highly efficient transcription activities in the cultured cells and mouse organs [16]. The VA-RNAI expression by Ad-CAL2 might be directly or indirectly influenced by the strong transcriptional activity of the CA promoter, although the CA promoter in the E1-deleted region is more than 5k bp far from the VA-RNAI gene.

The data in Figure 3 demonstrated that the expression profile of VA-RNAI was similar to those of the other Ad genes in the liver following intravenous administration of an Ad vector. These data made us hypothesize that leaky expression of Ad genes would be involved in VA-RNAI expression from an Ad vector genome. In order to test this hypothesis, we used Ad-E4-122aT-CAL2, which shows large reductions in leaky expression of Ad genes, including the pIX, E2A, E4, hexon, penton, and fiber genes, in hepatocytes in an miR-122a–dependent manner [14]. Ad-E4-122aT-CAL2 expressed approximately 70% lower levels of VA-RNAI as well as the E4 gene than Ad-CAL2 (Figure 3B). RNA polymerase III drives VA-RNAI expression [29]. We demonstrated that RNA polymerase III inhibitor reduced VA-RNAI expression levels in the cultured cells [8]. However, the data in the present study suggest that products of the E4 gene or other Ad genes would contribute to VA-RNAI expression from a replication-incompetent Ad vector genome. The E4 gene products have multi-functions and regulate the expression of not only viral genes but also host genes at the transcriptional and/or post-transcriptional level [30,31]. Cellular and/or viral proteins involved in the transcription of VA-RNA would be down-regulated in the cells following transduction with Ad-E4-122aT-CAL2. Further study is

required in order to elucidate the VA-RNAI expression mechanism following transduction with an Ad vector.

In summary, we quantitatively analyzed the expression profiles of VA-RNAI following transduction with replication-incompetent Ad vectors using real-time RT-PCR analysis. VA-RNAI was significantly expressed in cultured cells and mouse organs following treatment with the Ad vector, although WT-Ad exhibited much higher levels of VA-RNAI expression than an Ad vector. In addition, suppression of the leaky expression of the Ad genes resulted in the reduction in VA-RNAI copy numbers in the liver following intravenous administration, suggesting that certain Ad genes would induce VA-RNAI expression. The present findings will help to elucidate Ad vector-mediated tissue damage and will promote the development of safer Ad vectors.

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