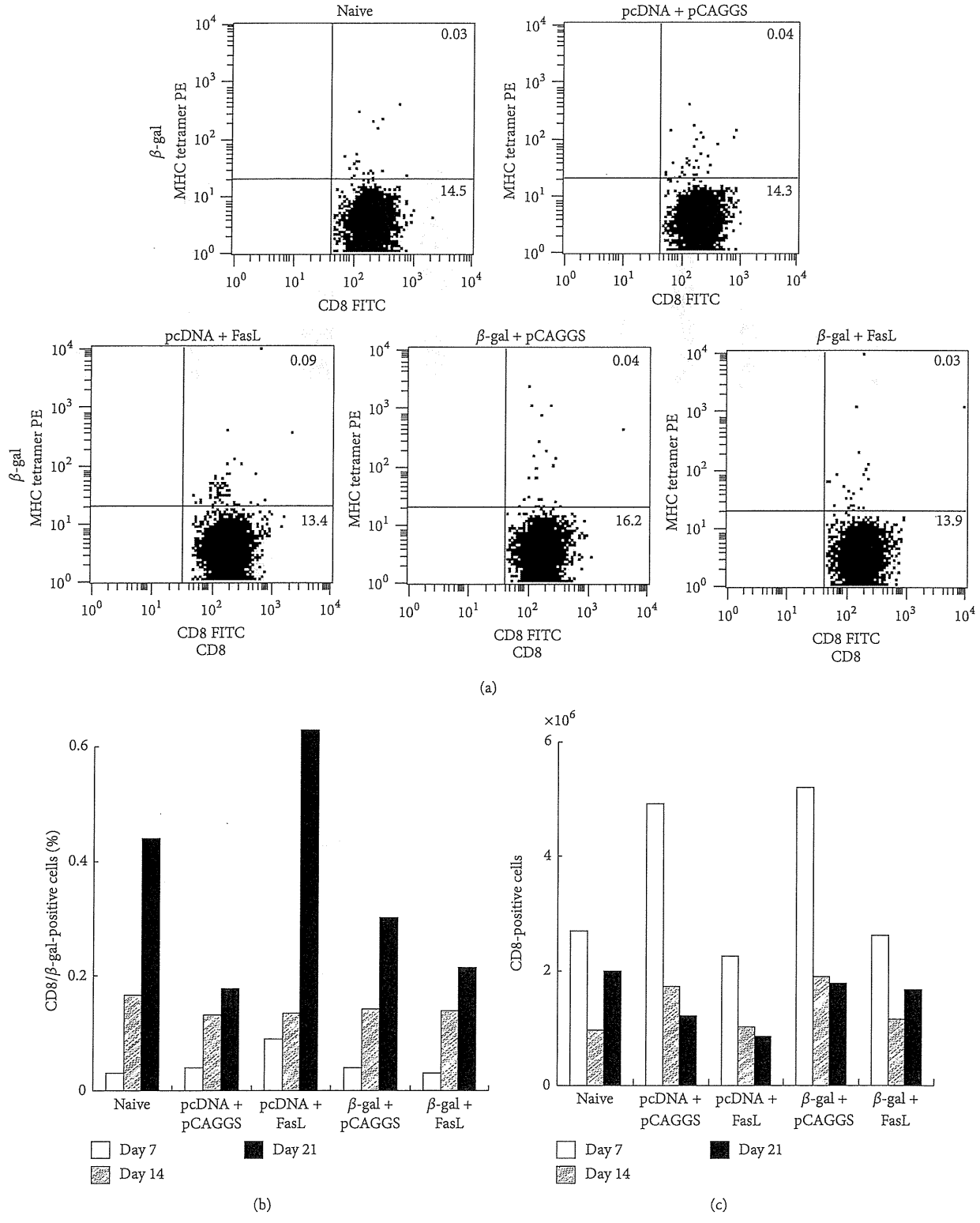

FIGURE 3: Representative data on frequency of β-gal-specific $CD8^+$ T cells in inguinal lymph nodes. BALB/c mice were pretreated with cardiotoxin and then uninjected (naive) or injected with pcDNA3 + pCAGGS, pcDNA3 + pCAGGS/FasL, pcDNA3/β-gal + pCAGGS, or pcDNA3/β-gal + pCAGGS/FasL (50 μg DNA for each). (a) Representative cell surface staining profiles of the lymph nodes 7 days after DNA immunization. The number indicates a percentage of each fraction. (b) Percentages of $CD8^+$/β-gal-positive T cells in respective mice on days 7, 14, and 21 after DNA immunization. (c) Percentages of $CD8^+$ T cells in respective mice on days 7, 14, and 21 after DNA immunization.

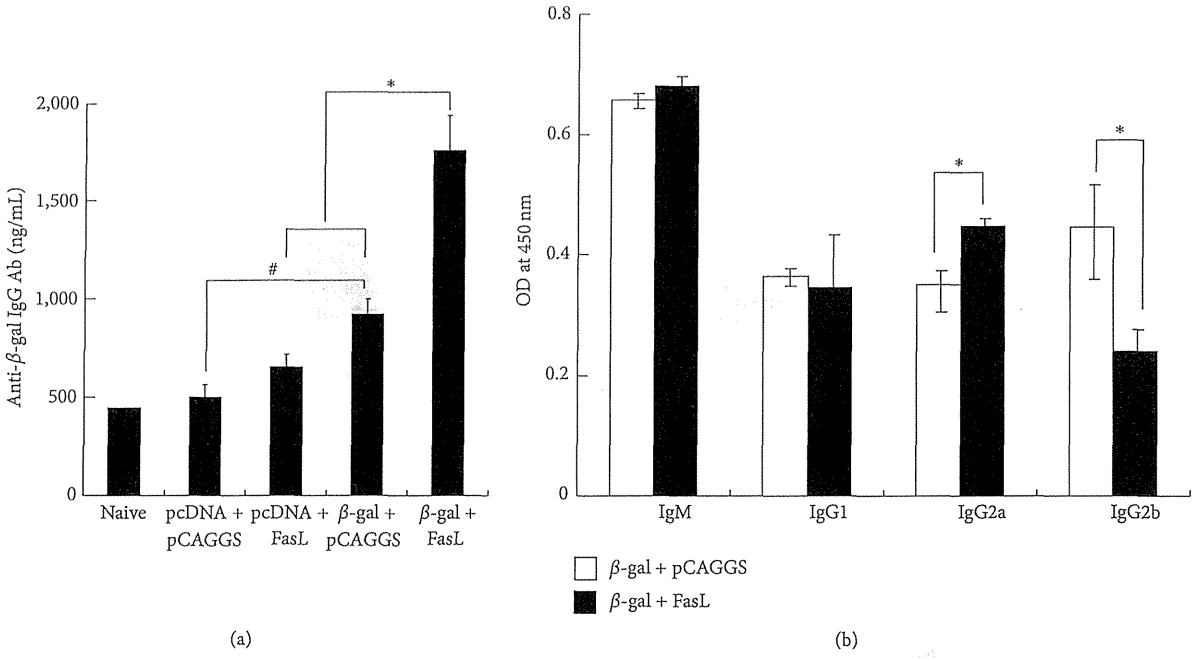

FIGURE 4: Production of ant-β-gal Ab after DNA immunization. BALB/c mice were treated with cardiotoxin followed by DNA immunization, pcDNA3 + pCAGGS, pcDNA3 + pCAGGS/FasL, pcDNA3/β-gal + pCAGGS, or pcDNA3/β-gal + pCAGGS/FasL (50 μg DNA for each). (a) Concentrations of anti-β-gal IgG Ab in the mice 14 days after DNA immunization were measured with an ELISA assay ($n = 3$). $^{\#}P < 0.05$, $^{*}P < 0.01$.

as interleukin-6, which is produced by cardiotoxin injection, potentiate B cell differentiation. Tissue destruction can therefore be crucial not only for integrating plasmid DNA but also for conditioning microenvironment for Ab production.

5. Conclusion

We demonstrated that administration of FasL DNA together with DNA encoding a putative tumor antigen gene produced antitumor effects on the antigen-expressing tumor cells *in vivo*. Cardiotoxin pretreatments enhanced expression of the DNA-encoded gene in muscle. The antitumor responses were not attributable to antigen-positive $CD8^+$ T cells but associated with enhanced Ab production in particular IgG_{2a} subtype. The present study indicates a role of FasL DNA in augmentation of humoral immunity and suggests a potential application of FasL in DNA-mediated vaccine.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] M. A. Kutzler and D. B. Weiner, "DNA vaccines: ready for prime time?" *Nature Reviews Genetics*, vol. 9, no. 10, pp. 776–788, 2008.

[2] E. Ragonnaud and P. Holst, "The rationale of vectored gene-fusion vaccines against cancer: evolving strategies and latest evidence," *Therapeutic Advances in Vaccines*, vol. 1, no. 1, pp. 33–47, 2013.

[3] Y. Xu, E. Yang, J. Wang et al., "Prime-boost bacillus Calmette-Guérin vaccination with lentivirus-vectored and DNA-based vaccines expressing antigens Ag85B and Rv3425 improves protective efficacy against *Mycobacterium tuberculosis* in mice," *Immunology*, vol. 143, no. 2, pp. 277–286, 2014.

[4] H. Liu, S. Geng, C. Feng et al., "A DNA vaccine targeting p42.3 induces protective antitumor immunity via eliciting cytotoxic $CD8^+$ T lymphocytes in a murine melanoma model," *Human Vaccines and Immunotherapeutics*, vol. 9, no. 10, pp. 2196–2202, 2013.

[5] J. Ni, V. Schirrmacher, and P. Fournier, "The hemagglutinin-neuraminidase gene of Newcastle disease virus: a powerful molecular adjuvant for DNA anti-tumor vaccination," *Vaccine*, vol. 28, no. 42, pp. 6891–6900, 2010.

[6] K. Li, H. Gao, L. Gao et al., "Adjuvant effects of interleukin-18 in DNA vaccination against infectious bursal disease virus in chickens," *Vaccine*, vol. 31, no. 14, pp. 1799–1805, 2013.

[7] S. Nagata and P. Golstein, "The Fas death factor," *Science*, vol. 267, no. 5203, pp. 1449–1456, 1995.

[8] M. Waldner, C. C. Schimanski, and M. F. Neurath, "Colon cancer and the immune system: the role of tumor invading T cells," *World Journal of Gastroenterology*, vol. 12, no. 45, pp. 7233–7238, 2006.

[9] Y. Tada, J. O-Wang, Y. Takiguchi et al., "Cutting edge: a novel role for Fas ligand in facilitating antigen acquisition by dendritic cells," *Journal of Immunology*, vol. 169, no. 5, pp. 2241–2245, 2002.

[10] P. Mroz, A. Szokalska, M. X. Wu, and M. R. Hamblin, "Photodynamic therapy of tumors can lead to development of systemic antigen-specific immune response," *PLoS ONE*, vol. 5, no. 12, Article ID e15194, 2010.

[11] M. Wang, P. W. Chen, V. Bronte, S. A. Rosenberg, and N. P. Restifo, "Anti-tumor activity of cytotoxic T lymphocytes elicited with recombinant and synthetic forms of a model tumor-associated antigen," *Journal of Immunotherapy with Emphasis on Tumor Immunology*, vol. 18, no. 3, pp. 139–146, 1995.

[12] J. A. Nemzek, J. Siddiqui, and D. G. Remick, "Development and optimization of cytokine ELISAs using commercial antibody pairs," *Journal of Immunological Methods*, vol. 255, no. 1-2, pp. 149–157, 2001.

[13] C.-J. Wu, S.-C. Lee, H.-W. Huang, and M.-H. Tao, "In vivo electroporation of skeletal muscles increases the efficacy of Japanese encephalitis virus DNA vaccine," *Vaccine*, vol. 22, no. 11-12, pp. 1457–1464, 2004.

[14] A. L. Mathes and R. Lafyatis, "Role for toll-like receptor 3 in muscle regeneration after cardiotoxin injury," *Muscle and Nerve*, vol. 43, no. 5, pp. 733–740, 2011.

[15] A. Rosato, A. Zoso, G. Milan et al., "Individual analysis of mice vaccinated against a weakly immunogenic self tumor-specific antigen reveals a correlation between CD8 T cell response and antitumor efficacy," *Journal of Immunology*, vol. 171, no. 10, pp. 5172–5179, 2003.

[16] W.-F. Cheng, C.-N. Lee, M.-C. Chang, Y.-N. Su, C.-A. Chen, and C.-Y. Hsieh, "Antigen-specific $CD8^+$ T lymphocytes generated from a DNA vaccine control tumors through the Fas-FasL pathway," *Molecular Therapy*, vol. 12, no. 5, pp. 960–968, 2005.

[17] R. Geiben-Lynn, J. R. Greenland, K. Frimpong-Boateng, N. van Rooijen, A.-H. Hovav, and N. L. Letvin, "$CD4^+$ T lymphocytes mediate in vivo clearance of plasmid DNA vaccine antigen expression and potentiate $CD8^+$ T-cell immune responses," *Blood*, vol. 112, no. 12, pp. 4585–4590, 2008.

[18] S. Dharmapuri, L. Aurisicchio, A. Biondo, N. Welsh, G. Ciliberto, and N. la Monica, "Antiapoptotic small interfering RNA as potent adjuvant of DNA vaccination in a mouse mammary tumor model," *Human Gene Therapy*, vol. 20, no. 6, pp. 589–597, 2009.

[19] M. Petráčková, V. Lučanský, and V. Vonka, "Tumor protective activity of $CD4^+$ but not of $CD8^+$ T cells in DNA-vaccinated mice challenged with bcr-abl-transformed cells," *Clinical and Developmental Immunology*, vol. 2013, Article ID 923107, 5 pages, 2013.

[20] F. Nimmerjahn and J. V. Ravetch, "Immunology: divergent immunoglobulin G subclass activity through selective Fc receptor binding," *Science*, vol. 310, no. 5753, pp. 1510–1512, 2005.

[21] M. J. Pokrass, M. F. Liu, M. A. Lindorfer, and R. P. Taylor, "Activation of complement by monoclonal antibodies that target cell-associated β_2-microglobulin: implications for cancer immunotherapy," *Molecular Immunology*, vol. 56, no. 4, pp. 549–560, 2013.

[22] S. Nimal, M. S. Thomas, and A. W. Heath, "Fusion of antigen to Fas-ligand in a DNA vaccine enhances immunogenicity," *Vaccine*, vol. 25, no. 12, pp. 2306–2315, 2007.

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ARTICLE

Suppression of leaky expression of adenovirus genes by insertion of microRNA-targeted sequences in the replication-incompetent adenovirus vector genome

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Leaky expression of adenovirus (Ad) genes occurs following transduction with a conventional replication-incompetent Ad vector, leading to an induction of cellular immunity against Ad proteins and Ad protein-induced toxicity, especially in the late phase following administration. To suppress the leaky expression of Ad genes, we developed novel Ad vectors by incorporating four tandem copies of sequences with perfect complementarity to miR-122a or miR-142-3p into the 3′-untranslated region (UTR) of the E2A, E4, or pIX gene, which were mainly expressed from the Ad vector genome after transduction. These Ad vectors easily grew to high titers comparable to those of a conventional Ad vector in conventional 293 cells. The leaky expression of these Ad genes in mouse organs was significantly suppressed by 2- to 100-fold, compared with a conventional Ad vector, by insertion of the miRNA-targeted sequences. Notably, the Ad vector carrying the miR-122a–targeted sequences into the 3′-UTR of the E4 gene expressed higher and longer-term transgene expression and more than 20-fold lower levels of all the Ad early and late genes examined in the liver than a conventional Ad vector. miR-122a–mediated suppression of the E4 gene expression in the liver significantly reduced the hepatotoxicity which an Ad vector causes *via* both adaptive and non-adaptive immune responses.

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INTRODUCTION

Replication-incompetent adenovirus (Ad) vectors are widely used in not only clinical gene therapy but also basic researches. Theoretically, Ad genes should not be expressed following transduction with a replication-incompetent Ad vector because the E1A gene, which is crucial for the transcription of other Ad genes, is deleted from the Ad genome. However, Ad genes are indeed expressed from the vector genome, resulting in an induction of cellular immunity against Ad proteins as well as Ad protein-induced toxicity. Such Ad protein-induced cellular immunity and toxicity frequently cause tissue damages and/or an elimination of Ad vector-transduced cells, leading to short-lived transgene expression.[1,2] The Leaky expression of Ad genes have been observed for E2A,[1,3–5] E4,[5] pIX,[5,6] hexon,[3] and fiber.[3,4]

In order to suppress the leaky expression of Ad genes, various types of replication-incompetent Ad vectors have been developed. Ad vectors in which not only E1 genes but also E2A and/or E4 genes were deleted[2–4,7–10] have also been developed. The E2A- and/or E4-deleted Ad vectors showed significant reduction in the leaky expression of viral proteins,[2–4] resulting in a decreased cytotoxic T lymphocyte (CTL) response,[2] diminished hepatotoxicity,[2,9,10] and increased *in vivo* transgene expression persistence.[2] pIX gene-deleted Ad vectors have also been developed.[11] The E2A-, E4-, and/or pIX-deleted Ad vectors are highly valuable and promising; however, special packaging cell lines complementing not only E1 gene products but also the E2A, E4, and/or pIX gene products are necessary for the production of these Ad vectors. It is relatively difficult to generate special packaging cells expressing these Ad genes at levels high enough for high titer production of E2A-, E4-, and/or pIX-deleted Ad vectors. The titers of these Ad vectors using these packaging cells were often lower than that of a conventional Ad vector.[4,7,10] A helper-dependent Ad (HD-Ad) vector that lacks all viral coding regions has also been developed. The HD-Ad vector shows reduced inflammation in the organs, and persistent transgene expression following intravenous administration.[12,13] However, the production systems of HD-Ad vectors are constrained by technical complexity and are limited to the production of low titers of HD-Ad vectors. In addition, the complete removal of helper virus contamination is a difficult and complicated procedure. The development

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of safe and efficient Ad vectors that can be easily produced at high titers by a conventional method using normal 293 cells is necessary for gene therapy and, even more, for basic researches.

In order to develop a replication-incompetent Ad vector of the type described above, a microRNA (miRNA)-regulated gene expression system was utilized to suppress the leaky expression of Ad genes in this study. Several groups, including our own, have demonstrated that insertion of miRNA-targeted sequences into the 3′-untranslated region (UTR) of a transgene reduced the expression levels of the transgene in the cells, with the extent of reduction being dependent on the cellular expression levels of the corresponding miRNA.[14–18] Thus we hypothesized that incorporation of the complementary sequences for miR-122a or miR-142-3p, which respectively exhibit liver- or spleen-specific expression,[14,19,20] into the 3′-UTR of the E2A, E4, or pIX genes suppressed the leaky expression of Ad genes in an miRNA-dependent manner and that high titer production of an Ad vector was achieved using conventional 293 cells.

RESULTS

Construction of a replication-incompetent Ad vector carrying the miRNA-targeted sequences for suppression of the leaky expression of Ad genes

In order to suppress the leaky expression of Ad genes, four tandem copies of sequences with perfect complementarity to miR-122a or miR-142-3p, which respectively exhibit liver- or spleen-specific expression but almost undetectable levels of expression in 293 cells, were inserted into the 3′-UTR of the E2A, E4, or pIX gene of the replication-incompetent Ad vector genome (Figure 1). The liver is the main organ where intravenously administered Ad vectors accumulate. The spleen is largely involved in the innate and acquired immune responses following Ad vector administration. The insertion sites of miRNA-targeted sequences were behind the stop codon in the 3′-UTR of each gene; at bp 4032 in the pIX gene, bp 22442 in the E2A gene, and bp 32913 in the E4 gene. The E4 gene is composed of at least six open reading frames (ORFs), all of which share the common 3′-terminal sequences.[21] The miRNA-targeted sequences were inserted into the common 3′-terminal sequences of the E4 gene and upstream of the poly A signal sequences in order that expression of all the E4 ORFs would be suppressed by miRNA. The Ad vectors carrying the miRNA-targeted sequences were normally propagated in conventional 293 cells and exhibited titer productions comparable to those of a conventional Ad vector, Ad-L2,[22] which is a conventional E1-deleted Ad vector (Table 1). Real-time RT-PCR analysis demonstrated that the Ct values for miR-122a and miR-142-3p in 293 cells were above 34 (data not shown). Rapid amplification of 3′-cDNA ends (3′-RACE) analysis confirmed that four tandem copies of sequences with perfect complementarity to miR-122a or miR-142-3p were inserted into the 3′-UTR of the E2A, E4 (including all the E4 ORFs) or pIX genes (data not shown). No mutations were found in the miRNA-targeted sequences.

miRNA-mediated suppression of the leaky expression of the Ad genes *in vitro*

In order to examine whether leaky expression of the Ad genes was suppressed by insertion of the miRNA-targeted sequences in an miRNA-dependent manner, HuH-7 cells, which highly express miR-122a,[23] were transduced with the luciferase-expressing Ad vectors, and the Ad gene expression levels were determined 12 hours after transduction. All the Ad vectors examined exhibited similar levels of luciferase production in HuH-7 cells (data not shown). Ad-E2A-122aT-L2 mediated 2.2-fold lower E2A gene expression than did Ad-L2 (Figure 2a). Ad-E4-122aT-L2 and Ad-pIX-122aT-L2 exhibited 4- and 6.5-fold lower E4 and pIX gene expression levels, respectively, than did Ad-L2. Insertion of the miR-142-3p–targeted sequences in the 3′-UTR also reduced the expression levels of the E4 and pIX genes in spite of the undetectable levels of miR-142-3p expression in HuH-7 cells, probably due to non-specific suppression *via* the insertion of miRNA-targeted sequences; however, the levels of suppression of the E4 and pIX genes realized by insertion of the miR-142-3p–targeted sequences were significantly lower than those by insertion of the miR-122a–targeted sequences. A reduction in E4 gene expression was also found for Ad-pIX-122aT-L2. It was unclear why the E4 gene expression was reduced for Ad-pIX-122aT-L2. The expression levels of Ad genes other than those described above were not significantly reduced in HuH-7 cells. The E2A, E4, and pIX gene expressions were also significantly suppressed in K562 cells, which is a human chronic myelogenous leukemia cell line highly expressing miR-142-3p,[24] by insertion of the miR-142-3p–targeted sequences in the 3′-UTR of these Ad genes (Supplementary Figure S1). Note that all the E4 ORFs can be detected by the primers for the E4 gene used in this study. The E4 mRNA levels in the graph represent the sum of each E4 ORF mRNA level.

In order to examine whether the suppression of the leaky expression of Ad genes by insertion of the miRNA-targeted sequences was miRNA-dependent, miR-122a was inhibited by pre-treatment with a locked nucleic acid (LNA)-modified antisense oligonucleotides (ASO) against miR-122a. The average expression levels of the E2A, E4, and pIX genes by Ad-L2 were elevated by transfection with the LNA-modified ASO against miR-122a *via* an unknown mechanism (Figure 2b). By contrast, more than threefold elevation in the expression of the E2A, E4, and pIX genes was found by the LNA-modified ASO against miR-122a in the cells treated with the Ad vectors containing the miR-122a–targeted sequences. The control LNA-modified ASO failed to restore the miR-122a–mediated suppression of these Ad genes. These results indicate that the reduction in the leaky expression levels of these Ad genes by insertion of the miR-122a–targeted sequences was miRNA-dependent.

Suppression of the leaky expression of Ad genes in mouse organs following intravenous administration

Next, to examine whether *in vivo* leaky expression of Ad genes in the organs was suppressed by incorporation of the miRNA-targeted sequences, Ad gene expression levels in the liver and spleen were determined by real-time RT-PCR following intravenous administration of Ad vectors. Among the Ad genes examined (E2A, E4, pIX, hexon, penton base, and fiber genes), the pIX gene exhibited the highest level of expression in the liver following intravenous administration of Ad-L2 (Supplementary Figure S2a). The pIX gene expression levels were approximately 40-fold lower than the expression level of a cytomegalovirus (CMV) promoter-driven luciferase gene in the E1-deleted region of the Ad vector genome. The expression levels of the other Ad genes were similar.

Insertion of the miR-122a–targeted sequences into the 3′-UTR of the Ad genes reduced the leaky expression of the corresponding Ad genes in the liver (Figure 2c). The expressions of the E2A, E4, and pIX genes by Ad-E2A-122aT-L2, Ad-E4-122aT-L2, and Ad-pIX-122aT-L2, respectively, in the liver were significantly suppressed by 20- to 30-fold, compared with the corresponding expressions by Ad-L2. The expression of the Ad late genes (hexon, penton base, and fiber genes), which did not possess miR-122a–targeted sequences in the 3′-UTR, was also decreased in the livers of mice

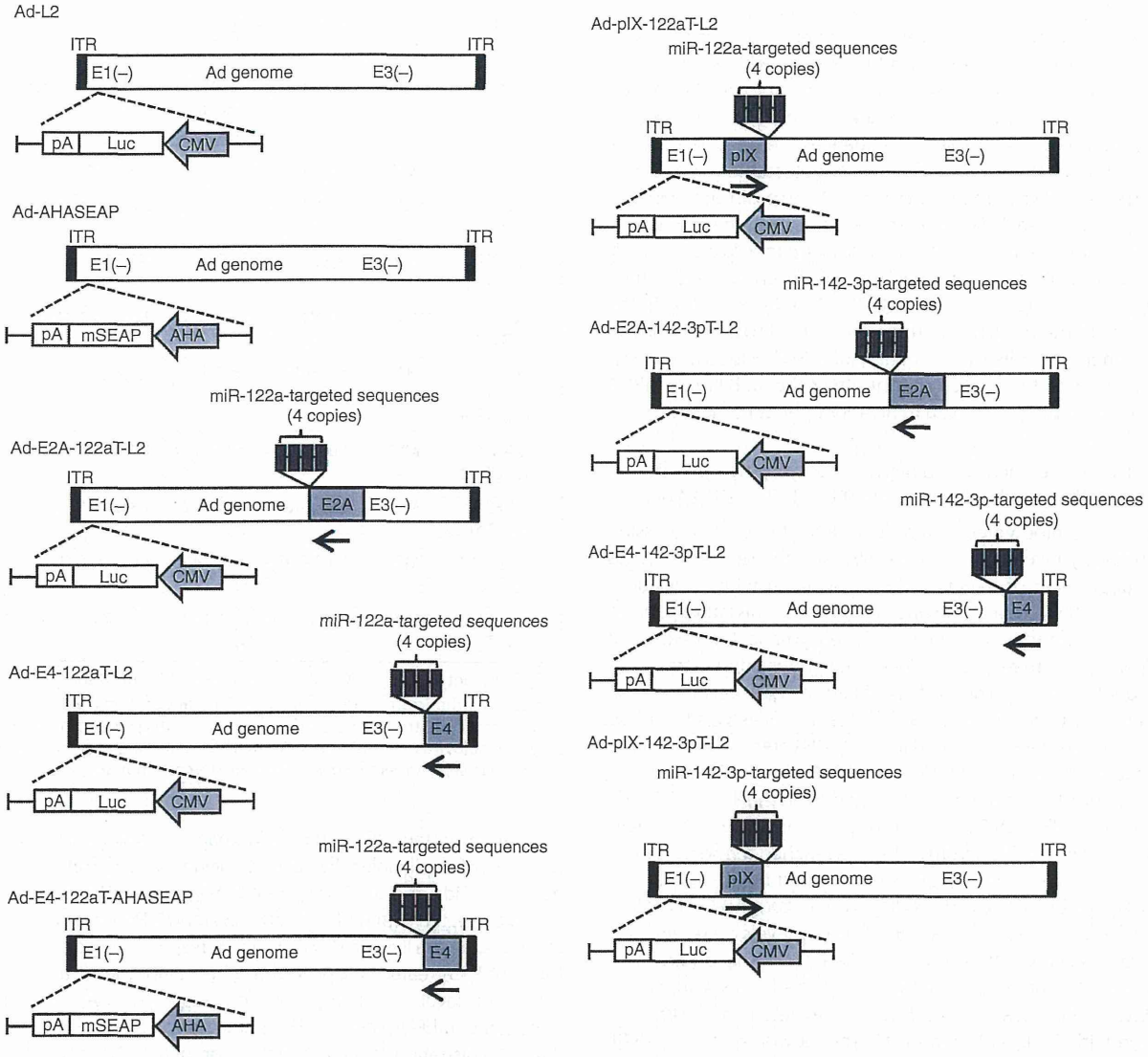


Figure 1 Schematic diagrams of replication-incompetent Ad vectors used in this study. A luciferase expression cassette was inserted into the E1-deleted region in Ad-L2, Ad-E2A-122aT-L2, Ad-E4-122aT-L2, Ad-pIX-122aT-L2, Ad-E2A-142-3pT-L2, Ad-E4-142-3pT-L2, and Ad-pIX-142-3pT-L2. A murine secreted embryo alkaline phosphatase (mSEAP) expression cassette was inserted into the E1-deleted region in Ad-AHASEAP and Ad-E4-122aT-AHASEAP. AHA, a *synthetic promoter composed of apolipoprotein E enhancer, the hepatocyte control region, and human α1-antitrypsin promoter; CMV, cytomegalovirus* promoter; ITR, inverted terminal repeat; 122aT, miR-122a–targeted sequences; 142-3pT, miR-142-3p–targeted sequences.

treated with Ad-E2A-122aT-L2 and Ad-E4-122aT-L2. In particular, the livers of mice treated with Ad-E4-122aT-L2 exhibited larger reductions—more than 20-fold in the expression of not only the E4 gene but also all the other Ad genes examined compared with livers treated with the other Ad vectors (Figure 2c). It is well known that the E4 gene products are required to express the E2A and late genes.[3,21,25] The E2A gene product also regulates the expression of the viral late genes.[4] We consider that miRNA-mediated suppression of the E2A or E4 gene expression resulted in a reduction of the Ad late gene expression in the liver. The livers treated with Ad-E4-142-3pT-L2 and Ad-pIX-142-3pT-L2 showed a reduction in the expression of the Ad late genes in spite of the almost undetectable levels of miR-142-3p expression in the liver. Ad-E4-142-3pT-L2 and Ad-pIX-142-3pT-L2 induced a reduction in the average E2A mRNA levels in the liver, although the reductions in the E2A mRNA levels were not statistically significant. A non-specific reduction in the E2A mRNA expression by Ad-E4-142-3pT-L2 or Ad-pIX-142-3pT-L2 could have caused the reduction in the Ad late gene mRNA levels in the liver.

The leaky expression levels of the Ad genes in the spleen were 50- to 5,000-fold lower than those in the liver (Supplementary Figure S2b). The highest level of leaky expression in the spleen was found for the E4 gene, followed by the E2A, pIX, and fiber genes, following intravenous administration of Ad-L2. Ad-E4-142-3pT-L2 and Ad-pIX-142-3pT-L2 mediated approximately twofold lower levels of the E4 gene and pIX gene, respectively, than did Ad-L2 in the spleen (Figure 2d). Ad-E2A-142-3pT-L2 administration failed to suppress the E2A gene expression in the spleen. It is unclear why the E2A gene expression was not reduced in the spleens of mice treated with Ad-E2A-142-3pT-L2. Overall, the levels of miR-142-3p–mediated suppression of the leaky expression of Ad genes in the spleen were lower than the corresponding levels of miR-122a–-mediated suppression in the liver, probably due to the lower levels

of miR-142-3p expression in the spleen, compared with miR-122a expression in the liver. Alternatively, the Ad vectors might transduce not only blood cells but also non-blood cells, which have negligible levels of miR-142-3p expression, in the spleen. The expressions of most of the Ad genes examined were also reduced in the spleen when miR-122a–targeted sequences were inserted in the 3′-UTR, although the spleen exhibited undetectable level of miR-122a expression. We confirmed that miR-142-3p did not suppress the expression of renilla luciferase gene possessing the miR-122a–targeted sequences in the 3′-UTR in the *in vitro* reporter gene assay (data not shown). It remains unclear why insertion of the miR-122a–targeted sequences in the 3′-UTR led to the reduction in the expressions of the Ad genes in the spleen. The 3′-UTR of an mRNA plays an important role in the stability of mRNA.[26] Insertion of the miR-122a–targeted sequences might affect the stability of mRNA for Ad genes in the spleen in an miRNA-independent manner.

Reduction in Ad vector-induced hepatotoxicity by incorporation of the miR-122a–targeted sequences into the 3′-UTR of the E4 gene

In order to examine whether a reduction in the leaky expression of Ad genes by incorporation of miRNA-targeted sequences leads to the suppression of hepatotoxicity associated with replication-incompetent Ad vectors after intravenous administration, serum alanine aminotransferase (ALT) levels, enzymatic biomarkers of hepatotoxicity, were measured after intravenous administration of Ad vectors (Figure 3a). The profiles of serum ALT levels contained two peaks, as previously reported.[13,27] The first peak was found on day 2. Serum ALT levels were significantly elevated on day 2 in the mice treated with Ad-L2, Ad-E2A-122aT-L2, Ad-E2A-142-3pT-L2, and Ad-pIX-142-3pT-L2. On the other hand, Ad-E4-122aT-L2, Ad-E4-142-3pT-L2, and Ad-pIX-122aT-L2 exhibited significantly lower ALT levels than Ad-L2 on day 2. The serum ALT levels reached the second peak on day 10. The serum ALT levels were elevated by all the Ad vectors examined, but among the Ad vectors tested, Ad-E4-122aT-L2 induced the lowest levels of serum ALT; the levels were approximately twofold lower than those by Ad-L2. On the other hand, Ad-pIX-122aT-L2 and Ad-pIX-142-3pT-L2 induced statistically significant elevations in the serum ALT levels 10 days after administration, compared with Ad-L2. The reason for the elevation of serum ALT levels by Ad-pIX-122aT-L2 and Ad-pIX-142-3pT-L2 remains unclear. Previous studies have demonstrated that pIX has various other functions in addition to its role as a capsid cement.[28] For example, pIX acts as a transcriptional activator in the nucleus, and pIX might be involved in Ad vector-induced hepatotoxicity. Lower ALT levels were induced by Ad-E4-122aT-L2 than by Ad-L2 at the dose of 2×10^9 infectious unit (IFU)/mouse (data not shown). Other hepatotoxicity parameters, i.e., serum alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and leucine aminopeptidase (LAP) levels, were also significantly higher in mice treated with Ad-L2, compared with the mice receiving Ad-E4-122aT-L2 (Figure 3b).

Next, in order to compare the hepatotoxicity profiles of Ad-L2 and Ad-E4-122aT-L2, histopathological examination of the liver sections was performed 10 days after Ad vector administration (Figure 3c). Many vacuolated cells were observed in the livers of mice treated with Ad-L2. Moreover, there were several necrotic areas in the sections. On the other hand, the livers of Ad-E4-122aT-L2-treated mice exhibited a much lower level of vacuolation than those of Ad-L2-treated mice.

To examine the influence of Ad vector-mediated hepatotoxicity on the expression of liver-specific genes, albumin mRNA levels in the liver were quantified by real-time RT-PCR (Figure 3d). Albumin, which plays an important role in the maintenance of oncotic pressure and transport of small molecules such as calcium, unconjugated bilirubin, free fatty acids, and cortisol, is abundantly expressed in hepatocytes. The mice treated with Ad-L2 showed 53% and 37% reductions in albumin mRNA in the liver on days 10 and 15, respectively, compared with those of PBS-treated mice. On the other hand, the albumin mRNA expression levels in the livers of mice treated with Ad-E4-122aT-L2 were comparable to those of PBS-treated mice. These results indicate that administration of a replication-incompetent Ad vector possessing the miR-122a–targeted sequences into the 3′-UTR of the E4 gene resulted in significantly lower hepatotoxicity than treatment with a conventional Ad vector. A reduction in the hepatotoxicity induced by Ad-E4-122aT-L2 would lead to the higher copy numbers of Ad vector genome remaining in the liver. We found that the genome copy number of Ad-E4-122aT-L2 was approximately threefold higher than those of the other Ad vectors 15 days following administration (Figure 3e).

Table 1 Ad vectors used in this study

Vector name	*Promoter*	*Transgene*	*miRNA-targeted sequences*	*Virus particle*	*Infectious unit*
Ad-L2	CMV	Luciferase	–	2.2×10^{12}	3.2×10^{11}
Ad-AHASEAP	AHA	mSEAP	–	2.2×10^{12}	3.4×10^{11}
Ad-E2A-122aT-L2	CMV	Luciferase	miR-122a	2.7×10^{12}	4.0×10^{11}
Ad-E4-122aT-L2	CMV	Luciferase	miR-122a	2.8×10^{12}	4.2×10^{11}
Ad-E4-122aT-AHASEAP	AHA	mSEAP	miR-122a	1.7×10^{12}	2.6×10^{11}
Ad-pIX-122aT-L2	CMV	Luciferase	miR-122a	2.9×10^{12}	4.3×10^{11}
Ad-E2A-142-3pT-L2	CMV	Luciferase	miR-142-3p	4.3×10^{12}	5.8×10^{11}
Ad-E4-142-3pT-L2	CMV	Luciferase	miR-142-3p	3.8×10^{12}	5.5×10^{11}
Ad-pIX-142-3pT-L2	CMV	Luciferase	miR-142-3p	3.7×10^{12}	5.3×10^{11}

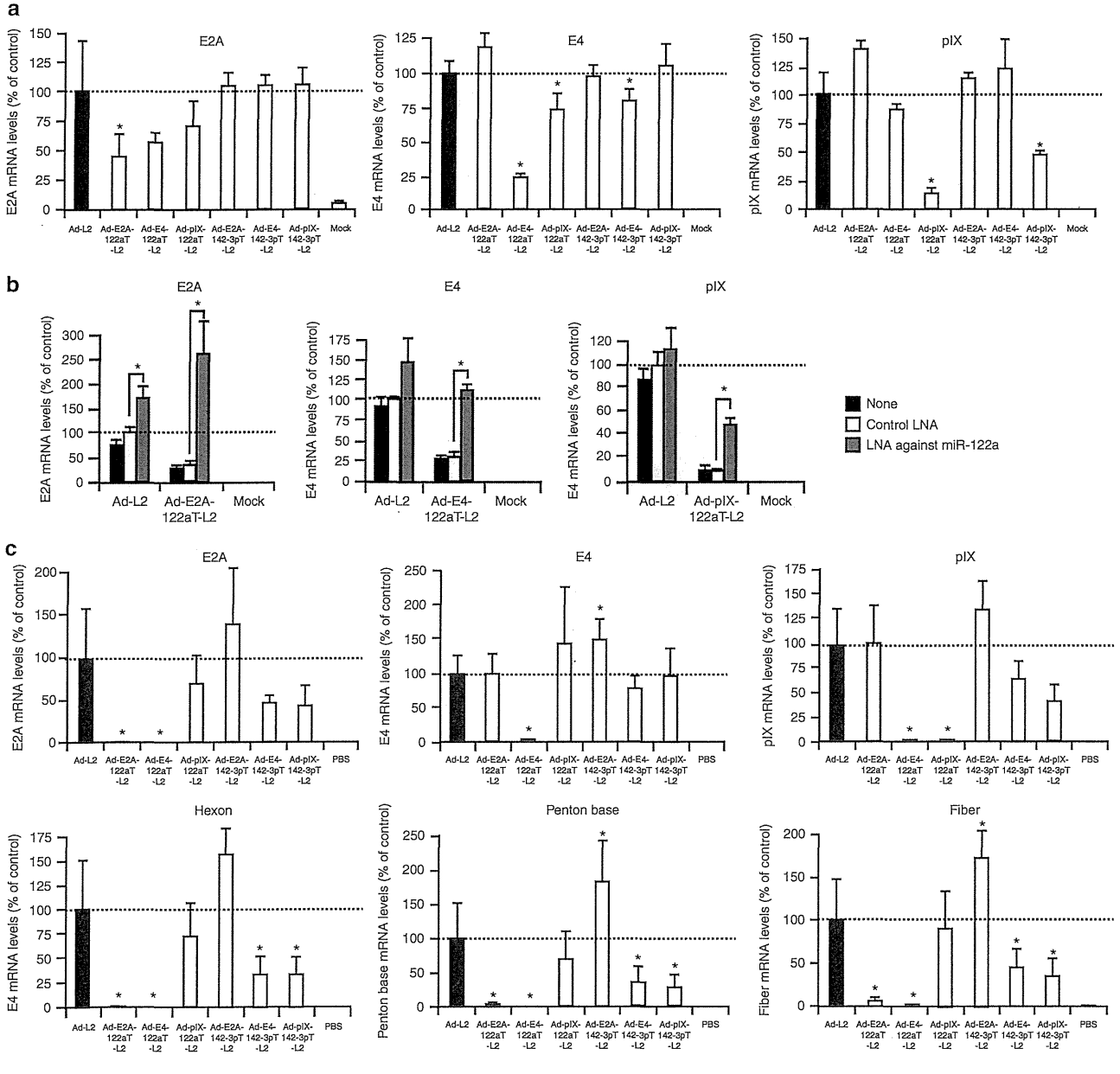
Total amount of titers recovered from 20 plates of 150-mm dishes.
AHA, a synthetic promoter composed of apolipoprotein E enhancer, the hepatocyte control region, and human α1-antitrypsin promoter; CMV, cytomegalovirus promoter; mSEAP, murine secreted embryonic alkaline phosphatase; –, Ad vector does not contain the corresponding sequences.

Immune responses against Ad proteins following Ad vector administration

In order to determine whether suppression of the E4 gene expression by incorporation of the miR-122a–targeted sequences in the 3′-UTR of the E4 gene would lead to a low level of cellular immune responses against Ad protein, the numbers of CTLs against the hexon, which is one of the major capsid proteins and the dominant epitope of Ad vector,[29] in the splenocytes were determined 15 days following administration by an intracellular cytokine staining assay. All the Ad vectors induced elevation in the numbers of hexon-specific $CD8^+$ T cells producing IFN-γ, but these cell numbers were not significantly different between the Ad vectors examined (Figure 4a).

We next examined the infiltration of lymphocytes into the liver 10 days following administration of Ad-L2 and Ad-E4-122aT-L2 (Figure 4b). Dramatic increases in the numbers of $CD4^+$ and $CD8^+$ T cells in the liver were found following administration of Ad-L2 and Ad-E4-122aT-L2. However, the numbers of $CD4^+$ and $CD8^+$ cells infiltrated into the liver were comparable between the mice treated with Ad-L2 and those treated with Ad-E4-122aT-L2. Furthermore, there were no significant differences in the mRNA levels of IFN-γ or chemokines, including CCL3, CXCL2, and CXCL10, in the livers of mice injected with Ad-L2 and Ad-E4-122aT-L2 on day 10, although administration of both Ad vectors significantly elevated the expression of IFN-γ and all the chemokines examined in the liver (Figure 4c). The liver mRNA levels of other chemokines, including CCL2, CCL4, CCL5, and CX_3CL were also comparable between mice receiving Ad-L2 and Ad-E4-122aT-L2 (Supplementary Figure S3). There were no significant differences in anti-Ad antibody levels in the serum for all the Ad vectors tested on day 14 (Figure 4d) and day 28 (data not shown).

Next, we hypothesized that the liver hepatocytes transduced with Ad-E4-122aT-L2 might be less susceptible to Ad-specific CTL attack, due to the lower levels of Ad antigen presentation, compared with the hepatocytes transduced with Ad-L2, even though the levels of Ad-specific CTL induction in the spleen and infiltration of Ad-specific CTL in the liver were comparable between the mice receiving Ad-L2 and those receiving Ad-E4-122aT-L2. In order to examine this hypothesis, primary mouse hepatocytes transduced with Ad-L2 or Ad-E4-122aT were incubated with splenocytes isolated from the mice receiving a conventional Ad vector (Ad-null). Significant LDH releases were found in both the hepatocytes transduced with Ad-L2 and the hepatocytes transduced with Ad-E4-122aT; however, the hepatocytes transduced with Ad-L2 showed a 1.8- to 3.2-fold higher LDH release than the hepatocytes transduced with Ad-E4-122aT (Figure 4e). These results indicate that the hepatocytes transduced with Ad-L2 were more susceptible to Ad-specific CTL attack than the hepatocytes transduced with Ad-E4-122aT.



Hepatotoxicity profile of Ad-E4-122aT-L2 in immune-incompetent mice

In order to examine whether suppression of the E4 gene expression in the liver by insertion of the miR-122a–targeted sequences would result in a reduction in immune-independent hepatotoxicity induced by replication-incompetent Ad vectors, serum ALT levels were measured following intravenous administration into Rag2/Il2rγ double-knockout mice, which have global defects in both cellular and humoral immunity due to the lack of T, B, and natural killer (NK) cells.[30,31] Rag2/Il2rγ double-knockout mice exhibited significant elevation in serum ALT levels following intravenous administration of Ad-L2 (Figure 5). On the other hand, no increases in the serum ALT levels were found in Ad-E4-122aT-L2-treated mice. These results indicate that the Ad vector-induced hepatotoxicity *via* a non-adaptive immune response was almost completely eradicated by miR-122a–mediated suppression of the E4 gene expression, and further that E4 gene expression in the liver is one of the main causes of replication-incompetent Ad vector-mediated hepatotoxicity.

In vivo transgene expression by the Ad vectors containing miRNA-targeted sequences in the 3′-UTR of Ad genes

In order to evaluate the *in vivo* transgene expression levels induced by the Ad vectors carrying miRNA-targeted sequences, luciferase expression in the liver was examined 2 days following administration (Figure 6a). The luciferase expression levels in the liver induced by the Ad vectors carrying miRNA-targeted sequences, with the exceptions of Ad-E2A-122aT-L2 and Ad-E4-122aT-L2, were comparable to those by Ad-L2. Ad-E4-122aT-L2–mediated luciferase expression in the liver was 15-fold lower than that mediated by Ad-L2. The lower luciferase expression levels of Ad-E4-122aT-L2 in the liver were probably due to the significant suppression of E4 gene expression. Previous studies demonstrated that the E4 gene products, especially the E4 ORF3 gene product, enhanced the transcriptional activity of a CMV promoter.[9,10,32]

To circumvent the influence of suppressing the E4 gene expression on the CMV promoter activity, the liver-specific synthetic promoter composed of apolipoprotein E enhancer, the hepatocyte control region, and human α1-antitrypsin (AHA) promoter was used for transgene expression. The transcriptional silencing was not observed in the AHA promoter, which made it possible to properly evaluate the influence of Ad vector-mediated hepatotoxicity on the transgene expression profile. In order to exclude the influence of immune responses to transgene products on the transgene expression profile and to examine whether the suppression of hepatotoxicity by insertion of the miR-122a–targeted sequences in the 3′-UTR of the E4 gene improves the transgene expression profiles, the murine secreted embryonic alkaline phosphatase (mSEAP) gene was inserted into the Ad vector genome as a reporter gene. mSEAP is a secreted form of murine embryonic alkaline phosphatase, which is an endogenous protein in mice. Ad-AHASEAP and Ad-E4-122aT-AHASEAP exhibited the comparable mSEAP expression on day 2, indicating that the E4 gene expression did not affect the AHA promoter activity (Figure 6b). Ad-AHASEAP–mediated mSEAP expression levels were gradually increased, and they reached a plateau at 10 days after administration. Subsequently, the mSEAP expression levels induced by Ad-AHASEAP gradually declined. On the other hand, the serum mSEAP levels induced by Ad-E4-122aT-AHASEAP were maintained for at least 149 days. In addition, the mSEAP expression levels by Ad-E4-122aT-AHASEAP were 1.5- to 34.1-fold higher than those by Ad-AHASEAP. These results indicate that suppression of the E4 gene expression in the liver led to the higher and longer-term transgene expression.

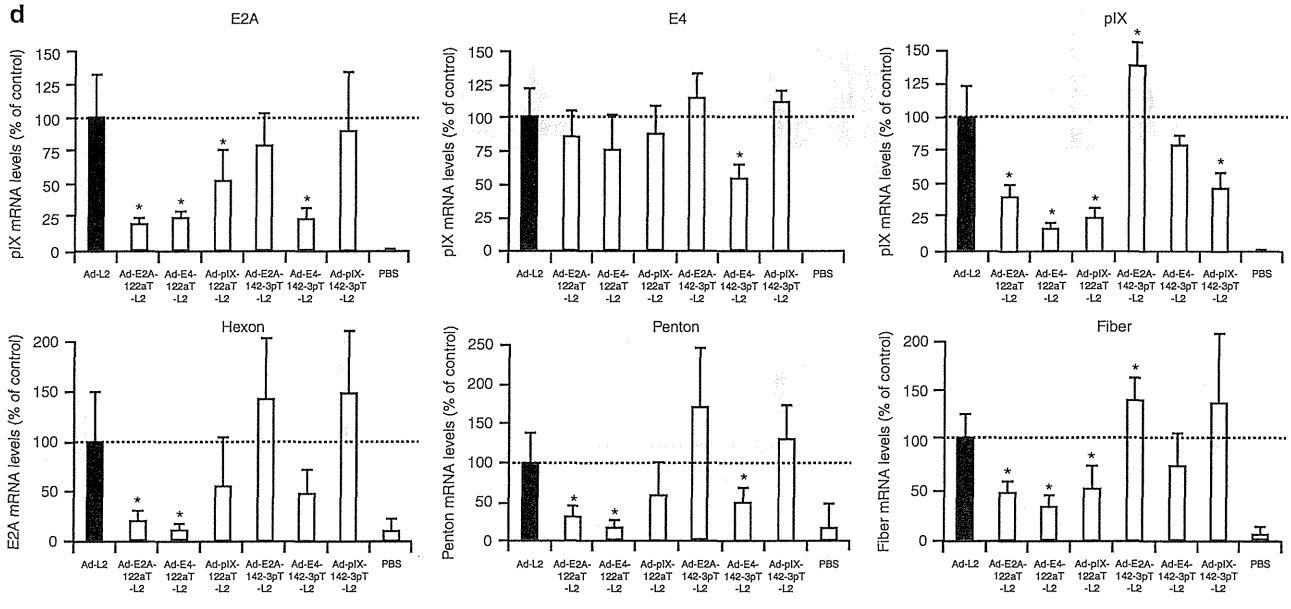


Figure 2 *Suppression of the leaky expression of Ad genes in culture cells and mouse organs by insertion of the miRNA-targeted sequences.* (**a**) HuH-7 cells were transduced with Ad vectors at an MOI of 10 for 1 hour and harvested at 12 hours after transduction. (**b**) Restoration of the leaky expression of Ad genes in HuH-7 cells by LNA-modified ASO complementary to miR-122a. HuH-7 cells were transfection with LNA-modified ASO complementary to miR-122a or an LNA control at 10 nmol/l. Twenty-four hours after transduction, HuH-7 cells were transduced with Ad vectors at an MOI of 10 for 1 hour, and harvested at 12 hours after transduction. The Ad gene expression levels in the cells transduced with Ad vectors were determined by real-time RT-PCR. (**c**,**d**) C57BL/6 mice were intravenously administered Ad vectors at 1×10^{10} IFU/mouse. Two days after administration, (**c**) the livers and (**d**) the spleens were harvested. The Ad gene expression levels in the cells and mice transduced with Ad vectors were determined by real-time RT-PCR. The data are expressed as the mean values ± SD (**a**,**b**: $n = 4$; **c**,**d**: $n = 5$–6). *$P < 0.05$ in comparison with (**a**,**c**,**d**) Ad-L2 or the (**b**) LNA control.