Replication-incompetent adenovirus (Ad) vectors are promising gene delivery vehicles, especially for hepatocytes, due to their superior hepatic tropism; however, in vivo application of an Ad vector often results in hepatotoxicity, mainly due to the leaky expression of Ad genes from the Ad vector genome. In order to reduce the Ad vector-induced hepatotoxicity, we previously developed an Ad vector containing the sequences perfectly complementary to a liver-specific microRNA (miRNA), miR-122a, in the 3′-untranslated region (UTR) of the E4 gene. This improved Ad vector showed a significant reduction in the leaky expression of Ad genes and hepatotoxicity in the mouse liver and primary mouse hepatocytes; however, the safety profiles and transduction properties of this improved Ad vector in human hepatocytes remained to be elucidated. In this study, we examined the transgene expression and safety profiles of Ad vectors with miR-122a-targeted sequences in the 3′-UTR of the E4 gene in human hepatocytes from chimeric mice with humanized liver.

**Key words** adenovirus vector; primary human hepatocyte; cytotoxicity; transduction

### INTRODUCTION

Replication-incompetent adenovirus (Ad) vectors have attracted much attention as gene delivery vehicles in not only gene therapy studies but also basic researches due to their various advantages, which include efficient transduction, a relatively large capacity for transgenes, and high titer production. The E1A gene, which is crucial for the Ad replication cycle, is genetically deleted from the Ad vector genome in a replication-incompetent Ad vector. Theoretically, the deletion of the E1A gene makes it impossible to transcribe other Ad genes in the Ad vector genome. However, other Ad genes in the Ad vector genome are slightly transcribed following transduction, resulting in Ad vector-induced cytotoxicity and immune responses against Ad proteins.1–6 In particular, Ad vector-induced hepatotoxicity has been reported in clinical and nonclinical studies, because Ad vectors efficiently accumulate in the liver following systemic administration.1,2,5–7 In order to overcome these problems, next-generation Ad vectors, including Ad vectors with the E2A and/or the E4 deletion in addition to the E1/E3 deletion, and helper-dependent Ad vectors, have been developed so far, but these Ad vectors are often difficult to produce at high titers.8–11

We previously developed a novel Ad vector containing four copies of sequences perfectly complementary to miR-122a, which is a hepatocyte-specific microRNA (miRNA), in the 3′-untranslated region (UTR) of the E4 gene in order to suppress the leaky expression of Ad genes from the Ad vector genome in the hepatocytes.4,12–14 This novel Ad vector mediated significant reductions in the Ad gene expression and cytotoxicity.

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1. These authors contributed equally to this work.

2. Present address: Laboratory of Biochemistry, Faculty of Pharmacy, Osaka Ohtani University; Osaka 584–8540, Japan.

3. To whom correspondence should be addressed. e-mail: sakurai@phs.osaka-u.ac.jp; mizuguchi@phs.osaka-u.ac.jp
icity in the human hepatocellular carcinoma cell line Huh-7 cells, primary mouse hepatocytes, and mouse liver, leading to efficient transgene expression; however, it remained to be elucidated whether this Ad vector with miR-122a-targeted sequences in the 3'-UTR of the E4 gene also showed superior transgene expression profiles without apparent cytotoxicity in human hepatocytes. Hepatocytes are multi-functional cells, and play highly crucial roles in various functions of the liver, including protein synthesis, transformation of carbohydrates, detoxification, modification, and excretion of exogenous and endogenous substances, and synthesis of cholesterol and lipids.\(^{15}\) Hepatocytes are an important target for gene therapy of various diseases, including hemophilia, ornithine transcarbamylase deficiency, and viral hepatitis.\(^{13,16–19}\) In addition, primary human hepatocytes are often used in studies of drug metabolism and toxicology.\(^{20}\) Therefore, methods for the safe and efficient delivery and expression of foreign genes in human hepatocytes are urgently needed not only for gene therapy studies but also gene function analyses.

In this study, we examined the transgene expression and cytotoxicity profiles of Ad vectors containing miR-122a-targeted sequences in the 3'-UTR of the E4 gene in primary human hepatocytes isolated from humanized-liver mice. Insertion of miR-122a-targeted sequences in the 3'-UTR of the E4 gene improved the transgene expression and cytotoxicity profiles of Ad vectors in the primary human hepatocytes, indicating that an Ad vector containing miR-122a-targeted sequences in the 3'-UTR of the E4 gene efficiently delivers transgenes to primary human hepatocytes without apparent toxicity.

**MATERIALS AND METHODS**

**Ad Vectors** An AHA promoter (a liver-specific apolipoprotein E enhancer-hepatocyte control region-human α1-antitrypsin promoter)-driven mouse secreted alkaline phosphatase (mSEAP) expression cassette-containing conventional Ad vector, Ad-AHASEAP and an Ad vector with miR-122a-targeted sequences in the 3'-UTR of the E4 gene in primary human hepatocytes isolated from humanized-liver mice. Insertion of miR-122a-targeted sequences in the 3'-UTR of the E4 gene improved the transgene expression and cytotoxicity profiles of Ad vectors in the primary human hepatocytes, indicating that an Ad vector containing miR-122a-targeted sequences in the 3'-UTR of the E4 gene efficiently delivers transgenes to primary human hepatocytes without apparent toxicity.

**RESULTS**

**Transgene Expression Levels of Ad-E4-122aT-AHASEAP and Ad-E4-122aT-CASEAP in Primary Human Hepatocytes**

![Image of text content]
In order to examine whether suppression of the leaky expression of Ad genes from the Ad vector genome improved the transduction properties of Ad vectors in primary human hepatocytes, Ad vectors containing the miR-122a-targeted sequences in the 3'UTR of the E4 gene were added to the primary human hepatocytes at a MOI of 30. A previous study demonstrated that transduction efficiencies of an Ad vector in primary human hepatocytes reached a plateau at a MOI of 25. Both the conventional Ad vector, Ad-GFP, and improved Ad vector, Ad-E4-122aT-CMVGFP, produced almost 100% GFP-positive cells at 48 h after transduction, indicating that the primary human hepatocytes were highly susceptible to Ad vector-mediated transduction (Fig. 2a). Next, we performed a time-course study of transgene expression in the primary human hepatocytes using mSEAP-expressing Ad vectors (Fig. 2b). In order to obtain higher transgene expression, we used the CA promoter instead of the CMV promoter because the CA promoter was stronger than the CMV promoter. We also tested the AHA promoter, which is a hepatocyte-specific promoter. mSEAP production levels by Ad-E4-122aT-CASEAP and Ad-CASEAP were higher than those by Ad-AHASEAP and Ad-E4-122aT-AHASEAP by about 1.4-fold on day 2, and by about 6-fold on day 4, indicating that the CA promoter mediated more efficient transcription than the AHA promoter in primary human hepatocytes.

Leaky Expression of Ad Genes from Ad Vectors in Primary Human Hepatocytes Next, in order to examine whether insertion of miR-122a-targeted sequences in the 3'UTR of the E4 gene suppressed the leaky expression of Ad genes from the Ad vector genome, Ad gene expression levels in the primary human hepatocytes were measured by real-time RT-PCR analysis following Ad vector transduction. Ad gene expression levels in the Ad-E4-122aT-CASEAP-treated and Ad-E4-122aT-CASEAP-treated primary human hepatocytes were substantially lower than those in the Ad-AHASEAP-treated and Ad-CASEAP-treated cells, although there were no statistically significant differences in the Ad gene expression levels between the Ad-E4-122aT-AHASEAP-
treated and the Ad-AHASEAP-treated cells (Fig. 3). The mRNA levels of the E2A, E4, and hexon genes following transduction with Ad-E4-122aT-CASEAP were approximately 91-fold, 11-fold, and 1200-fold lower than those following transduction with Ad-CASEAP, respectively. The mRNA levels of the E2A, E4, and hexon genes following transduction with Ad-E4-122aT-AHASEAP were approximately 10-fold, 3-fold, and 22-fold lower than those following transduction with Ad-AHASEAP, respectively. These results indicate that insertion of miR-122a-targeted sequences in the 3'-UTR of the E4 gene largely reduced the leaky expression of Ad genes from the Ad vector genome in primary human hepatocytes. In addition, Ad-CASEAP exhibited approximately 3.4-fold and 4.8-fold higher levels of the E2A and E4 gene expression in the primary human hepatocytes compared to Ad-AHASEAP, respectively. These data suggested that the transcriptional activities of the promoters driving the transgene expression in the Ad vector genome would affect the leasy expression levels of Ad genes.

**Ad Vector-Induced Cytotoxicity in Primary Human Hepatocytes** Next, in order to examine whether leaky expression of Ad genes from the Ad vector genome resulted in Ad vector-mediated cytotoxicity in primary human hepatocytes, the morphologies of the primary human hepatocytes were observed following Ad vector transduction at an MOI of 100. No apparent morphological changes in the primary human hepatocytes were not found on day 4 following transduction with any of the types of Ad vectors (Fig. 4a); however, Ad-CASEAP significantly induced a severe damage on the primary human hepatocytes on day 10. Ad-E4-122aT-CASEAP slightly induced cytotoxicities in the primary human hepatocytes on day 10. No cytotoxicity was evident following transduction with Ad-AHASEAP or Ad-E4-122aT-AHASEAP. Severe cytotoxicity was not induced by any of the Ad vectors transduced at MOIs of 25 or 50 (data not shown). High levels of cytotoxicity were also found in Ad-CASEAP-treated cells in WST-8 assay (Fig. 4b). These results suggested that leaky expression of Ad genes from the Ad vector genome partly contributed to the Ad vector-mediated cytotoxicity in primary human hepatocytes, and that suppression of the leaky expression of Ad genes by miR-122a-mediated post-transcriptional gene silencing mitigated the Ad vector-mediated cytotoxicity.

**DISCUSSION**

In this study, we examined the transduction properties of an Ad vector possessing sequences perfectly complementary to a liver-specific miRNA, miR-122a, in the 3'-UTR of the E4 gene in primary human hepatocytes. This study demonstrated that while a conventional Ad vector containing the CA promoter, Ad-CASEAP, induced severe cytotoxicity in the primary human hepatocytes at 10 d after transduction at an MOI of 100, insertion of miR-122a-targeted sequences in the 3'-UTR of the E4 gene improved not only the Ad vector-mediated cytotoxicity but also the transgene expression profiles of an Ad vector in the primary human hepatocytes by suppressing the leaky expression of Ad genes. These findings indicated that an Ad vector with miR-122a-targeted sequences in the 3'-UTR of the E4 gene is an efficient gene delivery vehicle for primary human hepatocytes. Development of an efficient gene delivery vehicle to primary human hepatocytes has been eagerly anticipated because human hepatocytes are an important research target for both gene therapy studies and basic studies. An efficient gene delivery vehicle to primary human hepatocytes in vitro makes it possible to genetically manipulate primary human hepatocytes by over-expressing foreign genes and knocking down/out target genes. In a previous work, we successfully achieved genome editing in the primary human hepatocytes by efficient delivery of a CRISPR-Cas9 system using an Ad vector with miR-122a-targeted sequences in the 3'-UTR of the E4 gene.31

Insertion of miR-122a-targeted sequences in the 3'-UTR of the E4 gene resulted in the reduction in the mRNA levels of not only the E4 gene but also the E2A and hexon genes in the primary hepatocytes. This is probably because the E4 gene products have various functions, including enhancement of the export of virus mRNA from the nucleus and late viral protein production.32 The E4 gene products are involved in the expression of the E2A and hexon genes.

The hexon mRNA levels were comparable in Ad-AHASEAP-treated and Ad-CASEAP-treated cells, however, the hexon mRNA levels were approximately 8-fold higher than the E2A and E4 mRNA levels following transduction.
with Ad-AHASEAP and Ad-CASEAP (data not shown). The mechanism of leaky expression of the hexon gene would be somewhat different from that of the E2A and E4 genes.

This study suggests that transgene expression levels in the primary human hepatocytes were correlated with Ad vector-mediated cytotoxicity levels. Significant differences in the mSEAP levels were not found on days 2 and 4 between conventional Ad vectors and Ad-E4-122aT vectors containing the same transgene expression cassette (Fig. 2b). Apparent morphological changes were not observed on day 4 (Fig. 4a). On the other hand, apparent cytotoxicities were found on day 6 in Ad-CASEAP-treated cells. Significant differences in the mSEAP levels were found on day 7 between Ad-AHASEAP and Ad-E4-122aT-AHASEAP, and between Ad-CASEAP and Ad-E4-122aT-CASEAP. These data indicate that suppression of Ad vector-mediated cytotoxicity is important for improvement of transgene expression profiles.

The mechanism of Ad protein-induced cytotoxicity via a non-adaptive immune response is highly complex and remains to be clarified. Among the Ad proteins, the E4 gene products have been demonstrated to contribute to Ad vector-induced cytotoxicity.\(^{10,33-35}\) Moreover, among the several E4 gene products, the E4 open reading frame (ORF) 4 was shown to induce apoptosis via caspase activation in a cell line-specific manner.\(^{36}\) In addition, the E4 ORF4 preferentially induced apoptosis in transformed cells, not non-transformed cells.\(^{37}\)

In the present study, we did not find significant up-regulation of apoptosis-related genes, including tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or Bcl-2-associated X protein (Bax), following transduction with Ad vectors in the primary human hepatocytes in this study (data not shown). Other mechanisms different from the classical apoptosis pathway and/or the other E4 ORF products are likely be involved in Ad vector-mediated in vitro cytotoxicity in the primary human hepatocytes.

As described above, leaky expression of Ad genes from the Ad vector genome is involved in Ad vector-induced cytotoxicity. Ad-CASEAP mediated higher levels of the E2A and E4 gene expression in the primary human hepatocytes than Ad-AHASEAP, and thereby led to more severe morphological changes in the primary human hepatocytes than Ad-AHASEAP. These data indicate that we should be mindful of the risk of Ad vector-mediated cytotoxicity when using a strong promoter for transgene expression. Ad-E4-122aT-CASEAP slightly induced the cytotoxicity on day 10, by contrast, we did not find apparent morphological changes in Ad-AHASEAP-treated cells on day 10 (Fig. 3). We did not find statistically significant differences in the leaky expression levels of the Ad genes in Ad-AHASEAP-treated or Ad-E4-122aT-CASEAP-treated cells (Fig. 4). Differences in the leaky expression levels of other Ad genes except for the E2A, E4, and hexon genes might explain the differences in the cytotox-
icity levels between Ad-AHASEAP-treated and Ad-E4-122aT-CASEAP-treated cells. For example, the pIX mRNA levels in Ad-E4-122aT-CASEAP-treated cells were approximately 30-fold higher than those in Ad-AHASEAP-treated cells (data not shown). Furthermore, high levels of mSEAP expression might be involved in the cytotoxicity in Ad-E4-122aT-CASEAP-treated cells. Approximately 10-fold higher levels of mSEAP expression were found in Ad-E4-122aT-CASEAP-treated cells than in Ad-AHASEAP-treated cells (Fig. 1b).

mSEAP production levels in Ad-E4-122aT-AHASEAP-treated cells were 1.5-fold higher than those in Ad-AHASEAP-treated cells on day 7. Although we did not find the apparent differences in the cell morphology or survival between Ad-AHASEAP and Ad-E4-122aT-AHASEAP, mRNA levels of hepatocyte-specific genes, including the CYP3A4 and albumin genes, in Ad-E4-122aT-AHASEAP-treated cells were significantly higher than those in Ad-AHASEAP-treated cells (data not shown). These data indicated that Ad-E4-122aT-AHASEAP-induced cytotoxicity levels were lower than those by Ad-AHASEAP. Lower levels of cytotoxicity in Ad-E4-122aT-AHASEAP-treated cells than those in Ad-AHASEAP-treated cells resulted in the higher levels of mSEAP production by Ad-E4-122aT-AHASEAP than by Ad-AHASEAP on day 10.

In summary, we demonstrated that an Ad vector containing miR-122a-targeted sequences in the 3′-UTR of the E4 gene showed superior transgene expression profiles and safety profiles in the primary human hepatocytes. This Ad vector is an efficient gene delivery vehicle for primary human hepatocytes in not only gene therapy studies but also gene function analysis.
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Conflict of Interest YI and CT are paid employees of PhoenixBio Co., Ltd. PXB-mice and PXB-cells are products of PhoenixBio Co., Ltd.

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