2.5 min. The mobile phase was then maintained at 90% Buffer B for 3 min before being changed back to 10% at 3.01 min for a 4 min equilibration. The mass spectrometer was operated in positive mode for parent compound and metabolite measurement.

Estimation of pharmacokinetic parameters: The plasma concentrations of the compounds and metabolites in each group at the indicated time were given as an average ($n = 3$, each time point). The pharmacokinetic parameters for the parent compound and its metabolite were estimated from the plasma concentration-time data by a noncompartmental approach using the program WinNonlin 6.1 (Pharsight, Mountain View, CA). The peak concentration in serum (C_{max}) and the corresponding time of maximum concentration (T_{max}) were obtained from the original data. The area under the serum concentration-time curve from time 0 to 420 min (AUC_{last}) was calculated by the trapezoidal rule and the AUC_{inf_obs} with extrapolation to infinity by dividing the last measured concentration by λ. The elimination rate constant (λ) was determined as the slope of linear regression for the terminal log-linear portion of the concentration *versus* time curve, and the elimination half-life ($T_{1/2}$) was calculated from $0.693/\lambda$.

Statistical analysis: Statistical significance ($p < 0.05$) was determined using Student's *t*-test. Data are presented as means ± S.D.

Results

Development of an Ad vector expressing human CYP3A4: In order to introduce the human CYP3A4 gene into the mouse liver, an Ad vector expressing human CYP3A4 was developed because Ad vectors are known to possess strong hepatotropism (**Fig. 1**). A CA promoter, which is a fusion promoter composed of the cytomegalovirus (CMV) enhancer and chicken β-actin promoter,[26] was selected for human CYP3A4 expression. A CA promoter is the most efficient promoter in the mouse liver.[27] In addition, an Ad vector possessing four copies of miR-122a-targeted sequences in the 3′-UTR of the E4 gene (Ad-E4-122aT) was used for delivery of the human CYP3A4 gene in order to avoid Ad vector-mediated hepatotoxicity. We previously demonstrated that Ad-E4-122aT exhibited a significant reduction in the leaky expression of Ad genes in the liver by miR-122a-mediated post-transcriptional silencing and Ad vector-mediated hepatotoxicity.[21] Apparent elevation in the serum AST and ALT levels, which is the representative hepatotoxicity marker, was not found 2 or 4 days following intravenous administration of Ad-E4-122aT-hCYP3A4 (data not shown).

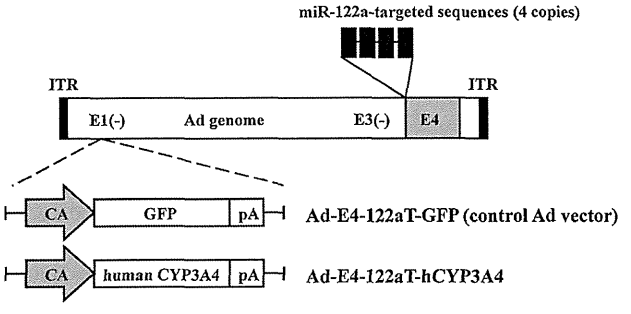

Fig. 1. Structure of the Ad vectors used in this study
A GFP expression cassette was inserted into the E1-deleted region in Ad-E4-122aT-GFP. A human CYP3A4 expression cassette was inserted into the E1-deleted region in Ad-E4-122aT-hCYP3A4. CA, a fusion promoter composed of a CMV enhancer and chicken β-actin promoter; 122aT, miR-122a-targeted sequences; ITR, inverted terminal repeat.

Over-expression of human CYP3A4 in HepG2 cells: Next, in order to examine whether *in vitro* transduction with Ad-E4-122aT-hCYP3A4 up-regulates human CYP3A4 activity, human CYP3A4 mRNA, protein and activity levels were determined following transduction with Ad-E4-122aT-hCYP3A4 in HepG2 cells. Dose-dependent over-expression of human CYP3A4 was observed in HepG2 cells at the mRNA, protein and activity levels (**Fig. 2**). These results indicate that Ad vector-mediated over-expression of human CYP3A4 conferred up-regulation of human CYP3A4 activity *in vitro*.

Human CYP3A4 protein and activity levels in the mouse liver following Ad vector administration: In order to determine the protein levels of human CYP3A4 in the livers of mice treated with Ad-E4-122aT-hCYP3A4, Western blot analysis was carried out. No human CYP3A4 expression was found in the livers of PBS- and Ad-E4-122aT-GFP-administered mice (**Fig. 3A**). Human CYP3A4 protein levels were dependent on the dose of Ad-E4-

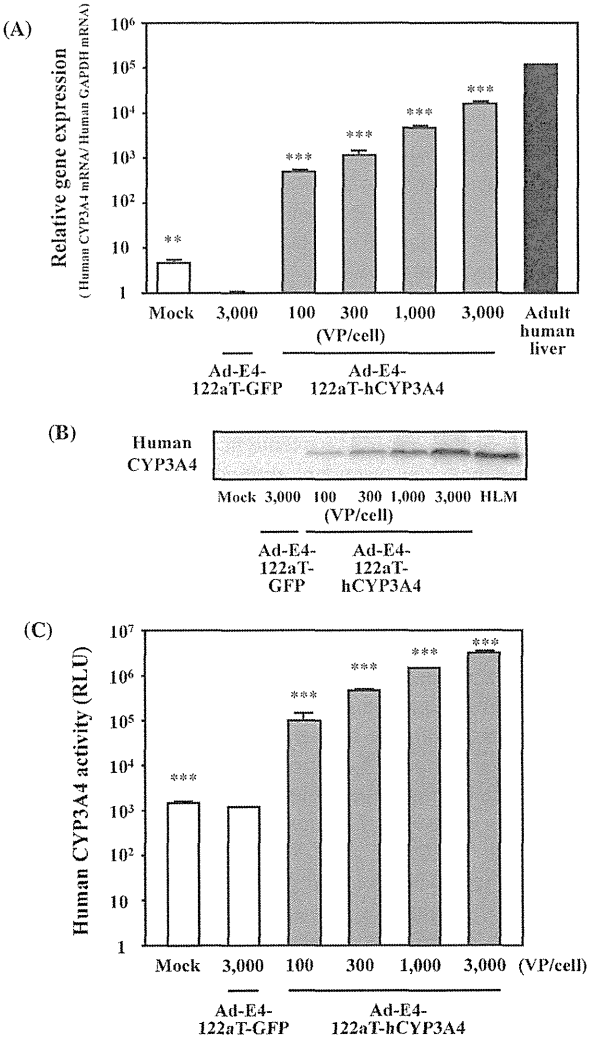

Fig. 2. Dose-dependent over-expression of human CYP3A4 in HepG2 cells transduced with Ad-E4-122aT-hCYP3A4
Levels of human CYP3A4 mRNA (A), protein (B) and activity (C) were determined in HepG2 cells 2 days after transduction with Ad-E4-122aT-GFP or Ad-E4-122aT-hCYP3A4. Data represent the mean ± S.E. ($n = 4$). **$p < 0.01$, and ***$p < 0.001$, compared with HepG2 cells transduced with Ad-E4-122aT-GFP. Human liver microsomes (HLMs) were used as a positive control.

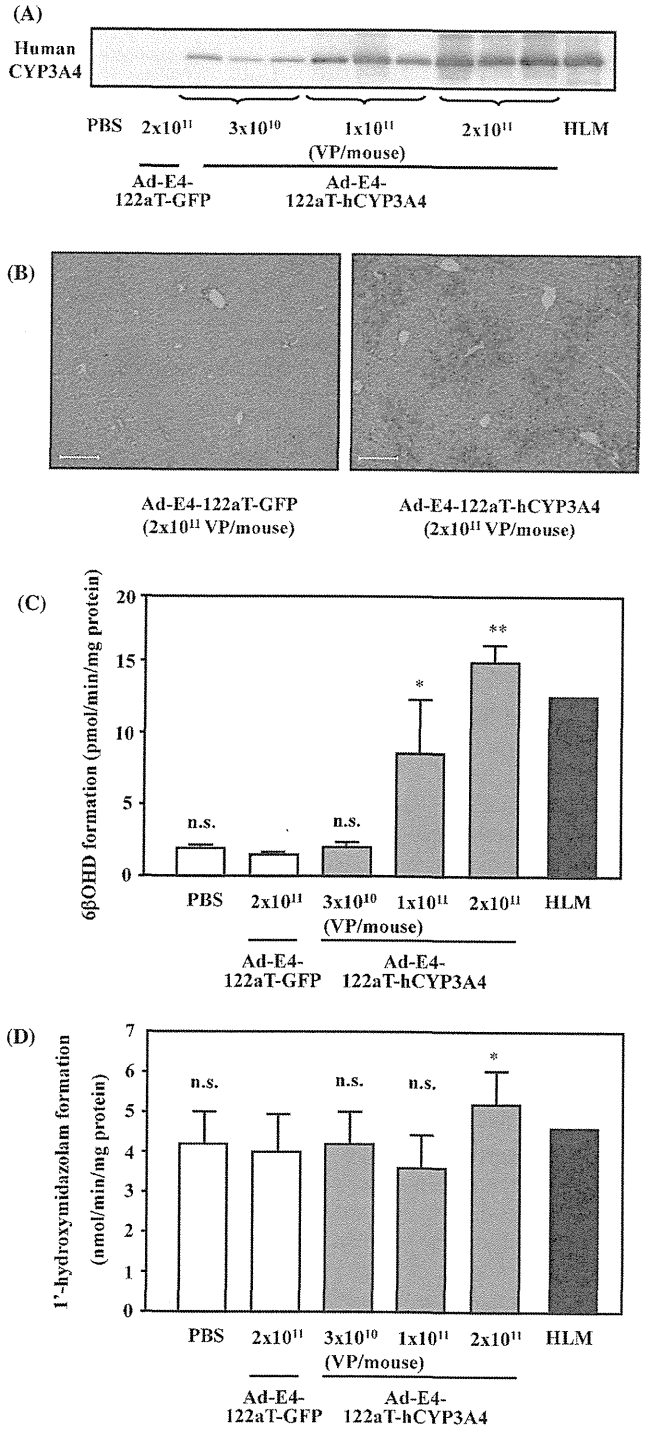

Fig. 3. Human CYP3A4 protein and activity levels in the mouse liver following Ad vector administration

Human CYP3A4 protein levels in the mouse liver following Ad vector administration were evaluated by Western blot (A) and immunohistochemical analysis (B). Human CYP3A4 activity was determined by the rate of formation of 6β-hydroxydexamethasone (6βOHD) (C) and 1′-hydroxymidazolam (D) in mouse liver microsomes. Ad-E4-122aT-hCYP3A4 was intravenously administered to mice at a dose of 3×10^{10}, 1×10^{11}, and 2×10^{11} VPs/mouse *via* the tail vein. The livers microsomes were prepared 2 days after Ad vector administration. White bars indicate 200 μm. Data represent the mean ± S.D. ($n = 3$). *$p < 0.05$, and **$p < 0.01$, compared with the liver microsomes of mice treated with Ad-E4-122aT-GFP. Human liver microsomes (HLMs) were used as a positive control.

122aT-hCYP3A4. Human CYP3A4 protein levels in the livers of mice treated with 2×10^{11} VPs of Ad-E4-122aT-hCYP3A4 were comparable to those in the human liver microsomes. Human CYP3A4 expression levels were also evaluated by immunohistochemical staining of mouse liver sections following Ad vector administration. There were human CYP3A4-positive cells throughout the liver sections of mice treated with 2×10^{11} VPs of Ad-E4-122aT-hCYP3A4 (**Fig. 3B**). These results indicate that 2×10^{11} VPs of Ad-E4-122aT-hCYP3A4 conferred to the mouse liver the ability to express human CYP3A4 protein levels comparable to those in human liver microsomes.

Next, to examine whether the human CYP3A4 protein expressed in the livers of Ad-E4-122aT-CYP3A4-administered mice was functional, mouse liver microsomes were prepared, and human CYP3A4 activities in the liver microsomes were evaluated 2 days after Ad vector administration. The concentration of 6β-hydroxydexamethasone (6βOHD) was measured for the determination of human CYP3A4 activity in the liver microsomes. Metabolism of dexamethasone to 6βOHD is catalyzed mainly by human CYP3A4.[28] The rate of 6βOHD formation by human liver microsomes is much higher than that in mouse liver microsomes.[29] As expected, only a small amount of 6βOHD was formed in the liver microsomes of PBS- and Ad-E4-122aT-GFP-administered mice (**Fig. 3C**). There was no increase in the formation of 6βOHD in the livers of mice treated with 3×10^{10} VPs of Ad-E4-122aT-CYP3A4 despite the significant expression of human CYP3A4 in the liver microsomes. However, the formation of 6βOHD was approximately 4- and 7-fold increased in the liver microsomes of mice treated with Ad-E4-122aT-CYP3A4 at doses of 1×10^{11} and 2×10^{11} VPs/mouse, respectively. In particular, the levels of 6βOHD formed in the liver microsomes of mice administered Ad-E4-122aT-CYP3A4 at a dose of 2×10^{11} VPs/mouse were similar to those formed in humans. We also evaluated human CYP3A4 activity by measuring the metabolism of midazolam in the liver microsomes of mice treated with Ad-E4-122aT-CYP3A4. Midazolam is commonly used as a probe for CYP3A activity in humans, and is metabolized by CYP3A to its primary metabolite, 1′-hydroxymidazolam.[30] The formation of 1′-hydroxymidazolam was slightly but significantly increased (1.3-fold) in mice treated with Ad-E4-122aT-CYP3A4 at a dose of 2×10^{11} VP/mouse, but not in mice treated with Ad-E4-122aT-CYP3A4 at doses of 1×10^{11} or 3×10^{10} VPs/mouse, compared with the mice receiving Ad-E4-122aT-GFP (**Fig. 3D**).

Next, in order to examine the duration of human CYP3A4 activities in the mouse liver following Ad vector administration, human CYP3A4 activities in the mouse liver were assessed at 2, 4, 6, 8, and 14 days after administration of 2×10^{11} VPs of Ad-E4-122aT-hCYP3A4. The highest human CYP3A4 activity was observed at 4 days after administration; the formation rate of 6βOHD in the liver microsomes on day 4 was slightly but significantly higher than that in human liver microsomes (**Fig. 4**). Human CYP3A4 activities in the mouse liver were maintained for at least 8 days after Ad-E4-122aT-hCYP3A4 administration. On day 14, although the formation rate of 6βOHD was reduced by 50% compared with that on day 4, the level of human CYP3A4 activity in the mouse liver microsomes was still 63% of that in the human liver microsomes. These results indicate that human CYP3A4 activity comparable to that in human liver microsomes is maintained in the mouse liver for at least 8 days following intravenous administration of Ad-E4-122aT-hCYP3A4.

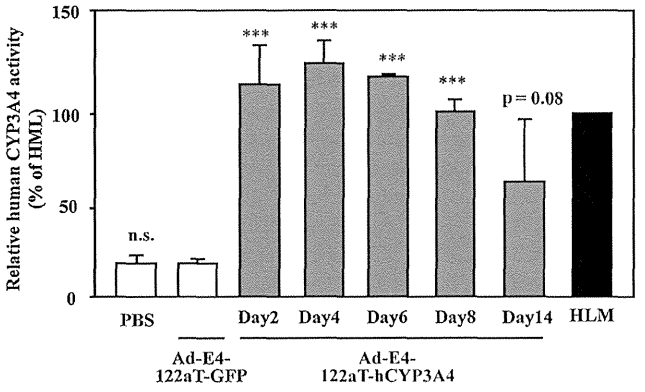

Fig. 4. Duration of human CYP3A4 activities in the mouse liver following Ad vector administration

Human CYP3A4 activities in the mouse liver following Ad vector administration were assessed by measuring the formation rate of 6βOHD in mouse liver microsomes. Ad-E4-122aT-hCYP3A4 was intravenously administered to mice at a dose of 2×10^{11} VPs/mouse *via* the tail vein. The liver microsomes were prepared on the indicated days after Ad vector administration. Values are presented as the mean $\pm$ S.D. ($n = 3$). ***$p < 0.001$, compared with mice treated with Ad-E4-122aT-GFP. Human liver microsomes (HLMs) were used as a positive control.

Table 1. Pharmacokinetic parameters estimated for dexamethasone in Ad vector-administered mice

Dexamethasone	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	AUC_{last} (ng h/mL)	AUC_{inf_obs} (ng h/mL)
PBS	6.8	0.17	783	2,709	5,087
Ad-E4-122aT-GFP (2×10^{11} VP/mouse)	5.4	1.0	799	2,443	3,903
Ad-E4-122aT-hCYP3A4 (3×10^{10} VP/mouse)	4.5	0.17	779	2,327	3,461
Ad-E4-122aT-hCYP3A4 (1×10^{11} VP/mouse)	3.0	0.17	987	1,853	2,276
Ad-E4-122aT-hCYP3A4 (2×10^{11} VP/mouse)	3.5	0.17	855	1,624	2,092
6βOH-dexamethasone	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	AUC_{last} (ng h/mL)	AUC_{inf_obs} (ng h/mL)
PBS	3.9	1.0	9.00	37.3	54.2
Ad-E4-122aT-GFP (2×10^{11} VP/mouse)	3.8	1.0	8.00	28.1	39.1
Ad-E4-122aT-hCYP3A4 (3×10^{10} VP/mouse)	4.1	1.0	8.00	37.4	55.0
Ad-E4-122aT-hCYP3A4 (1×10^{11} VP/mouse)	4.4	0.17	11.0	40.5	59.6
Ad-E4-122aT-hCYP3A4 (2×10^{11} VP/mouse)	4.4	0.17	28.0	74.3	106

***In vivo* pharmacokinetics profiles of human CYP3A4 substrates in mice over-expressing human CYP3A4:** Next, in order to examine whether Ad vector-mediated over-expression of human CYP3A4 in the mouse liver alters the *in vivo* pharmacokinetics of human CYP3A4 substrates, dexamethasone and midazolam were orally administered to mice treated with Ad-E4-122aT-CYP3A4 at a dose of 2 mg/kg. Subsequently, the blood concentrations of dexamethasone, midazolam, and their metabolites were measured. The AUC of 6βOHD in mice treated with Ad-E4-122aT-GFP was decreased by 72%, compared with that for the mice receiving PBS (**Table 1**), probably due to the decline in endogenous metabolic activity in the liver by transduction with a high titer of the Ad vector in the liver, although there was no apparent difference in the formation rate of 6βOHD between the liver microsomes of mice receiving PBS and those receiving Ad-E4-122aT-GFP, as shown in **Figure 3C**. The AUC of 6βOHD was 1.4-fold and 2.7-fold elevated in mice treated with Ad-E4-122aT-hCYP3A4 at a dose of 3×10^{10} VPs/mouse and 2×10^{11} VPs/mouse, respectively, compared with the mice receiving Ad-E4-122aT-GFP (**Fig. 5B** and **Table 1**). The $T_{1/2}$ of dexamethasone and the C_{max} of 6βOHD in mice receiving Ad-E4-122aT-hCYP3A4 were largely lower and higher than those in mice receiving Ad-E4-122aT-GFP, respectively (**Table 1**). These results indicate that Ad vector-mediated over-expression of human CYP3A4 in the mouse liver alters the *in vivo* pharmacokinetics of human CYP3A4-specific metabolites in mice. On the other hand, the AUCs for midazolam and the primary metabolite of midazolam, 1′-hydroxymidazolam, were not significantly different between the mice treated with Ad-E4-122aT-hCYP3A4 and Ad-E4-122aT-GFP (**Figs. 5C**, **5D** and **Table 2**).

Discussion

In this study, we used an Ad vector to generate a human CYP3A4-expressing animal that could be used for the evaluation of drug metabolism in humans. For this purpose, a human CYP3A4-expressing Ad vector (Ad-E4-122aT-hCYP3A4) was generated using a novel Ad vector that exhibits a reduction in Ad vector-mediated hepatotoxicity. A single administration of Ad-E4-122aT-hCYP3A4 to mice at dose of 2×10^{11} VPs/mouse achieved levels of human CYP3A4 activity in the liver microsomes of the Ad-CYP3A4-administered mice that were similar to those in human liver microsomes. In addition, pharmacokinetic experiments revealed that the AUC of 6βOHD was 2.7-fold elevated in the Ad-E4-122aT-hCYP3A4-administered mice, compared with the mice receiving a control Ad vector. To the best of our knowledge, this is the first report demonstrating the production of mice possessing hepatic activity of human CYP3A4 at levels similar to those in the human liver by the intravenous administration of an Ad vector expressing human CYP3A4, although several studies have reported that various types of human CYP activities are up-regulated in cultured cell lines, including HepG2 cells, by transduction with Ad vectors expressing human CYP enzymes.[31,32]

An Ad vector was used for over-expression of human CYP3A4 in the mouse liver in this study. An Ad vector was considered suitable for this purpose because Ad vectors are known to have strong hepatotropism. Several groups, including ours, have demonstrated that more than 90% of the injected dose of an Ad vector accumulates in the liver within several minutes following intravenous administration into mice.[16,33,34] Although transgenic animals expressing human CYP enzymes have been developed,[10–12] there remain several concerns with the resulting strains; the integration site and integrated copy numbers of the transgene cannot be adequately controlled, leading to unpredictable expression in transgenic mice. On the other hand, Ad vector-mediated over-expression of human CYP enzymes in the livers of experimental animals has several advantages for evaluating CYP-mediated drug metabolism. First, human CYP enzyme activities in the liver can be easily controlled by altering the injected doses of an Ad vector. It is well known that CYP-mediated drug metabolism exhibits considerable interindividual variation, due in part to genetic polymorphisms. For example, in the case of human CYP2D6, which is known to be highly polymorphic, more than 100

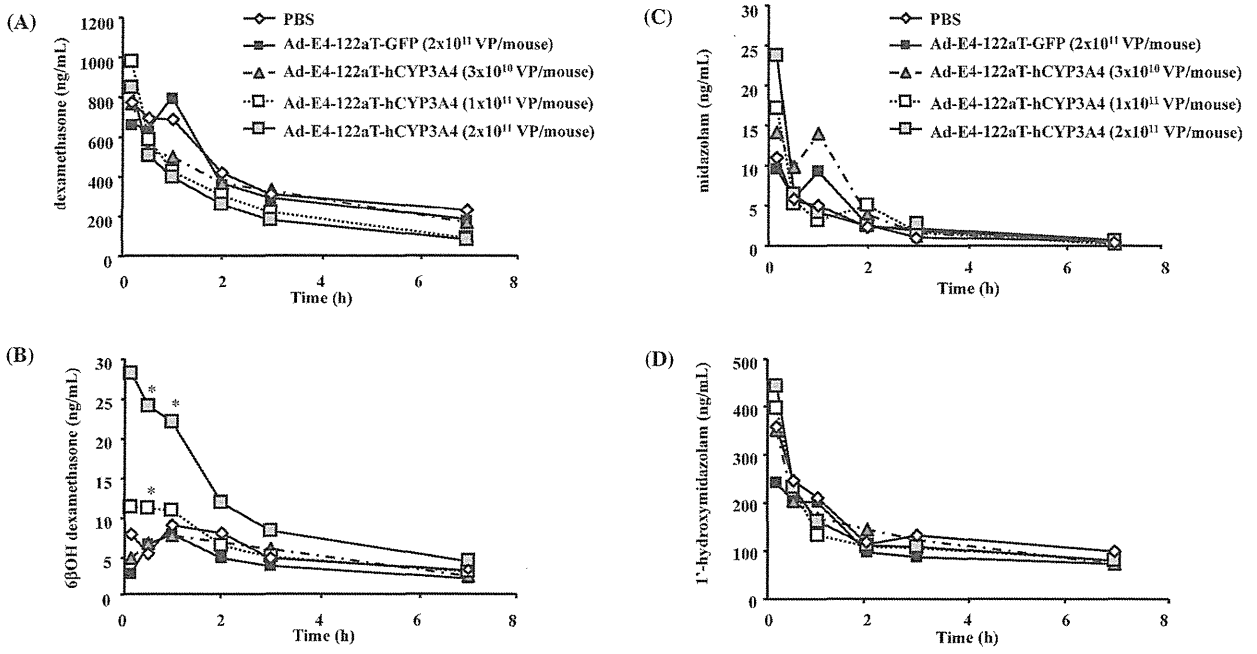

Fig. 5. *In vivo* blood clearance profiles of human CYP3A4 substrates in mice overexpressing human CYP3A4
Mice were transduced with 3×10^{10}, 1×10^{11}, and 2×10^{11} VPs of Ad-E4-122aT-hCYP3A4 or 2×10^{11} VPs of Ad-E4-122aT-GFP. Forty-eight hours after Ad vector administration, mice were orally administered dexamethazone and midazolam (2 mg/10 mL). Blood samples were taken at the indicated time points. Plasma concentration *versus* time curves of (A) dexamethasone, (B) 6βOHD, (C) midazolam and (D) 1'-hydroxymidazolam are shown. Data represent the mean ($n = 3$). *$p < 0.05$, compared with mice treated with Ad-E4-122aT-GFP.

Table 2. Pharmacokinetic parameters estimated for midazolam in Ad vector-administered mice

Midazolam	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	AUC_{last} (ng h/mL)	AUC_{inf_obs} (ng h/mL)
PBS	4.7	0.17	11.0	17.4	24.2
Ad-E4-122aT-GFP (2×10^{11} VP/mouse)	2.2	0.17	10.0	18.5	21.6
Ad-E4-122aT-hCYP3A4 (3×10^{10} VP/mouse)	0.71	0.17	14.0	22.0	24.0
Ad-E4-122aT-hCYP3A4 (1×10^{11} VP/mouse)	2.2	0.17	17.0	14.4	20.8
Ad-E4-122aT-hCYP3A4 (2×10^{11} VP/mouse)	3.1	0.17	24.0	21.0	25.4
1'-Hydroxymidazolam	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	AUC_{last} (ng h/mL)	AUC_{inf_obs} (ng h/mL)
PBS	5.6	0.17	359	977	1,763
Ad-E4-122aT-GFP (2×10^{11} VP/mouse)	12	0.17	243	743	1,962
Ad-E4-122aT-hCYP3A4 (3×10^{10} VP/mouse)	4.9	0.17	351	879	1,386
Ad-E4-122aT-hCYP3A4 (1×10^{11} VP/mouse)	11	0.17	398	816	2,044
Ad-E4-122aT-hCYP3A4 (2×10^{11} VP/mouse)	10	0.17	444	856	1,991

Mice were intravenously administered with Ad-E4-122aT-hCYP3A4. Forty-eight hours after Ad vector administration, mice received midazolam (2 mg/kg) by oral administration. Values represent the mean ($n = 3$).

allelic variants of CYP2D6 have been discovered to date (http://www.cypalleles.ki.se/).[35] Five to 10% of Caucasians are poor metabolizers, resulting in individual variation in response to many drugs due to impaired metabolism by CYP2D6.[36] In human CYP-possessing animal models created by Ad vector administration, certain CYP poor and extensive metabolizer phenotypes are easily prepared by altering the Ad vector doses. Second, several types of human CYP enzymes can be simultaneously over-expressed by using an Ad vector possessing multiple human CYP genes. The combination of human CYP enzymes over-expressed in the liver can be modified by changing the human CYP genes in the Ad vector genome. Furthermore, Ad vectors are able to efficiently transduce the livers of various types of animals which are available for drug development studies, including mice, rats, dogs, and rabbits.[37–39] Due to these superior properties of Ad vectors as gene delivery vehicles, we concluded that an Ad vector would be suitable for the generation of experimental animals expressing human CYP genes in the liver.

In this study, there was no significant increase in the production of 6βOHD in the liver microsomes derived from mice treated with Ad-E4-122aT-hCYP3A4 at a dose of 3×10^{10} VPs/mouse, compared with mice treated with a control Ad vector, despite the apparent human CYP3A4 protein in the livers of mice administered Ad-E4-122aT-hCYP3A4 at a dose of 3×10^{10} VPs/mouse in the Western blotting analysis (**Figs. 3A** and **3C**). Although there was no significant elevation of 6βOHD formation in the liver microsomes of mice receiving 3×10^{10} VPs of Ad-E4-122aT-hCYP3A4, we consider certain levels of CYP3A4 activity to have been present in the livers of mice even at the dose of 3×10^{10} VPs/mouse of Ad-E4-122aT-hCYP3A4; however, the levels of human CYP3A4 activity generated by 3×10^{10} VPs of Ad-E4-122aT-hCYP3A4 would be much lower than the intrinsic 6βOHD formation activity in the livers of mice treated with 2×10^{11} VPs of Ad-E4-122aT-GFP, which would account for the lack of a statistically significant increase in 6βOHD formation in the transduced liver microsomes, compared with the liver microsomes of mice treated with a control Ad vector. In this study, we used the

rate of 6βOHD formation as a marker of human CYP3A4 activity. Although 6βOHD is a human CYP3A4-specific metabolite of dexamethasone, 6βOHD is slightly formed in the mouse liver microsomes (accounting for 5.8% of total metabolites formed) due to the endogenous mouse CYP activity in the liver.[29] In contrast, significant levels of human CYP3A4 activity were found even at 100 VPs/cell in HepG2 cells in this study. HepG2 cells, which are commonly used to evaluate hepatotoxicity induced by bioactivable compounds, showed almost undetectable CYP activities.[32] Thus, an Ad vector-mediated increase in human CYP3A4 enzymatic activity was clearly detected in HepG2 cells.

In this study, human CYP3A4 activity in the mouse liver was also evaluated by measuring the metabolism of midazolam; however, no significant increase in the formation of the primary metabolite of midazolam, 1-hydroxymidazolam, was observed in the mice receiving Ad-E4-122aT-hYCP3A4, compared with the mice treated with a control Ad vector in the *in vivo* pharmacokinetic experiments (**Figs. 5C** and **5D**), although slight but significant elevation of 1′-hydroxymidazolam formation was found in the liver microsomes of mice receiving 2×10^{11} VP of Ad-E4-122aT-hCYP3A4 (**Fig. 3D**). It remains unclear why midazolam metabolism did not appear to be increased in mice receiving Ad-E4-122aT-hCYP3A4; however, the *in vitro* metabolism experiment of midazolam using liver microsomes demonstrated that similar levels of 1′-hydroxymidazolam were produced in the liver microsomes of humans and PBS-treated mice, indicating that midazolam metabolic activities in the human and mouse liver are comparable. Mouse CYP3A and CYP2C metabolize midazolam to 1′-hydroxymidazolam.[40] Over-expressed human CYP3A4-mediated metabolism of midazolam might be lower than mouse endogenous CYP-mediated metabolism of midazolam. In a previous study, the human CYP3A4-transgenic mice and wild-type mice exhibited no significant difference in the metabolism of orally administered midazolam.[10] On the other hand, when midazolam was intravenously administered to human CYP3A4-transgenic mice that showed an approximately 2-fold higher human CYP3A4 expression in the liver than wild-type mice, a higher metabolism of midazolam was observed in the livers of human CYP3A4-transgenic mice, compared with wild-type mice.[11] Elevation of midazolam metabolism *in vivo* might be achieved by increasing the dose of Ad-E4-122aT-hCYP3A4, since higher doses of Ad-E4-122aT-hCYP3A4 mediate higher human CYP3A4 expression in the liver than is observed in the human liver. The findings described above also suggest that human CYP expression levels in experimental animals have a major effect on drug metabolism, and that it might be difficult to provide appropriate expression levels of human CYPs in transgenic animals. On the other hand, Ad vector-mediated human CYP expression levels in the organs can be easily controlled by increasing or decreasing the dose of an Ad vector. Recently, two studies demonstrated the development of human CYP3A4-humanizing transgenic mice and showed that these mice would be useful for evaluating human CYP3A4-mediated drug metabolism.[41,42] The human CYP3A4-expressing mice in our present study possess mouse endogenous CYP activity, which might disrupt the evaluation of human CYP-mediated drug metabolism. Intravenous administration of an Ad vector expressing human CYP genes into mouse CYP gene-knockout mice would generate a better animal model for the evaluation of drug metabolism in humans, compared with wild-type mice receiving a human CYP gene-expressing Ad vector.

A novel Ad vector, in which four copies of sequences complementary to miR-122a, a liver-specific microRNA, are incorporated into the 3′-untranslated region of the E4 gene to suppress the leaky expression of Ad genes in the liver, was used for the over-expression of human CYP3A4 genes. We previously demonstrated that this Ad vector showed a significant reduction in the leaky expression of Ad genes in the liver and Ad vector-mediated hepatotoxicity.[21] This Ad vector was thus considered suitable for over-expression of human CYP3A4 in the liver. In order to confer to the mouse liver a level of human CYP3A4 activity comparable to that in the human liver microsomes, 2×10^{11} VPs of an Ad vector expressing human CYP3A4 was required; however, intravenous administration of a conventional Ad vector expressing human CYP3A4 at such a high dose induced a significant increase in serum ALT levels 2 days after administration (data not shown). On the other hand, Ad-E4-122aT-hCYP3A4 did not induce any apparent hepatotoxicity. This property would also be advantageous for the evaluation of human CYP3A4-mediated drug metabolism.

In conclusion, we succeeded in the development of mice exhibiting hepatic activity of human CYP3A4 at levels similar to those in the human liver by intravenous administration of a novel Ad vector expressing human CYP3A4. This novel Ad vector expressing human CYP3A4 would be a powerful tool for evaluating human CYP3A4-mediated drug metabolism in the livers of experimental animals.

Acknowledgments: The authors thank Sayuri Okamoto, Eri Hosoyamada, Aki Nonaka, Hayato Matsui (Osaka University, Osaka, Japan), and Nobue Hirata (National Institute of Biomedical Innovation, Osaka, Japan) for their help. The CA promoter was kindly provided by Dr. Junichi Miyazaki (Osaka University, Osaka, Japan).

References

1) Gonzalez, F. J. and Gelboin, H. V.: Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins. *Drug Metab. Rev.*, **26**: 165–183 (1994).
2) Guengerich, F. P.: Cytochromes P450, drugs, and diseases. *Mol. Interv.*, **3**: 194–204 (2003).
3) Guengerich, F. P.: Cytochrome p450 and chemical toxicology. *Chem. Res. Toxicol.*, **21**: 70–83 (2008).
4) Kaplowitz, N.: Idiosyncratic drug hepatotoxicity. *Nat. Rev. Drug Discov.*, **4**: 489–499 (2005).
5) Bogaards, J. J., Bertrand, M., Jackson, P., Oudshoorn, M. J., Weaver, R. J., van Bladeren, P. J. and Walther, B.: Determining the best animal model for human cytochrome P450 activities: a comparison of mouse, rat, rabbit, dog, micropig, monkey and man. *Xenobiotica*, **30**: 1131–1152 (2000).
6) Martignoni, M., Groothuis, G. M. and de Kanter, R.: Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opin. Drug Metab. Toxicol.*, **2**: 875–894 (2006).
7) Powley, M. W., Frederick, C. B., Sistare, F. D. and DeGeorge, J. J.: Safety assessment of drug metabolites: implications of regulatory guidance and potential application of genetically engineered mouse models that express human P450s. *Chem. Res. Toxicol.*, **22**: 257–262 (2009).
8) Smith, D. A. and Obach, R. S.: Metabolites and safety: What are the concerns, and how should we address them? *Chem. Res. Toxicol.*, **19**: 1570–1579 (2006).
9) FDA: Guidance for Industry: Safety Testing of Drug Metabolites, 2008.
10) Granvil, C. P., Yu, A. M., Elizondo, G., Akiyama, T. E., Cheung, C., Feigenbaum, L., Krausz, K. W. and Gonzalez, F. J.: Expression of the human CYP3A4 gene in the small intestine of transgenic mice: in vitro metabolism and pharmacokinetics of midazolam. *Drug Metab. Dispos.*, **31**: 548–558 (2003).
11) van Herwaarden, A. E., Smit, J. W., Sparidans, R. W., Wagenaar, E., van der Kruijssen, C. M., Schellens, J. H., Beijnen, J. H. and Schinkel,

A. H.: Midazolam and cyclosporin a metabolism in transgenic mice with liver-specific expression of human CYP3A4. *Drug Metab. Dispos.*, **33**: 892–895 (2005).
12) Yu, A. M., Fukamachi, K., Krausz, K. W., Cheung, C. and Gonzalez, F. J.: Potential role for human cytochrome P450 3A4 in estradiol homeostasis. *Endocrinology*, **146**: 2911–2919 (2005).
13) Cheung, C. and Gonzalez, F. J.: Humanized mouse lines and their application for prediction of human drug metabolism and toxicological risk assessment. *J. Pharmacol. Exp. Ther.*, **327**: 288–299 (2008).
14) Kovesdi, I., Brough, D. E., Bruder, J. T. and Wickham, T. J.: Adenoviral vectors for gene transfer. *Curr. Opin. Biotechnol.*, **8**: 583–589 (1997).
15) Mizuguchi, H. and Hayakawa, T.: Targeted adenovirus vectors. *Hum. Gene Ther.*, **15**: 1034–1044 (2004).
16) Sakurai, F., Mizuguchi, H., Yamaguchi, T. and Hayakawa, T.: Characterization of in vitro and in vivo gene transfer properties of adenovirus serotype 35 vector. *Mol. Ther.*, **8**: 813–821 (2003).
17) Bai, J. and Cederbaum, A. I.: Adenovirus-mediated expression of CYP2E1 produces liver toxicity in mice. *Toxicol. Sci.*, **91**: 365–371 (2006).
18) Guengerich, F. P.: Cytochrome P-450 3A4: regulation and role in drug metabolism. *Annu. Rev. Pharmacol. Toxicol.*, **39**: 1–17 (1999).
19) Koch, I., Weil, R., Wolbold, R., Brockmoller, J., Hustert, E., Burk, O., Nuessler, A., Neuhaus, P., Eichelbaum, M., *et al.*: Interindividual variability and tissue-specificity in the expression of cytochrome P450 3A mRNA. *Drug Metab. Dispos.*, **30**: 1108–1114 (2002).
20) Mian, A., Guenther, M., Finegold, M., Ng, P., Rodgers, J. and Lee, B.: Toxicity and adaptive immune response to intracellular transgenes delivered by helper-dependent vs. first generation adenoviral vectors. *Mol. Genet. Metab.*, **84**: 278–288 (2005).
21) Shimizu, K., Sakurai, F., Tomita, K., Nagamoto, Y., Nakamura, S., Katayama, K., Tachibana, M., Kawabata, K. and Mizuguchi, H.: Suppression of the leaky expression of adenovirus genes following transduction with a replication-incompetent adenovirus vector by incorporating microRNA-targeted sequences into the 3′-untranslated region of the E2A, E4, or pIX gene. *Mol. Ther. Methods Clin. Dev.*, in press (2014).
22) Mizuguchi, H. and Kay, M. A.: Efficient construction of a recombinant adenovirus vector by an improved in vitro ligation method. *Hum. Gene Ther.*, **9**: 2577–2583 (1998).
23) Sakurai, F., Kawabata, K., Yamaguchi, T., Hayakawa, T. and Mizuguchi, H.: Optimization of adenovirus serotype 35 vectors for efficient transduction in human hematopoietic progenitors: comparison of promoter activities. *Gene Ther.*, **12**: 1424–1433 (2005).
24) Maizel, J. V., Jr., White, D. O. and Scharff, M. D.: The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology*, **36**: 115–125 (1968).
25) Emoto, C., Yamato, Y., Sato, Y., Ohshita, H., Katoh, M., Tateno, C., Yokoi, T., Yoshizato, K. and Iwasaki, K.: Non-invasive method to detect induction of CYP3A4 in chimeric mice with a humanized liver. *Xenobiotica*, **38**: 239–248 (2008).
26) Niwa, H., Yamamura, K. and Miyazaki, J.: Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene*, **108**: 193–199 (1991).
27) Xu, Z. L., Mizuguchi, H., Ishii-Watabe, A., Uchida, E., Mayumi, T. and Hayakawa, T.: Strength evaluation of transcriptional regulatory elements for transgene expression by adenovirus vector. *J. Control. Release*, **81**: 155–163 (2002).
28) Gentile, D. M., Tomlinson, E. S., Maggs, J. L., Park, B. K. and Back, D. J.: Dexamethasone metabolism by human liver in vitro. Metabolite identification and inhibition of 6-hydroxylation. *J. Pharmacol. Exp. Ther.*, **277**: 105–112 (1996).
29) Tomlinson, E. S., Maggs, J. L., Park, B. K. and Back, D. J.: Dexamethasone metabolism in vitro: species differences. *J. Steroid Biochem. Mol. Biol.*, **62**: 345–352 (1997).
30) Wandel, C., Bocker, R. H., Bohrer, H., deVries, J. X., Hofmann, W., Walter, K., Kleingeist, B., Neff, S., Ding, R., *et al.*: Relationship between hepatic cytochrome P450 3A content and activity and the disposition of midazolam administered orally. *Drug Metab. Dispos.*, **26**: 110–114 (1998).
31) Hosomi, H., Fukami, T., Iwamura, A., Nakajima, M. and Yokoi, T.: Development of a highly sensitive cytotoxicity assay system for CYP3A4-mediated metabolic activation. *Drug Metab. Dispos.*, **39**: 1388–1395 (2011).
32) Tolosa, L., Gomez-Lechon, M. J., Perez-Cataldo, G., Castell, J. V. and Donato, M. T.: HepG2 cells simultaneously expressing five P450 enzymes for the screening of hepatotoxicity: identification of bioactivable drugs and the potential mechanism of toxicity involved. *Arch. Toxicol.*, **87**: 1115–1127 (2013).
33) Kalyuzhniy, O., Di Paolo, N. C., Silvestry, M., Hofherr, S. E., Barry, M. A., Stewart, P. L. and Shayakhmetov, D. M.: Adenovirus serotype 5 hexon is critical for virus infection of hepatocytes in vivo. *Proc. Natl. Acad. Sci. USA*, **105**: 5483–5488 (2008).
34) Waddington, S. N., McVey, J. H., Bhella, D., Parker, A. L., Barker, K., Atoda, H., Pink, R., Buckley, S. M., Greig, J. A., *et al.*: Adenovirus serotype 5 hexon mediates liver gene transfer. *Cell*, **132**: 397–409 (2008).
35) Kim, J., Lim, Y. R., Han, S., Han, J. S., Chun, Y. J., Yun, C. H., Lee, C. H. and Kim, D.: Functional influence of human CYP2D6 allelic variations: P34S, E418K, S486T, and R296C. *Arch. Pharm. Res.*, **36**: 1500–1506 (2013).
36) Yu, A. M., Idle, J. R. and Gonzalez, F. J.: Polymorphic cytochrome P450 2D6: humanized mouse model and endogenous substrates. *Drug Metab. Rev.*, **36**: 243–277 (2004).
37) Chuah, M. K., Schiedner, G., Thorrez, L., Brown, B., Johnston, M., Gillijns, V., Hertel, S., Van Rooijen, N., Lillicrap, D., *et al.*: Therapeutic factor VIII levels and negligible toxicity in mouse and dog models of hemophilia A following gene therapy with high-capacity adenoviral vectors. *Blood*, **101**: 1734–1743 (2003).
38) Jaffe, H. A., Danel, C., Longenecker, G., Metzger, M., Setoguchi, Y., Rosenfeld, M. A., Gant, T. W., Thorgeirsson, S. S., Stratford-Perricaudet, L. D., *et al.*: Adenovirus-mediated in vivo gene transfer and expression in normal rat liver. *Nat. Genet.*, **1**: 372–378 (1992).
39) Kozarsky, K. F., McKinley, D. R., Austin, L. L., Raper, S. E., Stratford-Perricaudet, L. D. and Wilson, J. M.: In vivo correction of low density lipoprotein receptor deficiency in the Watanabe heritable hyperlipidemic rabbit with recombinant adenoviruses. *J. Biol. Chem.*, **269**: 13695–13702 (1994).
40) Perloff, M. D., von Moltke, L. L., Court, M. H., Kotegawa, T., Shader, R. I. and Greenblatt, D. J.: Midazolam and triazolam biotransformation in mouse and human liver microsomes: relative contribution of CYP3A and CYP2C isoforms. *J. Pharmacol. Exp. Ther.*, **292**: 618–628 (2000).
41) van Herwaarden, A. E., Wagenaar, E., van der Kruijssen, C. M., van Waterschoot, R. A., Smit, J. W., Song, J. Y., van der Valk, M. A., van Tellingen, O., van der Hoorn, J. W., *et al.*: Knockout of cytochrome P450 3A yields new mouse models for understanding xenobiotic metabolism. *J. Clin. Invest.*, **117**: 3583–3592 (2007).
42) Hasegawa, M., Kapelyukh, Y., Tahara, H., Seibler, J., Rode, A., Krueger, S., Lee, D. N., Wolf, C. R. and Scheer, N.: Quantitative prediction of human pregnane X receptor and cytochrome P450 3A4 mediated drug-drug interaction in a novel multiple humanized mouse line. *Mol. Pharmacol.*, **80**: 518–528 (2011).