Human CD46 is expressed on almost all human cells, leading to broad tropism of Ad35 vectors in human cells, including coxsackievirus-adenovirus receptor-negative cells.[8,12] However, intravenous administration of Ad35 vectors resulted in inefficient transduction in the organs of human CD46-transgenic (CD46TG) mice and cynomolgus monkeys, which express CD46 in a pattern similar to that of humans.[13–15] These results indicate that CD46 does not successfully serve as a receptor for intravascularly injected Ad35 vectors and that Ad35 vectors are unsuitable for intravascular transduction. However, this property of Ad35 vectors would suggest a potential advantage, in that unwanted transduction would not occur in organs other than the organs targeted following direct injection of Ad35 vectors when draining from injected sites into the bloodstream. These properties suggest that Ad35 vectors would be suitable for gene transfer by local administration into the organs. In this study, we examined the transduction properties of Ad35 vectors following intraorgan administration in nonhuman primates, that is, cynomologus monkeys.

A previously constructed Ad35 vector expressing β-galactosidase (Ad35LacZ)[15] was locally administered at a dose of 1.5×10^{11} vector particles (VP) per point (high dose) or 3×10^{10} VP per point (low dose) in the following eight organs of two cynomolgus monkeys (designated no. 8 and no. 9; no. 8 received the high dose of Ad35LacZ and no. 9 received the low dose): liver, cerebrum, eyeball (vitreous body), quadriceps femoris muscle, pancreas, kidney, spleen and nasal cavity. Four days after administration, the tissues around the injection sites (approximately $40 \times 40 \times 10$ mm^3 with a central focus at the injection point) were collected and subjected to an analysis of β-galactosidase expression and histological pathology. The health condition of the monkeys was also monitored until necropsy.

Overall, both monkeys did well during the experiment. There were no apparent abnormalities in body temperature or heart rate, although no. 8, the high-dose monkey, exhibited slight reductions in blood pressure and body weight. Both monkeys apparently exhibited increased serum levels of aspartate aminotransferase and creatine phosphokinase on days 0–2 after injection. Mild decreases in hemoglobin levels and increases in levels of lactate dehydrogenase and C-reactive protein were also found in both animals. However, these changes were probably due to the operation. The levels of alanine aminotransferase, alkaline phosphatase, albumin, glucose, calcium, chloride and sodium in the serum were mostly within the normal ranges.

After the direct injection of the Ad35 vectors, the transduction profiles were assessed by immunostaining of β-galactosidase in the tissue sections; Table 1 summarizes the results. A detailed transduction profile in each organ is described below.

Liver

Direct injection of Ad35LacZ to the liver caused tissue damage around the injection site (Figures 1a and b). Infiltration of inflammatory cells, necrotic focus and regenerated bile duct epithelial cells were observed. Immunostaining of the liver sections revealed that hepatocytes were mainly transduced with Ad35LacZ in both no. 8 and no. 9 monkeys (Figures 2a and b). A higher level of β-galactosidase was expressed in the liver of no. 8 than in that of no. 9. The transduced cells were predominantly distributed around the injection point (approximately 1×1 mm^2) and were not found outside the periphery of the injection site. β-galactosidase was not expressed in the liver lobes, which were not injected with Ad35LacZ. β-galactosidase-expressing cells were mainly found on the border region between the normal and damaged areas. Direct injection of naked plasmid DNA or Ad5 vectors into mouse liver also resulted in the localized distribution of transgene-expressing cells around the injection points.[16,17] The liver would not allow dispersion of locally injected Ad vectors in the tissue.

Table 1 β-galactosidase expression in the organs following direct injection of Ad35LacZ into organs

	No. 8 (high dose)	*No. 9 (low dose)*
Liver	+++	+
Cerebrum	+++	+
Eyeball	+	−
Femoral muscle	+	+
Pancreas	−	++
Kidney	−	++
Spleen	−	−
Nasal cavity	−	−

+++, strong positive; ++, moderate positive; +, weakly positive; −, negative.

Cerebrum

Ad35LacZ was stereotaxically injected into the left frontal lobe of the cerebrum. After infusion of the high dose of Ad35LacZ, softening of the tissue, which appeared necrotic, was widely observed in the left basal ganglia (Figure 1c). Neutrophils were infiltrated into the necrotic area. In contrast, injection of a low dose of Ad35LacZ resulted in no apparent toxicity, although slight bleeding was found around the artery (Figure 1d). Transduced cells, which appeared to be microglia, were found around the softening regions of both no. 8 and no. 9 animals, although the latter had fewer transduced microglia (Figures 2c and d). There were no β-galactosidase-expressing cells in the right hemisphere of the brain, which was infused with phosphate-buffered saline buffer (data not shown).

Eye

Ad35LacZ was infused into the vitreous body for inoculation into the eyeball. The high dose induced invasion by inflammatory cells, including macrophages and neutrophils, into the ciliary body, iris and retina (Figure 1e). Necrotic changes were also found in all layers of the retina. The low dose caused similar damage to the eyeball. The high dose mediated transduction in the conjunctival epithelial cells (Figure 2e). β-galactosidase expression was not observed in other areas. After injection into the vitreous body, Ad35LacZ might be drained from it and transduce the conjunctival epithelial cells. Bora *et al.*[18] demonstrated that human CD46 was hardly expressed in eye tissues, suggesting that these tissues are refractory to Ad35 vectors. We did not find β-galactosidase expression in the eye of no. 9 animal. Phosphate-buffered saline injection did not result in

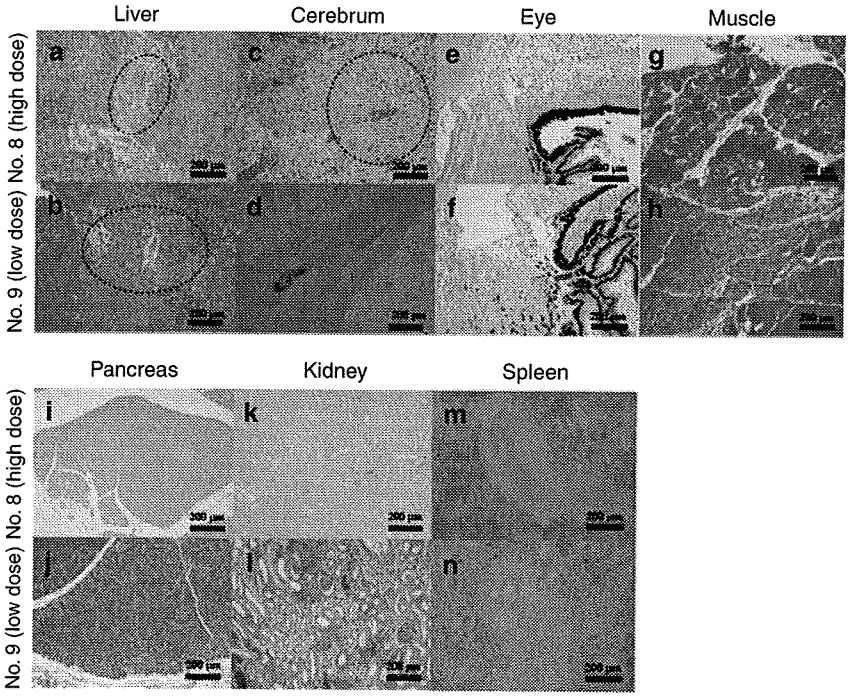

Figure 1 Tissue histology in the organs of cynomolgus monkeys 4 days after intraorgan injection of Ad35LacZ. (**a** and **b**) The liver, (**c** and **d**) cerebrum, (**e** and **f**) eyeball, (**g** and **h**) skeletal muscle, (**i** and **j**) pancreas, (**k** and **l**) kidney and (**m** and **n**) spleen. Young male cynomolgus monkeys (*Macaca fascicularis*) were housed and handled in accordance with the rules for animal care and management of the Tsukuba Primate Center and with the guiding principles for animal experiments using nonhuman primates formulated by the Primate Society of Japan. The animals (approximately 3 years of age, 1.9 and 2.2 kg) were certified free of intestinal parasites and seronegative for simian type-D retrovirus, herpesvirus B, varicella-zoster-like virus and measles virus. The protocol of the experimental procedures was approved by the Animal Welfare and Animal Care Committee of the National Institute of Biomedical Innovation (Osaka, Japan). The liver, cerebrum, eyeball, nasal cavity, pancreas, kidney, skeletal muscle and spleen of cynomolgus monkeys were each injected with Ad35LacZ suspended in 200 μl (100 μl for eyeball) of phosphate-buffered saline at a dose of 1.5×10^{11} vector particles (VP) per point (monkey no. 8) or 3×10^{10} VP per point (monkey no. 9). Four days after injection, tissue sections were hematoxylin–eosin stained by a routine method. Dotted-line circles in (**b**) and (**c**) indicate the necrotic area in the liver and the softening area in the cerebrum, respectively.

transgene expression or apparent abnormality in the eyeball (data not shown).

Femoral muscle

Severe inflammation did not occur after intramuscular injection of the high dose, although we found slight damage to the muscle fibers (Figure 1g). In contrast, the low dose induced more severe inflammation (Figure 1h). Infiltration of neutrophils and macrophages was seen in the muscle of no. 9. It is currently unclear why the low dose induced higher levels of damage. A slight difference in the injection point might affect Ad35 vector-induced inflammatory responses in the muscle. β-galactosidase expression was found only in the cells that appeared to be macrophages and/or fibroblasts located among the muscle fibers in both monkeys (Figures 2g and h). No muscle fibers expressed β-galactosidase in either monkey. It remains to be elucidated why intramuscular injection of Ad35 vectors mediated poor transduction in muscle fibers of cynomolgus monkeys. Ad35 vectors transduced the muscle following intramuscular injection in wild-type mice and in CD46TG mice.[12,14] The transduction mechanism and efficiencies of Ad35 vectors in muscle fibers might differ among species, and the muscle of nonhuman primates might be more refractory to transduction than that of rodents. Thirion *et al.*[19] demonstrated that Ad vectors would transduce human, rat and mouse primary muscle cells through different pathways. Danko *et al.*[20] reported that transgene expression levels by intramuscular injection of naked DNA were lower in dogs and nonhuman primates than in rodents. On the other hand, several studies demonstrated the utility of Ad35 vectors as vaccine vectors that express antigen by intramuscular administration in mice and nonhuman primates.[3,4] Macrophages and/or dendritic cells transduced with Ad35 vectors might play important roles in transgene-specific immune responses by intramuscular injection of Ad35 vectors.

Pancreas

Injection into the pancreas caused no severe damage to that organ in either monkey (Figures 1i and j). We did not find transduced cells in the pancreas of no. 8; in contrast, β-galactosidase was apparently expressed in exocrine acinar cells of no. 9 in the pancreatic lobules (Figures 2i and j). Chemiluminescence assay of β-galactosidase also revealed significant levels of β-galactosidase expression in the pancreas of no. 9 but not in that of no. 8 (data not shown). Wang *et al.*[21] also demonstrated that direct injection of conventional Ad vectors and adenoassociated virus vectors into murine pancreas achieved efficient transduction in acinar cells. Pancreatic acinar cells would be susceptible to Ad vectors.

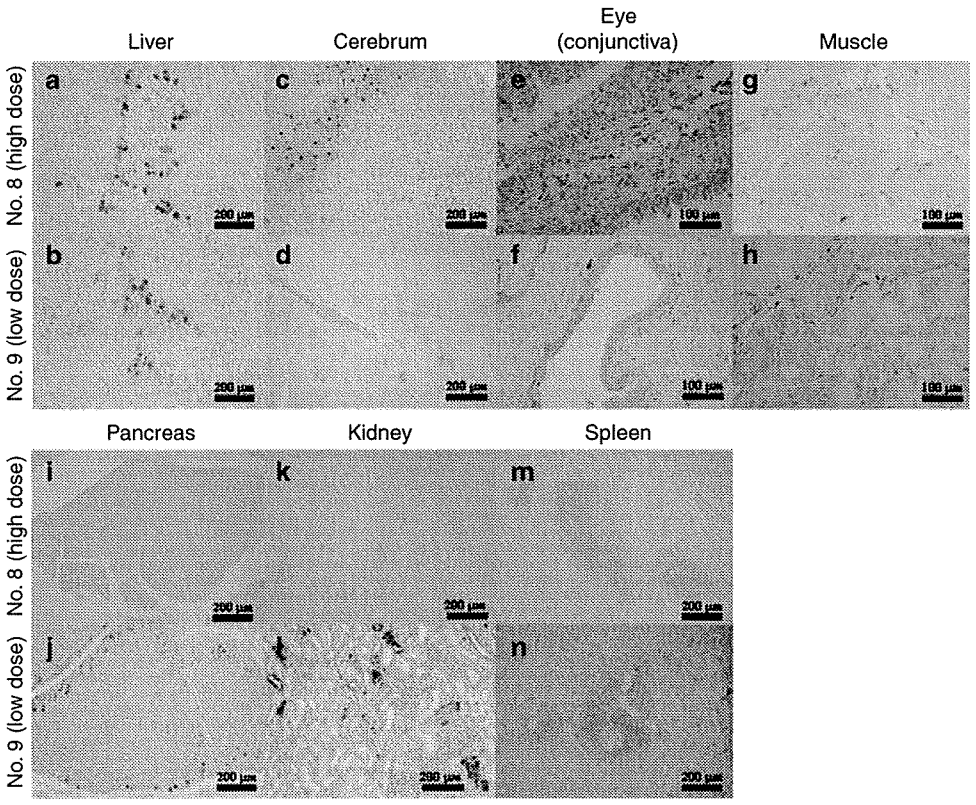

Figure 2 β-galactosidase expression in the organs of cynomolgus monkeys 4 days after intraorgan injection of Ad35LacZ. (**a** and **b**) The liver, (**c** and **d**) cerebrum, (**e** and **f**) eyeball, (**g** and **h**) skeletal muscle, (**i** and **j**) pancreas, (**k** and **l**) kidney and (**m** and **n**) spleen. Ad35LacZ was locally administered in the organs of cynomolgus monkeys at the low (3×10^{10} vector particles (VP) per point) or high dose (1.5×10^{11} VP per points) as described in Figure 1. Four days after injection, the tissues were collected for analysis of β-galactosidase expression and histological pathology. Immunostaining of β-galactosidase was performed using anti-β-galactosidase antibody (Abcam, Cambridge, UK).

Kidney

Ad35LacZ injection to the left kidney induced infiltration by inflammatory cells, including lymphocytes, into the interstitial tissue of the kidney (Figures 1k and l). The right kidney, which was injected with phosphate-buffered saline, did not exhibit β-galactosidase expression or inflammatory responses (data not shown). The high dose did not mediate β-galactosidase expression, but the low dose led to apparent transduction (Figures 2k and l). The renal tubular epithelial cells were mainly transduced with Ad35LacZ. In the kidney, compared with the other organs, transduced cells were more widely spread around the injection points. Refractoriness to the high dose and massive β-galactosidase expression by the low dose in the pancreas and kidney together form a major conundrum in this study. The differences in transduction efficiencies might be due to the slight differences in injection sites. Especially, Ad35LacZ may have been drained into the renal tubule of no. 9 following injection into the kidney, leading to efficient transduction in the renal tubule epithelial cells. Ad35 was originally identified in the kidney and causes cystitis,[22] indicating the tropism of Ad35 for renal epithelial cells.

Spleen and nasal cavity

Unexpectedly, direct injection of Ad35LacZ to the spleen did not induce inflammatory responses such as hyperplasia (Figures 1m and n). There was no β-galactosidase expression in the spleen of either monkey (Figures 2m and n). For transduction in the mucosal membrane of the nasal cavity, Ad35 vector suspensions were instilled into the nasal cavity of each monkey, but neither one showed β-galactosidase expression or cellular damage in the mucosal membrane of the nasal cavity (data not shown).

Other organs

β-galactosidase production in the lung, heart, thymus, bone marrow, lymph node, bladder and testis, which were not injected with Ad35LacZ, were examined by chemiluminescence assay. None of these organs showed detectable β-galactosidase expression (data not shown).

Next, we determined the blood concentrations of Ad35LacZ genome DNA in the blood using quantitative real-time PCR to examine whether or not Ad35LacZ locally injected to the organs was drained from the injection site into the bloodstream. The Ad35 vector DNA was detected in the blood as soon as 6 h post-injection, then gradually decreased (Figure 3). However, the blood-clearance kinetics of Ad35LacZ following intraorgan injection were slower than those following intravenous administration, which were previously reported,[23] although the total amounts of Ad35 vector doses in this study (no. 8: 1.5×10^{11} VP $\times$ 8 points; no. 9: 3×10^{10} VP $\times$ 8 points) were comparable to or lower than those in the previous study in which Ad35LacZ was intravenously infused in cynomolgus monkeys ($0.4–2 \times 10^{12}$ VP per kg, 1.88–2.96 kg).[23] Ad35 vector

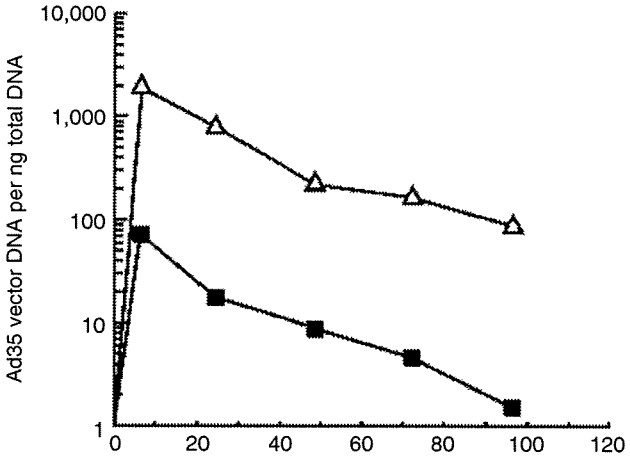

Figure 3 Blood concentrations of Ad35 vectors in cynomolgus monkeys following intraorgan administration. Ad35LacZ was locally administered in the organs of cynomolgus monkeys at the low (3×10^{10} vector particles (VP) per point, closed square) or high dose (1.5×10^{11} VP per points, open triangle) as described in Figure 1. Blood was collected at the indicated post-injection time points (6, 24, 48, 72 and 96 h post-injection). Total DNA, including Ad vector DNA, was isolated from the blood, and the Ad vector DNA contents were measured by quantitative TaqMan PCR assay, as previously described.[23]

DNA was still detectable 4 days after injection. These results suggest that Ad35 vectors or Ad35 vector DNA remaining in the injection sites might be gradually released from the injection sites and drained into the bloodstream.

Furthermore, to examine whether or not Ad35LacZ draining into the bloodstream was accumulated in the organs, we determined the Ad35 DNA contents in the portions of the liver and spleen that were away from the respective injection sites. The liver and spleen play crucial roles in the clearance of systemically injected Ad vectors. The Ad35 vector DNA was not detected in those portions of the liver in no. 9, but was detected in the portions of the liver in no. 8 and in those of the spleen in both monkeys (data not shown). These results suggest that Ad35LacZ or the Ad35 vector DNA draining into the systemic circulation would be taken up by the liver and spleen. We further assessed the Ad35 DNA contents in the lungs, heart, thymus and bone marrow, in which Ad35 vectors were not directly infused. Ad35 vector DNA was detected in the lungs and heart of no. 8 but not in those of no. 9 (data not shown). We did not detect Ad35 vector DNA in the thymus or bone marrow of either monkey. Considering that intravascularly injected Ad35 vectors did not efficiently transduce organs,[15] organs must not be transduced with Ad35LacZ, which is drained into the bloodstream and taken up by the organs.

In most cases of cancer gene therapy using Ad vectors, the vectors are administered directly to the tumor regions.[24–26] When used as vaccine vectors, on the other hand, Ad vectors are intramuscularly injected.[27,28] In addition, Ad vectors are intramyocardially injected in angiogenic gene therapy.[29,30] Thus, direct infusion of Ad vectors to organs is one of the most frequent application methods in clinical settings. However, there has been little information about the transduction properties of these vectors following direct injection into organs. This study demonstrated that different types of cells were transduced with Ad35 vectors depending on the organ after direct infusion into the organ. The differences in the histological structures and cell types comprising the organs would explain the differences in transduction properties of locally injected Ad35 vectors. This study provides important information for clinical study by intraorgan injection of Ad35 vectors, although the characteristics of the organs (structure, cell types and so on) differ different between normal tissue and diseased areas.

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Transduction Properties of Adenovirus Serotype 35 Vectors After Intravenous Administration Into Nonhuman Primates

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Adenovirus serotype 35 (Ad35) vectors have shown promise as effective gene delivery vehicles. However, the transduction profiles of Ad35 vectors in conventional mice allow only a limited estimation of transduction properties of these vectors, because the mouse analog of the subgroup B Ad receptor, CD46, is restricted to the testis. In order to assess the transduction properties of Ad35 vectors more completely, we performed transduction experiments using cynomolgus monkeys, which ubiquitously express CD46 in a pattern similar to that in humans. *In vitro* transduction experiments demonstrated that cultured cells from the cynomolgus monkey were efficiently transduced with Ad35 vectors. In contrast, after intravenous administration into live monkeys hardly any evidence of Ad35 vector–mediated transduction was found in any of the organs, although Ad35 vector genomes were detected in various organs. Less severe histopathological abnormalities were found in the Ad35 vector–infused monkeys than in the conventional Ad5 vector–injected monkeys. In the latter, serious tissue damage and inflammatory responses, such as hepatocyte necrosis and lymphatic hyperplasia in the colon, were induced. Both Ad35 and Ad5 vectors caused similar hematological changes (increase in $CD3^+$ cells, and decrease in $CD16^+$ cells and $CD20^+$ cells) in peripheral blood cells. These results should provide valuable information for the clinical application of Ad35 vectors.

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INTRODUCTION

Human adenoviruses (Ads) are nonenveloped, double-stranded DNA viruses that are composed of 51 serotypes.[1,2] Among the 51 serotypes, the conventional Ad vectors that are most widely used, including for human clinical trials, are constructed based on the subgroup C Ad serotype 5 (Ad5). Ad5 vectors have several advantages as gene delivery vehicles, but clinical and preclinical studies have revealed three major disadvantages of Ad5 vectors. First, target cells that are important for gene therapy, including malignant tumor cells and dendritic cells, express nil or insufficient levels of a cellular receptor for Ad5, the coxsackievirus-adenovirus receptor. The transduction efficiencies of Ad5 vectors depend to a large extent on the expression levels of coxsackievirus-adenovirus receptor, leading to refractoriness of coxsackievirus-adenovirus receptor-negative cells to Ad5 vectors.[3] Second, >50% of adults are seropositive for Ad5 because natural infection with Ad5 is common.[4,5] Pre-existing anti-Ad5 antibodies not only largely inhibit Ad5 vector–mediated transduction, but may also enhance the toxicities induced by Ad5 vectors.[6] Third, inflammatory responses are systemically and rapidly induced by intravascular administration of Ad5 vectors, leading to tissue damage, and this can be fatal to the host.[7–10]

In order to address these problems, we as well as others have developed a replication-incompetent subgroup B Ad serotype 35 (Ad35) vector.[11–15] Ad35 vectors utilize human CD46 as a cellular receptor.[16,17] Human CD46 is ubiquitously expressed on almost all human cells, leading to a wide tropism of Ad35 vectors. In addition, pre-existing anti-Ad5 immunity does not hamper Ad35 vector–mediated transduction, and seroprevalence for Ad35 is much lower than that for Ad5 (refs. 13,14). Ad35 vectors have properties that make them very promising prospects for use as transduction vehicles, but the transduction efficiencies of Ad35 vectors in conventional mice are lower than those of Ad5 vectors.[12,14] Conventional mice seem inappropriate as animal models for Ad35 vectors because mouse CD46 is expressed only in the testis.[18] In addition, there is low homology between human CD46 and mouse CD46. We considered that transduction experiments with Ad35 vectors should be performed using nonhuman primates so as to properly evaluate the transduction properties of Ad35 vectors. The CD46 of nonhuman primates is ubiquitously expressed in a similar pattern to humans, and shows high homology to human CD46.[19]

In this study, we examined the transduction profiles of Ad35 vectors after intravenous administration into nonhuman primates, *i.e.*, cynomolgus monkeys. Ad35 vector–induced immune responses and the blood concentrations of Ad35 vectors were

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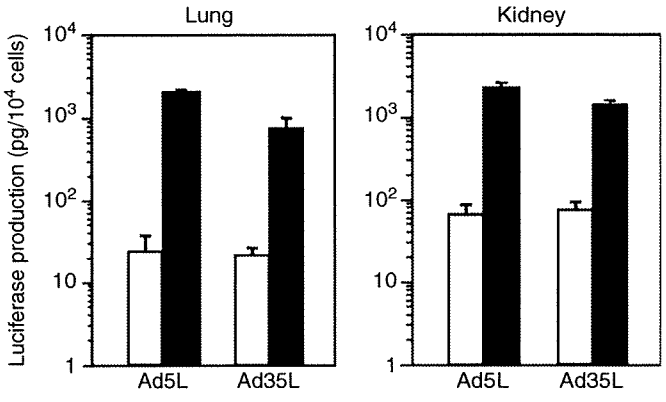

Figure 1 *In vitro* transduction efficiencies of Ad35 and Ad5 vectors in cultured cells of cynomolgus monkey. Luciferase production in primary lung and kidney cells following Ad vector transduction. Primary lung and kidney cells isolated from cynomolgus macaque embryos were transduced with Ad35L or Ad5L at 300 (open bar) and 3,000 vector particles/cell (closed bar) for 1.5 hours. After a 48-hour culture, luciferase production in the cells was measured by luminescence assay. The data are expressed as the mean values ± SD (n = 4). Luciferase expression in the mock-infected cells was less than the detectable level. Ad, adenovirus.

analyzed for 4 days after the injection. Necropsy was performed 4 days after the injection to examine the transduction efficiencies, tissue accumulations of Ad35 vectors, and histopathological changes in the organs after injection.

RESULTS

In vitro transduction in cultured cynomolgus monkey cells

First, to examine whether cynomolgus monkey cells were susceptible to Ad35 vectors, primary lung and kidney cells isolated from embryonic cynomolgus monkeys were transduced with a firefly luciferase–expressing Ad35 vector (Ad35L) and a conventional Ad5 vector (Ad5L). Both Ad35L and Ad5L vectors were shown to mediate efficient transduction in the cells from both organs (Figure 1). Ad35 vectors also efficiently transduced the cynomolgus monkey T-cell line HSC-F (**Supplementary Figure S1**). These results indicate that cynomolgus monkey cells are susceptible to Ad35 vectors. However, peripheral blood mononuclear cells of cynomolgus monkeys were almost refractory to Ad35 vectors (data not shown).

Blood clearance of Ad vectors

Next, the six cynomolgus monkeys (designated #1–#6) were administered either a β-galactosidase-expressing Ad35 vector (Ad35LacZ) or an Ad5 vector (Ad5LacZ) through the femoral vein (**Supplementary Table S1**). The blood clearances of the Ad vectors were examined using a quantitative real-time polymerase chain reaction. Both Ad35LacZ and Ad5LacZ vectors were rapidly cleared from the blood circulation within 24 hours after the injection (Figure 2a and b). We did not find any apparent differences between the blood-clearance kinetics of Ad35LacZ and Ad5LacZ. Assuming that the entire Ad vector DNA in the blood was completely recovered from the blood samples, there would remain 0.12% and 0.09% of the injected Ad35LacZ in the blood of monkey #6 at 3 and 6 hours after injection, respectively. The lower levels of

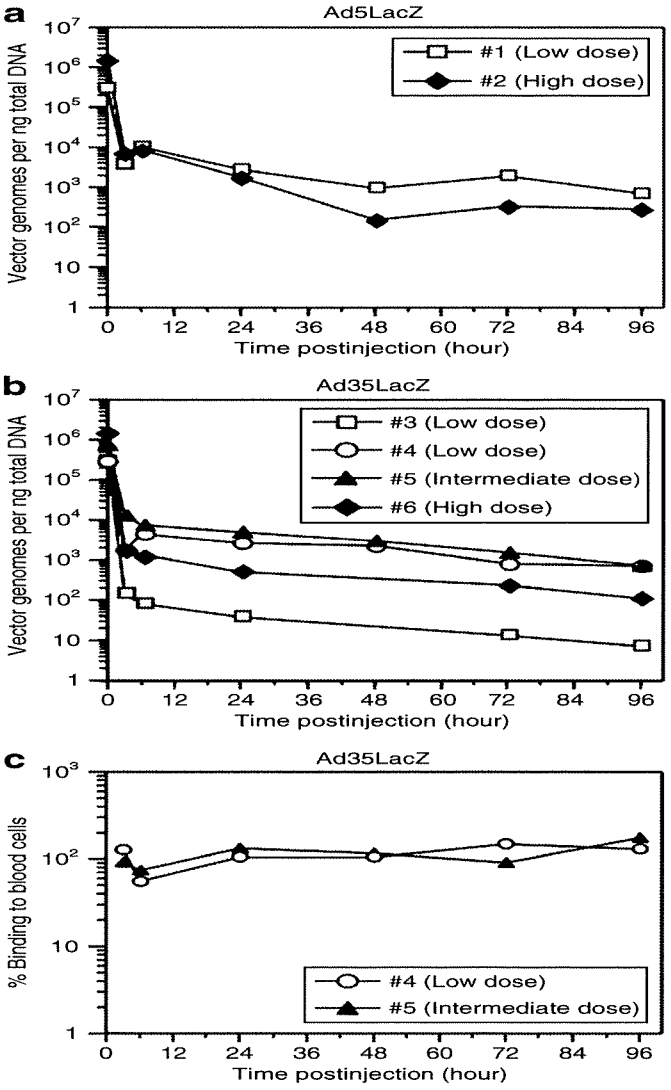

Figure 2 Persistence of adenoviral (Ad) vectors in the blood of cynomolgus monkeys following systemic administration. (**a**) Ad vector DNA concentrations in the blood after intravenous administration. Cynomolgus monkeys were intravenously infused with Ad35LacZ or Ad5LacZ at low [0.4 × 10^{12} vector particles (VP)/kg], intermediate (1.0 × 10^{12}VP/kg), or high (2 × 10^{12}VP/kg) doses. Blood was collected at the indicated time points after injection (3, 6, 24, 48, 72, and 96 hours after injection). Total DNA, including Ad vector DNA, was isolated from the blood, and the Ad vector DNA contents were measured using quantitative TaqMan polymerase chain reaction (PCR) assay. The concentrations of the Ad vectors in the blood at the zero time point were calculated based on the total number of Ad vector particles infused and the estimated circulating blood volume (65 ml/kg). Ad vector DNA was not detected in the blood before injection. (**b**) Percentages of blood cell–associated Ad35lacZ remaining in the blood after systemic administration in cynomolgus monkeys. After isolating the blood as described, blood cells were washed twice with phosphate-buffered saline buffer and the amounts of Ad35LacZ associated with blood cells were evaluated using TaqMan PCR as described earlier. The percentages were calculated as follows: 100 × (the amounts of Ad35 vector DNA associated with blood cells)/(the amounts of Ad35 vector DNA recovered from whole blood).

Ad35LacZ remaining in the blood of monkeys #3 and #6 than those in monkeys #4 and #5 might have been partly because of the low infectious titer-to-particle ratio of the vector batch of Ad35LacZ injected into monkeys #3 and #6. The infectious titer-to-particle

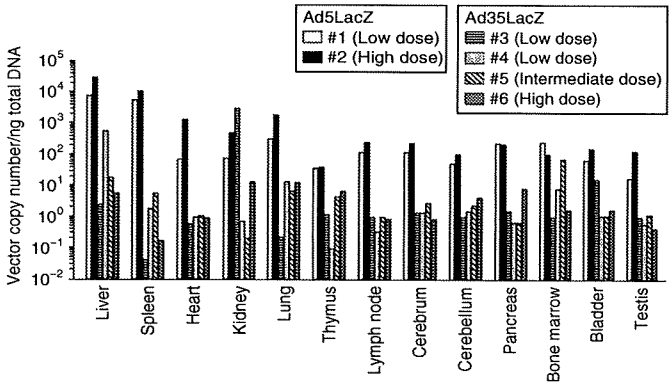

Figure 3 *In vivo* tissue distributions of adenoviral (Ad) vector DNA in cynomolgus monkeys after systemic administration. Ad35LacZ or Ad5LacZ was intravenously administered into cynomolgus monkeys as described for **Figure 2**. Four days after the injection, necropsy was performed, and Ad vector DNA contents were measured using quantitative TaqMan polymerase chain reaction analysis. The Ad vector DNA was not detected in the organs of mock-infected animals.

ratio of the Ad35LacZ used in monkeys #3 and #6 was lower than that used in monkeys #4 and #5 (data not shown). Noninfectious Ad particles might be more easily degraded in the blood or taken up by phagocytic cells.

Further, we examined whether the Ad35 vectors were associated with blood cells in the blood stream after the injection. The majority of Ad35LacZ remaining in the blood was associated with blood cells at all the time points (Figure 2c). Similarly, assuming the complete recovery of the Ad vector DNA as described earlier, 1.5% of the injected Ad35LacZ would be associated with blood cells in monkey #5 at 3 hours after the injection. The levels of Ad35LacZ associated with blood cells remained constant during the study. These results suggest that Ad35 vectors may bind to blood cells, or be taken up by blood cells after the injection.

Tissue distribution of Ad vectors

In order to examine the biodistribution of Ad35 and Ad5 vectors in cynomolgus monkeys after intravenous administration, Ad DNA contents in the organs were assessed (Figure 3). The Ad35 vector DNA was mainly found in the liver, lung, and kidney; however, the levels of Ad35 vector DNA were one to five orders of magnitude lower in almost all organs than the levels of Ad5 vector DNA, which was found mainly in the liver and spleen. Ad35LacZ was also less efficiently accumulated in the organs that exhibited low levels of Ad5LacZ accumulation, such as the thymus and testis.

Ad vector–mediated transgene expression in organs

In order to evaluate the *in vivo* transduction efficiencies of Ad35 and Ad5 vectors, β-galactosidase expression in the organs was examined. Ad5LacZ efficiently transduced the organs (Figure 4a). The highest level of β-galactosidase production was found in the liver, followed by the spleen. Liver parenchymal cells and spleen marginal zone cells were mainly transduced by Ad5LacZ in these organs (Figure 4b). On the other hand, Ad35 vector–mediated β-galactosidase expression in the organs at all doses was approximately equal to, or slightly above, the levels in

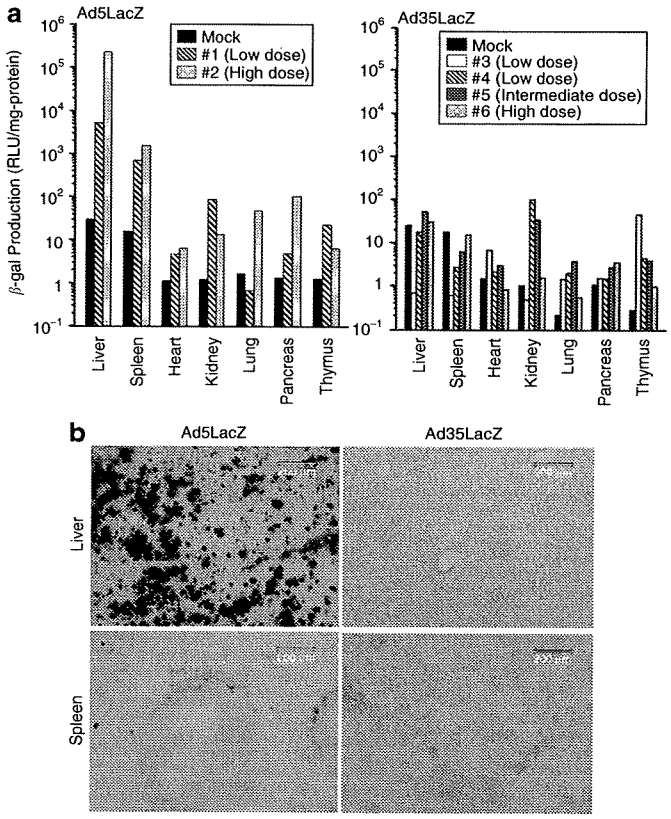

Figure 4 Adenoviral (Ad) vector–mediated transgene expression in cynomolgus monkeys after systemic administration. (**a**) Chemiluminescence analysis of β-galactosidase production in cynomolgus monkeys after systemic administration of Ad35LacZ or Ad5LacZ. Ad35LacZ or Ad5LacZ was intravenously injected into cynomolgus monkeys as described for **Figure 2**. Four days after injection, the organs were collected, and β-galactosidase production in the organs was assessed using a chemiluminescence assay. (**b**) X-gal staining of tissue sections of cynomolgus monkeys receiving Ad5LacZ or Ad35LacZ. Four days after intravenous administration of Ad35LacZ or Ad5LacZ at a high dose (2×10^{12} vector particles/kg), tissues were collected, and X-gal staining was performed as described in Materials and Methods. RLU, relative light units.

mock-infected animals. X-gal-positive cells were not found in the tissue sections of the liver or spleen of the Ad35LacZ-infused monkeys. These results indicate that Ad35 vectors show much lower transduction activity than Ad5 vectors after systemic delivery in cynomolgus monkeys.

Serum chemistry profiles

Next, we measured the levels of serum biochemical markers to assess Ad vector–induced tissue/organ damage. Almost all the markers were increased following Ad vector injection; however, overall, the markers examined appeared to be more elevated in the monkeys receiving Ad5LacZ than in those receiving Ad35LacZ (Figure 5a). Aspartate aminotransferase (AST) levels were elevated as early as 3 hours after the injection, and peaked at 24 hours in most cases. The peak levels of AST in Ad35LacZ-injected monkeys #3, #4, #5, and #6 were 6.1-, 4.8-, 8.2-, and 3.8-fold higher than the preinjection levels, respectively. By contrast, Ad5LacZ-infused monkeys (#1 and #2) showed 4.9- and 27.5-fold increases in AST at the peak points, respectively. Significant elevations in alanine

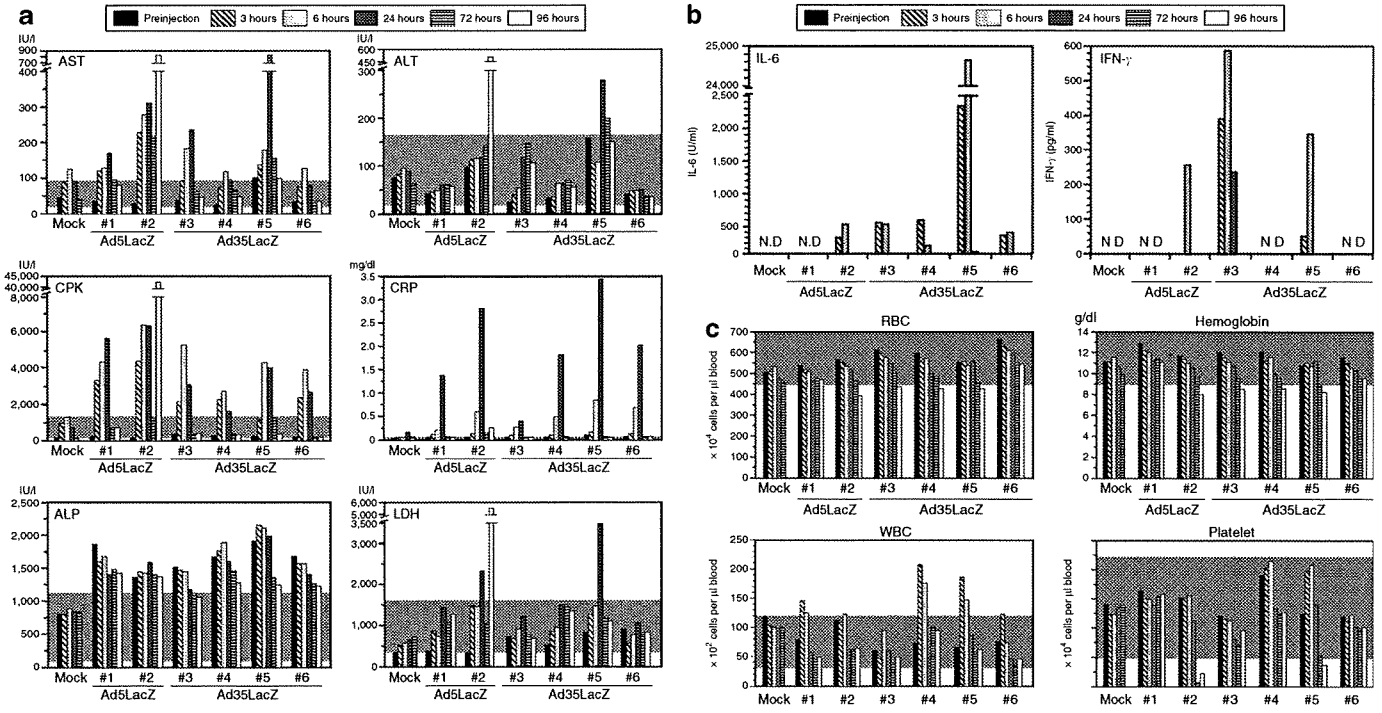

Figure 5 Blood analysis after adenoviral (Ad) vector administration to cynomolgus monkeys. (**a**) Serum marker levels, (**b**) inflammatory cytokine productions, and (**c**) blood cell counts in the peripheral blood after Ad vector administration. The gray area in the graphs of serum markers and blood cell counts indicates the normal range for adult cynomolgus monkeys. Ad35LacZ or Ad5LacZ was intravenously injected into cynomolgus monkeys and blood was collected as described for **Figure 2**. Serum marker levels and blood cell counts were measured using routine methods. Inflammatory cytokine levels were examined using enzyme-linked immunosorbent assay. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CPK, creatine phosphokinase; CRP, C-reactive protein; IFN-γ, interferon-γ; IL-6, interleukin-6; LDH, lactate dehydrogenase; ND, not detected (under the limit of detection); RBC, red blood cell; WBC, white blood cell.

aminotransferase were also found in several of the monkeys, but the alanine aminotransferase levels were within the normal range at almost all the time points. Creatine phosphokinase (CPK) levels sharply rose to a peak 6 or 24 hours after injection. CPK in the Ad35 vector–injected monkeys #3, #4, #5, and #6 showed 14.2-, 9.7-, 16.3-, and 17.7-fold increases at the peak points. On the other hand, the Ad5 vector–injected monkeys #1 and #2 exhibited 16.6- and 40.9-fold elevations in CPK at 6 hours after the injection. Dramatic increases in AST, alanine aminotransferase, and CPK levels in monkey #2 at 96 hours after injection was possibly caused by a slight expression of Ad5 E2 and/or E4 proteins. E4 protein was expressed in the liver 4 days after injection of conventional Ad vectors in mice, leading to liver damage.[20] Levels of C-reactive protein were also sharply increased in all the Ad vector–injected animals. A high dose of Ad35LacZ and Ad5LacZ caused 29-fold (#6) and 56.2-fold (#2) increases in C-reactive protein levels 24 hours after injection, respectively. Alkaline phosphatase levels gradually decreased over the first 96 hours after injection. Alkaline phosphatase levels at preinjection were higher than the normal range in the monkeys. This is because young cynomolgus monkeys (<4 years of age) often have alkaline phosphatase levels >1,000 IU/L. Apparent increases in lactate dehydrogenase were found in monkeys #2 and #5. The lactate dehydrogenase levels in the other animals were within the normal range. There were no abnormalities in the other parameters, including serum albumin, glucose, cholesterol, calcium, sodium, potassium, and chloride (data not shown).

Inflammatory cytokine induction

In order to examine the innate immune responses after Ad vector injection, inflammatory cytokine levels in the serum were measured (Figure 5b). Interleukin-6 (IL-6) was rapidly induced with a peak at 3 or 6 hours after the injection in all the animals except in monkey #1. There were no apparent differences in IL-6 levels between Ad35LacZ-treated and Ad5LacZ-treated animals, except that monkey #5 produced an extremely high level of IL-6. The levels of interferon-γ were also elevated and reached a peak at 6 hours after the injection in monkeys #2, #3, and #5. Tumor necrosis factor-α was not detected in any of the animals (data not shown).

Hematological profiles

In order to evaluate the influence of Ad vector injection on the hematological profiles, we examined the changes in peripheral blood cell counts (Figure 5c). The changes in the levels of red blood cells and hemoglobin were marginal, but the levels gradually decreased after injection in all the monkeys, including a mock-infected animal, probably because of the collection of large volumes of blood samples (>5 ml/time point) every day. Ad35LacZ-injected monkeys #3, #4, and #5, and Ad5LacZ-injected monkey #2 showed a rapid decline in platelet levels beginning at 24 hours after the injection. A transient increase in the platelet levels was found 3 and 6 hours after the injection in monkey #5. It remains unclear why the platelet levels increased in monkey #5; however, the previous study also reported an initial increase in the platelet levels after Ad5 vector injection in nonhuman primates.[21] A rapid

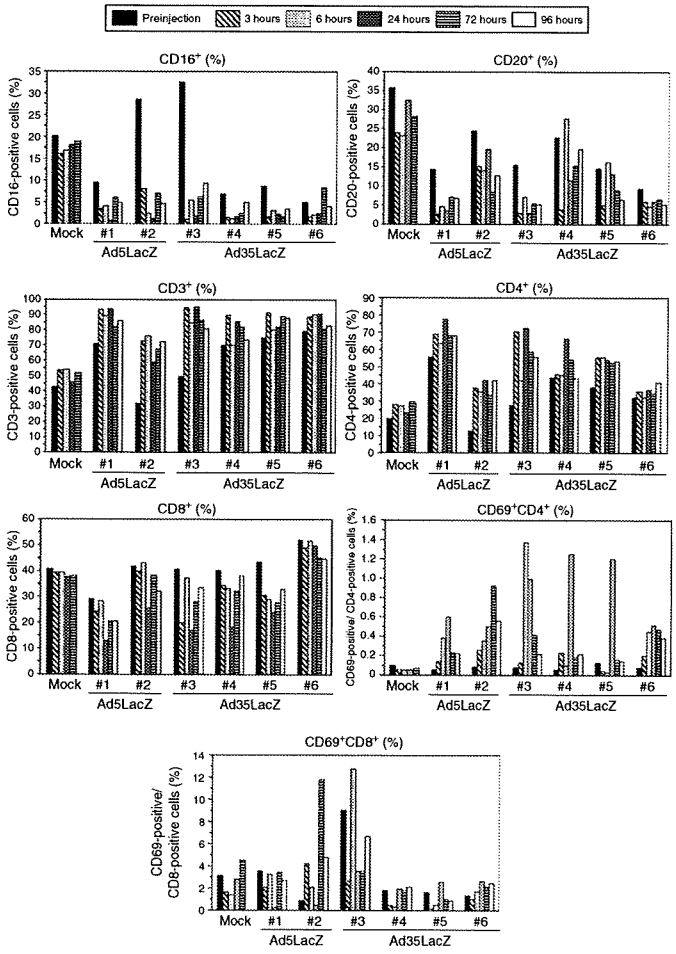

Figure 6 Profiles of peripheral blood lymphocyte subsets after systemic administration of adenoviral (Ad) vectors in cynomolgus monkeys. Ad35LacZ or Ad5LacZ was intravenously injected into cynomolgus monkeys and blood was collected as described for **Figure 2**. Peripheral blood mononuclear cells were stained with monoclonal antibodies following hemolysis, and fluorescence-activated cell sorting analysis was performed for evaluation of profiles of lymphocyte subsets.

elevation in the white blood cells was observed in the Ad vector–injected monkeys. The elevated white blood cells level returned to normal at 24 hours after the injection.

Next, we examined which types of blood cells were increased or decreased after Ad vector injection (Figure 6). The Ad vector injection induced a rapid decline in the percentages of $CD16^+$ cells (natural killer cells, granulocytes, and monocytes). Monkeys #2 and #3 showed sharp decreases of 71 and 97% of $CD16^+$ cells, respectively, at 3 hours after the injection. The percentages of $CD20^+$ cells (B cells) quickly dropped in all the monkeys, including a mock-infected monkey. In contrast, the $CD3^+$ cell (T-cell) levels were sharply elevated in the animals receiving the Ad vectors. We found a 1.1- to 2.3-fold increase in $CD3^+$ cell levels at 3 hours after the injection. $CD8^+$ cells did not increase, but rather decreased after the injection; however, increases in $CD4^+$ cells were found in the Ad vector–injected monkeys. The $CD4^+$ cell levels were 1.1- to 3.4-fold elevated compared with the preinjection levels, with a peak at 24 hours after the injection, in most of the animals. The administration of Ad vectors also increased

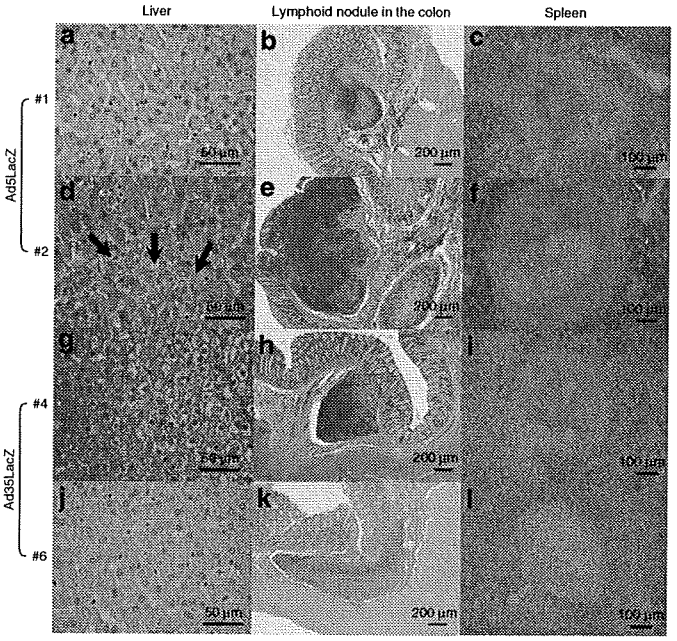

Figure 7 Histopathological analysis of liver, lymphoid nodules in the colon, and spleen. Representative histological sections of the liver (**a, d, g, j**), lymphoid nodules in the colon (**b, e, h, k**), and spleen (**c, f, i, l**) from animals killed 4 days after systemic injection of a low or high dose of Ad35LacZ (monkeys #4 and #6) or Ad5LacZ (#1 and #2). The arrows indicate necrosis of hepatocytes.

$CD69^+CD4^+$ cells (activated $CD4^+$ cells) more predominantly than $CD69^+CD8^+$ cells. Both $CD29^+CD4^+$ cells (memory helper T cells) and $CD29^-CD4^+$ cells (naive helper T cells) increased in the Ad vector–injected animals (data not shown). These results indicate that, overall, both Ad35 and Ad5 vectors cause similar changes in hematological profiles after systemic infusion.

Clinical observation and histopathological examinations

In order to perform a safety assessment of the Ad vectors, the health condition of the animals was monitored until necropsy. None of the Ad vector–infused monkeys showed any apparent abnormalities in appetite, body weight, body temperature, or heart rate. However, the low dose of Ad35LacZ (#3) induced vomiting 3 hours after the injection, and a skin rash was observed in monkey #2 on day 2.

In order to further evaluate the safety profiles of Ad vectors, organ histopathology was examined during necropsy. There were no obvious changes in the spleens of monkeys #1 and #3–#6, or in the livers in any of the animals. However, splenomegaly was found in monkey #2. The whitish nodules at the cut surface of the spleen in monkey #2 were the largest among those of all the monkeys examined. Marked swelling of the lymph nodules, especially in the colon and mesentery, was also found in monkey #2.

Microscopic analysis of tissue sections revealed that no apparent damage and inflammation were found in the liver of monkey #1 (Figure 7a). Although slight hyperplasia in the spleen white pulp occurred in this monkey (Figure 7c), no obvious changes were found in the lymphatic nodules of the colon (Figure 7b). In contrast, severe damage and inflammation, including necrosis of hepatocytes (Figure 7d, arrows) and infiltration of lymphocytes

into the Glisson's sheath (data not shown) were found in monkey #2. Furthermore, apparent severe hyperplasia in the lymphoid nodules of the colon (Figure 7e) and spleen white pulp (Figure 7f) had been induced in monkey #2. On the other hand, the livers of Ad35LacZ-treated monkeys exhibited almost no damage or inflammation (Figure 7g and j). In addition, Ad35LacZ induced hyperplasia in lymphoid nodules of the colon (Figure 7h and k) was only slightly developed and less serious than that induced by the high dose of Ad5LacZ. These results suggest that Ad5 vectors may cause more severe damage and/or inflammation in the liver and lymphoid nodules of the colon than Ad35 vectors. The spleen white pulp developed only slight hyperplasia in monkey #4 (Figure 7i), in contrast, the high dose of Ad35LacZ induced severe hyperplasia in the spleen white pulp (Figure 7l). The level of hyperplasia in spleen white pulp of monkey #6 appeared to be slightly more severe than that of monkey #2. The monkeys #3 and #5 did not show apparent abnormalities in the spleen or colon, although slight vacuolation in hepatocytes and infiltration of lymphocytes in the Glisson's sheath was found (data not shown). Hyperplasia in spleen white pulp and lymphatic follicles in the mesenteric, axillary, and inguinal lymph nodes (data not shown) occurred dose-dependently in the Ad35-injected animals as well as in the Ad5-injected ones.

DISCUSSION

In this study, subgroup B Ad35 vectors were intravenously infused into cynomolgus monkeys in order to evaluate the *in vivo* fundamental transduction properties of Ad35 vectors more thoroughly. Cynomolgus monkey CD46 and the CD46 of other nonhuman primates, have significant homology with human CD46 (ref. 19). In particular, short consensus repeats 1 and 2 (which are crucial for Ad35 binding to CD46),[22–24] of the CD46 of the cynomolgus monkey show high homology (85%) with those in human CD46. In addition, we confirmed that the monkey cells used in this study were highly stained with anti-human CD46 monoclonal antibody M177, which is specific for short consensus repeat 2, and that the antibody M177 significantly inhibited Ad35 vector–mediated transduction in the cynomolgus monkey cells (data not shown). The amino acid sequences important for Ad35 binding to CD46 (refs. 23,24) are also well conserved in cynomolgus monkey CD46. These results indicate that cynomolgus monkey CD46 serves as a cellular receptor for Ad35, at least in the context of *in vitro* transduction.

In this study, four and two cynomolgus monkeys were intravenously injected with the Ad35 and Ad5 vectors, respectively. We must exercise caution in interpreting the results because the sample size is small, as is natural in nonhuman primate studies. Overall, there are no dose responses in several transduction profiles of both Ad35 and Ad5 vectors, including blood concentration of Ad vectors and inflammatory cytokine production. The variations in the transduction profiles suggest that these profiles may depend largely on the specific Ad vector batch and on the differences between individuals, such as health conditions and genetic backgrounds, as well as on Ad vector doses. In the clinical trials using Ad vectors, inflammatory responses were dramatically different between patients receiving the same vector dose.[10] Gene therapy studies, both preclinical and clinical, should be performed with considerable caution in view of these individual differences. Further studies, including toxicogeomics, would be necessary in order to clarify which parameters play the most crucial roles in this entire process of transduction. Such studies would enable prediction of profiles of Ad vector–mediated transduction, and associated toxicities.

Although efficient transduction was achieved using Ad35 vectors *in vitro*, transduction of Ad35 vectors in the organs *in vivo* was hardly detectable after systemic infusion (Figure 4). In addition, the levels of Ad35 vector genome in the organs were one to five orders lower than those of the Ad5 vector genome (Figure 3). Previous studies demonstrated that, after systemic injection, Ad35 vectors were poor at transducing CD46-transgenic (CD46TG) mice, which ubiquitously express human CD46 in all the organs.[25,26] Chimeric Ad5 vectors containing Ad35 fiber protein also mediated much lower transgene expression in baboons than conventional Ad5 vectors did.[27] These results indicate that Ad35 vectors cannot transduce organs efficiently when introduced into the blood stream. There are two possible explanations for the poor transduction activity of Ad35 vectors after systemic administration. First, Ad35 vectors might be more susceptible than Ad5 vectors to degradation in the blood or in intracellular compartments such as endosomes/lysosomes after internalization. Fiber-substituted Ad5 vectors containing a fiber protein of Ad35 remain for a longer time in late endosome/lysosomal compartments after internalization than Ad5 vectors do.[28] Ad35 vectors might exhibit similar intracellular trafficking to the fiber-substituted Ad5 vectors, leading to high susceptibility to intracellular degradation. Second, Ad35 vectors might not be able to gain access to CD46 after systemic injection. CD46 is predominantly expressed on the basolateral sides of cells,[29,30] making it inaccessible to Ad35 vectors. Ad35 vectors which are not able to bind to CD46 on the cell surface would be phagocytosed into phagocytic cells, such as liver Kupffer cells, leading to degradation.

It is well known that erythrocytes of cynomolgus monkeys express CD46 (ref. 19) and that Ad35 causes hemagglutination of monkey erythrocytes.[31] Ad35 vectors might induce hemagglutination in the blood vessels after the injection, and this might lead to hemolysis and a decrease in the transduction efficiencies of Ad35 vectors. A large percentage of the Ad35 vectors recovered from the blood after the injection were associated with blood cells (Figure 2c). However, lactate dehydrogenase (a marker of hemolysis) levels in the sera of Ad35LacZ-injected animals at most of the time points were within normal levels and comparable with those in the sera of animals injected with Ad5LacZ, which does not induce hemagglutination of monkey erythrocytes. These results suggest that hemagglutination by Ad35 vectors would have, at most, a minimal influence on the transduction profiles of Ad35 vectors.

As mentioned earlier, CD46TG mice as well as cynomolgus monkeys were only poorly transduced with Ad35 vectors after intravenous administration, thereby suggesting that the transduction profiles of Ad35 vectors in CD46TG mice would correspond to those in primates and that CD46TG mice might be suitable as a small animal model for the study of Ad35 vectors. The profiles of inflammatory cytokine production by Ad35 vectors in cynomolgus monkeys were also approximately similar to those in CD46TG mice. Intravenous

infusion of Ad35 vectors resulted in levels of inflammatory cytokine production comparable to those induced by Ad5 vectors in the monkeys (Figure 5b) as well as in CD46TG mice.[32]

Histopathological analysis demonstrated that tissue damage and inflammatory responses, including hepatocyte necrosis, were less severe in all the Ad35 vector–infused monkeys than in the Ad5 vector–injected ones (Figure 7). Previous studies also demonstrated that Ad35 vectors are less immunogenic than Ad5 vectors in mice,[33,34] and this may result in the higher safety profiles of Ad35 vectors as compared to Ad5 vectors. It remains to be elucidated why Ad35 vectors produce less severe side effects than Ad5 vectors. Ad5 vectors were more widely distributed in most organs than Ad35 vectors, suggesting that Ad5 vectors may cause tissue damage and inflammatory responses throughout the whole body. On the other hand, Ad35LacZ induced much higher levels of IL-6 and interferon-γ in monkeys #5 and #3, respectively, than in the other Ad35LacZ-infused monkeys (Figure 5b), although no severe damage or inflammation was observed in these two animals. It remains unclear why such high levels of inflammatory cytokines were induced by Ad35 vectors in these animals; however, previous studies have indicated that the high levels of inflammatory cytokine induction might be involved in tissue damage.[9,35] It is important to pay attention to Ad35 vector–induced innate immune responses.

The poor transduction efficiencies of Ad35 vectors in organs after systemic administration could constitute another potential advantage in their use, namely, that locally administered Ad35 vectors would not cause unwanted side effects in organs other than the targeted organs, when draining from injected sites into the blood stream. This is in contrast to Ad5 vectors which, after injection into local tissues, have been shown to drain into the blood stream in large quantities and cause unwanted side effects in the liver and other organs.[36,37] We previously demonstrated that intramuscular injection of Ad35 vectors led to efficient transduction at the injected sites,[12] and thus local injection of Ad35 vectors would be expected to mediate efficient transduction at the injected sites without side effects in other organs.

In summary, we have demonstrated the transduction properties of Ad35 vectors after intravenous administration in nonhuman primates. Systemic infusion of Ad35 vectors did not result in detectable levels of transgene expression in the organs. Also, the tissue damage was less severe in the animals receiving Ad35 vectors than in those receiving Ad5 vectors, although two monkeys produced marked inflammatory cytokines after receiving Ad35 vectors. Further studies are in progress, focusing on the local injection of Ad35 vectors, and the results of these studies may further clarify the potential utility of Ad35 vectors.

MATERIALS AND METHODS

Ad vectors. An Ad5 vector and an Ad35 vector containing a β-galactosidase expression cassette, Ad5LacZ and Ad35LacZ, respectively, were prepared using an improved *in vitro* ligation method.[38–40] Briefly, for preparation of Ad5LacZ, pHMCMV6-LacZ, which was constructed by insertion of the β-galactosidase gene derived from pCMVβ (Clontech, Palo Alto, CA) into pHMCMV6,[39] was digested with I-*Ceu*I and PI-*Sce*I, and then ligated with I-*Ceu*I- and PI-*Sce*I-digested Ad5 vector plasmid pAdHM4.[39] The resulting plasmid was digested with *Pac*I and transfected into 293 cells with Superfect (Qiagen, Valencia, CA). The vector plasmid for Ad35LacZ was constructed in a similar manner, but using pHMCMV6-LacZ and pAdMS18.[25] The resulting plasmid was digested with *Sbf*I and transfected into 293-E1B cells,[25] which are a 293 transformant stably expressing Ad35 E1B-55K protein. The viruses were prepared using a standard method, and purified by $CsCl_2$ step gradient ultracentrifugation followed by $CsCl_2$ linear gradient ultracentrifugation. Determination of virus particle titers was accomplished spectrophotometrically using the methods of Maizel *et al.*[41] Luciferase-expressing Ad5 and Ad35 vectors, Ad5L and Ad35L, were constructed as explained earlier.[11]

In vitro *transduction.* Lung and kidney primary cells, isolated from embryonic cynomolgus monkeys and cultured in Roswell Park Memorial Institute-1640 medium supplemented with 10% fetal bovine serum, antibiotics, and L-glutamine, were seeded in a 96-well dish at 1×10^4 cells/well. On the following day, they were transduced with Ad5L or Ad35L at 300 and 3,000 vector particles/cell for 1.5 hours. After a 48-hour culture period, luciferase production in the cells was measured using a luciferase assay system (PicaGene LT2.0; Toyo Inki, Tokyo, Japan).

Animals. Young male cynomolgus monkeys (*Macaca fascicularis*) were housed and handled in accordance with the rules for animal care and management of the Tsukuba Primate Center and the guiding principles for animal experiments using nonhuman primates formulated by the Primate Society of Japan. The animals (~3 years of age, 1.88–2.96 kg) were certified free of intestinal parasites and seronegative for simian type-D retrovirus, herpesvirus B, varicella-zoster-like virus, and measles virus. The protocol of the experimental procedures was approved by the Animal Welfare and Animal Care Committee of the National Institute of Biomedical Innovation (Osaka, Japan).

In vivo *transduction.* Cynomolgus monkeys were sedated with ketamine (5–10 mg/kg) and injected with phosphate-buffered saline (mock), or Ad5LacZ or Ad35LacZ at 2×10^{12} vector particles/kg (high dose), 1×10^{12} vector particles/kg (intermediate dose), or 0.4×10^{12} vector particles/kg (low dose) through the saphenous vein at a rate of ~2 ml/minutes. Blood was collected for analysis at 3, 6, 24, 48, 72, and 96 hours after injection. Four days after vector administration, the monkeys were killed and the tissues were collected. Tissue samples were subjected to analysis as described in the later text.

β-Galactosidase assay and X-gal staining. β-Galactosidase activity in the organs was measured using Galacto-Light Systems (Applied Biosystems, Foster City, CA) as earlier described.[42] Protein concentrations were determined with a Bio-Rad assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. X-gal staining of tissue sections was performed as earlier described.[43]

Blood clearance and tissue distribution of Ad vectors. Blood clearance analysis of Ad vectors was performed using a real-time polymerase chain reaction assay, as earlier described.[8,40] Briefly, total DNA, including the Ad vector DNA, was isolated from whole blood samples. After isolation, the total DNA concentrations were determined, and the Ad DNA contents were quantified using a TaqMan fluorogenic detection system (ABI Prism 7700 sequence detector; Perkin-Elmer Applied Biosystems, Foster City, CA).

The association of Ad35 vectors to blood cells circulating in the blood stream was evaluated using a real-time polymerase chain reaction assay. Blood samples collected at the indicated time points were washed two times with phosphate-buffered saline immediately after isolation to remove unbound Ad35 vectors. After washing, total DNA was extracted from blood cells and the Ad35 DNA contents were assessed as described earlier.

The Ad DNA contents in each organ were similarly quantified using a real-time polymerase chain reaction assay, as described earlier, after isolation of the total DNA from each organ using a Automatic Nucleic Acid Isolation System (NA-2000; KURABO, Osaka, Japan).

Histopathology. For routine histopathology, tissues were fixed in 10% formalin at the time of necropsy, and processed for paraffin embedding.

Sections of 4-μm thickness were cut and stained with hematoxylin and eosin. The tissue sections were examined under a microscope.

Analysis of inflammatory cytokines, serum chemistry profiles, and hematology parameters. Blood was drawn from the saphenous veins of all the monkeys prior to vector administration and at 3, 6, 24, 72, and 96 hours after vector administration. Blood samples were collected into separate tubes containing either EDTA or no anticoagulant, for hematology and for determination of inflammatory cytokines and serum chemistry, respectively. Serum samples for analysis of inflammatory cytokines and serum chemistry were separated by centrifugation (4 °C, 2,500 rpm, 15 minutes), stored in a freezer at −80 °C, and thawed at the time of measurement. The levels of inflammatory cytokines (IL-6 and interferon-γ) in serum samples were measured using enzyme-linked immunosorbent assay (BioSource, Camarillo, CA). The serum chemistry parameters, which were measured with an automated chemistry analyzer AU400 (OLYMPUS, Tokyo, Japan), included AST, alanine aminotransferase, CPK, alkaline phosphatase, lactate dehydrogenase, and C-reactive protein. The hematology parameters that were determined included white blood cells, red blood cells, hemoglobin, platelets, $CD3^+$ cells, $CD4^+$ cells, $CD8^+$ cells, $CD16^+$ cells, $CD20^+$ cells, $CD29^+$ cells, and $CD69^+$ cells.

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SUPPLEMENTARY MATERIAL

Figure S1. *In vivo* transduction efficiencies of Ad35 and Ad5 vectors in cultured cynomolgus monkey T-cell line H-SCF.

Table S1. Dosing of cynomolgus macaques with β-galactosidase-expressing Ad vectors in this study.

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Efficient osteoblast differentiation from mouse bone marrow stromal cells with polylysin-modified adenovirus vectors

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ABSTRACT

Bone marrow stromal cells (BMSCs) are expected to be a source for tissue regeneration because they can differentiate into multiple cell types. Establishment of efficient gene transfer systems for BMSCs is essential for their application to regenerative medicine. In this study, we compared the transduction efficiency in mouse primary BMSCs by using fiber-modified adenovirus (Ad) vectors, and demonstrated that AdK7, which harbors a polylysin (K7) peptide in the C-terminus of the fiber knob, could efficiently express a transgene in BMSCs. Notably, AdK7 robustly drove transgene expression in more than 90% of the BMSCs at 3,000 vector particles/cell. Furthermore, we showed that *in vitro* and *in vivo* osteogenic potential of BMSCs was dramatically promoted by the transduction of Runx2 gene using AdK7. These results indicate that this transduction system could be a powerful tool for therapeutic applications based on BMSCs.

Because bone marrow stromal cells (BMSCs) containing mesenchymal stem cells (MSCs) can be easily isolated from adult tissues and efficiently expanded *in vitro*, and can differentiate into multiple cell types [1,2], BMSCs are expected to be an ideal source of cells for the regeneration of tissues. However, it is difficult to obtain a large amount of pure differentiated cells from BMSCs because of their low differentiation efficiency. The cell transition from stem cells to lineage-committed cells involves many transcription factors that promote or suppress cellular differentiation [3]. Thus, to develop an efficient method for differentiating from BMSCs into specialized cells, we planned to combine the transduction of a functional gene, which promotes cellular differentiation, with stimulation by chemical reagents. To do this procedure, it is essential to develop efficient transduction systems for BMSCs.

Among the various types of gene delivery vectors, adenovirus (Ad) vectors have been widely used for gene transfer studies, since they can achieve high transduction efficiency and transduce both dividing and non-dividing cells [4]. Although Ad vector-mediated transduction into BMSCs has been performed, the transduction efficiency was found to be lower than those of many other cell lines [5,6]. This is due to the low levels of coxsackievirus and adenovirus receptor (CAR), which mediates adenovirus entry, on the cell surface [5,6]. To overcome this problem, we and others have generated several types of fiber-modified Ad vectors, which mediate efficient gene transduction into the cells expressing very low levels of CAR [7,8]. Transduction efficiency was improved in various types of the cells by the insertion of Arg-Gly-Asp (RGD) peptide or 7-tandem lysine residues (KKKKKKK: K7) peptide, which targets αv integrins or heparan sulfates, respectively, on the cell surface, into the fiber knob of the Ad vector [7,8]. In particular, we previously reported that polylysin-modified Ad vector (AdK7) is the most suitable vector for transduction into human bone marrow-derived MSCs (hMSCs) [9].

In this study, we initially investigated the transduction efficiency of mouse primary BMSCs by using fiber-modified Ad vectors. We next examined whether the osteogenic potential of BMSCs was promoted by using Ad vector-mediated transduction of a runt-related transcription factor 2 (Runx2) gene, which is known as a master gene for osteoblastogenesis [10,11].

Materials and methods

Ad vectors. Ad vectors were constructed using an improved *in vitro* ligation method [12,13]. The CA (cytomegalovirus (CMV) enhancer/β-actin promoter) promoter [14]-driven β-galactosidase

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(LacZ)-expressing plasmid, pHMCA-LacZ [15], was digested with I-*Ceu*I/PI-*Sce*I and inserted into I-*Ceu*I/PI-*Sce*I-digested pAdHM15-RGD [16] or pAdHM41-K7 (C) [8], resulting in pAdRGD-CA-LacZ, pAdK7-CA-LacZ, respectively. The CMV or the human elongation factor (EF)-1α promoter-driven LacZ-expressing plasmid, pHMCMV-LacZ [15] or pHMEF-LacZ [15], respectively, was also digested with I-*Ceu*I/PI-*Sce*I and ligated into I-*Ceu*I/PI-*Sce*I-digested-pAdHM41-K7 (C), resulting in pAdK7-CMV-LacZ or pAdK7-EF-LacZ, respectively. The CA promoter-driven mouse Runx2-expressing plasmid, pHMCA-Runx2, was generated by inserting a mouse Runx2 cDNA, which is derived from pCMV-Runx2 (a kind gift from Dr. S. Takeda, Tokyo Medical and Dental University, Tokyo, Japan) [17], into pHMCA5. pHMCA-Runx2 was also digested with I-*Ceu*I/PI-*Sce*I, and inserted with pAdHM4 [12] or pAdHM41-K7 (C), resulting in pAd-CA-Runx2 or pAdK7-CA-Runx2, respectively. Ad vectors (Ad-CA-LacZ, AdRGD-CA-LacZ, AdK7-CA-LacZ, AdK7-CMV-LacZ, AdK7-EF-LacZ, Ad-CA-Runx2, and AdK7-CA-Runx2) were generated and purified as described previously [18]. Determination of virus particle (VP) and biological titer were determined using by a spectrophotometrical method [19] and by means of an Adeno-X Rapid Titer Kit (Clontech, Palo Alto, CA), respectively. The ratio of the biological-to-particle titer was 1:14 for Ad-CA-LacZ, 1:35 for AdRGD-CA-LacZ, 1:42 for AdK7-CA-LacZ, 1:25 for AdK7-CMV-LacZ, 1:32 for AdK7-EF-LacZ, 1:17 for Ad-CA-Runx2, and 1:28 for AdK7-CA-Runx2.

Mouse primary BMSCs. Primary BMSCs were harvested from female C57BL/6 mice (8 weeks; Nippon SLC, Shizuoka, Japan) as below. Femora and tibias were isolated and placed in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO)/20% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin. Bone marrow was obtained by flushing these bones, and cells recovered from the bones of one animal were then seeded into a 150 mm tissue culture plate. Medium was changed every 2 days to remove non-adherent cells, and adherent cells were cultured until reaching confluence. At confluence, BMSCs were passaged after digestion with 0.25% trypsin/1 mM EDTA. BMSCs (passage 4–12) were subsequently used for further analysis.

LacZ assay. BMSCs (1×10^4 cells) were plated in 24-well plates. The next day, they were transduced with the indicated doses of Ad vectors for 1.5 hr. Two days later, X-gal staining and β-gal luminescence assays were performed as described previously [18].

Osteoblasts differentiation. BMSCs (1×10^4 cells) were plated in 24-well plates. Cells were transduced with 3000 VP/cell of Ad vector for 1.5 hr. After aspirating the viral solution, osteogenic differentiation medium, consisting of growth medium (DMEM/20% FBS) containing 50 μg/mL ascorbic acid 2-phosphate (Sigma), 5 mM β-glycerophosphate (Sigma), and 100 nM dexamethasone (Wako, Osaka, Japan), was added. The medium was replaced every 3 days.

von Kossa staining, calcium quantitation. Cells were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) and stained with $AgNO_3$ by the von Kossa method. To measure calcium deposition, cells were washed twice with PBS and decalcified with 0.5 M acetic acid, and cell culture plates were rotated overnight at room temperature (R/T). Insoluble material was removed by centrifugation. The supernatants were then assayed for calcium with the calcium C-test Wako kit (Wako). DNA in pellets was extracted using the DNeasy tissue kit (Qiagen), and calcium content was then normalized to DNA.

ALP assay. Cells were lysed in 10 mM Tris–HCl (pH 7.5) containing 1 mM $MgCl_2$ and 0.1% Triton X-100, and the lysates were then used for assay. Alkaline phosphatase (ALP) activity was measured using the LabAssay ALP kit (Wako) according to the manufacturer's instructions. The protein concentration of the lysates was determined using a Bio-Rad assay kit (Bio-Rad laboratories, Hercules, CA), and ALP activity was then normalized by protein concentration.

RT-PCR. RT-PCR was performed as described previously [18]. The sequences of primers were as follows: Runx2(F), 5′-CCT CTG ACT TCT GCC TCT GG-3′; Runx2(R), 5′-CAG CGT CAA CAC CAT CAT TC-3′; osterix(F), 5′- CTT AAC CCA GCT CCC TAC CC-3′; osterix(R), 5′- TGT GAA TGG GCT TCT TCC TC-3′; bone sialoprotein(F), 5′- AAA GTG AAG GAA AGC GAC GA-3′; bone sialoprotein(R), 5′-GTT CCT TCT GCA CCT GCT TC-3′; osteocalcin(F), 5′-GCG CTC TGT CTC TCT GAC CT -3′; osteocalcin(R), 5′-TTT GTA GGC GGT CTT CAA GC-3′; collagen Iα1(F), 5′-CAC CCT CAA GAG CCT GAG TC-3′; collagen Iα1 (R), 5′-GCT ACG CTG TTC TTG CAG TG-3′, GAPDH(F), 5′-ACC ACA GTC CAT GCC ATC AC-3′; GAPDH(R), 5′-TCC ACC ACC CTG TTG CTG TA-3′.

Western blotting. Western blotting was performed as described previously [18]. Briefly, lysates (20 μg) were subjected to 12.5% polyacrylamide gel and were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). After blocking with Immunoblock (DS Pharma Biomedical, Osaka, Japan) at R/T for 1 hr, the membrane was exposed to rabbit anti-Runx2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4 °C overnight, followed by horseradish peroxidase-conjugated secondary antibody at R/T for 1 hr. The band was visualized by ECL Plus Western blotting detection reagents (Amersham Bioscience, Piscataway, NJ) and the signals were read using a LAS-3000 imaging system (FUJIFILM, Tokyo, Japan). All blots were stripped and reblotted with antibody against β-actin (Sigma) for normalization.

In vivo heterotopic bone formation. BMSCs (2×10^6 cells, passage 8–9) were transduced with AdK7-CA-LacZ, AdK7-CA-Runx2, or Ad-CA-Runx2, at 3000 VP/cells for 1.5 hr. The next day, cells were collected by trypsin, and resuspended in 150 μl of PBS, and then injected into the hind limb biceps muscle of nude mice (Nippon SLC) (2animal/ group). At 4–5 weeks after injection, mice were anesthetized by isofluorane and bone formation was analyzed with a microcomputed tomography (microCT) system (eXplore Locus CT System; GE Healthcare, London, ON, Canada). Both an X-ray image and a three-dimensional reconstitution image were obtained by using the microCT system.

Results

Optimization of transduction efficiency in BMSCs by using various types of Ad vectors

To optimize Ad vectors for transduction into BMSCs, we prepared three LacZ-expressing Ad vectors, Ad-CA-LacZ, AdRGD-CA-LacZ, and AdK7-CA-LacZ. We investigated the transduction efficiency of these Ad vectors in BMSCs at the indicated vector dose. X-gal staining showed that LacZ-positive cells were less than 10% even at a dose of 3000 vector particles (VP)/cell in Ad-CA-LacZ (Fig. 1A). On the other hand, more than 90% of the cells expressed LacZ at the same dose in AdK7-CA-LacZ. A luminescence assay revealed that, at 3000 VP/cell, the LacZ expression level in the cells transduced with AdRGD-CA-LacZ or AdK7-CA-LacZ was increased by about 5- or 50-fold, respectively, in comparison with that in the cells transduced with Ad-CA-LacZ (Fig. 1B). These results were quite similar to those of our previous report, in which efficient transduction in hMSCs was achieved by using AdK7 [9], and our data clearly demonstrated that AdK7 is a suitable vector for transduction into both mouse BMSCs and hMSCs.

We and others reported that the choice of promoters is important for transduction efficiency, especially in immature cells [15,18,20,21]. Thus, we examined the transduction efficiency by comparing the promoter activities in BMSCs. In addition to the CA promoter, we prepared LacZ-expressing AdK7 under the control of the CMV promoter or the EF-1α promoter (AdK7-CMV-LacZ or AdK7-EF-LacZ, respectively). A luminescent assay showed that the CA promoter represented the highest transgene expression

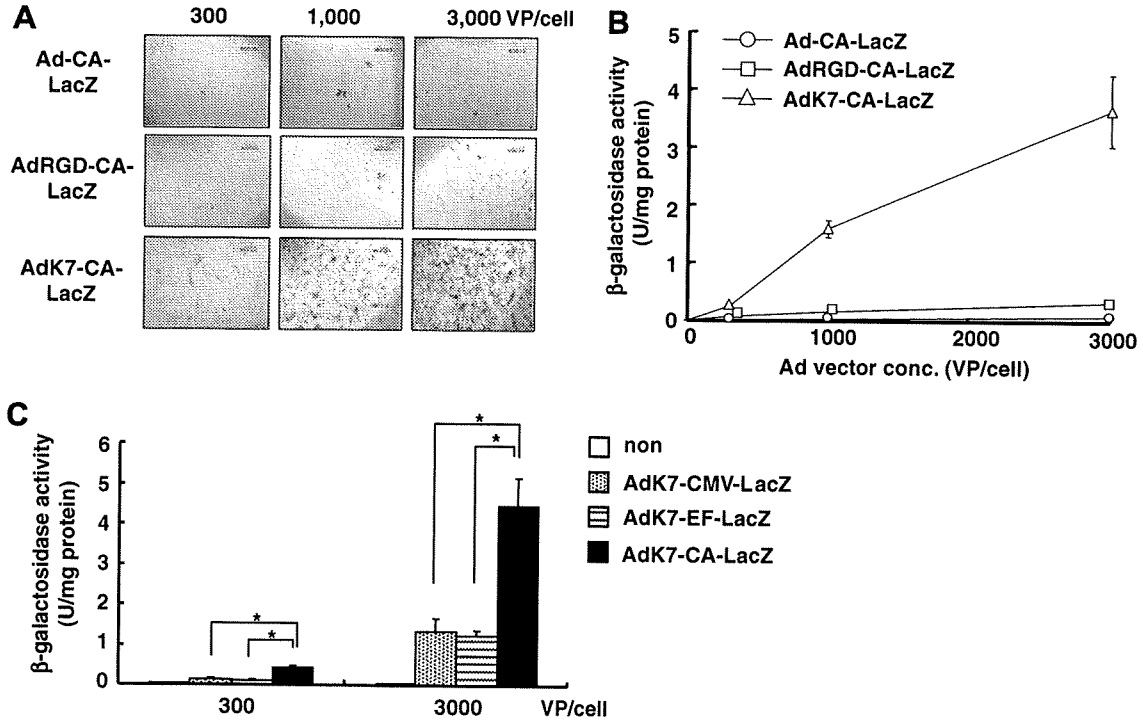

Fig. 1. Gene transduction efficiency in mouse primary BMSCs by various types of Ad vectors. Mouse BMSCs were transduced with the indicated doses of LacZ-expressing Ad vectors. Two days later, (A) X-gal staining and (B) luminescence assay were performed. Similar results of X-gal staining were obtained in three independent experiments. Scale bar indicates 200 μm. (C) Optimization of promoter activity in BMSCs using LacZ-expressing AdK7. BMSCs were transduced with the indicated dose of each Ad vector, and LacZ expression in the cells was measured. The data are expressed as mean ± S.D. ($n = 3$). $^{*}p < 0.01$.

among the three types of the promoters (Fig. 1C). These results demonstrate that AdK7 containing the CA promoter is the most effective at attaining high transduction efficiency in mouse BMSCs.

We also investigated the cytotoxicity in BMSCs transduced with AdK7-CA-LacZ. Almost no difference in cell number between non-transduced cells and AdK7-CA-LacZ-transduced cells was observed on day 2 after transduction (data not shown), indicating that AdK7 is an excellent vector with high transduction activity and low cytotoxicity in BMSCs.

Efficient osteoblast differentiation in vitro and in vivo by fiber-modified Ad vectors

Because an efficient method for transduction into BMSCs could be established by using AdK7 containing the CA promoter, we expected that efficient differentiation into specialized cells from BMSCs might be achieved by using this Ad vector. To test this, we generated mouse Runx2-expressing Ad vectors, AdK7-CA-Runx2 and Ad-CA-Runx2, because a Runx2 gene is both necessary and sufficient for mesenchymal cell differentiation towards osteoblast lineage [3]. Western blot analysis showed that Runx2 protein levels in AdK7-CA-Runx2-transduced cells were quite higher than those in non-, AdK7-CA-LacZ-, or Ad-CA-Runx2-transduced cells (Fig. 2).

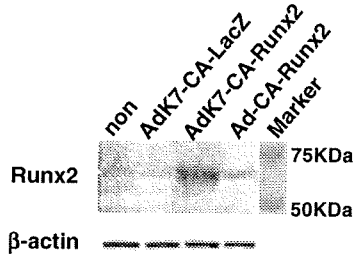

Fig. 2. Runx2 expression in Ad vector-transduced BMSCs. Cell lysates were isolated from BMSCs 2 days after the transduction, and Western blotting was performed.

We next assessed osteoblast differentiation by measuring alkaline phosphatase (ALP) activity, which is a marker of early osteoblast differentiation. After transduction with Ad vector, BMSCs were cultured in osteogenic differentiation medium for the indicated number of days. As shown in Fig. 3A, the ALP activity levels in AdK7-CA-Runx2-transduced cells were extremely increased in comparison with control cells. Notably, AdK7-CA-Runx2 mediated approximately 50-fold higher ALP activity than non-transduction or AdK7-CA-LacZ on day 5 after transduction. These results indicated that early osteoblast differentiation of BMSC was facilitated by AdK7-CA-Runx2. Because mature osteoblasts are known to be specialized in the production of extracellular matrix and the mineralization [22], we next examined the matrix mineralization in BMSCs. von Kossa staining revealed that matrix mineralization in AdK7-CA-Runx2-transduced cells was dramatically increased in comparison with non-, AdK7-CA-LacZ, or Ad-CA-Runx2-transduced cells (Fig. 3B, left). Furthermore, we observed a significant elevation of calcium deposition in AdK7-CA-Runx2-transduced cells even on day 5 after transduction, while neither non-transduced cells nor AdK7-CA-LacZ-transduced cells showed mineralization until day 15 (Fig. 3B, right). Ad-CA-Runx2 mediated slightly higher levels of calcium deposition than non-transduced or AdK7-CA-LacZ-transduced cells, but significantly lower levels than AdK7-CA-Runx2-transduced cells. Additionally, we found that the expression levels of marker genes characteristic of osteoblast differentiation, such as Runx2, osterix, bone sialoprotein, osteocalcin, and type I collagen, were also increased in AdK7-CA-Runx2-transduced cells (Fig. 3C). These results demonstrated that a conventional method using only osteogenic differentiation medium is

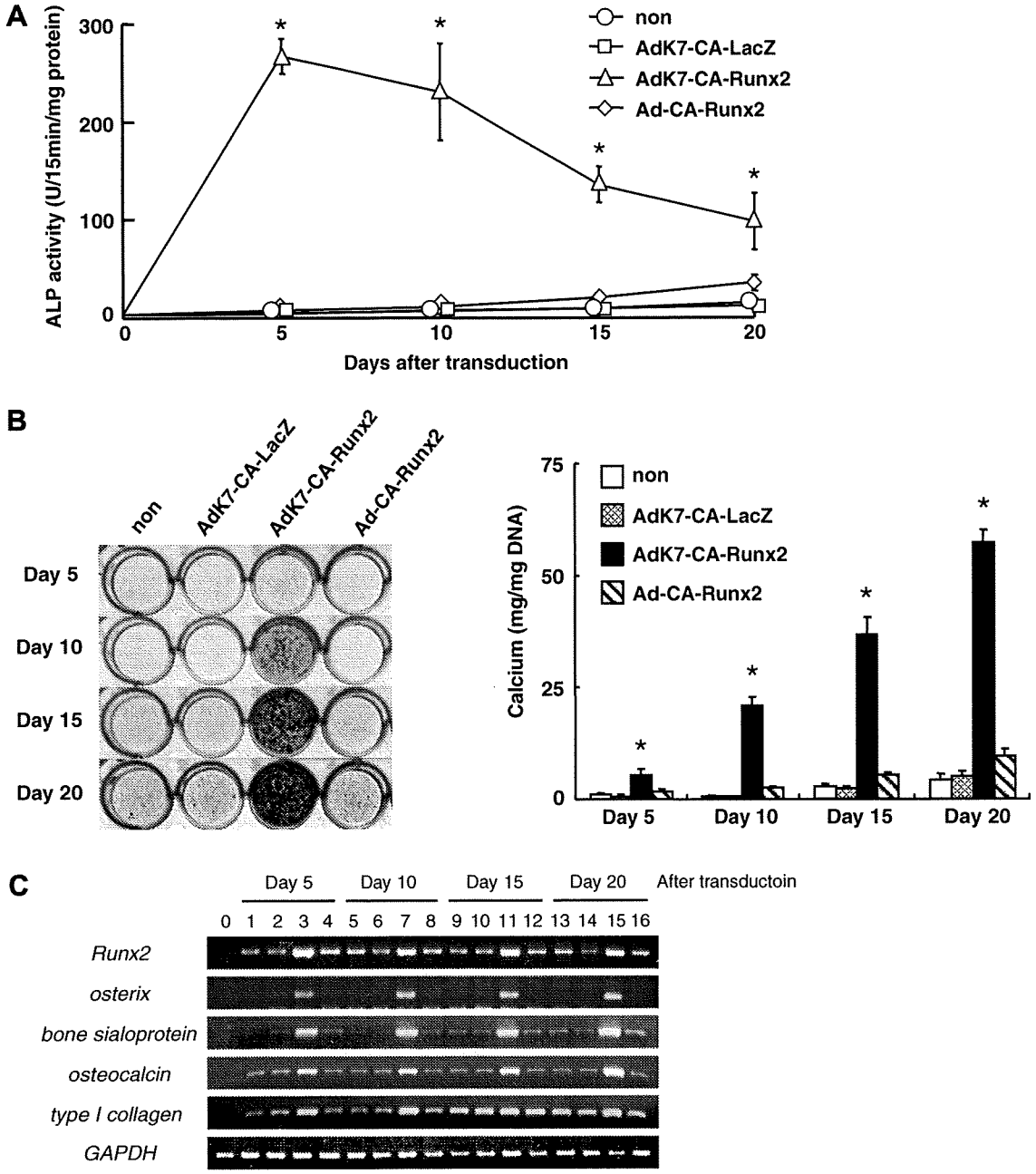

Fig. 3. Promotion of *in vitro* osteoblastic differentiation in AdK7-CA-Runx2-transduced BMSC. After transduction with each Ad vector at 3000 VP/cell for 1.5 hr, BMSCs were cultured for the indicated number of days. (A) ALP activity, (B, left) matrix mineralization, and (B, right) calcium deposition in the cells was determined. The data are expressed as mean ± S.D. ($n = 3$). $^{*}p < 0.01$ as compared with non-, AdK7-CA-LacZ-, or Ad-CA-Runx2-transduced cells. (C) RT-PCR was performed using primers for Runx2, osterix, bone sialoprotein, osteocalcin, collagen type I, and GAPDH. Lane 0: non-treated BMSCs; lanes 1, 5, 9, and 13: BMSCs with osteogenic supplements (OS); lanes 2, 6, 10, and 14: BMSCs with OS plus AdK7-CA-LacZ; lanes 3, 7, 11, and 15: BMSCs with OS plus AdK7-CA-Runx2; lanes 4, 8, 12, and 16: BMSCs with OS plus Ad-CA-Runx2.

not enough for efficient osteoblast differentiation, and that, by efficient Runx2 transduction using AdK7, osteoblastogenesis of BMSCs could be dramatically accelerated *in vitro*.

Finally, to examine whether the increased levels of Runx2 expression in BMSCs could enhance the osteogenic potential of BMSC *in vivo*, BMSCs transduced with each Ad vector were injected into the hind limb biceps muscle of nude mice. Microcomputed tomography analysis revealed that no bone formation was observed in non-, AdK7-CA-LacZ-, or Ad-CA-Runx2-transduced cells, while new bone was detected in mice injected with AdK7-CA-Runx2-transduced cells (Fig. 4), indicating that AdK7-CA-Runx2-transduced BMSCs efficiently differentiated into mature osteoblasts *in vivo*. These results clearly showed that AdK7-CA-Runx2 could facilitate the osteogenic potential of BMSCs both *in vitro* and *in vivo*.

Discussion

Because genetic manipulation is considered to be a powerful tool to promote cellular differentiation, it is necessary to establish efficient methods for transduction into BMSCs. Many researchers have reported that transduction efficiency of rat or human MSC was increased by using fiber-modified Ad vectors, such as AdRGD or Ad vectors containing Ad35 fiber knob and

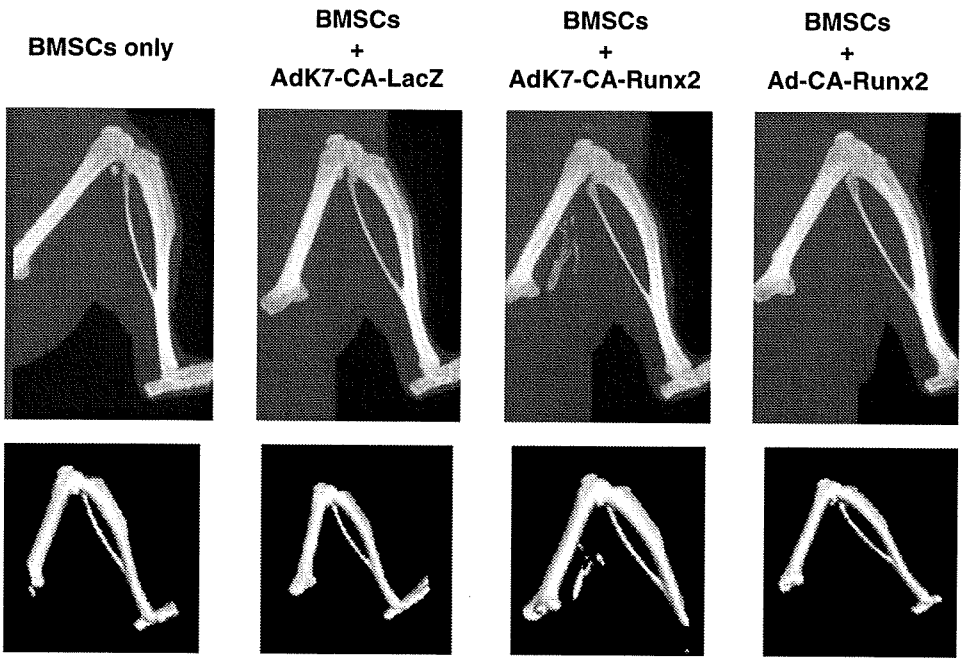

Fig. 4. *In vivo* ectopic bone formation of mouse BMSCs by AdK7-mediated Runx2 gene transduction. BMSCs were transduced with indicated Ad vectors at 3000 VP/cell. On the following day, cells were injected into the hind limb biceps muscle of nude mice. Four weeks later, bone formation was analyzed by the microCT system. Similar results were obtained in two independent experiments. Upper: X-ray images; lower: 3D reconstitution images.

shaft (AdF35) [23–25]. In this study, we demonstrated that AdK7 could express a transgene in BMSCs more efficiently than conventional Ad vector or AdRGD (Fig. 1A and B). Similarly, we have previously shown that the highest transduction efficiency in hMSC could be achieved by using AdK7, but not AdRGD or AdF35 [9]. Therefore, our data indicate that AdK7 is the most appropriate vector for various mesenchymal cells. We also found that the CA promoter showed higher gene expression in BMSCs than did the CMV or EF-1α promoter (Fig. 1C). This appears to be due to the potent activity of the CA promoter in immature cells [18,20]. Hence, we conclude that AdK7 containing the CA promoter is the most suitable vector for transduction into BMSCs.

We demonstrated that osteoblastogenesis of BMSCs was dramatically promoted by using AdK7-mediated Runx2 transduction (Figs. 3 and 4). This is the first study to report the usefulness of AdK7 in the field of stem cell differentiation. Runx2 is known to regulate osteoblastogenesis by controlling the expression of multiple osteoblast marker genes [10]. Because Runx2 protein and mRNA were highly expressed for more than 20 days in AdK7-CA-Runx2-trasduced cells (Figs. 2 and 3C), the expression of marker genes and ALP activity would be increased and would thereby enhance both *in vitro* and *in vivo* osteogenic ability. On the other hand, osteoblast differentiation could not be facilitated by AdK7-CA-Runx2 when osteogenic supplements were removed (data not shown), suggesting that osteogenic supplements were required for matrix mineralization, although differentiation efficiency was low when using only osteogenic supplements. Thus, efficient osteoblast differentiation of BMSCs would be achieved by the synergistic effect of both osteogenic supplements and efficient Runx2 transduction.

Unlike the case with AdK7-CA-Runx2, almost no osteoblast differentiation was seen in Ad-CA-Runx2-transduced cells. However, several groups reported that the osteogenic potential of MSCs was enhanced by Runx2 transduction using the conventional Ad vectors [26,27]. This difference would be attributable to the difference in transduction efficiency in BMSCs using the conventional Ad vector, because they showed that approximately 30–40% of the cells expressed transgenes by conventional Ad vector at 250–500 infectious units (ifu)/cell. Although we could not obtain high transduction efficiency using the conventional Ad vector, we showed that more than 90% of the cells were transduced by using AdK7-CA-LacZ at only 71 ifu/cell (3000 VP/cell) (Fig. 1A), without any decrease in viability (data not shown). Our results indicate that vector doses can be reduced by using AdK7, leading to a decrease in cytotoxicity to the cells. Therefore, AdK7, but not other fiber-modified Ad vectors or conventional Ad vectors, would contribute to safe regenerative medicine procedures.

In summary, we succeeded in developing efficient methods both for transducing mouse BMSCs and differentiating osteoblasts from BMSCs. Recently, many researchers have reported that mesenchymal stem/stromal cells could be isolated from adipose or placental tissues [28,29]. Because these mesenchymal cells are shown to possess mostly the same properties as BMSCs, AdK7 could probably be applied to these cells. Thus, our transduction methods can be a valuable tool for therapeutic applications based on adult mesenchymal stem/stromal cells.

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Chromosomal instability in human mesenchymal stem cells immortalized with human papilloma virus E6, E7, and hTERT genes

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Abstract Human mesenchymal stem cells (hMSCs) are expected to be an enormous potential source for future cell therapy, because of their self-renewing divisions and also because of their multiple-lineage differentiation. The finite lifespan of these cells, however, is a hurdle for clinical application. Recently, several hMSC lines have been established by immortalized human telomerase reverse transcriptase gene (hTERT) alone or with hTERT in combination with human papillomavirus type 16 E6/E7 genes (E6/E7) and human proto-oncogene, Bmi-1, but have not so much been characterized their karyotypic stability in detail during extended lifespan under in vitro conditions. In this report, the cells immortalized with the hTERT gene alone exhibited little change in karyotype, whereas the cells immortalized with E6/E7 plus hTERT genes or Bmi-1, E6 plus hTERT genes were unstable regarding chromosome numbers, which altered markedly during prolonged culture. Interestingly, one unique chromosomal alteration was the preferential loss of chromosome 13 in three cell lines, observed by fluorescence in situ hybridization (FISH) and comparative-genomic hybridization (CGH) analysis. The four cell lines all maintained the ability to differentiate into both osteogenic and adipogenic lineages, and two cell lines underwent neuroblastic differentiation. Thus, our results were able to provide a step forward toward fulfilling the need for a sufficient number of cells for new therapeutic

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