Expansion of Donor Hepatocytes After Recombinant Adenovirus-Induced Liver Regeneration in Mice

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Hepatocyte transplantation is a potential form of therapy for patients with genetic hepatodeficiency disorders. Unfortunately, hepatocellular transplantation has been limited because of the relatively low numbers of donor cells that can ultimately take up residence in the host liver. To give the donor cells a proliferative stimulus, a recombinant adenovirus vector that expresses a nonsecreted urokinase (urokinase-type plasminogen activator) was transduced into the livers of recipient animals before transplantation. Because urokinase production in hepatocytes causes the slow turnover of hepatocytes, 2 days after adenovirus-mediated gene transfer into the livers of recipient mice, 2×10^6 congenic donor cells tagged with β-galactosidase (β-Gal) reporter were implanted via the portal vein. As a result, on average, 8.6% of the recipient hepatocytes in the livers were derived from donor cells—a 20-fold increase compared with control animals in which no proliferative stimulus was present. (HEPATOLOGY 1997;25:884-888.)

Since the general acceptance of organ transplantation as treatment for a variety of diseases, more attention is being directed toward the development of techniques that allow for transplantation of individual cells. There has been increasing interest in transplantation of hepatocytes as a potential therapeutic alternative for orthotopic liver transplantation in some clinical situations.[10] It has the advantage of being less invasive, and hepatocytes from a single donor can be transplanted into several recipients, which may ultimately help resolve the dilemma of the chronic shortage of donor livers. Furthermore, hepatocyte transplantation has potential benefit in the treatment of disorders in which a specific hepatic function is lacking, such as inborn errors of metabolism resulting from the absence of an enzyme.

From the time that reproducible methods have been developed for isolating viable hepatocytes, such as the “digestion-perfusion” technique of Seglen,[27] different sites of implantation have been investigated for their efficacy of transplantation in various animal models. Functionally active hepatocytes can be detected in the liver after intraportal transplantation.[22,24,28] Similarly, transplantation into the spleen results in functionally active hepatocytes migrating to the liver, where they are detected indefinitely.[9,11,24] Splenic and intraportal routes of administration have been the most successful methods of hepatocellular transplantation. Other ectopic areas that have been used include fat pads,[11] intraperitoneal after attachment to microcarriers,[6] solid supports,[1] or bioabsorbable polymers.[13]

The limiting factor in both the intraportal and the intrasplenic approach is the number of cells that can be injected without causing complications such as portal vein thrombosis, portal hypertension, and pulmonary emboli. Using genetically tagged donor mouse hepatocytes, it has been determined that the maximal percentage of engraftment of functional donor hepatocytes in the mouse is 0.5%.[24] This has been confirmed by a number of studies using autologous hepatocytes alone or after genetic modification with viral vectors in other animals[1,5,14] and in humans.[8] This type of *ex vivo* gene therapy has been limited in its efficacy because of the relatively low proportion of genetically modified cells that take up residence in the recipient livers.

In an attempt to increase the efficiency of hepatocyte transplantation, investigators have shown that inducing hepatocellular regeneration by performing a surgical partial hepatectomy results in a proliferative stimulus for the original hepatocytes as well as for the transplanted hepatocytes.[4,16,30] In a recently described model for liver regeneration using transgenic mice that express urokinase-type plasminogen activator, a chronic stimulus of hepatocyte injury allowed for the repopulation of up to 80% of the recipient mouse liver with syngenic donor cells after transplantation of relatively few hepatocytes.[25] This shows conclusively that proliferation of donor cells in a liver is possible.

A new method for inducing liver regeneration using a recombinant adenovirus that transiently expresses a modified, nonsecreted urokinase-type plasminogen activator (Ad.PGK-muPA) was developed.[19,20] The advantage of the modified urokinase molecule is that the nonsecreted protein does not cause transient hypocoagulation, which is seen with wild-type urokinase from the liver.[19,20] Administration of Ad.PGK-muPA results in asynchronous liver injury beginning within the first few days after gene transfer, followed by regeneration over a period of about 10 days.[19,20] During the period of regeneration, at any one day, 30% to 50% of the host hepatocytes were found to be replicating, as determined by nuclear ^{3}H-thymidine incorporation. This study shows that modified urokinase–induced liver degeneration in the recipient also allows for replication of non–adenovirus-containing donor hepatocytes. When congenic hepatocytes were transplanted during the period of liver regeneration, proliferation of these donor cells was observed, resulting in the reconstitution on average of 8.6% of the recipient liver. This model may have a number of scientific and clinical applications.

MATERIALS AND METHODS

Animals

A transgenic mouse line that contains the mouse metallothionein-I gene promoter plus flanking sequences fused to an *Escherichia*

Abbreviations: β-Gal, β-galactosidase; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

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coli β-galactosidase (β-Gal) structural gene (MT-*lac* Z), serving as congenic B6 hepatocyte donors, was previously described.[23] The animals were bred to homozygosity for the *lac* Z transgene and used in the described experiments.

Nontransgenic female recipient C57Bl/6 mice, aged 5 to 6 weeks (weight, $\approx$14 g) were purchased from Jackson Laboratories (Bar Harbor, ME). All studies were performed in accordance with the institutional guidelines of the University of Washington.

Recombinant Adenoviral Constructions

The recombinant adenoviral vector, expressing a modified urokinase (Ad.PGK-muPA).[19] Ad/RSV-hAAT,[15] and viral preparation[2] has been previously described. The viral titers ranged from 2 to 5×10^{11} plaque forming units/mL. The purified virus was stored in aliquots at -80°C and freshly diluted with serum free Dulbecco's modified Eagle media (Life Technologies, Gaithersburg, MD) before infusion. Both preparations tested negative for the presence of replication-competent virus by an earlier reported assay.[2]

Surgical Procedures

Portal Vein Cannulation. Female mice were anesthetized by an intraperitoneal administration of 0.3 to 0.5 mL of 20 mg/mL Avertin (2,2,2-Tribromoethanol; Aldrich Chemical, Milwaukee, WI). In all mice, an indwelling portal vein cannula was inserted as previously described.[29] The distal end of the cannula was tied off, tunneled through the peritoneum, and placed subcutaneously in a previously created pocket. Perfusion through the portal vein of both adenovirus and hepatocytes was performed under anesthesia after exposure of the cannula through a 3-mm-wide skin incision at the proximal site of the previously made abdominal incision.

Adenoviral Administration. One day after placement of the portal vein cannula, 5×10^9 plaque-forming units of adenovirus (Ad.PGK-muPA or Ad/RSV-hAAT [control]) in 200 μL serum-free Dulbecco's modified Eagle medium was administered into the portal vein through the cannula. Control animals were injected with 200 μL saline.

Hepatocyte Isolation. Donor hepatocytes were isolated from 5- to 10-week-old transgenic MT-*lac* Z C57Bl/6 mice by *in situ* collagenase perfusion (Collagenase D; Boehringer Mannheim Corporation, Indianapolis, IN) of the liver via the inferior vena cava.[18] Cells were filtered through a presterilized 85-μm nylon mesh, resuspended in Dulbecco's modified Eagle medium with 10% fetal calf serum and penicillin/streptomycin, and pelleted twice by centrifugation at 50*g* for 5 minutes. The viability of the cells, as quantified by trypan blue exclusion, varied between 80% and 90%. Hepatocytes with viabilities of less than 80% were excluded from transplantation. A portion of the freshly isolated MT-*lac* Z hepatocytes was plated in 6 cm Falcon Primaria dishes (Becton Dickinson, Franklin Lakes, NJ) in Williams' media E (Life Technologies) containing 10% fetal calf serum and penicillin/strep.

Transplantation of Hepatocytes. Two days after administration of the adenoviral vector or saline, hepatocyte transplantation was performed. Freshly isolated hepatocytes from MT-*lac* Z C57Bl/6 mice, diluted in 0.4 mL serum-free Dulbecco's modified Eagle medium, were infused through the portal vein cannula of congenic C57Bl/6 mice, at a flow rate of approximately 40 μL/min. One to 2×10^6 hepatocytes were infused into recipients.

Detection of Transplanted Hepatocytes

X-gal Staining. Mice that received MT-*lac* Z hepatocytes were killed 4 weeks after transplantation. To induce *lac* Z expression, mice were injected with 1 μg of $CdSO_4$ per kilogram of body weight at 20 hours and 6 hours before killing.[25] A portion of the liver was removed and embedded in OCT compound (Miles Inc., Elkhart, IN), frozen in methylbutane, cooled in liquid nitrogen, and stored at -80°C.

To detect β-Gal activity, 10-μm-thick frozen sections were cut, fixed with 1.25% gluteraldehyde in phosphate-buffered saline for 10 minutes, and stained overnight with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (Sigma, St. Louis, MO).[21] The sections were rinsed with phosphate-buffered saline counterstained with Nuclear Fast Red (Sigma), rinsed in water and then alcohol, placed in xylene, and coverslipped with Permount (Fisher Scientifics, Santa Clara, CA).

In each animal, from the remaining part of the liver, hepatocytes were isolated by collagenase perfusion (Collagenase D, 0.3 mL/min), plated, and cultured on Falcon Primaria plates. The cells were stained with X-Gal within 12 hours after seeding as described.[21] After incubation overnight with the X-gal solution, the cultured hepatocytes were randomly counted and scored for β-Gal expression. At least 1,000 cells were counted from random fields for each liver analyzed.

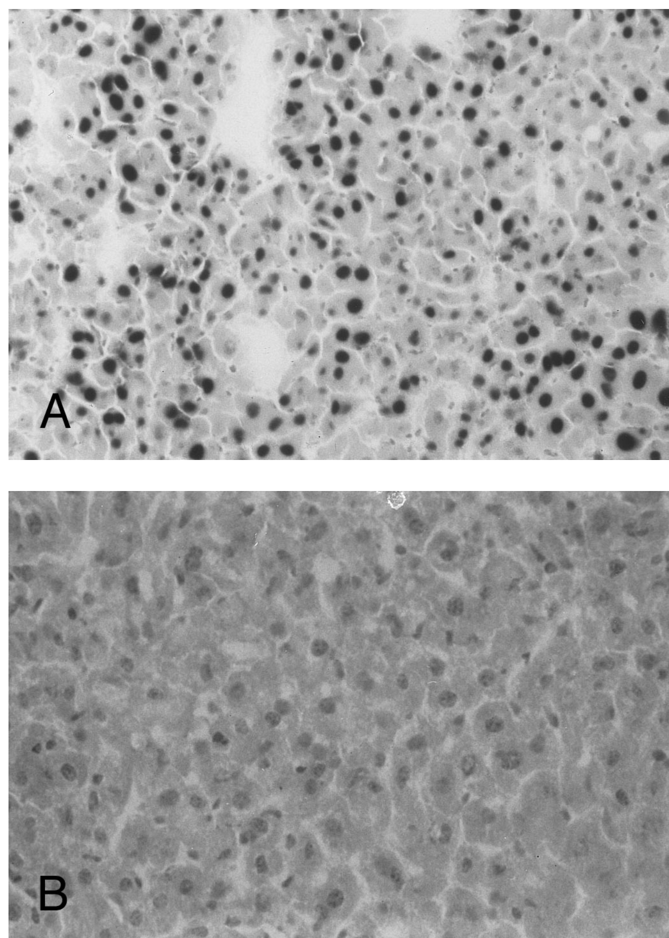

FIG. 1. Histochemical staining of livers from (A) a transgenic MT-*lac* Z C57Bl/6 mouse used for donor cells and (B) a normal C57Bl/6 mouse.

DNA Blot Analysis. Total liver DNA from three female recipient mice was isolated 4 weeks after transplantation with male hepatocytes, and analyzed by Southern blot analysis as described[29] using α ^{32}P-labeled DNA probe (pY353/B) containing mouse Y-specific chromosomal DNA[3] (a gift from Richard Palmiter), allowing detection of male (donor) hepatocytes. Ten micrograms of genomic DNA was cut with *Hin*dIII. For a standard curve, known amounts of male hepatocyte DNA were mixed with female liver DNA. The relative radiolabeled signals were quantitated on a Model 400S phosphorimager (Molecular Dynamics, Sunnyvale, CA) for relative quantitation of the bands.[29]

RESULTS

MT-*lac *Z C57Bl/6 Donor Mice. To determine the number of cells that would stain blue from MT-*lac* Z C57Bl/6 mice, frozen sections and liver cell suspensions from MT-*lac* Z transgenic liver were analyzed for β-Gal expression after cadmium induction. X-Gal staining of both liver sections (Fig. 1A) and cultured hepatocytes showed about 95% blue nuclear-stained hepatocytes, whereas control liver tissue and hepatocytes from nontransgenic mice did not stain blue (Fig. 1B).

Transplantation of MT-*lac *Z Hepatocytes. For all transplantation experiments, liver cell suspensions were freshly prepared from MT-*lac* Z C57Bl/6 mice. The cellular viability after selection of hepatocytes by centrifugation, as measured

by trypan blue exclusion, ranged from 80% to 90%. A recombinant adenovirus that expressed a nonsecreted form of urokinase (Ad.PGKmuPA) was used before transplantation because expression the modified urokinase in hepatocytes results in hepatocellular destruction. To give the transplanted hepatocytes a proliferative stimulus, transplantation was performed 2 days after the administration of Ad.PGK-muPA in the recipient mice because, after modified uPA induced hepatocellular degeneration, liver regeneration begins on day 3 and persists for more than 1 week.[19,20] The control mice were injected with a control adenovirus (Ad/RSV-hAAT) or with saline 2 days before transplantation of the hepatocytes.

To quantitate the efficiency of cell reconstitution after transplantation, hepatocytes from the recipient mice were isolated and cultured 4 weeks after transplantation. The 4-week time point was selected because 3 weeks after Ad.PGK-muPA administration, the livers return to normal by gross and histological examination.[19,20] Table 1 and Fig. 2 summarize the relative number of donor cells as determined by X-gal staining of the cultured hepatocytes. When 1×10^6 hepatocytes were transplanted, the recipient, on average, contained about 2% donor cells (Fig. 2). However, the best result was obtained when 2×10^6 hepatocytes were injected 2 days after Ad.PGK-muPA injection. In this group, on average, 8.6% of the hepatocytes stained blue. One mouse contained over 20% blue cells in the liver. Why this level of reconstitution occurred in one experimental recipient is not known, but may simply be related to normal variation between animals. In comparison, less than 0.5% of the hepatocytes stained blue when the same number of cells was injected in the two control groups (Table 1, Fig. 2).

To localize the transplanted hepatocytes, histological analysis was performed on liver sections stained with X-Gal (Fig. 3). The relative numbers of X-gal–positive cells determined by this method was similar to those obtained by counting the isolated hepatocytes. The donor hepatocytes were randomly distributed throughout the liver parenchyma in all animals examined. Notably, clusters of donor cells were found throughout the livers of mice treated with Ad.PGK-muPA, but rarely were clusters of two cells or mostly isolated single cells found in the controls. Occasionally, clusters of up to eight cells were found in the livers of mice that underwent regeneration, suggesting clonal expansion of transplanted cells.

TABLE 1. Hepatocyte Transplantation in Adenovirus-Treated Animals

Treatment	Amount of Viable Cells Transplanted	Donor No.	Number Positive Cells in Recipient Liver Hepatocyte Suspension (%)
Ad.PGK-muPA	1.6×10^6	1	6.6
	1.8×10^6	2	6.8
	1.8×10^6	2	5.2
	1.8×10^6	2	4.1
	1.8×10^6	3	22.2
	1.8×10^6	3	6.8
Ad.RSV-hAAT (control)	1.8×10^6	2	0.4
	1.8×10^6	2	0.01
	1.8×10^6	2	0.5
	1.8×10^6	3	0.8
Saline (control)	1.6×10^6	1	0.01
	1.8×10^6	3	0.5
	1.8×10^6	3	0.3
	1.8×10^6	3	0.2

NOTE. Hepatocytes were isolated from recipient mice 4 weeks after transplantation of 2×10^6 (viable and nonviable) MT-*lac* Z–positive hepatocytes. X-Gal staining was performed within 12 hours after the initial plating of the hepatocytes. The cells were counted in random fields for the presence of β-Gal activity by blue staining.

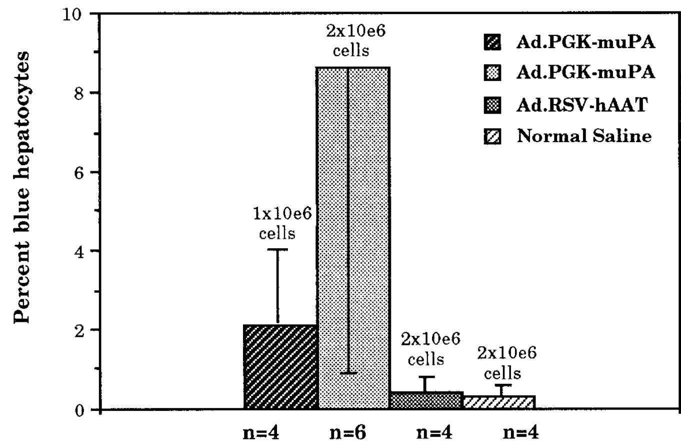

FIG. 2. Effects of Ad.PGK-muPA–induced liver regeneration on the efficiency of donor hepatocyte transplantation. Mice were given Ad.PGKmuPA, Ad/RSVhAAT, or normal saline 2 days before hepatocyte transplantation. Mice were killed 4 weeks after transplantation; hepatocytes were isolated, cultured, and stained with X-gal. At least 1,000 hepatocytes from each animal were counted from random fields and scored for the presence of positive (=donor) hepatocytes. The mean of the percentage of positive cells of four to six mice per experiment with the standard deviations are presented. Individual results for each animal are given in Table 1.

To confirm these results with a second method, three female mice were given Ad.PGKmuPA or saline before transplantation of 2×10^6 male hepatocytes, and were killed about 1 month after transplantation. From these mice, liver DNA was isolated and analyzed for the relative amount of Y-chromosome DNA (Fig. 4). The results showed that, with modified urokinase–induced liver regeneration, 5% to 6% of the DNA was derived from a male and thus represents transplanted donor cells, whereas a faint band representing less than 0.5% DNA was detected in two control animals. This method of quantitation probably underestimates the true number of transplanted cells because hepatocytes only make up about 70% of the total number of cells in the liver.[12] Thus, liver DNA contains about 30% DNA from nonhepatic cells. Because purified hepatocytes were used for transplantation and total liver DNA, (not hepatocyte DNA) was used for the Southern blot quantitation, the corrected number of donor-derived hepatocytes detected by this method was about 7% to 8.5%. Nevertheless, between different experiments, the relative numbers of donor hepatocytes detected in the recipient liver by DNA quantitation and counting X-gal–positive cells were similar.

DISCUSSION

Hepatocyte transplantation can be considered as a potential alternative treatment for metabolic diseases and, in some cases, orthotopic liver transplantation. Previous studies showed that the number of donor hepatocytes that could be engrafted into the livers of normal recipients was less than 0.5% of the original liver mass.[24] Similar results have been shown in dogs and rabbits using autologous cells transduced with a retroviral vector.[5,14] Other investigators have shown that performing a partial surgical hepatectomy leads to proliferation of the transplanted hepatocytes, resulting in a slightly higher percentage of engraftment of donor cells into the recipient.[16,30]

We now describe a strategy in which the efficient gene transfer properties of the adenoviral vector were used to introduce a modified urokinase gene into hepatocytes. This results in asynchronous hepatocyte degeneration, most likely

FIG. 3. Histology of livers from animals after transplantation of MT-*lac* Z hepatocytes. Ten-micrometer liver sections from representative animals described in Fig. 2. Mice were treated with (A) Ad.PGK-muPA (low magnification), (B) Ad.PGK-muPA (high magnification), and (C) Ad/RSVhAAT (control adenovirus). (D) Normal saline. In (C) and (D), positive cells were not found in most of the fields.

(although not proven to be) caused by intracellular conversion of the hepatocyte-derived plasminogen to plasmin.[19,20] Plasmin is a protease that results in the activation of a number of proteins and which is thought to toxic within cells. Hepatocellular deficiency stimulates hepatocellular regeneration. Thus, the regenerative stimulus, induced by the urokinase-mediated hepatocyte degeneration, was used to stimulate the donor hepatocytes to proliferate *in vivo.* Administration of Ad.PGK-muPA before transplantation of 2×10^6 hepatocytes resulted, on average, in 8.6% repopulation of the recipient liver with donor hepatocytes. This was 20-fold more donor cells than obtained with the infusion of the same number of hepatocytes after administration of a control adenovirus or saline.

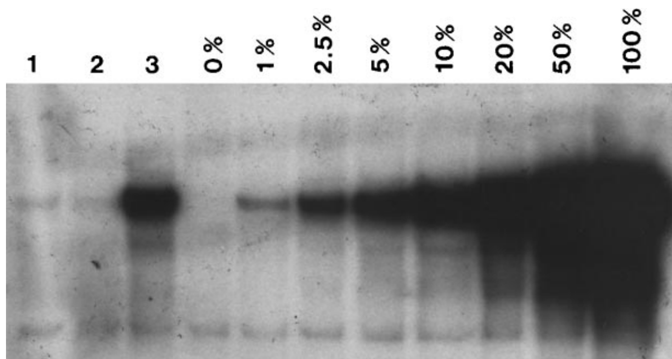

FIG. 4. DNA quantitation. Ten micrograms of *Hin*d III digested total DNA from the livers of mice obtained at 4 weeks, treated with normal saline (lanes 1 and 2) or Ad.PGK-muPA (lane 3), 2 days before transplantation with 2×10^6 male hepatocytes. The percentage of male mouse DNA mixed with female mouse DNA is shown above the lanes.

Assuming that the number of hepatocytes in an adult mouse is 1×10^8, we were able to transplant only 2% of the liver mass by infusing 2×10^6 donor cells. At best, transplantation of 2% of the original liver mass alone results in reconstitution of 0.5% of the endogenous liver.[24] If we assume that this means that, on average, only 25% of the transplanted hepatocytes survive *in vivo,* then only 5×10^5 donor hepatocytes were responsible for repopulating the regenerating liver. This would require at least three to four cell divisions to generate 8×10^6 hepatocytes (8% of original liver mass). If, on the other hand, Ad.PGKmuPA increased the number of viable cells that were able to implant successfully into the host liver, fewer cell divisions would be required to obtain the level of reconstitution observed. Even if all the transplanted cells survived, on average, they would require two rounds of cell division to reach the detected number of cells. In support of multiple rounds of hepatocyte cell divisions was the finding of liver sections in which clonal groups of up to eight blue-stained cells were present, whereas liver sections of the control groups showed blue staining of only single or, rarely, groups of two hepatocytes. Finally, the nonlinear dose response in that injecting half the number of cells resulted in a four-fold decrease in the final number of donor

cells in the recipient's liver is consistent with donor cells replicating more than once after transplantation.

Ultimately, a greater number of transplanted cells may be stably implanted by giving a larger number of cells at each transplantation. Our preliminary studies suggest that the number of cells that can be transplanted at a single infusion is limited because of compromise of the portal vasculature circulation, resulting in hepatic necrosis and/or pulmonary infarcts. Our mouse model, in which a permanent access to the portal vein exists, is suitable for trying to give multiple infusions of hepatocytes. Thus, the ability to give several infusions of lower numbers of cells may be possible. In support of these studies, a previous report showed that, in a rat model, multiple transplantations of a lower number of hepatocytes resulted in a higher efficiency of donor hepatocyte engraftment.[26]

Another potential way to increase the reconstitution of the recipient liver with donor cells is to increase the amount of Ad.PGK-muPA given right before transplantation. The dosage of adenovirus given in the present study was enough to transduce 80% to 90% of the hepatocytes.[17] By increasing the dose of Ad.PGKmuPA two-fold (1×10^{10} plaque-forming units), a dose at which 100% of cells were transduced,[17] the mortality increased from less than 5% to near 100%.[20] The high mortality may be overcome by infusion of a sufficient number of cells early enough to supply enough hepatic function until regeneration is complete. Thus, a number of parameters can, in theory, be adjusted to increase the number of donor hepatocytes in the liver.

This technology offers new ideas toward increasing the number of donor cells or genetically modified hepatocytes that can ultimately be used for liver reconstitution in both animal and human applications. There are a number of issues that limit using this approach in humans. Most important is the possibility of producing irreversible liver injury with uncontrolled intracellular urokinase production. With the recent development of promoters that can control gene expression by low-dose drug administration,[7] it may be possible to titer the amount of liver injury in the future.

The model of expanded donor cell repopulation has several theoretical advantages. First, this method or a similar method can be used to ultimately increase the number of genetically modified hepatocytes used in an *ex vivo* gene therapy protocol. Second, Ad.PGKmuPA administration can be used in immunodeficient animals as a means of studying hepatocellular xenotransplantation. Finally, because adenovirus vectors can transduce the livers of many species, liver repopulation can be studied in animals other than mice.

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