Protective Effect of Recombinant Adeno-Associated Virus 2/8-Mediated Gene Therapy from the Maternal Hyperphenylalaninemia in Offsprings of a Mouse Model of Phenylketonuria

Phenylketonuria (PKU) is an autosomal recessively inherited metabolic disorder caused by a deficiency of phenylalanine hydroxylase (PAH). The accumulation of phenylalanine leads to severe mental and psychomotor retardation, and the fetus of an uncontrolled pregnant female patient presents with maternal PKU syndrome. We have reported previously on the cognitive outcome of biochemical and phenotypic reversal of PKU in a mouse model, *Pah^enu2*, by the AAV serotype 2-mediated gene delivery of a human PAH transgene. However, the therapeutic efficacy had been limited to only male PKU mice. In this study, we generated a pseudotyped recombinant AAV2/8-hPAH vector and infused it into female PKU mice through the hepatic portal vein or tail vein. Two weeks after injection, complete fur color change to black was observed in female PKU, as in males. The PAH activity in the liver increased to 65-70% of the wild-type activity in female PKU mice and to 90% in male PKU mice. Plasma phenylalanine concentration in female PKU mice decreased to the normal value. In addition, the offsprings of the treated female PKU mice can rescue from the harmful effect of maternal hyperphenylalaninemia. These results indicate that recombinant AAV2/8-mediated gene therapy is a potential therapeutic strategy for PKU.

Key Words : *Phenylketonurias; Hyperphenylalaninemia; Mice; Dependovirus; Gene Therapy*

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INTRODUCTION

Phenylketonuria (PKU; MIM 261600) is an autosomal recessive metabolic disorder caused by a deficiency of phenylalanine hydroxylase (PAH; EC 1.14.16.1) (1, 2). PAH (L-phenylalanine-4-monooxygenase; EC 1.14.16.1), expressed predominantly in liver, catalyzes the conversion of the essential amino acid phenylalanine into tyrosine using tetrahydrobiopterin (BH₄) as a cofactor (3). The dysfunction of this enzyme results in the accumulation of phenylalanine and its abnormal derivatives in the blood and other tissues. Untreated PKU patients exhibit severe mental retardation, cognitive problems, growth failure, and other symptoms such as hypopigmentation due to a reduction of melanin synthesis (4, 5). In addition, increased concentration of blood phenylalanine caused by noncompliance to dietary control during pregnancy has an effect on offspring, leading to birth defects, the so-called maternal PKU syndrome (6).

To ameliorate PKU-related symptoms, a strict lifelong dietary restriction of phenylalanine is recommended (7). Gene transfer technology has been used to deliver recombinant PAH gene into somatic cells and restore functional protein (8-11), which can replace the usual dietary treatment. We have previously demonstrated that recombinant adeno-associated virus serotype 2 (rAAV-2)-mediated delivery of a human PAH transgene efficiently induces biochemical and phenotypic reversal in an animal PKU model (*Pah^enu2* mouse), which resembles the metabolic and biochemical abnormality of human PKU (12). However, route-independent and long-term therapeutic effects in females were not achieved in our earlier study. Recently, other studies demonstrated the complete correction of hyperphenylalaninemia in both males and females by the rAAV 2/8 vector (13, 14). AAV serotype 8 is an attractive candidate for liver-targeted gene therapy (15, 16). However, their observations were confined to the correction in the adult PKU mouse. The clinical use of gene therapy should completely overcome any possible complications such as maternal PKU syndrome, so that female PKU mice reproduce health offspring without birth defects or growth abnormalities. Here, we report pseudotyped rAAV2/8-mediated gene therapy that rescues from the effect of maternal PKU to provide long-term and sex-independent therapeutic correction of PKU mice.

MATERIALS AND METHODS

Recombinant AAV construction and production

The AAV-2-based human PAH expression vector plasmid, pAAV2-EF-hPAH-WPRE, was created as described previously (12). The pAAV2-EF-hPAH-WPRE is a full-length human PAH cDNA, phPAH247 (ATCC, Manassas, VA, U.S.A.)-containing vector harboring the human elongation factor 1-α promoter (EF), woodchuck hepatitis virus post-transcriptional element (WPRE), and bovine growth hormone poly (A) signal. The rep2/cap2 plasmid or rep2/cap8 plasmid (p5E18-VD2/8, kindly provided by James M. Wilson) (15) was used to package the expression vector. The rAAV2/2-hPAH and rAAV2/8-hPAH vectors were produced by the triple plasmid transfection method, purified by cesium chloride (Sigma-Aldrich, St. Louis, MO, U.S.A.) density gradient ultracentrifugation (17). The rAAV genomic titer was determined by real-time quantitative PCR using the ABI7700 (PerkinElmer/Applied Biosystems, Foster City, CA, U.S.A.), in which the signal from aliquots of the test material is compared with a standard signal generated using the linearized pAAV2-EF-hPAH-WPRE plasmid.

Cell culture and animal experiments

The human embryonic kidney cell line, 293T, was propagated in Dulbecco modified Eagle medium supplemented with heat-inactivated 10% fetal bovine serum and antibiotics. The animal protocol was approved by the animal experiment review committee of the Ewha Medical Research Institute, Ewha Womans University, Korea. A pair of PAH-deficient mice, *Pah^enu2*, were purchased from the Jackson Laboratory (Bar Harbor, ME, U.S.A.), maintained, and bred to obtain sufficient numbers of *Pah^enu2* homozygotic mice for this study (18). Mice had unlimited access to water and a diet containing 18.0% protein without phenylalanine restriction (Rat & Mouse 18%, PMI Nutrition International, Brentwood, MO, U.S.A.) throughout the entire experimental period. Five- to six-week-old mice were anesthetized with ketamine-xylazine and infused with 2×10^{12} viral particles of rAAV2/2-hPAH or rAAV2/8-hPAH through the hepatic portal vein. For tail vein injection, 3×10^{12} viral particles of rAAV2/8-hPAH was infused. At least three *Pah^enu2* homozygote mice were grouped and treated for each experiment.

Plasma phenylalanine assay and PAH enzyme assay

Plasma phenylalanine concentration and PAH enzyme activity in the liver were measured using our previous method (12). Quantitative analysis of plasma phenylalanine and tyrosine concentrations was carried out using high performance liquid chromatography (Biochrom20, Pharmacia, Cambridge, England). The PAH enzyme activity was assayed by thin layer chromatography to measure the production of [^{14}C]tyrosine from [^{14}C]phenylalanine. The radioactivity of [^{14}C]phenylalanine and converted [^{14}C]tyrosine was quantified using the ImageQuant (Amersham, Buckinghamshire, U.K.), after visualization with a PhosphoImager system (Fuji, Tokyo, Japan).

The effect of gene therapy on maternal PKU syndrome

Three five- to six-week-old female homozygous *Pah^enu2* mice were injected with 3×10^{12} viral particles of rAAV2/8-hPAH once through the tail vein. After two weeks, each female mouse was mated with a heterozygous male mouse. Control wild type and untreated female homozygous mice were also mated with heterozygous male mice. Females were checked daily for evidence of a vaginal plug, and on day 18.5 gestation (18.5 dpc), the pregnant female mice were sacrificed and the abdominal cavity was opened. The abortion rate, crown-rump length, and fetal weight were determined as described previously (19-21). Crown-rump length was measured with vernier calipers after removing each fetus from their gestational sacs (20). To calculate the abortion rate, the fetuses were individually dissected from the uterine lining after the gestational sacs were counted. The abortion (resorption) sites could be identified by their small size accompanied by a necrotic, hemorrhagic appearance, compared with normal fetuses and placentas. The percentage abortion rate was calculated as the ratio of resorption sites to total implantation sites (resorption plus normal implantation sites) as described previously (21). Polymerase chain reaction amplification with viral-specific primers was used to identify the transmission of viral particles to placentae and fetuses. The forward primer for the human elongation factor 1-α promoter was 5′-CGTCCAGGCACCTCGATTAGT-3′, and the reverse primers were 5′-CAGAGAGTTTCCTGCCCA-AG-3′ for rAAV2/8-hPAH and 5′-GTAGGTCAGGGT-GGTCACGA-3′ for rAAV2/8-eGFP.

Statistical analysis

The statistical significance levels of differences between groups were determined using Student's t-test. The data were presented as mean $\pm$ SD.

RESULTS

PAH activities and plasma phenylalanine concentration in PKU mice

PAH activity in livers of the rAAV-hPAH-treated PKU mice six weeks after treatment recovered to $58.43 \pm 5.73\%$ and $89.25 \pm 8.79\%$ of the wild-type activity in female and male PKU mice, respectively (Fig. 1A). Basal plasma pheny-

lalanine concentration in untreated seven- to eight-month-old female mice was $2,891 \pm 336.2$ μM, which was higher than in untreated male mice ($1,800.6 \pm 233.7$ μM). Plasma phenylalanine concentration in female PKU mice infused with high titer rAAV2/8-hPAH vectors (2×10^{12} viral particles via the portal vein) decreased markedly to 110 ± 34.5 μM at two weeks and showed continued significant phenylalanine clearance for six month after rAAV2/8-hPAH infusion (Fig. 1B). The plasma phenylalanine concentration in female PKU mice infused 3×10^{12} viral particles via the tail

vein also decreased to the values of wild type mice. However, there was no significant reduction of plasma phenylalanine concentration in rAAV2/2-hPAH infused female PKU mice. Plasma phenylalanine level at 6 months after rAAV2/2-hPAH treatment in female Pah^{enu2} was not measured since PAH enzyme activity peaks at 5 weeks after rAAV2/2-hPAH injection (12).

The fur color changed from grayish to black in the treated female PKU mice from one week after delivery of rAAV2/8-hPAH, similar to the pattern observed in treated male PKU
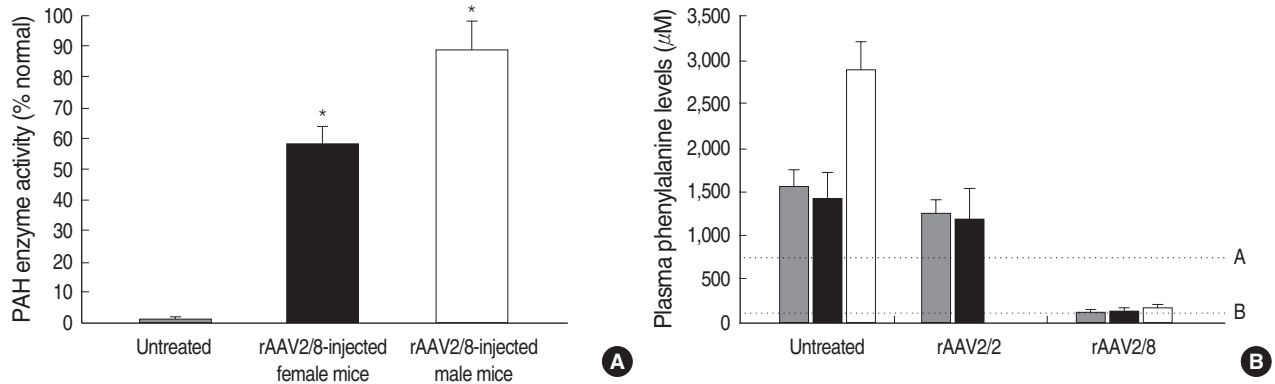

Fig. 1. (A) PAH enzyme activity in the livers of Pah^{enu2} mice six weeks after rAAV-hPAH administration. Enzymatic activity is represented as a percentage of that found in wild-type mice. The dotted bar represents PAH activities in homozygous Pah^{enu2} mice; the black bars, rAAV2/8-hPAH-injected homozygous female mice; the opened bars, rAAV2/8-hPAH-injected homozygous male mice. (B) Plasma phenylalanine concentration in female Pah^{enu2} mice after rAAV2/2-hPAH or aAAV2/8-hPAH treatment. The dotted bars represent plasma phenylalanine concentrations in homozygous female Pah^{enu2} mice 2 weeks after treatment; the black bars, 6 weeks after treatment; the open bars, 6 months after treatment. The dotted line A represents the recommended maximum value by the National Society for PKU (U.K.); dotted line B, plasma concentration in wild-type control mice. Values are presented as the mean $\pm$ SD.
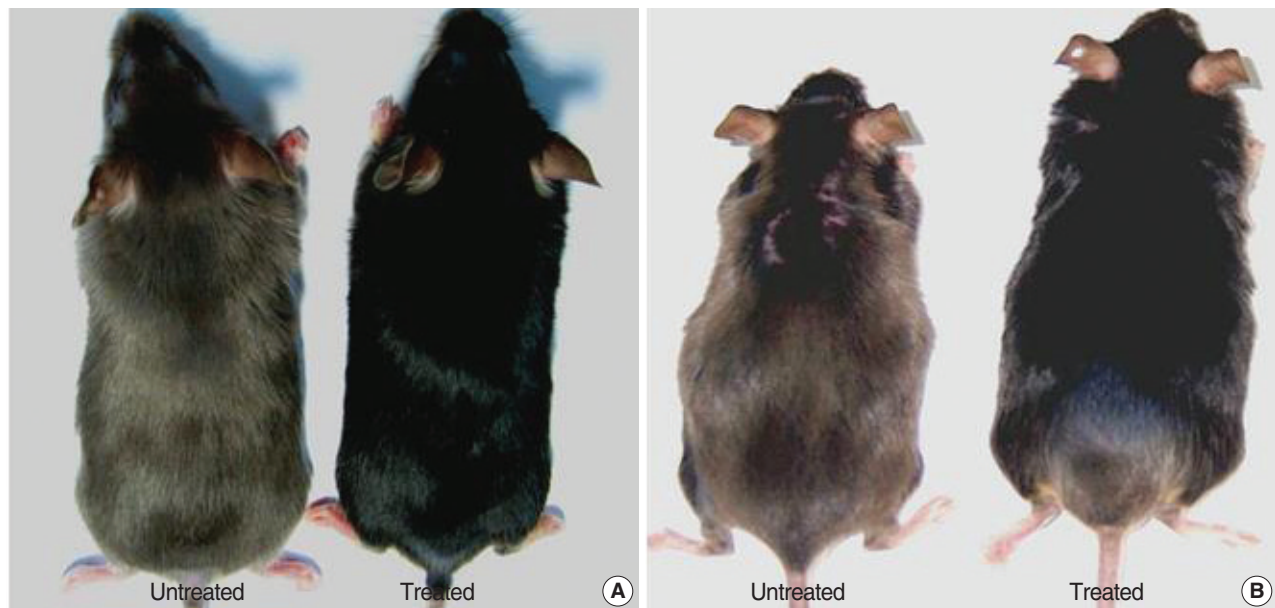*, $p < 0.001$ vs. untreated Pah^{enu2} mice (n=3 for each experiment).

Fig. 2. Fur color change to black in rAAV2/8-hPAH-treated Pah^{enu2} mice. (A) Female Pah^{enu2} mice infused with 2×10^{12} viral particles through the portal vein displayed pigmentation six weeks after injection. (B) Pregnant female Pah^{enu2} mice administered with 3×10^{12} viral particles through the tail vein exhibited the fur color change four weeks after injection.

mice (Fig. 2A). Sustained fur color changes were observed over the entire six-month experimental period.

Effect of rAAV2/8-hPAH treatment on pregnant PKU mice

At 18.5 dpc, plasma phenylalanine concentration was $106.3 \pm 19.3\ \mu$M in rAAV2/8-hPAH-treated pregnant PKU mice and $1,199 \pm 63.6\ \mu$M in untreated pregnant PKU mice. The fur color change also occurred in pregnant female PKU mice treated with rAAV2/8-hPAH (Fig. 2B). The crown-rump length and body weight of fetuses from treated PKU mice were recovered to the wild-type values (Fig. 3A, B). The mean crown-rump length of fetuses from untreated pregnant PKU mother was 1.746 ± 0.203 cm. The mean crown-rump length of fetuses from rAAV2/8-hPAH treated moth-

er was 2.081 ± 0.269 cm, which is recovered to the value from wild-type mother, 2.070 ± 0.220 cm. The mean weight of fetuses from untreated and wild-type mother was 774 ± 144 mg and 916 ± 67 mg respectively. The mean weight of fetuses from rAAV2/8-hPAH treated mother was $1,040 \pm 258$ mg, that is also recovered to wild-type value. The spontaneous abortion rate of homozygous PKU female mice was $30.95 \pm 14.27\%$. The spontaneous abortion rate of treated PKU mice was $4.55 \pm 5.89\%$, that was also normalized to the wild-type level, $2.27 \pm 4.55\%$ (Fig. 3C). The rAAV-hPAH genome was not detected in the placentae and fetuses of treated PKU mice (data not shown). The genotype of fetuses did not affect the development parameters, such as crown-rump length and body weight, of fetuses (Fig. 4).
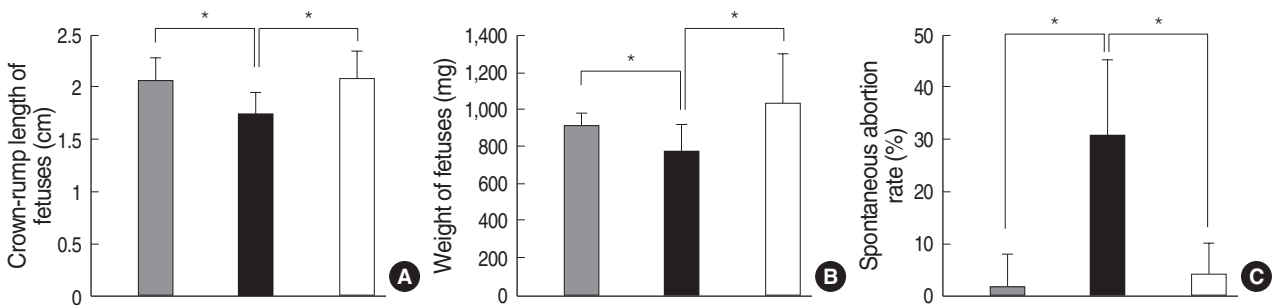

Fig. 3. Analysis of offspring of homozygous PKU female mice. Mean values of crown-rump length (A), weight (B), and spontaneous abortion rates (C) of the fetuses by maternal status. The dotted bars represent offspring of wild type mothers; the black bars, offspring of untreated homozygous mothers; the opened bars, offspring of rAAV2/8-hPAH-injected homozygous mothers.
*p<0.001 vs. wild type and untreated mothers, treated and untreated mothers (n=38 for fetuses from 4 wild type mothers, n=29 for fetuses from 5 untreated mothers, and n=21 for fetuses from 3 treated mothers).

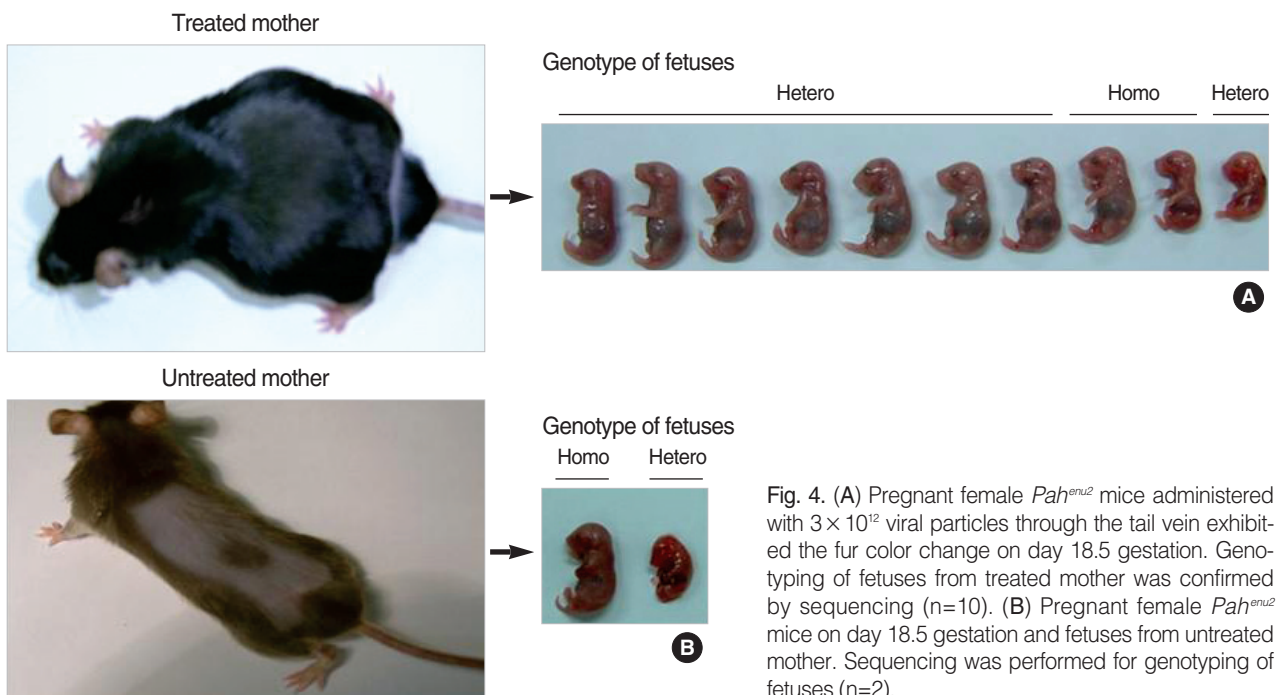Fig. 4. (A) Pregnant female *Pah^enu2* mice administered with 3×10^{12} viral particles through the tail vein exhibited the fur color change on day 18.5 gestation. Genotyping of fetuses from treated mother was confirmed by sequencing (n=10). (B) Pregnant female *Pah^enu2* mice on day 18.5 gestation and fetuses from untreated mother. Sequencing was performed for genotyping of fetuses (n=2).

DISCUSSION

Gene therapy for patients with PKU would be a useful therapeutic candidate to replace dietary restriction. The recombinant AAV vector is the most attractive candidate for therapeutic gene delivery because of its long-term expression and minimal antigenicity. Gene therapy using recombinant AAV vectors containing the PAH transgene has been reported several times (11-14). Earlier recombinant AAV-2 based gene therapy showed remarkable biochemical and phenotypic corrections in treated PKU mice, including our previous study (12). However, the therapeutic efficiency was limited to male PKU mice, and rAAV 2-based gene therapy did not show efficient therapeutic outcome in female PKU mice. This sexual dimorphism probably originated in the characteristics of the recombinant AAV vector itself and from the characteristics of the pathophysiology of PKU. Davidoff et al. (22) reported that the transduction of murine liver by recombinant AAV depends on an androgen-dependent pathway and that the liver transduction efficiency is influenced by the route of administration of the rAAV vector. Intrahepatic injection of the rAAV vector is associated with the fastest and strongest onset of transgene expression (23). More recently, Chen et al. (24) reported that the underlying mechanism responsible for the observed sexual dimorphism in PKU mice after AAV vector-mediated gene therapy is BH4 suppression by estrogen, suggesting that the estrogen-suppressed steady-state levels of BH4 in mouse hepatocytes is limiting.

Several AAV serotypes have been studied to overcome the low efficiency of liver transduction of rAAV-2-mediated gene transfer. The recombinant AAV-2 vector pseudotyped with the AAV-8 capsid greatly improves liver transduction and increase transgene expression levels (15, 16). The pseudotyped AAV2/8 vector has also been used in gene therapy of PKU and has achieved long-term complete correction of PKU in a mouse model (13, 14). These were fascinating and promising studies of PKU treatment. However, their observations were limited to adult PKU mice. Therefore, we were interested in investigating whether the therapeutic efficiency of rAAV2/8-hPAH in a PKU mouse model could be extended to the offspring of homozygous PKU mice, i.e., whether rAAV-hPAH treatment could overcome maternal PKU syndrome.

We found that portal vein infusion of pseudotyped rAAV2/8 vector into female PKU mice achieved very strong PAH expression and complete correction of plasma phenylalanine concentration, in accordance with other studies (13, 14). The PAH enzyme activity in the liver of female PKU mice was more than 65% of wild-type value. Although the PAH activity in the treated female mice was still lower than in the treated male mice, this value was sufficient to achieve phenylalanine clearance in the female PKU mice. The plasma phenylalanine clearance in the rAAV2/8-hPAH-treated female PKU mice was almost normal, which had not been achieved by

rAAV2/2-hPAH vector. The observed fur color change accompanied the recovery of PAH activity.

Because of excessive blood phenylalanine concentration in the pregnant PKU homozygous female Pah^{enu2} mothers, their offspring have severe problems, such as growth failure, heart defects, mental retardation, and stillbirth (7, 18, 25). This suggests that the offspring of PKU mother treated with rAAV2/8-hPAH gene therapy could be free from the effect of maternal PKU.

Interestingly, we observed that the offspring of treated female PKU mice can escape from the complications of maternal hyperphenylalaninemia. The pregnant females were infused once with the rAAV2/8-hPAH vector through the tail vein two weeks before conception. The offspring of treated PKU mice showed no growth retardation and the spontaneous abortion rate of treated females recovered to the values of wild-type pregnant mice. No viral vector sequence was detected in the fetuses, indicating this therapeutic effect comes from the normalized blood phenylalanine concentration in pregnant PKU mice. The genotypes of fetuses were not correlated with the developmental phenotypes of fetuses, demonstrating that the genotype of fetus is not a critical factor in determining the pregnancy outcome in maternal PKU syndrome. It is noteworthy, considering that maternal genotype is an important factor predicting the pregnancy outcome in maternal PKU syndrome when dietary treatment is discontinued (26). The fur color change also observed in the pregnant PKU mice corresponded with PAH activity in the liver of treated PKU mice (12). The PAH activity in the rAAV2/8-hPAH-treated PKU mice was sufficient to prevent the blood phenylalanine excess in the pregnant PKU mice, although these mice were in an unfavorable environment of high estrogen levels during pregnancy. The high transduction efficiency of the rAAV2/8 vector can completely overcome the obstacles of the etiology of sexual dimorphism (22, 24). This result also indicates that the single tail vein injection of rAAV2/8-hPAH into female PKU mice can achieve high transduction efficiency in the liver.

Since the mating of PKU mice was performed at two weeks after rAAV-hPAH viral injection, the probability of germline transmission is considered extremely low according to previous reports (27-29). AAV infectious particles were present only as long as day four after viral injection in rabbit and were not detected thereafter (29). No AAV vector sequences were detected from rabbit semen collected at time from seven to ninety days following intramuscular injection of 1×10^{13} rAAV vector genomes per kg (27). In addition, the viral genome was not detected in the placentae and fetuses of treated PKU mice in our study.

Like other studies using rAAV2/8-mediated gene therapy (13, 14), we observed the complete correction of plasma phenylalanine concentration in treated male and female PKU mice. More importantly, the offspring of the treated female PKU mice were free of the effect of maternal hyperpheny-

lalaninemia. These results demonstrate that pseudotyped rAAV2/8 vector-mediated gene therapy is a promising therapeutic strategy for the clinical control of PKU and other inherited metabolic disorders.

ACKNOWLEDGMENTS

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