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Low-Dose Gene Therapy for Murine PKU Using Episomal Naked DNA Vectors Expressing PAH from Its Endogenous Liver Promoter

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Limited duration of transgene expression, insertional mutagenesis, and size limitations for transgene cassettes pose challenges and risk factors for many gene therapy vectors. Here, we report on physiological expression of liver phenylalanine hydroxylase (PAH) by delivery of naked DNA/minicircle (MC)-based vectors for correction of homozygous *enu2* mice, a model of human phenylketonuria (PKU). Because MC vectors lack a defined size limit, we constructed a MC vector expressing a codon-optimized murine *Pah* cDNA that includes a truncated intron and is under the transcriptional control of a 3.6-kb native *Pah* promoter/enhancer sequence. This vector, delivered via hydrodynamic injection, yielded therapeutic liver PAH activity and sustained correction of blood phenylalanine comparable to viral or synthetic liver promoters. Therapeutic efficacy was seen with vector copy numbers of <1 vector genome per diploid hepatocyte genome and was achieved at a vector dose that was significantly lowered. Partial hepatectomy and subsequent liver regeneration was associated with >95% loss of vector genomes and PAH activity in liver, demonstrating that MC vectors had not integrated into the liver genome. In conclusion, MC vectors, which do not have a defined size-limitation, offer a favorable safety profile for hepatic gene therapy due to their non-integration in combination with native promoters.

INTRODUCTION

Phenylketonuria (PKU; OMIM 261600) is an inborn error of metabolism caused by deficiency of hepatic phenylalanine hydroxylase (PAH; EC 1.14.16.1) due to autosomal recessively inherited mutations in the *Pah* gene. PAH catalyzes the conversion of L-phenylalanine (L-Phe) to L-tyrosine using molecular oxygen and the cofactor tetrahydrobiopterin.[1] In the absence of liver PAH activity, L-Phe accumulates in blood and other tissues causing damage to the developing brain, but the liver itself is unaffected by PAH deficiency. PKU in humans comprises a clinical continuum of elevated blood L-Phe concentrations with levels up to 600 μmol/L on an unrestricted diet, commonly classified as mild hyperphenylalaninemia, between 600–1,200 μmol/L as mild PKU, and those over 1,200 μmol/L as classic PKU (normal blood L-Phe = 50–100 μmol/L) with the degree of L-Phe elevation correlating generally to the specific *Pah* genotype of the patient. Contemporary therapy for classical PKU is based on dietary L-Phe restriction; however, future treatments may include enzyme substitution with enteral or parenteral administration of the bacterial enzyme phenylalanine ammonia lyase or gene augmentation therapy using viral or non-viral vector systems to target liver or other organs (for more details see Blau et al.[1] and Thöny[2]). The *Pah*^enu2/enu2 mouse, a model of human PKU, faithfully replicates the physiological and behavioral abnormalities associated with untreated human patients, including hyperphenylalaninemia, hypopigmentation, growth retardation, maternal PKU syndrome, and cognitive deficits.[3] We (and others) have used this model of human PKU extensively in the past to study various aspects of this metabolic disease and for development of novel therapies including gene therapy.

Gene therapy has great potential for treatment of many inherited disorders by directly addressing the genetic defect, and several recent treatment successes have been reported.[4] Development of novel gene transfer vectors that direct tissue-specific, persistent, and high levels of transgene expression but with minimal toxicity from anti-vector immune responses or insertional mutagenesis remains an important objective. Vectors that do not integrate into the host genome have the advantage of low toxicity and avoidance of potential germline transmission but at the same time may fail to produce long-term therapeutic efficacy in mitotically active tissues because of gradual loss of vector DNA and transgene expression. For liver-directed gene therapy, the most advanced or promising vector system today is recombinant adeno-associated virus (AAV) that has yielded mixed success for

therapy of hemophilia in ongoing clinical trials.[4] Some of the remaining challenges for clinical use of recombinant AAV are the requirement for high (and expensive) vector doses, the limited cargo capacity for an expression cassette, the non-persistent expression due to immune-mediated vector elimination, the inability to (re)administer the vector due to anti-capsid antibody, and the repeatedly observed oncogenic potential for insertional mutagenesis and development of hepatocellular carcinomas in mice.[5,6] It is thus a vital aspect for the success of liver-directed gene therapy to develop vector systems that allow persistent and safe expression. We are exploring potential alternative vector systems to AAV for liver gene therapy and have previously reported on long-term safe and efficacious treatment of PKU mice through delivery of non-viral naked minicircle DNA (MC-DNA)-vectors via a single hydrodynamic tail vein injection without adverse effects.[7] The MC-DNA vectors employed in that study was entirely devoid of any bacterial or viral sequences and only contained a minimal expression cassette that expressed the mouse *Pah*-cDNA from a synthetic liver promoter (P3). While delivery of naked DNA is still challenging, and for liver-targeting currently only possible in an experimental setting with mice or pigs (by hydrodynamic injections), the MC vector system has great potential and avoids most of the problems related to viral vectors (for a broader discussion see Yin et al.,[8] Gaspar et al.,[9] and Stoller et al.[10]).

In the present study, we aimed to further characterize and improve the MC-DNA vectors for gene therapy of murine PKU as a model for a genetic liver defect. Experimental studies included in vivo testing for episomal maintenance of MC vectors, codon-optimization, addition of a 5′-intron, and expression under potentially "physiological" conditions from its endogenous *Pah* promoter. The latter may have several advantages, as use of the endogenous promoter restricts transgene expression to liver thereby helping to avoid potential immunogenicity of the transgene product and also likely reduces the copy number of vector genomes per cell necessary for persistent, long-term physiologically relevant expression. Although the use of tissue-specific promoters has been the central focus for the development of gene therapy vectors (for a review, see Toscano et al.[11]), the choice of natural or endogenous promoters was hampered by limited genome size capacity in AAV vectors. The lack of any defined genome size limit for MC vectors has allowed us to study liver PAH expression from a gene transfer vector that utilized the endogenous *Pah* promoter. We found that the use of the native promoter, along with codon-optimization and inclusion of a truncated intron in the cDNA, lead to robust, long-term liver PAH expression in PKU mice, and we concluded that MC vectors have an overall favorable safety profile for therapy of genetic liver defects.

RESULTS

Loss of Episomal MC Vector and PAH Activity after Partial Hepatectomy and Liver Regeneration in MC-Treated PKU Mice

MCs are considered to be passively maintained as non-integrating (and non-replicating) vectors and thus bear little risk for insertional mutagenesis. To investigate whether MCs in our vector-treated PKU mice remained episomal and thus subject to elimination

following hepatocyte turnover, we performed partial hepatectomy (pHx) and examined untreated (control) versus MC-treated mice before and after liver regeneration. Past experience has shown that the restoration of liver PAH activity to only 5% of wild-type activity was sufficient to maintain blood L-Phe levels below a defined therapeutic level (≤ 360 μmol/L). We have previously shown that hydrodynamic tail vein injection of 3×10^{13} copies of MC.PKU20 expressing *mPah*-cDNA under control of the synthetic liver-specific promoter (P3) was sufficient to persistently lower the blood L-Phe level in PKU mice.[7] Here, we injected five PKU mice with vector MC.PKU37 (3×10^{13} particles) that is identical to vector MC.PKU20 except for expressing an N-terminally Flag-tagged PAH. As depicted in Figure 1A and Table 1, untreated PKU mice remained hyperphenylalaninemic (L-Phe >1,200 μmol/L) while blood L-Phe levels decreased to below the therapeutic level of 360 μmol/L in MC-treated PKU mice. Twenty-eight days after gene delivery, pHx removing two-thirds of the liver was performed (see Figure S2). As indicated by the ratio of liver/body weight in Table 1, livers regenerated to the original mass,[12] and concomitantly, blood L-Phe levels in MC-treated mice increased back up to 1,219 ± 328 μmol/L (for all five mice) that was similar to L-Phe levels in control animals (1,315 μmol/L) or to pre-treatment levels in MC-treated mice. Liver tissue from three treated mice (and control) was collected for molecular analysis by pHx at day 28 and again at euthanasia (day 49). As shown in Table 1, pHx in MC-treated mice resulted in a >95% loss of PAH enzyme activity (i.e., from 58% ± 36% down to 2.3% ± 2.3% compared to wild-type) and 95% loss of vector copy number per hepatocyte (i.e., from 86.6 ± 38.5 copies to 4.5 ± 4.0 copies). The loss of MC-DNA was further confirmed by histological examination where we found 17.7% ± 8.1% positively stained hepatocytes expressing Flag-PAH protein mouse liver at pHx while there were only 1.9% ± 0.4% positively stained cells 3 weeks after pHx (Figures 1B–1F, respectively). The one-tailed p value of t test for Flag-positive cell counting in Figure 1B was not significant due to the large variation of the number of positive hepatocytes between the two mice analyzed. H&E staining illustrated that no liver damage or necrosis was observed during the entire experiment (Figures 1G and 1H). The re-rise of blood L-Phe levels to pre-treatment values, concomitant with the loss of MC-DNA and hepatic PAH expression in MC-treated mice, confirms that MC vectors do not integrate into the genome but rather are maintained as episomes that corroborates our previous observations from Southern blot analyses[7] (see also Discussion).

Expression of PAH from Its Native Endogenous *Pah* Promoter-Enhancer Corrects Hyperphenylalaninemia

A 10-kb fragment upstream of the mouse *Pah* gene has initially been characterized in in vitro studies to harbor the essential liver-specific promoter elements for PAH expression, including four DnaseI hypersensitive sites (HSSI-HSSIV).[13–15] Further refinement revealed that the critical sites for liver expression can be narrowed down within a transcription-start upstream-fragment comprising the hypersensitive sites HSSI to HSSIII, including the full HSSIII enhancer site corresponding to a liver-specific and hormone-inducible enhancer that was localized within a 410-bp fragment between kb −3.5 and −3.2
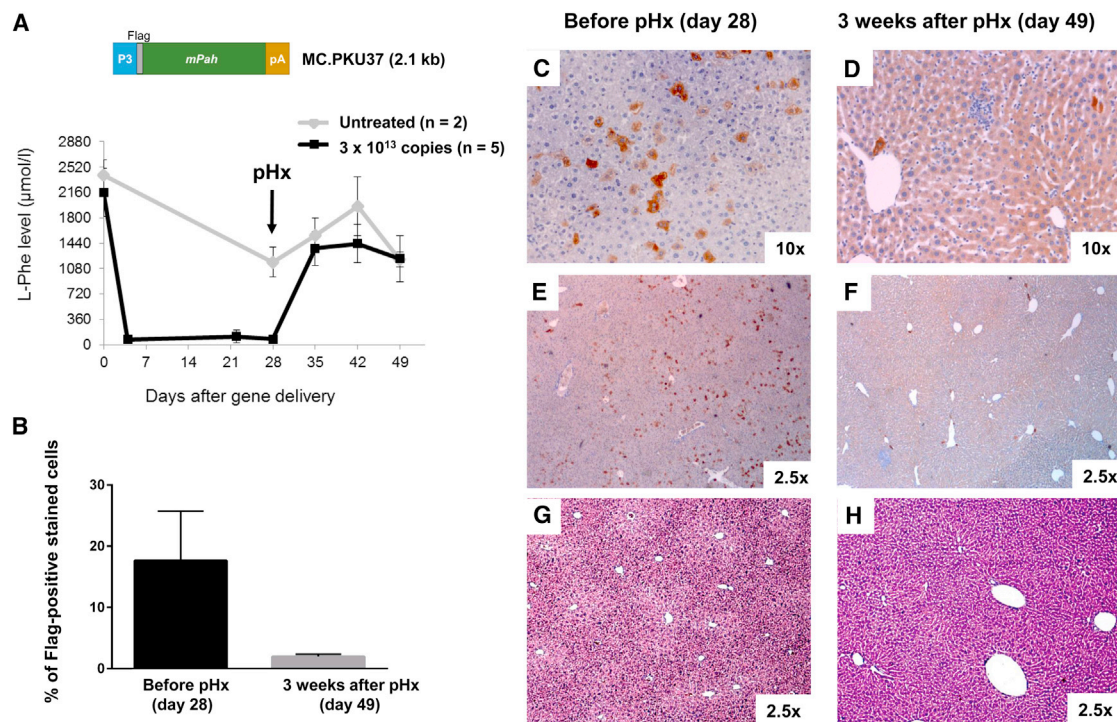
Figure 1. MC Vectors Are Maintained as Episomes and Are Lost during Hepatocyte Turnover

(A) Five PKU mice were treated with 3×10^{13} copies of vector MC.PKU37 expressing the Flag-tagged PAH. At day 28, two-thirds of the mouse liver were removed and 3 weeks later, after liver regeneration, mice were sacrificed (at day 49). For control, two untreated PKU mice were included for pHx. Blood L-Phe levels were followed and depicted as the mean ± SD. (B–H) Livers from two of the five MC.PKU37-treated mice shown in (A) were processed for histological analyses. (B) Mean ± SD was calculated for the percentage of Flag-positively stained cells from five random fields in 2.5× magnification of each mouse. (C–F) Histology of liver sections that were stained with anti-Flag antibody followed by hematoxylin counterstaining. Representative examples of a random field in 2.5× and 10× magnifications after liver resection at day 28 (C and E) and 3 weeks after pHx (D and F). Histological examination of liver sections after H&E staining (G) at pHx (day 28) and (H) 21 days after surgery (day 49).

(Figure 2A).[13] Based on these observations, we amplified the entire ∼3.6 kb 5′-fragment including part of exon 1 from mouse genomic DNA and placed it in frame upstream of the *mPah*-cDNA to generate MC.PKU43 (for more details see the Supplemental Materials and Methods and Figure S1). This so-called P6 promoter-enhancer was tested for liver (and kidney)-specific expression of PAH activity from an MC vector. PKU mice were injected with 3×10^{13} copies of MC.PKU43 via tail vein and euthanized 80 days later for organ collection. We detected PAH enzyme activity exclusively in liver with 12% ± 3% of wild-type (Table 2).

Next, we performed a dose-escalation study in PKU mice to test for the minimal amount of vector required for therapeutic efficacy of the P6 promoter-enhancer construct. For comparison, we used identical MC vectors expressing the *mPah* either from the synthetic liver promoter P3 (MC.PKU20) or the classical "modified cytomegalovirus (CMV) enhancer/chicken β-actin" (CBA) promoter (MC.PKU38). The latter was also used in our earlier AAV-vector-based liver-directed gene therapy experiments to correct PKU in mice.[16,17] Various doses of all three vectors were infused via hydrodynamic tail vein injection for delivery to hepatocytes, followed by monitoring blood L-Phe level over a period of 80 days until euthanasia. As illus-

trated in Figures 2B–2D and Table 2, we found that the therapeutic threshold for all three MC vectors was a dosage of 6×10^{12} copies per injection as this resulted in blood L-Phe levels below the defined therapeutic threshold of 360 μmol/L over the entire therapy period of 80 days. Lower amounts of injected DNA vector also resulted in a sharp response by lowering the high blood L-Phe but to a level still considered to be (mildly) hyperphenylalaninemic. On euthanasia, we found 1.3–8.1 MC vector genomes per diploid hepatocyte genome and 7%–13% liver PAH activity for all vectors at a dose of 6×10^{12} copies. Based on the observation that only ∼18% of all hepatocytes of a mouse liver are transduced with MC-DNA (see Figure 1B and Viecelli et al.[7]), it can be calculated that between 8–47 copies of MC-DNA were present per transduced (diploid) hepatocyte.

The similar outcome for these three vectors was somewhat surprising as we infused the same number of vector copies but each vector was considerably different in total size so that 6×10^{12} copies were equivalent to DNA doses of 15.4 μg, 25.2 μg, and 38.6 μg total DNA administered for vector MC.PKU20 (2.1 kb), MC.PKU38 (3.5 kb), and MC.PKU43 (5.5 kb), respectively. From this observation, we conclude that MC vector copy number administered rather than the total DNA amount is the more important dosing parameter. Furthermore, we

Table 1. Liver Analysis of Treated PKU Mice after pHx

Mouse and Treatment	pHx (No. of Mice)	PAH Enzyme Activity (% of WT)	Blood L-Phe (μmol/L)	DNA Copy Number Per Diploid Genome	Ratio Liver/ Body Weight
PKU, untreated	before day 28 (n = 1)	<0.2	1,383	NA	ND
	after day 49 (n = 1)	<0.2	1,315	NA	2.9
PKU, MC.PKU37-treated	before day 28 (n = 3)	58 ± 36	70 ± 18	86.6 ± 38.5	ND
	after day 49 (n = 3)	2.3 ± 2.3	1,227 ± 386	4.5 ± 4.0	4.3 ± 0.18

Two untreated PKU mice were used as control, one for histological staining and the other one for molecular analysis, while five PKU mice were treated with 3×10^{13} copies of vector MC.PKU37 (77 μg) expressing the Flag-tagged PAH under control of the synthetic liver-specific promoter P3. The resected liver lobes from three mice were collected before pHx (day 28) and after liver regeneration (day 49) for various analyses, including PAH activity, blood L-Phe levels, vector copy number per diploid genome, and as a mass of regeneration the ratio of liver-over-body weight. pHx, partial hepatectomy; NA, not applicable; ND, not determined; WT, wild-type.

did not observe any sex-dependent difference in therapeutic efficacy (for L-Phe levels, PAH enzyme activity, and copy number per hepatocytes).

We continued to monitor a few MC-treated mice beyond 80 days post injection in order to evaluate long-term effects including mice treated with MC.PKU38 followed out to 179 days, 339 days for MC.PKU43, and up to 353 days for MC.PKU20. Blood L-Phe levels (not shown) remained either below the therapeutic threshold or were mildly hyperphenylalaninemic at the time point of euthanasia (Table 2). Overall, our data demonstrate robust and life-long liver PAH expression and therapeutic efficacy following treatment with MC vector containing the native P6 promoter-enhancer. In conclusion, MC vectors that have no defined size limitations can express their therapeutic transgene from a large endogenous or natural promoter-enhancer element with comparable efficiency like synthetic or viral promoters commonly used for expression from gene therapeutic vectors with limited cargo capacity. Overall, our data showed that regarding expression of PAH and therapeutic efficacy, the natural endogenous P6 promoter-enhancer revealed robust and life-long performance.

Codon-Optimization and Inclusion of an Intron Fragment in the *Pah*-cDNA Improves Therapeutic Efficacy at Significantly Reduced MC Vector Dosage

In a next step, we aimed to further improve the mouse *Pah*-cDNA expression cassette of vector MC.PKU20 through codon-optimization (*mcoPah*) and inclusion of a 5′ intervening sequence. While the design of codon optimization is done with support from computational methods, we also validated efficient splicing of the artificial intron in vitro and in vivo. We chose to evaluate a truncated version of *Pah* intron 1 as the entire IVS-1 of the mouse *Pah* gene is 6.2 kb long. Our first construct that included a so-called fragment IVS-1A was not efficiently spliced (as tested in vitro; see the Supplemental Information). In a second attempt, IVS-1B, which retains 300 bp from the 5′ end and 520 bp from the 3′ end of *Pah* IVIS1 but lacks 5.4 kb from the native intron, was introduced between exons 1 and 2 in the *Pah* cDNA. The combined alterations of codon-optimization and intron inclusion resulted in vector MC.PKU28 (Figure 3A; see Supplemental Materials and Methods and Figure S1 for further details). Hydrody-

namic injection into PKU mice and subsequent liver cDNA analysis revealed correct splicing from vector MC.PKU28 (Figure 3B). Next, we injected PKU mice with different doses of MC.PKU28 and analyzed treatment performance over a period of 80 days and in comparison with MC.PKU20. We found that therapeutic correction of blood L-Phe was achieved with injection of 6×10^{11} MC.PKU28 copies, a dose that is 10-fold lower than that needed to achieve efficacy with MC.PKU20 (Figure 3A; Table 2). A further decrease in dose down to 3×10^{11} copies of MC.PKU28 still yielded a clear response in lowering the blood L-Phe levels; however, this dose did not achieve the therapeutic threshold of 360 μmol/L blood L-Phe. Nevertheless, excellent long-term performance of vector MC.PKU28 was further demonstrated by a single mouse injected with 3×10^{13} vector copies and remained at normal blood L-Phe levels for a period of 414 days (see Table 2).

We again measured the MC-DNA copy number remaining in liver after the treatment period of 80 days. Mice that had received 6×10^{11} vector copies of MC.PKU28 and had exhibited a robust therapeutic response retained on average 0.3 ± 0.1 copies per diploid genome in liver. Successful treatment with MC.PKU20 required a 10-fold higher vector dose and yielded 9 ± 4.3 copies of MC.PKU20 per diploid liver genome 80 days after injection. The finding of therapeutic efficacy with a 10-fold lower vector copy number retained in liver is in agreement with the results from the vector-titration studies and thus confirms that our codon-optimized intron containing vector MC.PKU28 is superior to the parental MC-vector. Assuming again that 18% of all hepatocytes were transduced, ~1.8 copies of MC.PKU28 are statistically present per transduced diploid hepatocyte that is a value close to having two functional native *Pah* alleles (see Discussion).

Finally, we investigated the performance of vector MC.PKU44 that harbors the *mcoPah*-IVS-1B expression cassette under transcription control of the endogenous promoter-enhancer P6. The results are depicted in Figure 3C and Table 2. We found that injections between $3-6 \times 10^{11}$ copies of MC.PKU44 per animal was therapeutic and yielded 0.5–1 vector copy number per diploid genome in liver 80 days after injection. These results were similar to those for vector MC.PKU28 (see also Figure 4).
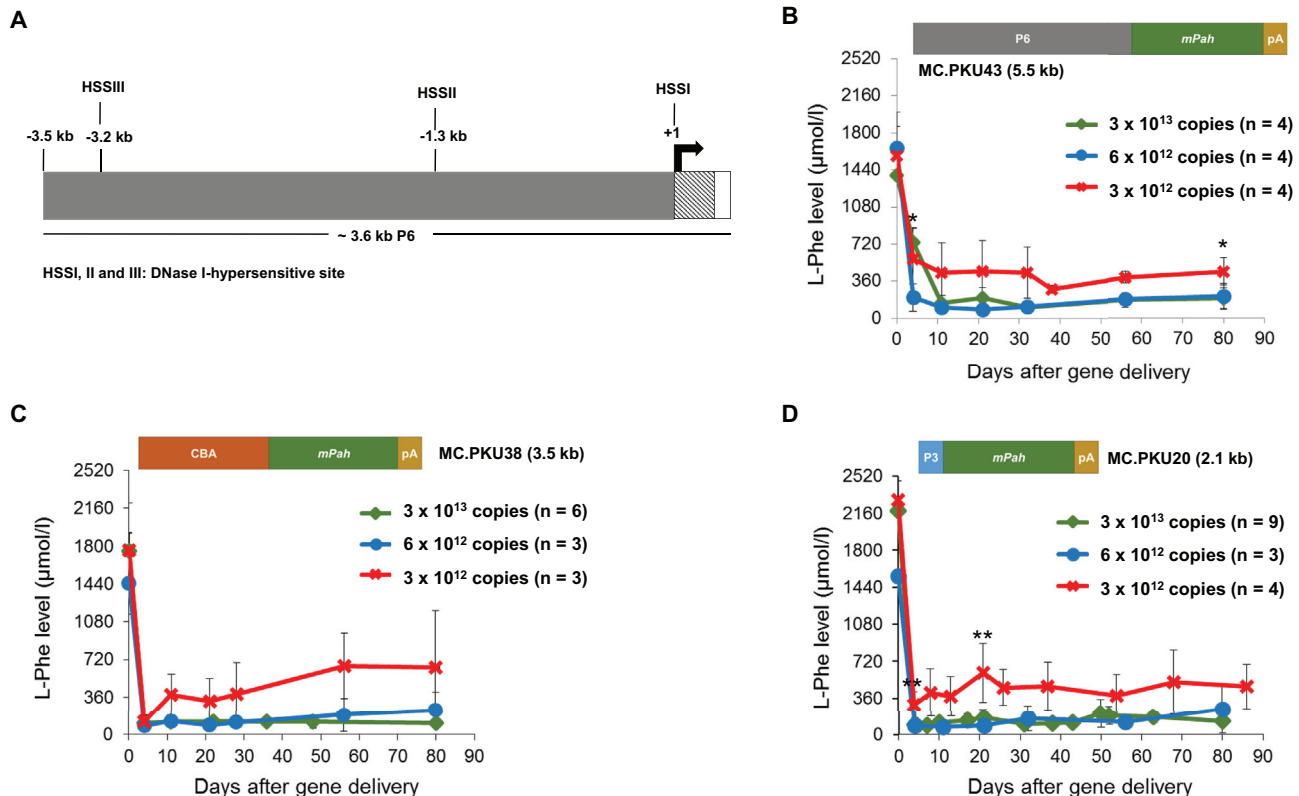
Figure 2. Physiological Expression and Thus Correction of Hyperphenylalaninemia in PKU Mice after Delivery of an MC-DNA Vector Driving Expressing of *Pah* by Its Natural Liver Promoter-Enhancer

(A) Schematic of the 3.6 kb murine *Pah* genomic promoter-enhancer fragment, termed P6, which was amplified and inserted into MC.PKU20 to replace the P3 promotor. The P6 promotor contains the coding part of exon 1 (open box), the 5′ untranslated region (striped box) and the 3.5-kb fragment upstream of 5′ untranslated region (gray box) including three DNase I-hypersensitive sites (HSSI, HSSII, and HSSIII). (B–D) Comparison of blood L-Phe level in PKU mice treated with three different vectors expressing *mPah* by different promoters, i.e., (B) endogenous mouse *Pah* promoter-enhancer P6, (C) modified cytomegalovirus enhancer/chicken β-actin (CBA) promoter, and (D) the synthetic liver promoter P3. PKU mice were infused with three different doses of MC-vectors, 3×10^{13}, 6×10^{12}, and 3×10^{12}. Note that in (D) data from mice treated with 3×10^{13} and 3×10^{12} copies, but not with 6×10^{12} copies of vector MC.PKU20, were taken from a previous publication and are shown for comparison.[7] Serum L-Phe levels in treated mice injected with 6×10^{12} and 3×10^{12} copies of different MC-DNA vectors in (B)–(D) were compared for statistically significant difference by one-tailed Student's t test at days 4, 21, and 80 following vector administration.

MC-DNA Vector Prepared by an Ultra-Pure versus Standard DNA Purification Method Had No Influence on the Efficacy of L-Phe Clearance in Mouse Liver

We previously reported that MC vector purification using a research grade DNA isolation kit yielded ∼97% pure MC-DNA with very minor contamination of parental plasmid, bacterial backbone, and/or bacterial chromosomal DNA.[7] To date, we have prepared all MC-DNA vectors for injection from bacterial cultures using commercially available endotoxin free DNA purification kits that utilize a classic alkaline lysis approach followed by anion-exchange resin column (see the Supplemental Materials and Methods). Although we had no indication for inflammatory or immune-stimulatory effects in MC-treated mice whatsoever,[7] we examined whether more highly purified[18] MC-DNA, prepared using PlasmidFactory's (PF) affinity chromatography-based MC production technology (see the Supplemental Materials and Methods), would exhibit better therapeutic performance. As displayed in Figures S3A and S3B, standard kit grade

MC.PKU28 DNA demonstrated that most of the MC DNA was present as a monomeric covalently closed circular (ccc) supercoiled molecule, but a significant amount was relaxed open circular monomer, dimer, trimmer, tetra-, and larger concatemers. In contrast, more than 98% of highly purified MC.PKU28 (PF) was in monomeric supercoiled ccc form with undetectable bacterial chromosomal DNA or bacterial endotoxin (see Figures S3C and S3D). The behavior of research (kit) grade versus highly purified MC-DNA was investigated through administration of MC.PKU20 and MC.PKU28 vectors to PKU mice where we found basically no difference in outcomes regardless of the MC-DNA purification method used (see Table 2).

DISCUSSION

The main novel findings of the present study are the observation that MC vectors are maintained in episomal state in vivo, and a native promoter sequence in combination with an optimized expression cassette

Table 2. Liver Analysis of Treated PKU Mice Sacrificed at Different Time Points following Gene Delivery

MC Vector Treatment (Size of Vector)	MC Vector Dosage: No. of Particles (µg of MC-DNA)	Time Point on Sacrification (No. of Mice)	PAH Enzyme Activity (% of WT)	Blood L-Phe (µmol/L)	DNA Copy Number Per Diploid Genome
Wild-type, untreated	NA	(n = 5)	100	53 ± 7	NA
PKU, untreated	NA	(n = 5)	<0.3	1,683 ± 245	NA
MC.PKU20 (2.1 kb)[a]	3×10^{13} (77)	day 52 (n = 1)	15	150	3.5
	3×10^{13} (77)	day 179 (n = 2)	9 ± 8	205 ± 66	2.5 ± 0.7
	6×10^{12} (15.4)	day 80 (n = 3)	11 ± 10	272 ± 287	4.9 ± 4.3
	3×10^{12} (7.7)	day 10 (n = 1)	3	532	1.5
	3×10^{12} (7.7)	day 353 (n = 3)	2 ± 0	464 ± 172	2.6 ± 0.8
MC.PKU20 (PF) (2.3 kb)	3×10^{12} (8.2)	day 80 (n = 2)	3 ± 0	470 ± 245	6.8 ± 0.2
	3×10^{11} (0.8)	day 15 (n = 2)	3 ± 1	1,265 ± 1	2.3 ± 1.2
MC.PKU28 (2.8 kb)	3×10^{13} (100)	day 48 (n = 1)	22	144	1.7
	3×10^{13} (100)	day 80 (n = 2)	47 ± 2	74 ± 11	2.9 ± 1.8
	3×10^{13} (100)	day 414 (n = 1)	10	127	0.9
	3×10^{12} (10)	day 80 (n = 3)	15 ± 7	79 ± 9	3.2 ± 0.4
	6×10^{11} (2)	day 80 (n = 3)	3 ± 1	178 ± 59	0.3 ± 0.1
	3×10^{11} (1)	day 21 (n = 1)	2	1,680	0.3
	3×10^{11} (1)	day 80 (n = 2)	3 ± 1	1,097 ± 190	0.1 ± 0
MC.PKU28 (3.0 kb)	3×10^{12} (10.5)	day 80 (n = 3)	23 ± 6	72 ± 3	12.7 ± 5.3
	3×10^{11} (1)	day 80 (n = 2)	2 ± 0	889 ± 287	1.2 ± 0.7
MC.PKU38 (3.5 kb)	3×10^{13} (126)	day 80 (n = 3)	25 ± 16	130 ± 73	1.4 ± 0.8
	3×10^{13} (126)	day 179 (n = 3)	12 ± 2	103 ± 14	5.5 ± 2.1
	6×10^{12} (25.2)	day 80 (n = 3)	13 ± 4	235 ± 174	1.3 ± 0.4
	3×10^{12} (12.6)	day 80 (n = 3)	5 ± 3	644 ± 539	1.0 ± 0.5
MC.PKU43 (5.5 kb)	3×10^{13} (193)	day 80 (n = 4)	12 ± 3	191 ± 105	8.5 ± 4.3
	3×10^{13} (193)	day 339 (n = 1)	2	609	2.8
	6×10^{12} (38.6)	day 80 (n = 4)	7 ± 5	213 ± 125	8.1 ± 6.4
	3×10^{12} (19.3)	day 32 (n = 2)	1 ± 1	443 ± 244	3.2 ± 2.1
	3×10^{12} (19.3)	day 80 (n = 2)	3 ± 0	454 ± 131	0.9 ± 0.8
MC.PKU44 (6.1 kb)	3×10^{12} (21.7)	day 80 (n = 3)	9 ± 2	186 ± 132	1.0 ± 0.2
	6×10^{11} (4.3)	day 80 (n = 5)	2 ± 1	475 ± 152	0.5 ± 0.4
	3×10^{11} (2.2)	day 80 (n = 4)	1 ± 1	761 ± 258	0.2 ± 0.1

PAH enzyme activity and copy numbers of vector genomes in whole liver extracts of PKU mice infused with various MC-DNA vectors and different doses were analyzed. Blood L-Phe levels correspond to time of sacrification. Note that L-Phe values during whole treatment periods are shown in Figures 1 and 2. The type and size of each construct, vector dosage, time point of euthanasia and the numbers of mice analyzed, and DNA copy numbers per diploid hepatocyte genome are indicated. Details of each construct are depicted in Figure S1. In brief, MC.PKU20 harbors the synthetic liver-specific promoter P3 and *Pah*-cDNA, MC.PKU28 a codon-optimized mouse *Pah* (*mcoPah*) and a truncated intervening sequence 1B (IVS-1B) driven by P3, MC.PKU38 the modified cytomegalovirus (CMV) enhancer/chicken β-actin promoter (CBA) and *Pah*-cDNA, MC.PKU43 the endogenous promoter-enhancer P6 and *Pah*-cDNA, and MC.PKU44 promotor P6, *mcoPah* and IVS-1B. PF: performance of high-quality purified MC-DNA vectors MC.PKU20 (PF) and MC.PKU28 (PF). NA, not applicable; WT, wild-type.
[a]Except for vector dose 6×10^{12}, results from MC.PKU20 were taken from a previous publication (see Viecelli et al.[7]) for comparison. Note that 3×10^{13} MC-vector copies of MC.PKU20 injected into an adult mouse (~25 g) is equivalent to ~1.2 × 10^{15} copies/kg.

can yield physiologically relevant PAH expression in *Pah*[enu2/enu2] mice, a model of human PKU. We have previously demonstrated through Southern blot analysis that MC-DNA does not integrate into liver genomic DNA.[7] Here, we employed pHx in MC-treated mice to induce rapid hepatocyte turnover and observed a ≥95% loss of MC-DNA and PAH activity. This result corroborates that MC-DNA is maintained as a non-integrated and non-replicating vector in vivo. Using similar approaches, evidence for episomal maintenance of plasmids[19,20] or S/MAR-containing MC vectors has been previously reported;[21] however, to our knowledge, this has never been convincingly demonstrated directly for MC vectors lacking S/MAR elements in mice that were corrected by gene therapy. Nevertheless, complete exclusion of MC vector integration at a very low frequency needs further proof using more sophisticated methods than

Southern blot or pHx. Non-integrating gene transfer vectors lack any significant risk of (random) insertional mutagenesis. Concerns regarding low level insertional mutagenesis and consequent hepatic malignancy have been raised even for recombinant AAV vectors, which are considered to be nonpathogenic and non-integrating.[22] On the other hand, gradual vector genome loss with normal hepatocyte turnover is a potential disadvantage of any vector system that relies on maintenance of episomes. This has not been a limitation for MC-DNA, as vector DNA (as well as silenced parental plasmid DNA) has been persistent in adult mouse liver out to at least 1 year following vector injection (see Table 2 and Viecelli et al.[7]). Presumably, administration of MC to newborn animals would not yield stable therapeutic correction as MC episomes would be rapidly eliminated with normal hepatocyte proliferation. However, even if MC
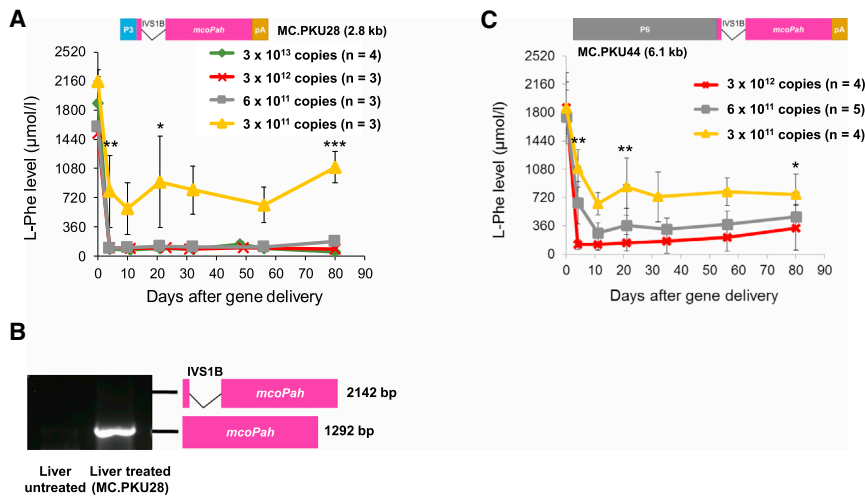
Figure 3. Inclusion of a Truncated IVS-1B in Combination with *Pah*-Codon-Optimization Allowed a 10-Fold Reduction of Vector Dosage to Treat PKU

(A) Vector MC.PKU28 harboring an intron-containing (IVS-1B) and codon optimization mouse *mcoPah* transgene under the control of promoter P3 was injected hydrodynamically into PKU mice at four different doses (i.e., 3×10^{13}, 3×10^{12}, 6×10^{11}, and 3×10^{11} vector copies). (B) Gene expression (cDNA) analysis shows correct splicing of *mcoPah*-IVS-1B in mouse liver treated with MC.PKU28. The expected lengths of potential non-spliced (2,142 bp) and correctly spliced (1,292 bp) cDNA-fragments are indicated. (C) Vector MC.PKU44 was generated by replacing P3 with the endogenous *Pah* promoter-enhancer P6 (see text). A dose-escalation study to treat PKU mice was done with 3×10^{12}, 6×10^{11}, and 3×10^{11} vector copies per infusion. Blood L-Phe was followed and depicted as the mean ± SD. Serum L-Phe levels in treated mice injected with 6×10^{11} and 3×10^{11} copies of different MC-DNA vectors in (A) and (C) were compared for statistically significant difference by one-tailed Student's t test at day 4, 21, and 80 following vector administration.

episomes are gradually lost from liver in adult mice, MC vectors, unlike viral vectors, can be readministered.[23]

Application of vectors that use native mammalian promoters to drive prolonged therapeutic transgene expression has broad potential in the field of gene therapy. Native promoters such as the human α1 antitrypsin (hAAT)[24] or albumin promoter,[25] as well as chimeric promoters, including the apolipoprotein E (Apo E) enhancer/hAAT promoters[26] or the synthetic mammalian liver-specific promoter P3,[7,27] have been used in both viral and non-viral vectors to successfully target liver. Constructs that incorporate regulatory elements from native promoters, for example low-density lipoprotein receptor (LDLR),[28] the Rpe65 and vitelliform macular dystrophy 2,[29] and the CD40L,[30] have achieved restricted expression (for review, see also Toscano et al.[11]), and expressing of the therapeutic protein driven by the endogenous promoter in Wiskott-Aldrich syndrome patients has shown a remarkable success.[31] Use of endogenous regulatory sequences likely insures tissue-specific and physiologically relevant therapeutic gene expression. In the case of treatment for PKU, introduction of two vector genomes per hepatocyte of a vector expressing the PAH cDNA from the PAH promoter replicates the natural state of biallelic PAH expression from the endogenous *Pah* genes. As described in the Results, we chose to test the 3.6 kb murine *Pah* gene-upstream promoter-enhancer element that harbors the HSSIII regulatory enhancer element and contains multiple protein binding sites, including sites for ubiquitous factors (NF1 and AP1) as well as for liver-enriched transcription factors hepatocyte nuclear factor 1 (HNF1) and CCAAT/enhancer binding protein (C/EBP).[13] HNF1 is a major regulatory element involved in PAH expression as it has been shown that the *Pah* gene fails to be expressed in HNF1α-deficient mice.[32] Following administration of our constructs MC.PKU43 and MC.PKU44, which include the native *Pah* regulatory elements,

we detected PAH expression only in liver. Because hydrodynamic tail vein injection restricts MC-DNA delivery essentially to liver only,[7] we cannot know from our experiment whether liver-restricted PAH expression is due to the vector delivery method or to the use of the endogenous liver-specific promoter sequence. Nevertheless, we showed that a 3.6-kb sequence of upstream genomic DNA was sufficient to confer therapeutic sustained transgene expression in PKU mice. Analysis of promoter performance demonstrated that blood L-Phe levels dropped below the therapeutic level at day 4 of treatment in the mice treated with MC-DNA vectors containing either the synthetic P3 (MC.PKU20) or the hybrid CBA promoter (MC.PKU38). In contrast, the L-Phe levels in mice receiving MC vectors expressing PAH from the P6 promoter in vector MC.PKU43 declined gradually and reached the same therapeutic level like the other promoters only after 11 days (Figure 2). The reason for that is unclear, although it has also been observed by others that the luciferase expression driven by a native human vitelliform macular dystrophy 2 promoter was lower initially compared to other viral promoters, but the activity gradually increased and sustained for a few months.[29] Overall, future therapeutic development of MC-DNA vectors is attractive due the lack of vector size limitations that allows the incorporation of larger therapeutic transgenes and native promoter that direct long-term physiologic gene expression.

Gene expression is modulated at many levels, including transcription, post-transcriptional RNA modification (RNA splicing and export), RNA stability, and finally mRNA translation. One important strategy to improve in vivo protein expression from a therapeutic transgene is to adjust codon usage to the codon preference of highly expressed host genes (reviewed in Gustafsson et al.[33]) and furthermore to optimize GC content. Computational algorithms that consider mRNA stability, but reduce mRNA secondary structure and eliminate repetitive
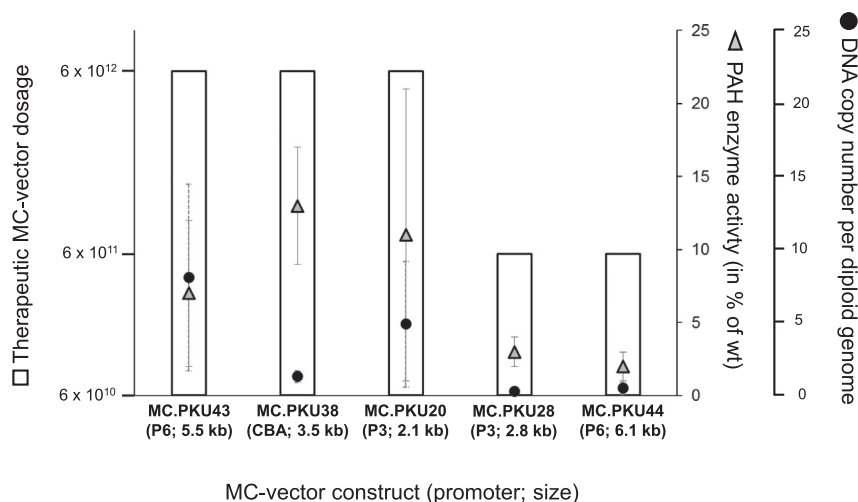
Figure 4. Summary of In Vivo Performance of MC Vector Constructs at Therapeutic Doses

Data in this graph were compiled from Figures 2 and 3, as well as from Table 2. The therapeutic MC-vector doses (i.e., number of particles injected) are given on the left side. Therapy was defined as blood L-Phe levels at 360 μmol/L or below. Treated mice were sacrificed 80 days after MC vector injection for liver analyses to determine PAH enzyme activity and DNA copy number per diploid genome, which are given on the right side. Note that the vector size between the two most effective constructs, MC.PKU28 and MC.PKU44, differ considerable due to different promoter fragments and may thus not be directly comparable (see also Discussion).

elements and CpG islands,[34] are available for planning optimization of codon usage, and many reported studies have illustrated that transgene expression can be dramatically improved through codon optimization.[35–37] For example, a study by Wang and colleagues[38] demonstrated 100-fold and 33-fold improvements in protein production and enzyme activity, respectively, following codon optimization of a human ornithine transcarbamylase (OTC) cDNA to achieve therapeutic effects at lower vector doses. Several ongoing human clinical trials for clotting factor IX deficiency (hemophilia B) have utilized similar codon optimization strategies.[39–41] Codon optimization of our mouse *Pah*-cDNA resulted in alteration to 20% of the base pairs in the native coding sequence (for details see the Supplemental Materials and Methods). Changes in codon usage were combined with insertion of a 850 bp-truncated IVS-1B intron fragment between exon 1 and exon 2. This strategy was based on a classic report by Palmiter et al.[42] demonstrating that inclusion of the first intron of the rat growth hormone gene was essential for high-level expression in transgenic systems. It was speculated that the first intron was particularly important because it was close to the transcriptional start site, and because the optimal sequence for attachment of the translational machinery might extend into the first intron. Splicing from promoter proximal introns has been shown to enhance transcription,[43] while splicing of terminal introns has been shown to increase the efficiency of polyadenylation.[44] The process of splicing might enhance mRNA stability in the nucleus or lead to more efficient transport into the cytoplasm,[45,46] resulting in greater accumulation of mature mRNA in the cytoplasm. Also, intronic sequences may protect pre-mRNA from degradation.[47] Taken together, all these data suggest that the combination of transgene codon optimization along with the incorporation of introns may significantly increase expression. The genome size limitations inherent to most gene transfer vectors preclude inclusion of all native introns of a therapeutic gene. As there is no genome size limit in MC vectors, it would have been possible to include the entire 6-kb intervening sequence 1 of *Pah* in an MC vector. However, construction of a mini-intron by removing regions that were not essential for splicing has also been shown to yield correct protein-dependent

splicing.[48] Moreover, a study from Jallat et al.[49] reported that including a 1.4-kb truncated fragment of the first intron of human factor IX into the expression cassette of transgenic mice produced much higher protein expression than a similar construct containing only the cDNA and demonstrated that the entire intervening sequence 1 was not necessary. We evaluated dose-dependent treatment efficacy only from a MC vector that included both codon optimization and inclusion of IVS-1B in combination. Our study showed that this optimized transgene yielded therapeutic efficacy at a dose (6×10^{11} vector copies per mouse) that was 10-fold lower than the dose required for the parental MC.PKU20 vector, which included the native *Pah* cDNA and lacked an intron (Figures 3A and 4; Table 2).

In conclusion, our study demonstrates that MC vectors have a favorable safety profile, including negligible liver toxicity or evidence of inflammation following hydrodynamic delivery (see Kattenhorn et al.[6]) and direct long-term, persistent therapeutic gene expression despite their non-integrating nature. The lack of a vector genome size limit allows the combined insertion of generally larger natural promoter-enhancer elements and intron sequences. A combination of these improvements will benefit the development of non-viral MC vectors for the treatment genetic liver defects.

MATERIALS AND METHODS

Gene Delivery and Animal Experiments

All animal experiments were approved by the State Veterinary Office of Zurich and were carried out according to the guidelines of the Swiss Law of Animal Protection, the Swiss Federal Act on Animal Protection (1978), and the Swiss Animal Protection Ordinance (1981). Young adult (7–12 weeks old; 16–22 g) female and male homozygous C57BL/6-*Pah*^enu2/enu2 ("PKU") mice were used for all experiments. Mice were maintained on standard chow and only for L-Phe determination, food was withdrawn 3–4 hr prior to tail vein bleeding. Blood was collected from tail veins on Guthrie filter cards, and L-Phe concentration was determined using tandem mass spectrometry. Minicircle

(MC)-DNA vectors at the indicated doses were infused into mice for liver targeting by hydrodynamic tail vein injection as described.[7]

Vector Construction and MC Production

Six out of eight MC-producer plasmids with various expression cassettes were constructed according to the description in the Supplemental Materials and Methods that included pMC.PKU20, pMC.PKU28, pMC.PKU37, pMC.PKU38, pMC.PKU43, and pMC.PKU44 (Figure S1). After transforming the MC-producer plasmids into *E. coli* ZYCY10P3S2T, corresponding MC vectors were generated and purified by using the QIAGEN endofree plasmid purification kit (QIAGEN AG) as previously described[7] and analyzed by agarose gel electrophoresis (example shown in Figure S3A) and/or capillary gel electrophoresis (CGE) (Figure S3C). More details of all expression cassettes and quality control are described in the Supplemental Materials and Methods.

Partial Hepatectomy

PKU mice that were either infused with saline as a negative control group or treated with vector MC.PKU37 expressing the Flag-tagged phenylalanine hydroxylase (PAH) (Viecelli et al.[7], also see Vector Construction and MC Production). Blood was collected periodically from tail veins to determine the L-Phe concentration. On day 28, all treated mice received standard 70% pHx. In brief, a midline incision was performed and the liver freed from ligaments. The pedicle of the caudate and left lobe was ligated (silk, 6/0) and resected as shown in Figure S2. After cholecystectomy (Prolene, 8/0), the middle lobe was ligated in two steps (silk 6/0) and resected. The resected lobes were either fixed in 4% formalin for immunohistochemistry staining or snap-frozen for further molecular analysis. Vascular and biliary structures of the right anterior and posterior lobe were preserved by this technique and visually controlled. Three weeks after pHx (on day 49), mice were sacrificed and livers were collected for either immunohistochemistry staining or molecular analysis.

PAH Enzyme Assay

PAH enzyme activity in whole liver extracts was measured according to a highly sensitive and quantitative assay using isotope-dilution liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS, API 4000, Sciex) developed in our laboratory.[50] Frozen tissues were lysed at +4°C in homogenization buffer as described[51] using a QIAGEN TissueLyser device (QIAGEN AG) according to our published method.[50]

RT-PCR for Liver cDNA Analysis of Correct Splicing

A total of 20–30 mg of mouse liver tissue was used for total RNA isolation using QIAmp RNA blood mini kit (QIAGEN AG) in accordance with the manufacturer's protocol. Random primed cDNA was prepared from 1 μg of total RNA using the Reverse Transcription kit (Promega). PCR amplification of the cDNA from exon 1 to exon 13 of *mcoPah* (vector MC.PKU28) was performed using the primers forward 5′-CATG GCAGCTGTTGTCCTGGAG-3′ and reverse 5′-GCCCACTT CGCTGTTGATGGAG-3′. The amplified product was separated by agarose gel electrophoresis, extracted using NucleoSpin Gel and PCR

clean-up (Macherey-Nagel), and analyzed by Sanger sequencing with the same forward and reverse primers (expected size of product was 1,292 bp if IVS-1B was correctly spliced, or 2,142 bp if the truncated intron IVS-1B was retained). Quantification of copy number by real-time qPCR is described in the Supplemental Materials and Methods.

Histological Studies

Flag-tagging was required for histochemical analysis to discriminate between the transgenic minicircle-derived wild-type PAH and the endogenous mutant PAH-p.Phe263Ser expressed from the *enu2* allele in PKU mice, as the (inactive) mutant protein is highly expressed in PKU (see also Viecelli et al.[7]). In brief, mouse livers were perfused through hepatic portal vein with 4% PBS-buffered paraformaldehyde. Subsequently, the organs were fixated with 10% formalin and preserved in ethanol 70% for paraffin embedding. The specimens were sectioned into 3-μm thick slices and stained with H&E for regular histo-pathological analysis. For immunohistochemistry, the primary DYKDDDDK tag antibody (1:500–1:1,000, cat #2368, Cell Signaling Technology) diluted in TBST-5% normal goat serum was used. The sections were then incubated with anti-rabbit SignalStain Boost Detection Reagent (cat #8114, Cell Signaling Technology) for 30 min at room temperature. The slides were stained using liquid DAB Substrate Chromogen (DAKO North America) and hematoxylin for counterstain. Sections were examined and images were taken at 2.5- and 10-fold magnifications using a Leica microscope and camera (Leica UTR 6000). The assessment of the Flag-tag was analyzed by a publicly available web application, called ImmunoRatio, which calculates the percentage of positively diaminobenzidine (DAB)-stained area out of the total hematoxylin-stained nuclear areas.[52]

More materials and methods are described in the Supplemental Materials and Methods.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Materials and Methods and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.omtn.2017.04.013.

AUTHOR CONTRIBUTIONS

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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