Published in final edited form as:
Mol Genet Metab. 2004 January ; 81(1): 3–8. doi:10.1016/j.ymgme.2003.09.010.

State-of-the-art 2003 on PKU gene therapy

Zhaobing Ding[a], **Cary O. Harding**[b], and **Beat Thöny**[a,*]

[a]Department of Pediatrics, Division of Clinical Chemistry and Biochemistry, University of Zürich, Steinwiesstrasse 75, Zürich CH-8032, Switzerland [b]Pediatrics, Molecular and Medical Genetics, Oregon Health and Science University, Portland, OR, USA

Abstract

Phenylketonuria (or PKU) is a well-known and widespread genetic disease for which many countries perform newborn screening, and life-long dietary restriction is still the ultimate and effective therapy. However, the diet is complicated, unpalatable, and expensive. The long-term effects of diet discontinuation in adults, except for the serious adverse effects of maternal hyperphenylalaninemia upon the developing fetus, have not been systematically studied, but congnitive decline and neurologic abnormalities have been anecdotally reported. Thus, alternative approaches for PKU therapy, including *gene therapy*, must be further explored. Here we summarize past present nonviral and viral gene transfer approaches, both in vitro studies and preclinical animal trials, to delivering the *PAH* gene into liver or other organs as potential alternatives to life-long phenylalanine-restricted dietary theraphy.

Keywords

Phenylketonuria; Hyperphenylalaninemia; Phenylalanine hydroxylase; Tetrahydrobiopterin; GTP cyclohydrolase I; Gene therapy; Recombinant adeno-associated virus

Introduction

Phenylketonuria (PKU) is an autosomal recessive genetic disorder with an average incidence of roughly 1 case in 10,000 Caucasian live births (OMIM 261600). It is caused by deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH; EC 1.14.16.1), responsible for converting phenylalanine to tyrosine, using molecular oxygen and tetrahydrobiopterin (BH_4) as a necessary cofactor to perform its catalytic activity [1,2]. This conversion is the rate-limiting step in phenylalanine catabolism in the liver. PAH deficiency leads to hyperphenylalaninemia (HPA), a dramatic increase in blood phenylalanine concentration (from <120 to >1200 μM). The inability to degrade phenylalanine present in dietary protein leads to the occurrence of urinary phenylalanine, phenylpyruvate, and phenylacetate. Mild forms of PKU accumulate phenylalanine in the blood at levels of 800–1200 μM. High levels of accumulated phenylalanine in PKU patients are toxic to the human body if left untreated, and is associated with an abnormal phenotype presenting with growth failure, microcephaly, seizures, and mental retardation. HPA can also be caused by the absence of the BH_4 cofactor due to deficiency of cofactor biosynthesis and regeneration, but this group of neurometabolic diseases will not be discussed here [3]. The biochemistry and genetics of PKU are well characterized (see for instance http://www.pahdb.mcgill.ca/ [4]). The clinical phenotype is defined and

*Corresponding author. Fax: +41-1-266-7169. *E-mail address:* E-mail: beat.thony@kispi.unizh.ch (B. Thöny).

treatment is also available. Moreover, PKU has been detected in most Western countries for many decades by newborn screening programs [5].

Although the underlying pathologies between hyperphenylalaninemia and abnormal brain function in PKU remain elusive, the relationship is undisputable. Children with severe PKU can have normal cognitive development when dietary treatment is initiated in early infancy and the blood phenylalanine level is maintained at near normal or normal levels [6]. Therefore an efficient method to reduce the phenylalanine level in the blood will eventually improve, or correct PKU. Woolf and Vulliamy [7] were the first to propose the use of a low phenylalanine diet as possible therapy for PKU patients. Following their suggestion, several important studies demonstrated that a phenylalanine-restricted diet significantly reduced the level of blood phenylalanine and improved the mental development and behavioral performance [8,9]. Dietary restriction treatment and newborn screening for PKU in developed countries have normalized the lives of PKU patients. However, there are still limitations to dietary treatments. Studies have shown that the restricted diet, which is not very palatable, offensive in odor and taste, and rigid has to be continued for a lifetime [10,11], that is, PKU patients have to modify their life styles significantly in comparison with healthy people. Although it is recognized that dietary treatment initiated early in life is successful in avoiding the severe mental retardation seen in untreated patients, the long-term outcome for adults remains unclear. Discontinuation of the restricted diet in adulthood is often accompanied by progressive loss of intellectual functions and by emotional disturbances [12]. Dietary restriction is an absolute requirement during pregnancy with PKU to prevent the occurrence of developmental abnormality and mental impairment in the offspring, a syndrome called maternal PKU [13–15]. In addition to the PKU patients themselves, the treatment inevitably also puts a heavy burden on the patients' families.

A promising approach towards eliminating the need for dietary restriction in PKU patients, is somatic gene therapy, in which a functional recombinant gene—in this case the *PAH* gene—is targeted to the affected tissue or organ such as the liver in vivo. Therefore, development of a safe and effective method of gene transfer for the treatment of PKU has been a top priority. In the past two decades, different approaches have been employed to study the possibility of somatic gene therapy in PKU animal models in vitro and in vivo (for older reviews on this subject see [16–18]). Other approaches, such as enzyme substitution, have also been developed as alternative treatments [19,20], but this is not the topic of this review.

PKU animal models

To experimentally explore new treatments for PKU, an appropriate animal model is required. An initial attempt was to generate a rat PKU model by administrating phenylalanine analogs as PAH inhibitors to suppress the enzyme activity. However, the results were complicated by the side effects of the inhibitors [21]. More successful was the chemical mutagenesis of a BTBR-mouse strain using the alkylating agent *N*-ethyl-*N*-nitrososurea (ENU), resulting in the isolation of a hyperphenylalaninemic mouse with a mutation called Pah^{enu2} [22]. The Pah^{enu2} allele was genetically mapped to the *Pah* locus, and sequence analysis revealed a miss-sense mutation at exon 7, a region that encodes the active site of the PAH enzyme, which is by far the most frequent mutation site for PKU in human [23]. Besides hyperphenylalaninemia, this mouse displays many symptoms found in human PKU patients, including slow growth, small head, hypopigmentation, behavior disturbances, and maternal PKU [22,24], and numerous studies from genetics and biochemistry have demonstrated the reliability of this animal model for human PKU [25]. Thus most PKU-animal studies, if not all, in somatic gene therapy and enzyme substitution have been carried out in this Pah^{enu2} mouse model and provided invaluable information on the biology and pathology of PKU. Nevertheless, and as will be mentioned below, recent unexpected results from gene therapy experiments with this

Pah^{enu2} mouse demand an alternative and better defined model, presumably by targeting the mouse gene for *Pah* via homologous recombination in embryonic stem cells. Furthermore, the BTBR-mouse strain seems to have limited fertility, suggesting breeding the *Pah*^{enu2} allele in another inbred mouse strain such as, for instance, C57BL/6 (BT and CH, personal observation).

Gene transfer vehicles

There are generally two approaches to carry transgenes into cells or tissues, using either viral or non-viral vectors. Both approaches have been employed to test the efficiency of gene delivery and transgene expression in vivo and in vitro [26,27]. Two different strategies have been explored to deliver the vectors to the target tissue, one is ex vivo, the other in vivo. In the ex vivo approach, cells were isolated and permanently transduced with a *PAH*-cDNA, followed by re-implantation of the transduced cells into the donor. In the in vivo approaches, recombinant vectors were infused directly into the portal vein or systemic circulation via the tail vein of the PKU mouse model.

Gene transfer with non-viral vectors

The transfer of foreign genes into tissues or organs by direct injection of naked plasmid-DNA has been demonstrated, leading to transgene expression and physiological or therapeutic responses [28]. To achieve better transfection of cells, naked DNA is often coated with cationic lipid to facilitate transport into nucleus. This kind of delivery system has advantages over other viral delivery systems, such as no size limitation of the DNA insert, and no cytopathic side-effects. The limited efficacy, however, is due to the very low gene transfer rate and the transient transgene expression [29].

In an earlier approach, the *PAH* gene was successfully delivered in vitro into hepatocytes by covalently attaching the DNA to the asialoglycoprotein, which is bound and internalized by the specific asialoglycoprotein receptor presented on the surface of the target cells. The transduction of cells by such DNA/protein complexes in vitro was greatly increased by the co-administration of a replication-defective adenovirus and reached an efficiency of 100% [30]. Although the low levels of PAH activity were reconstituted to nearly normal ranges, this method has produced very small portions of transduced hepatocytes in vivo [31].

A more recent approach was to inject naked plasmid DNA either via the portal vein or using the hydrodynamic technique through the tail vain into *Pah*^{enu2} mice (C.O.H., unpublished observations). The CMV-driven expression caused transient *Pah* expression that was not sustained beyond 24 h, and a mild decrease in serum phenylalanine levels. Furthermore, no effect was found if liver-specific promoters such as human albumin or nuclear elongation factor 1α was used.

Since PKU is probably a candidate for gene therapy in vivo, a significant improvement of the delivery system and persistent gene expression will be required before clinical application can be considered.

Liver gene transfer with viral vectors

Several types of viral vectors, including retroviral, adenoviral, and adeno-associated viral gene transfer vectors, have been examined for their hepatic correction potential in the PKU mouse model or its hepatocytes. So far only either short-term or sex (male)-specific treatment effects were observed in the PKU mouse.

Recombinant adenoviral vector

Recombinant adenoviral vectors are derived from the various serotypes of human adenoviruses. These viruses first cross the cell membrane via receptor-mediated endocytosis, and then shuttle into the endosomes. After lysing the endosomes, the viruses enter the nucleus, where they replicate episomally. Due to several advantages of adenovirus, including broad host cell ranges, extremely high efficiency of transduction, and the ability to transduce non-dividing cell types [26], a series of experiments had been conducted to explore the potential of recombinant adenoviruses as vectors for in vivo delivery of *PAH*-cDNA into liver. Fang et al. [32] described a recombinant adenoviral vector containing the human *PAH*-cDNA under control of the Rous sarcoma virus long-terminal repeat (RSV-LTR). The recombinant virus was infused into the liver through the portal vein of the PKU mouse. A significant increase of PAH activity was observed, leading to complete normalization of the serum phenylalanine levels in these PKU mice within one week of treatment. Unfortunately, the therapeutic effect of the adenoviral vector delivery did not persist beyond a few weeks, and repeated administration did not generate the original results due to the neutralizing antibodies against adenoviral vectors [33]. Furthermore, this study did not reveal other phenotypic changes such as for instance hypopigmentation. However, an important finding from this study is that 10–20% of normal PAH enzymatic activity is sufficient to restore normal serum phenylalanine levels. In another study, a recombinant adenoviral vector containing the strong CAG-hybrid promoter was constructed to drive expression of the human *PAH* gene in the PKU mouse to potentially enhance treatment efficacy [34]. Besides, suppression of the host immune response by administration of the immunosuppressant FK506 allowed repeated gene delivery, resulting in moderately prolonged *PAH* gene expression and reversal of hypopigmentation. Overall, both studies demonstrated the feasibility of gene therapy for PKU, but the adenoviral vector must be modified to reduce or eliminate expression of the adenoviral genes responsible for evoking immune responses. Improvement with second- and third-generation vectors was achieved but is still not yet satisfactory [26]. A more general problem to be solved is a sustained and prolonged trans-gene expression in vivo upon adenoviral gene transfer.

Recombinant retroviral vector

Recombinant retroviral vectors (from oncoretroviruses) are derived from the Moloney murine leukemia retrovirus by replacing all of the retroviral genes with therapeutic DNA. An early approach was the transduction via recombinant retroviral vectors of the *PAH*-cDNA into hepatocytes isolated from the PKU mouse [35,36]. The *PAH* gene was efficiently transferred and expressed at high levels in these primary hepatocytes. However, so far there is no report on successful in vivo or ex vivo retrovirus-mediated gene transfer in PKU mice. With the recent observation that retrovirus vectors induce leukemia-like disorders [26], it is unlikely that clinical trials for PKU with oncoretroviral vectors will have a future before this problem is solved.

Recombinant adeno-associated virus vector

The recombinant adeno-associated virus (rAAV) has recently emerged as an attractive vector for gene therapy, as it is non-pathogenic, can establish long-term transgene expression in a wide variety of tissues, is capable of transducing non-dividing cells, and elicits minimal immune responses as no viral genes are present in the vectors [26,37]. Hence, comparing with other viral vectors, rAAV vectors appear to be safer and more efficient, and the existence of different AAV serotypes expands the usefulness of the rAAV vectors, and leads to more specific and strong transgene expression with capsid pseudotypes from alternative serotypes in different organs and tissues [38]. These high promises are somewhat flawed by a recent report indicating that rAAV (type 2) vectors, despite low integration efficiency, may integrate

in murine hepatocytes, i.e., quiescent cells, preferentially into actively transcribed genes, and cause small genomic deletions, hence having the potential for dangerous side effects [39,40].

Regarding PKU gene therapy, Laipis et al. [41] recently published a preliminary communication on a rAAV containing the mouse *Pah*-cDNA, which was used for delivery to the liver of the PKU mouse by portal vein injection. Their results demonstrated the possibility of using a rAAV vector to correct PKU in the PKU-mouse model. Unexpectedly, only males responded by lowering phenylalanine to therapeutic levels, whereas females were unresponsive unless they were ovariectomized and treated with testosterone. As it cannot be excluded that this is a non-HPA specific effect as a result of the chemical mutagenesis it will be important to generate a new PKU mouse model by targeted deletion of the *Pah* gene and to repeat these rAAV gene therapy experiments. Nonetheless, the experiments demonstrate the high potential of gene therapy using rAAV, as it has already been shown for other monogenic diseases in human trials (including for instance haemophilia B or coagulation factor IX deficiency and cystic fibrosis [26]).

Heterologous, non-liver gene therapy for PKU

Some inherited metabolic disorders, for which the accumulated intermediate metabolite(s) is toxic at high concentration, can potentially be corrected by heterologous gene expression, i.e., enzymatic degradation in another tissue or organ than what is predicted from nature. As the pathology of PKU is largely caused by the high concentration of circulating phenylalanine and not by a local effect of PAH deficiency, such a strategy should also work for PKU. Several targeting tissues have been examined for heterologous expression of the *PAH* gene and potential efficacy for clearing the phenylalanine in the blood stream. Yet, due to the absence of the BH_4 cofactor for PAH in some tissues or organs, cofactor supply was a new obstacle in the heterologous *PAH*-gene expression. The de novo synthesis of the BH_4 cofactor starts from GTP and involves sequentially the enzymes GTP cyclohydrolase I (GTPCH), 6-pyruvoyl-tetrahydropterin synthase, and sepiapterin reductase. BH_4 can also be regenerated by two additional enzymes, pterin-4a-carbinolamine dehydratase and dihydropteridine reductase [42]. It turns out that only GTPCH and 6-pyruvoyltetrahydropterin synthase are highly regulated and/or not expressed constitutively in all tissues, whereas the other enzymes for BH_4 metabolism seem to be ubiquitously present [42–45].

Expression of human *PAH* in T lymphocytes was first investigated [46]. These authors found that T cells contain only small amounts of oxidized biopterin but significant dihydropteridine reductase activity. In vitro experiments showed that the intracellular biopterin content could be increased by exogenous BH_4 supplementation, and retrovirally transduced primary T cells from PKU patients produced high levels of human PAH activity. Unfortunately, no in vivo data of T lymphocytes or haematopoietic stem cell gene therapy of PKU in mice are available.

Pah expression in erythrogenic bone marrow has also been explored as potential therapy for PKU [47]. In this experiment, transgenic mice with bone marrow PAH activity expressed under the transcriptional control of the human β globin locus control region (LCR) were bred to Pah^{enu2} mice. The resulting progeny were homozygous for the Pah^{enu2} mutation and therefore lacked liver PAH activity but did express *Pah* in erythrogenic bone marrow under the control of the transgene. The progeny remained hyperphenylalaninemic despite the presence of PAH activity and sufficient BH_4 supply in bone marrow. The efficacy of bone marrow as a platform for phenylalanine hydroxylation in this model was likely limited by other fundamental physiologic properties such as insufficient flux of plasma through the *Pah*-expressing bone marrow compartment or insufficient phenylalanine transport into *Pah*-expressing cells.

The skin as another potential target tissue for PKU gene therapy is also under investigation. Primary human keratinocytes or skin fibroblasts, which both do not produce BH_4

endogenously, were engineered by transducing two independent retroviral vectors expressing PAH and GTPCH separately. GTPCH is the rate-limiting enzyme in de novo BH_4 biosynthesis in these cells, and co-transduction of human keratinocytes or fibroblasts with retroviral vectors carrying the *PAH* and the *GTPCH* genes resulted in phenylalanine clearance in vitro without additional BH_4 supplementation [48,49]. For skin-based gene therapy, keratinocytes (or fibroblasts) permanently expressing *PAH* and *GTPCH* cDNAs would be used for epidermal grafting. However, current limitations of such a 'metabolic sink' for phenylalanine by skin grafting are the limiting metabolic rate of clearance and the low rate of phenylalanine flux from the circulation into the skin graft.

Another attractive heterologous expression target tissue is the skeletal muscle, which comprises approximately 40% of total body weight in an adult human, is well vascularized, and more easily accessible through percutaneous techniques than the liver. To assess whether heterologous expression of *Pah* in muscle would lead to degradation of phenylalanine, the *Pah* gene was put under control of the muscle creatine kinase promoter, and was constitutively expressed in skeletal and cardiac muscle of transgenic mice generated by classical microinjection of eggs [50]. Breeding such mice to the Pah^{enu2} strain for homozygosity resulted in offspring lacking PAH activity in the liver but expressing *Pah* in the skeletal muscle. These animals had significantly elevated serum phenylalanine levels, which decreased only when BH_4 was supplemented by intraperitoneal injections, as the skeletal muscle lacks cofactor biosynthesis. Thus, *PAH* gene therapy in muscle might become a feasible approach provided that enough BH_4 cofactor is supplied. The latter problem can be overcome by co-expression of BH_4 biosynthesis genes, like *GTPCH*, together with the gene for *PAH*.

Summary and future perspectives

In a first phase of approaches, liver *PAH* gene transfer in vitro or in vivo was the dominating experimental philosophy, which failed due to poor efficiency of gene delivery into the liver and lack of sustained gene expression. In a second phase of studies, autologous non-hepatic gene targeting became attractive, yet with the additional challenge of overcoming the essential but limiting BH_4 cofactor supply. So far no studies were carried out employing lentiviruses, only oncoretroviruses were applied. Furthermore, besides gene replacement, targeted in situ gene repair that does not use viruses as delivery vehicles might also be envisaged to correct the errant PAH gene [51,52]. From all of the above, despite the different non-viral and viral gene transfer approaches that have been examined, none of them seem to hold great promise for future clinical trials until an appropriate gene transfer vector is designed. In addition to the problem of combining *PAH* with BH_4-cofactor gene expression in heterologous tissues, the delivery system and sustained expression are the main challenges to be overcome for PKU as for other gene therapies.

Acknowledgments

We thank Mr. M. Killen for help with the preparation of the manuscript. Z.D. was supported by a grant from the Swiss National Science Foundation (No. 31-64154.00 to B.T.).

References

1. Scriver CR, Eisensmith RC, Woo SLC, Kaufman S. The hyperphenylalaninemias of man and mice. Annu. Rev. Genet 1994;28:141–165. [PubMed: 7893121]
2. Scriver, CR.; Kaufman, S. Hyperphenylalaninemia: phenylalanine hydroxylase deficiency. In: Scriver, CR.; Beaudet, AL.; Sly, WS.; Valle, D.; Vogelstein, B., editors. The Metabolic and Molecular Bases of Inherited Disease. New York: McGraw-Hill; 2001. p. 1667-1724.

3. Blau, N.; Thöny, B.; Cotton, RGH.; Hyland, K. Disorders of tetrahydrobiopterin and related biogenic amines. In: Scriver, CR.; Beaudet, AL.; Sly, WS.; Valle, D.; Vogelstein, B., editors. The Metabolic and Molecular Bases of Inherited Disease. New York: McGraw-Hill; 2001. p. 1725-1776.
4. Scriver CR, Hurtubise M, Konecki D, Phommarinh M, Prevost L, Erlandsen H, Stevens R, Waters PJ, Ryan S, McDonald D, et al. PAHdb 2003: what a locus-specific knowledge-base can do. Hum. Mutat 2003;21:333–344. [PubMed: 12655543]
5. Guthrie R. The introduction of newborn screening for phenylketonuria. A personal history. Eur. J. Pediatr 1996;155:S4–S5. [PubMed: 8828599]
6. Waisbren SE, Mahon BE, Schnell RR, Levy HL. Predictors of intelligence quotient and intelligence quotient change in persons treated for phenylketonuria early in life. Pediatrics 1987;79:351–355. [PubMed: 3822635]
7. Woolf LI, Vulliamy DG. Phenylketonuria with a study of the effect upon it of glutamic acid. Arch. Dis. Child 1951;26:487–494. [PubMed: 14904091]
8. Bickel H. The first treatment of phenylketonuria. Eur. J. Pediatr 1996;155:S2–S3. [PubMed: 8828598]
9. Woolf LI. Treament of phenylketonuria with diet low in phenylalanine. Br. Med. J 1955;1:57–64. [PubMed: 13219342]
10. National Institutes of Health Consensus Development Conference Statement: phenylketonuria: screening and management. Pediatrics 2000;108:972–982.
11. Harding CO. Mommy, why can't I have a hamburger like the other kids? Gene Ther 2000;7:1969–1970. [PubMed: 11175306]
12. Smith I, Lobascher ME, Stevenson JE, Wolff OH, Schmidt H, Grubel-Kaiser S, Bickel H. Effect of stopping low-phenylalanine diet on intellectual progress of children with phenylketonuria. Br. Med. J 1978;2:723–726. [PubMed: 698696]
13. Koch R, Friedman E, Azen C, Hanley W, Levy H, Matalon R, Rouse B, Trefz F, Waisbren S, Michals-Matalon K, et al. The international collaborative study of maternal phenylketonuria: status report 1998. Eur. J. Pediatr 2000;159:S156–S160. [PubMed: 11043164]
14. Sheard NF. Importance of diet in maternal phenylketonuria. Nutr. Rev 2000;58:236–239. [PubMed: 10946561]
15. Platt LD, Koch R, Hanley WB, Levy HL, Matalon R, Rouse B, Trefz F, de la Cruz F, Guttler F, Azen C, et al. The international study of pregnancy outcome in women with maternal phenylketonuria: report of a 12-year study. Am. J. Obstet. Gynecol 2000;182:326–333. [PubMed: 10694332]
16. Eisensmith RC, Woo SL. Molecular genetics of phenylketonuria: from molecular anthropology to gene therapy. Adv. Genet 1995;32:199–271. [PubMed: 7741023]
17. Eisensmith RC, Woo SL. Gene therapy for phenylketonuria. Eur. J. Pediatr 1996;155:S16–S19. [PubMed: 8828602]
18. Eisensmith RC, Woo SL. Gene therapy for phenylketonuria. Acta Paediatr. Suppl 1994;407:124–129. [PubMed: 7766948]
19. Sarkissian CN, Shao Z, Blain F, Peevers R, Su H, Heft R, Chang TM, Scriver CR. A different approach to treatment of phenylketonuria: phenylalanine degradation with recombinant phenylalanine ammonia lyase. Proc. Natl. Acad. Sci. USA 1999;96:2339–2344. [PubMed: 10051643][see comments]
20. Levy HL. Phenylketonuria:old disease, new approach to treatment. Proc. Natl. Acad. Sci. USA 1999;96:1811–1813. [PubMed: 10051548]
21. Lane JD, Schone B, Langenbeck U, Neuhoff V. Characterization of experimental phenylketonuria. Augmentation of hyperphenylalaninemia with alpha-methylphenylalanine and p-chlorophenylalanine. Biochim. Biophys. Acta 1980;627:144–156. [PubMed: 6444271]
22. Shedlovsky A, McDonald JD, Symula D, Dove WF. Mouse models of human phenylketonuria. Genetics 1993;134:1205–1210. [PubMed: 8375656]
23. McDonald JD, Charlton CK. Characterization of mutations at the mouse phenylalanine hydroxylase locus. Genomics 1997;39:402–405. [PubMed: 9119379]
24. Cho S, McDonald JD. Effect of maternal blood phenylalanine level on mouse maternal phenylketonuria offspring. Mol. Genet. Metab 2001;74:420–425. [PubMed: 11749047]

25. McDonald JD, Andriolo M, Cali F, Mirisola M, Puglisi-Allegra S, Romano V, Sarkissian CN, Smith CB. The phenylketonuria mouse model: a meeting review. Mol. Genet. Metab 2002;76:256–261. [PubMed: 12208130]
26. Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. Nat. Rev. Genet 2003;4:346–358. [PubMed: 12728277]
27. Chesnoy S, Huang L. Structure and function of lipid–DNA complexes for gene delivery. Annu. Rev. Biophys. Biomol. Struct 2000;29:27–47. [PubMed: 10940242]
28. Niidome T, Huang L. Gene therapy progress and prospects: nonviral vectors. Gene Ther 2002;9:1647–1652. [PubMed: 12457277]
29. Lechardeur D, Lukacs GL. Intracellular barriers to non-viral gene transfer. Curr. Gene Ther 2002;2:183–194. [PubMed: 12109215]
30. Cristiano RJ, Smith LC, Woo SL. Hepatic gene therapy: adenovirus enhancement of receptor-mediated gene delivery and expression in primary hepatocytes. Proc. Natl. Acad. Sci. USA 1993;90:2122–2126. [PubMed: 8384712]
31. Wu GW, Chowdhury JR, Bommineni VR, Basu SK, Wu CH, Chowdhury NR. Fate of DNA targeted to hepatocytes by asialoglycoprotein polylysine conjugates. Proc. Assoc. Am. Physicians 1995;107:211–217. [PubMed: 8624854]
32. Fang B, Eisensmith RC, Li XH, Finegold MJ, Shedlovsky A, Dove W, Woo SL. Gene therapy for phenylketonuria: phenotypic correction in a genetically deficient mouse model by adenovirus-mediated hepatic gene transfer. Gene Ther 1994;1:247–254. [PubMed: 7584088]
33. Eisensmith RC, Woo SL. Somatic gene therapy for phenylketonuria and other hepatic deficiencies. J. Inherit. Metab. Dis 1996;19:412–423. [PubMed: 8884565]
34. Nagasaki Y, Matsubara Y, Takano H, Fujii K, Senoo M, Akanuma J, Takahashi K, Kure S, Hara M, Kanegae Y, et al. Reversal of hypopigmentation in phenylketonuria mice by adenovirus-mediated gene transfer. Pediatr. Res 1999;45:465–473. [PubMed: 10203136]
35. Peng H, Armentano D, MacKenzie-Graham L, Shen RF, Darlington G, Ledley FD, Woo SL. Retroviral-mediated gene transfer and expression of human phenylalanine hydroxylase in primary mouse hepatocytes. Proc. Natl. Acad. Sci. USA 1988;85:8146–8150. [PubMed: 3186716]
36. Liu TJ, Kay MA, Darlington GJ, Woo SL. Reconstitution of enzymatic activity in hepatocytes of phenylalanine hydroxylase-deficient. Somat. Cell. Mol. Genet 1992;18:89–96. [PubMed: 1312261]
37. Kootstra NA, Verma IM. Gene therapy with viral vectors. Annu. Rev. Pharmacol. Toxicol 2003;43:413–439. [PubMed: 12359866]
38. Rabinowitz JE, Rolling F, Li C, Conrath H, Xiao W, Xiao X, Samulski RJ. Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. J. Virol 2002;76:791–801. [PubMed: 11752169]
39. Nakai H, Montini E, Fuess S, Storm TA, Grompe M, Kay MA. AAV serotype 2 vectors preferentially integrate into active genes in mice. Nat. Genet 2003;34:297–302. [PubMed: 12778174]
40. Kay MA, Nakai H. Looking into the safety of AAV vectors. Nature 2003:251–424. [PubMed: 12867952]
41. Laipis, PJ.; Charron, CE.; Steele, HA.; Embury, JE.; Ross, K.; Knapp, J-M.; Porvasnik, SL.; Alexander, JJ.; Reyes, L.; Zori, RT. Mol. Ther; Abstracts of the 6th Annual Meeting of the American Society of Gene Therapy; June 4–8, 2003; Washington, DC, USA. 2003. p. S391-S392.
42. Thöny B, Auerbach G, Blau N. Tetrahydrobiopterin biosynthesis, regeneration, and functions. Biochem. J 2000;347:1–16. [PubMed: 10727395]
43. Leitner KL, Meyer M, Leimbacher W, Peterbauer A, Hofer S, Heufler C, Muller A, Heller R, Werner ER, Thony B, et al. The low tetrahydrobiopterin biosynthetic capacity of human monocytes is caused by exon skipping in 6-pyruvoyl tetrahydropterin synthase. Biochem. J. Pt. 2003
44. Werner-Felmayer G, Golderer G, Werner ER. Tetrahydrobiopterin biosynthesis, utilization and pharmacological effects. Curr. Drug Metab 2002;3:159–173. [PubMed: 12003348]
45. Hwu WL, Yeh HY, Fang SW, Chiang HS, Chiou YW, Lee YM. Regulation of GTP cyclohydrolase I by alternative splicing in mononuclear cells. Biochem. Biophys. Res. Commun 2003;306:937–942. [PubMed: 12821132]

46. Lin CM, Tan Y, Lee YM, Chang CC, Hsiao KJ. Expression of human phenylalanine hydroxylase activity in T lymphocytes of classical phenylketonuria children by retroviral-mediated gene transfer. J. Inherit. Metab. Dis 1997;20:742–754. [PubMed: 9427141]

47. Harding CO, Neff M, Jones K, Wild K, Wolff JA. Expression of phenylalanine hydroxylase (PAH) in erythrogenic bone marrow does not correct hyperphenylalaninemia in *Pah*^{enu2} mice. J. Gene Med 2003;5:984–993. [PubMed: 14601136]

48. Christensen R, Kolvraa S, Blaese RM, Jensen TG. Development of a skin-based metabolic sink for phenylalanine by overexpression of phenylalanine hydroxylase and GTP cyclohydrolase in primary human keratinocytes. Gene Ther 2000;7:1971–1978. [PubMed: 11175307]

49. Christensen R, Guttler F, Jensen TG. Comparison of epidermal keratinocytes and dermal fibroblasts as potential target cells for somatic gene therapy of phenylketonuria. Mol. Genet. Metab 2002;76:313–318. [PubMed: 12208136]

50. Harding CO, Wild K, Chang D, Messing A, Wolff JA. Metabolic engineering as therapy for inborn errors of metabolism-development of mice with phenylalanine hydroxylase expression in muscle. Gene Ther 1998;5:677–683. [PubMed: 9797873]

51. Kren BT, Chowdhury NR, Chowdhury JR, Steer CJ. Gene Therapy as an alternative to liver transplantation. Liver Transpl 2002;8:1089–1108. [PubMed: 12474147]

52. Liu L, Parekh-Olmedo H, Kmiec EB. The development and regulation of gene repair. Nat. Rev. Genet 2003;4:679–689. [PubMed: 12951569]