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DNA methylation in the pathophysiology of hyperphenylalaninemia in the PAH^{enu2} mouse model of phenylketonuria

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Abstract

Phenylalanine hydroxylase deficient phenylketonuria (PKU) is the paradigm for a treatable inborn error of metabolism where maintaining plasma phenylalanine (Phe) in the therapeutic range relates to improved clinical outcomes. While Phe is the presumed intoxicating analyte causal in neurologic damage, the mechanism(s) of Phe toxicity has remained elusive. Altered DNA methylation is a recognized response associated with exposure to numerous small molecule toxic agents. Paralleling this effect, we hypothesized that chronic Phe over-exposure in the brain would lead to aberrant DNA methylation with secondary influence upon gene regulation that would ultimately contribute to PKU neuropathology. The PAH^{enu2} mouse models human PKU with intrinsic hyperphenylalaninemia, abnormal response to Phe challenge, and neurologic deficit. To examine this hypothesis, we assessed DNA methylation patterns in brain tissues using methylated DNA immunoprecipitation and paired end sequencing in adult PAH^{enu2} animals maintained under either continuous dietary Phe restriction or chronic hyperphenylalaninemia. Heterozygous PAH$^{enu2/WT}$ litter mates served as controls for normal Phe exposure. Extensive repatterning of DNA methylation was observed in brain tissue of hyperphenylalaninemic animals while Phe restricted animals displayed an attenuated pattern of aberrant DNA methylation. Affected gene coding regions displayed aberrant hypermethylation and hypomethylation. Gene body methylation of noncoding RNA genes was observed and among these microRNA genes were prominent. Of particular note, observed only in hyperphenylalaninemic animals, was hypomethylation of miRNA genes within the imprinted Dlk1-Dio3 locus on chromosome 12.

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Conflicts of interest

None of the authors are conflicted.

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Aberrant methylation of microRNA genes influenced their expression which has secondary effects upon the expression of targeted protein coding genes. Differential hypermethylation of gene promoters was exclusive to hyperphenylalaninemic PAH^{enu2} animals. Genes with synaptic involvement were targets of promoter hypermethylation that resulted in down-regulation of their expression. Gene dysregulation secondary to abnormal DNA methylation may be contributing to PKU neuropathology. These results suggest drugs that prevent or correct aberrant DNA methylation may offer a novel therapeutic option to management of neurological symptoms in PKU patients.

Keywords

PAH^{enu2}; DNA methylation; Phenylketonuria; Toxicity

1. Introduction

Phenylalanine hydroxylase (PAH) deficient PKU is the paradigm for a treatable inborn error of metabolism and was the motivation for prospective newborn screening [1]. Newborn screening identifies PAH deficient patients to enable early intervention (i.e. dietary Phe restriction) such that severe neurologic manifestations are avoided. It has long been assumed that Phe is the intoxicating analyte in PKU but the mechanisms by which Phe over-exposure leads to neurological damage remains unclear despite significant investigation over recent decades.

We approached Phe intoxication leveraging observations in other toxic exposure scenarios. Altered DNA methylation, in response to toxic exposure, is a mechanism for cellular survival that is observed following small molecule intoxication encompassing numerous chemically diverse agents [2–12]. This response is not limited to toxic compounds (i.e. heavy metals) but has also been observed following exposure to excessively high levels of physiological compounds, the quintessential example being methylome repatterning in the fetus following glucose over-exposure in maternal diabetes [13–15]. Most toxic exposure is short term and of environmental origin. Hyperphenylalaninemia is intrinsic to the PAH deficient patient and in untreated or poorly treated classical disease, high-level Phe exposure is chronic. We hypothesized that chronic Phe toxicity could subvert protective aspects of DNA methylation leading to deleterious physiological consequences. Moreover, as DNA methylation is among the classic mediators of gene function (both normal and pathological); the hypothesis was extended to indicate aberrant DNA methylation would influence gene expression and in so contribute to PKU neuropathology. While deleterious consequences of aberrant DNA methylation are widely recognized in cancer systems [16–18], evidence of aberrant DNA methylation is becoming evident in diverse disorders including: rheumatoid arthritis, Legg-Calvé-Perthes disease, lung fibrosis, inflammatory bowel disease, and schizophrenia [19–25].

Clinical outcomes in PKU are significantly improved by maintaining blood Phe concentrations within the therapeutic window which is widely agreed to be $<360\ \mu M$ [26]. While maintaining Phe control in the neonatal period and childhood is particularly

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critical owing to active brain development, maintaining Phe control in adulthood is indicated owing to late-onset neurological and neuropsychiatric presentation. Further, the somatic impact of in utero Phe exposure to the offspring of poorly controlled PKU-affected women leads to maternal PKU syndrome. We have investigated DNA methylation in human PKU and a mouse model of maternal PKU syndrome [27,28]. In both systems, severe hyperphenylalaninemia led to increased differential DNA methylation with secondary effects on gene regulation. Another consistent observation in our previous studies is that maintaining blood Phe within the therapeutic range significantly attenuates both DNA methylation and aberrant gene regulation. Herein, we report assessment of DNA methylation in the PAH^{enu2} mouse which has served as a standard model to investigate PKU pathology and treatment [29–33]. These studies continue to support our observation that hyperphenylalaninemia leads to abnormal DNA methylation in the brain. Further, differential DNA methylation frequently involves microRNA (miRNAs) genes as was observed in previous investigations. We have identified epigenome dysfunction in brain tissue of the PAH^{enu2} mouse suggesting the neuropathology of Phe exposure is at least partially realized through aberrant DNA methylation leading to gene dysregulation.

2. Methods and materials

2.1. Animals, mating, and dietary Phe restriction

PAH^{enu2} mice were propagated in the Rangos Research Center animal facility at Children's Hospital of Pittsburgh under an approved protocol. To generate experimental animals, matings paired either a heterozygous $Pah^{enu2/WT}$ female with a homozygous male ($Pah^{enu2/enu2}$) or heterozygous female and a heterozygous male. To determine the phenotype of offspring, tail snippets were obtained for DNA extraction which was assessed for the Pah genotype with a novel melt profiling assay (see Supplemental Fig. 1). Female animals were selected for experimental analysis. After weaning, hyperphenylalaninemic experimental animals were generated by providing homozygous offspring a diet of normal mouse chow. Phe controlled animals were created by implementing Phe restriction immediately following weaning by providing Phe-free chow and water supplemented with 0.5 g/l Phe as described [27]. In Phe restricted animals, Phe concentration was periodically assessed by drawing blood from the mandibular vein, spotting blood on filter paper, and Phe concentration was assessed by a reference service. Heterozygous control animals were provided a normal diet. Animals were sacrificed by cervical dislocation at 7–10 months of age, brain tissue was dissected, and either used immediately or stored at −80 °C until utilized.

2.2. DNA preparation, library construction, Me-DIP, and paired end sequencing

Approximately 25 mg of tissue was dissected from the frontal lobe and used for preparation of nucleic acids. DNA was prepared with QiagenDNeasy Blood & Tissue reagents according to the manufacturer and quantified using both absorbance at 260 nm and picogreen staining. DNA fragmentation and library construction were performed as previously described [27,28]. Briefly, genomic DNA was sheared utilizing a Fisher 550 Sonic Dismembrator and fragment size (200–1000 bp fragments) was determined utilizing both an Agilent 2100 Bioanalyzer and gel electrophoresis. Sequencing libraries were constructed with Agilent SureSelectXT reagents according to the manufacturer. To enrich for inserts containing

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methylated DNA, methylated DNA immunoprecipitation (Me-DIP) was performed as described [27,28]. Briefly, libraries were incubated with biotinylated α-5-methyl-cytosine and immuno-complexes were captured on magnetic streptavidin [34]. Immunoprecipitate is purified by extraction with phenol-chloroformisoamyl alcohol, washed with 70% ethanol, and dried in a Savant Speed Vac. Enrichment of library inserts containing methylated DNA is demonstrated using PCR of the hypermethylated H19 gene and the non-methylated GADPH gene utilizing starting material and immunoprecipitate [34]. Sequencing utilized an Illumina HiSeq 2000 with 3 libraries assessed per lane as described [27,28]. RNA was prepared from brain frontal lobe tissue using Qiagen RNeasy Plus Universal Kit.

2.3. Me-DIP data analysis

Data analysis was performed as described [27,28]. Briefly, fastq format read files were mapped with Bowtie2 to the current mouse build (mm9). Areas with coverage >5 reads were considered. Methylation of coding gene sets was calculated from .bam files as average coverage of the total sum of exons represented in the protein coding locus, and methylation of non-protein coding genes was represented by the average coverage of the locus. Coverage was determined with RNASequnator software [35]. Differential methylation was determined using intelligent methods optimization by Efficiency Analysis [36]. This approach selects the most reproducible combination of transformation, normalization, test for differential coverage, and a learned threshold of significance via resampling to identify an optimal low-bias measure of differences in the average coverage between group-wise comparisons of experimental and control groups. For our comparisons, the J5 score [36] was highly reproducible. Worst-case false discovery risk was estimated in each comparison as False Discovery Risk = (R /m)(1-R / m) where R is the number of genes with differential coverage at the optimal threshold, and m = the total number of genes.

2.4. Gene expression analysis

Protein coding genes were assessed with the Agilent SurePrint G3 Mouse Gene Expression 60 K Microarray according to the manufacturer. Array analysis included four biological replicates per group and the following comparisons were made: 1. Hyperphenylalaninemic PAH^{enu2} vs. heterozygote controls and 2. Phe restricted PAH^{enu2} vs. heterozygote controls. Following data reduction of the experimental groups (hyperphenylalanimemic animals; Phe restricted animals) against heterozygous controls; manual comparison of differential gene expression between the experimental groups was performed. NcRNA genes were assessed using the Agilent Mouse miRNA Microarray, Release 19.0, 60 K according to the manufacturer. Microarray data analysis utilized GeneSpring GX Software Version 12.6 with the two color expression mode for protein coding genes or the miRNA mode for ncRNA arrays. Within an individual array, data from analysis of unique biological replicates was combined creating aggregate scores. Some genes (protein coding, miRNA) were confirmed by single-site RT-PCR (data not shown).

2.5. Identifying miRNA targets

MiRBase (mirbase.org) algorithms identify genes targeted by miRNAs utilizing a scale where scores 80 are likely to represent a bona fide miRNA:mRNA interaction [37–39]. MiRNA genes displaying differential methylation within coding regions and miRNA genes

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were the most prominent group therein. both methylome modification and altered expression were queried with consideration given to hits scoring 80. MiRNA genes are often clustered and expressed in a polycistronic message from which miRNAs are excised. Instances where a methylome modified miRNA gene(s) displays altered expression and is within a cluster of miRNA genes (especially so when the genes are coded on the same DNA strand), the expression of adjacent miRNA genes is considered. If expression of an adjacent miRNA(s) is up-regulated, putative targets for these miRNAs were identified. Again, only targets with a MirBase score 80 are considered. Concordance of miRNA expression was correlated to expression of high scoring mRNA target genes to determine potential interactions.

3. Results

3.1. Animal models

Dietary Phe restriction of female PAH^{enu2} animals maintains blood Phe at ~150–200 μM as previously described [27]. PAH^{enu2} females provided a normal diet have blood Phe of >1000 μM. Heterozygous (PAH$^{enu2/WT}$) females have a blood Phe of 60–70 μM. Me-DIP was utilized to assess five PAH^{enu2} homozygous animals provided normal chow (henceforth called PKU Group), five PAH^{enu2} homozygous animals under dietary Phe restriction (henceforth called Diet Group), and seven heterozygous (Pah$^{enu2/WT}$) control animals (henceforth called Control Group). These animals and additional experimental (PKU group, Diet group) and control heterozygous animals were utilized for gene expression (miRNA, protein coding) analysis.

3.2. Methylome assessment

Among the 17 Me-DIP libraries assessed the average number of reads was 56,535,027 with an average of 94.5% of reads mapping to the NCBI37/mm9 mouse genome assembly. No differences were observed in the number of reads or percent mapped reads between the PKU, Diet, and Control groups. Informatic analysis targeted genes, recognized genomic elements (promoters, enhancers, etc.), and predicted genes. Among all libraries, there were >23,400 annotated loci that were common among the three groups. To identify differential methylation, comparisons were made between the PKU Group vs. Controls Group and Diet Group vs. Control Group. Efficiency analysis determined the J5 statistical metric was optimal to discern differential coverage with an absolute value of J5 3.5 determining a significant difference. Following data reduction of the experimental groups (PKU group, Diet group) against controls, direct comparison between the PKU Group and Diet group identified shared sites of differential methylation and differential methylation that were unique to each group. Differential methylation includes the following: unique coverage in the experimental group (hypermethylation), loss of coverage in the experimental group (hypomethylation), and significant loss or gain in the depth of coverage within experimental groups. Broadly categorized, differential methylation involved either gene body methylation (hypermethylation and hypomethylation) or gene promoter hypermethylation.

Differential gene body methylation was observed in the PKU Group and the Diet Group. The PKU Group displayed more extensive gene body methylation with 566 differentially methylated loci compared to 269 differentially methylated loci in the Diet Group. Twenty-

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six genes were common to the PKU and Diet Groups. Supplemental Fig. 2 displays differential gene body methylation unique to each group and those shared between the groups. Aside from a greater absolute number of differentially methylated loci in the PKU group, the PKU and Diet Groups diverge concerning the degree of differential hypermethylation vs. hypomethylation. Hypermethylation in the PKU group involved 330 sites (58.3%) while in the Diet group hypermethylation accounts for 106 sites (39.4%). Small noncoding RNA genes frequently displayed

A significant site of hypomethylation, unique to the PKU Group, involved miRNA genes within the imprinted, maternally expressed Dlk1-Dio3 region on chromosome 12 which contains a cluster of 40 miRNA genes with Mir882 and Mir3072 representing the 5′ and 3′ boundaries of the cluster respectively [40]. Within a region of ~25 kb in the 3′ of the miRNA gene cluster (chromosome 12 109739119–109747960), we identified eight hypomethylated miRNA genes all of which displayed up-regulated expression (see Table 1). These hypomethylated miRNA gene in the Dlk1-Dio3 regions could be further subdivided into a 5′ cluster containing five immediately adjacent miRNA genes (Mir376a, Mir376b, Mir376c, Mir654, Mir543) housed within a 1130 bp region (109723848–109722718) and a second cluster of 3 miRNA genes (Mir369, Mir541, Mir3072) that are within the MirG locus. Within close proximity to these hypomethylated miRNA genes were an additional ten miRNA genes that displayed altered expression (see below and Table 1).

Table 2 identifies hypermethylated miRNA genes in the PKU group with upregulated expression. In the Diet Group these miRNA genes are neither hypermethylated nor are expression of these genes upregulated compared to heterozygous controls. Mir23a, Mir27a, and Mir24–2 are clustered within 403 bp on chromosome 8 and expressed from a common DNA strand. While only Mir23a was identified as hypermethylated, the adjacent Mir27a, and Mir24–2 are included as these too demonstrated up regulated expression. Within this cluster of miRNA genes, proximity is likely to influence adjacent genes similar to that observed among clustered miRNA genes with the Dlk1-Dio3 locus (Table 1).

Differential promoter hypermethylation was exclusive to the PKU group; no differentially hypermethylated gene promoters were observed in the Diet group. Among promoter hypermethylated genes in the PKU Group, protein coding genes were prominent but several pseudogenes, putative gene loci, and long noncoding RNA genes also displayed promoter methylation. Table 3 identifies promoter methylated genes with down-regulated expression that have defined neural function.

3.3. MiRNA expression and identification of miRNA targets

MiRNA expression was assessed in 9 biological replicates from each the PKU group and Diet group while 6 biological replicates were assessed in the Control group. Each miRNA array contains three separate arrays each of which assessed a unique biological replicate. Data from the three individual arrays are condensed into a single data set as described [27,28]. Within the imprinted Dlk1-Dio3 region, upregulated expression was observed in 18 miRNA genes which include the eight hypomethylated miRNA genes (Table 1) and ten additional miRNA genes adjacent to the hypomethylated genes. The miRNA genes with up-regulated expression were within two distinct clusters: 1. *Cluster 1* miRNA genes in

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an ~9 kb region in the MirG locus (Mir369, Mir541, Mir3072, Mir377, Mir154, Mir496a, Mir409) and 2. *Cluster 2* miRNA genes within an ~17 kb region 5′ to MirG (Mir376a, Mir376b, Mir376c, Mir654, Mir382, Mir539, Mir544, Mir667, Mir495, Mir666). In the Diet group, expression of the miRNA genes identified in Table 1 is equivalent to heterozygous controls.

MirBase is the premier database cataloging miRNA genes and their functions. We leveraged MirBase algorithms to identify high-scoring targets for over-represented miRNAs and compared these to our gene expression data. Only targets with a MirBase score 80 were considered as these have exceedingly high probability to be genuine biological targets [37–39]. MirBase scores in this study were 89 in most cases with one target (Table 1 Mir376b 3p) scoring 86. Mir369 and Mir654, both within the imprinted Dlk1-Dio3 region, target Sema5a. The semaphorins are secreted and membrane-associated proteins necessary to establish neural circuits via axon guidance. Recently, deletion of Sema5a has been identified in autistic patients [41]. Mir376c is likely targeting the Cntn4 gene which codes for contactin 4, an immunoglobulin family cell adhesion molecule with roles in formation and maintenance of neural networks. Further, toxicity induced methylation of the Cntn4 gene has been observed in response to ethanol over-exposure [46]. Mir544 likely targets Temn4 which codes for a transmembrane adhesion molecule whose expression is largely limited to the brain. Tenm4 regulates axon guidance, has roles in myelination, and germline mutations are causal in essential tremor [51]. Down-regulated potassium channel genes have been identified in this study and our other investigation of hyperphenylalaninemia-induced DNA methylation [27,28]. Mir382 targets Kcnd2 which has roles in astrocyte function and epilepsy [48–50]. The Kcna4 gene is targeted by Mir543 (Table 1) and Mir301 (Table 2). Down regulation of Kcna4 in the mouse brain has recently been associated stressors [44]. Chronic Phe exposure could represent another stressor that leads to gene down-regulation representing a potential cellular survival mechanism. In the Diet group all of the miRNA genes and the putative target genes described in Table 1 show expression equivalent to controls.

Table 2 identifies hypermethylated MiRNA genes with up-regulated expression. Mir27a is likely targeting the potassium channel the Kcnk2 gene. Kcnk2 null mice are phenotypically normal but have increased susceptibility to epilepsy. It was suggested that altered lipid metabolism could be causal in epileptic susceptibility. Mir24–2 is likely targeting the Nefm gene that codes for medium neurofilaments. In our human PKU study, we identified neurofilament gene involvement [28]. Mir301 is likely targeting the Kcn4a gene which was also identified to be a target of Mir543 as noted in Table 1.

3.4. Expression of promoter methylated genes

Table 3 identifies four promoter hypermethylated genes with down-regulated expression. Two genes Arhgap21 and Cdc42bpa function within the Cdc42 Rho GTPase system. Cdc42 is recognized to function in synaptic processes thus any dysfunction within this system could be detrimental to neurological function. The Pde10a gene codes for a dual specificity cyclic nucleotide phosphodiesterase that is highly enriched in the brain and in particular within spiny neurons. Down regulation of cyclic nucleotide phosphodiesterase activity

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could be pathological by slowing the termination of second messenger signaling. The Itpr1 gene, like Pde10a, is involved in second messenger signaling as a receptor for inositol 1,4,5-triphosphate. Receptor stimulation leads to release of calcium from the endoplasmic reticulum. Constitutional mutations in Itpr1 gene are causal in Spinocerebellar ataxia 15 [62].

4. Discussion

Neurological aspects of PAH deficient PKU have been investigated for decades. It is evident that Phe is the neuro-toxic insult as maintaining a low concentration of blood Phe improves outcomes and no secondary Phe metabolite with greater neuro-toxicity has been identified. Our investigation of Phe toxicity leveraged observations in other toxic exposures where altered DNA methylation was observed. Altered DNA methylation is a cellular survival response to protect against environmental exposure and until relatively recent times in the evolution of the human species, these exposures were acute and of environmental origin. Chronic environmental exposures, such as arsenic exposure in contaminated drinking water or exposure to chemicals in an industrial setting, are associated with epigenome dysfunction and produce clinical phenotypes [2,3]. This study and our previous investigations of human PKU and mouse MPKU syndrome also demonstrated altered DNA methylation [27,28]. This study utilized female Pah^{enu2} animals in both the experimental and control groups. Female Pah^{enu2} animals possess higher intrinsic hyperphenylalaninemia that males; however, both are within the range of classical PKU and as such we do not anticipate a gender bias. Heterozygous $Pah^{enu2/wt}$ animals were used as controls as both have equivalent circulating PHE concentrations. As these Phe exposure was the primary metric being assessed the heterozygote is a valid control. This investigation and our previous investigations continue to demonstrate that Phe-restriction decreases aberrant DNA methylation [27,28]. In this study, we have demonstrated dietary Phe restriction essentially eliminates promoter hypermethylation as this form of aberrant methylation was restricted to the PKU group. We were unable to demonstrate Phe restriction attenuates aberrant methylation in the human PKU brain as tissue from well controlled patients was not attainable. In the current study, we suspect further attenuation of aberrant DNA methylation would be observed if Pah^{enu2} homozygous pups were Phe restricted shortly after birth as opposed to initiating a Phe restriction when animals were weaned (day 20 of life). During the interim from birth to weaning, Phe from mother's milk induces significant hyperphenylalaninemia thus exposing the brain to toxic Phe concentrations during postnatal maturation. We suspect that some aberrant DNA methylation observed in the Diet group owes to Phe exposure early in life as the post-weaning regimen of dietary restriction is rigorous and maintains blood Phe within the therapeutic range (i.e. <360 μM). Persistent aberrant DNA methylation has been described in the offspring of obese women (ref). Additional studies will be required to determine if aberrant DNA methylation persists owing to severe hyperphenylalaninemia during the initial 20 days of life.

A common thread binding our three studies is differential methylation of noncoding RNA genes and the prominence of miRNA genes. In studies using Pah^{enu2} for MPKU and PKU studies both hypermethylation and hypomethylation of miRNA genes was observed. This study identified, in the hyperphenylalaninemic PKU group, hypomethylation of miRNA

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genes within the imprinted Dlk1-Dio3 region on chromosome 12. This region is orthologous to the imprinted Dlk1-Dio3 region at human chromosome 14q32. Re-assessment of data we published on methylation in human PKU identifies involvement of the Dlk1-Dio3 region. Within a 25 kb region in the 3′ of the miRNA cluster, eighteen miRNA genes were identified to have up-regulated expression and among these 8 miRNA genes display hypomethylation. Several miRNA genes were within the MirG locus. High-level expression of MirG is observed during central nervous system development (E9.5–E15.5); however, the role of MirG in postnatal central nervous system development and/or maintenance is unclear [63]. Knock-out of the maternal miR379/miR410 cluster (includes all miRNA genes in Table 1) creates mice with partially penetrant neonatal lethality with defects in energy homeostasis [64]. Parent of origin imprinting is a mechanism to down regulate gene expression by silencing genes on one chromosome. Prader–Willi syndrome is the quintessential example of an imprinting defect [65]. Our data suggests that demethylation of miRNA genes in the repressed paternal allele upregulates their expression to influence post-transcriptional gene regulation. Table 1 identifies genes, likely targeted by overabundance of specific miRNAs and genes with synaptic roles (Kcna4, Kcnd2, Sema5a) and genes involved in axonal processes (Cntn4, Tenm4) were identified. As there is a growing recognition of synaptic dysfunction in PKU, these data support anatomical and other studies in PAH^{enu2} that identify synaptic involvement [66,67]. Targets were not identified for all over-expressed miRNAs. It is possible that the transcriptome within a particular cell type(s) where the miRNA is being expressed may not provide an appropriate target. Additionally, algorithms to identify miRNA targets may not be optimal.

Hypermethylation of miRNA genes (in addition to other noncoding RNA genes) was observed across all of our published studies investigating the role of DNA methylation in the pathology of hyperphenylalaninemia [27,28]. Gene body hypermethylation is typically associated with gene upregulation. Table 2 identifies upregulated expression of the hypermethylated mir23a gene. Interestingly, reflecting the observed up-regulated genes in proximity to methylome modified sites (Table 1), the adjacent mir27a and mir24–2 genes also display upregulated expression. Mir23a, Mir27a, and Mir24–2 genes are within 403 bp on chromosome 8 thus concerted changes in expression are not unexpected. Mir24–2 is likely targeting the Nefm gene. Epigenome studies in the human PKU brain identified down-regulation of the NEFL and NEFH genes [28]. These data in mouse support those earlier observations. Modified expression of potassium channels has been observed in all our PKU epigenome studies. The Kcnk2 gene is among the most highly characterized potassium channels with recognized functions in neurological processes and disease. It is likely that Mir27a is targeting Kcnk2. Mir301 is likely targeting the Kcn4a gene which was also a likely target of Mir543 (Table 1) an upregulated gene is the imprinted Dlk1-Dio3 region.

The hypermethylation of gene promoters is exclusively observed in the hyperphenylalaninemic PKU group. Phe restricted PAH^{enu2} animals are chronically exposed to low-level hyperphenylalaninemia (PHE 150–200 μM); however, promoter hypermethylation is not observed. We assume that dysregulation of the system(s) responsible for promoter hypermethylation is activated by chronic high-level hyperphenylalaninemia. We made a similar observation in our MPKU studies as promoter methylated genes with down-regulated expression was restricted to pups born of

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severely hyperphenylalaninemic females [27]. Gene down-regulation mediated by promoter hypermethylation is widely recognized. Table 2 identifies differentially promoter methylated genes likely to participate in the neuropathology of PKU. The Pde10a gene displays promoter hypermethylation and two-fold down-regulated expression. Phosphodiesterase 10a is enriched in spiny neurons and has implicated roles in autism, Huntington's disease, and Parkinson disease [55,56]. The role of Pde10a in second messenger signaling makes this pathway attractive to PKU pathology. Arhgap21 and Cdc42bpa are components of the Cdc42 pathway of Rho GTPases. Roles for Rho GTPases in synaptogenesis and axon/ dendrite growth are established [59,61].

Finally, in both our mouse studies (MPKU, present study) and our methylome biomarker study in human PKU, we determined that the degree of aberrant hypermethylation increases with severity of hyperphenylalaninemia. In mouse studies, we identified aberrant promoter hypermethylation occurring only with protracted sever hyperphenylalaninemia. Our studies have also demonstrated that altered DNA methylation leads to gene dysregulation either directly by promoter methylation or via dysregulation of miRNAs. All PKU management strategies have sought to lower Phe either by direct dietary restriction or indirect means (i.e. Kuvan, PEG-PAL). Our studies suggest that the epigenome may be a potential therapeutic target to either block or correct consequences of hyperphenylalaninemia. Several demethylating agents are widely prescribed in a variety of cancers. We vision regimens of demethylating agents in management of PKU but these would be far less aggressive than regimens used in cancer applications. Demethylating agents would not correct hypomethylation of miRNA genes in the Dlk1-Dio3 locus; however, DNA methylation and histone epigenetics are inextricably linked. Active DNA demethylation pathways and ATP-dependent chromatin remodeling enzymes alter nucleosome composition contributing to methylome:histone cooperation [68]. Drugs targeting the histone epigenome are presently utilized and these may be candidates to address pathological DNA hypomethylation. Further studies are clearly required to clarify mechanisms of epigenome involvement in PKU pathology; however, our data suggests that the epigenome may provide a novel target to manage PKU and potentially other inborn errors with prominent small molecule intoxication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Upregulated microRNA genes within the imprinted Dlk1-Dio3 region.

MicroRNA gene	Hypometh.	Within MirG locus	Expression[a]	Targets	MirBase score	Target expression	Target function	Refs
Mir369 5p	Yes	Yes	+2.4	Sema5a[c]	93	−1.7	Synaptic, migration	[42,43]
Mir541 5p	Yes	Yes	+4.0	n.t.i				
Mir3072 5p	Yes	Yes	+2.6	n.t.i.				
Mir377 3p	No	Yes	+4.0	n.t.i				
Mir154 3p	No	Yes	+7.1	n.t.i				
Mir496a 3p	No	Yes	+7.1	n.t.i				
Mir409 5p	No	Yes	+3.1	n.t.i				
Mir543 3p	Yes	No	+3.7	Kcna4[b]	98	−2.1	Synaptic, down reg. stress, epilepsy	[44,45]
Mir376a 5p	Yes	No	+3.5	n.t.i.				
Mir376b 3p	Yes	No	+4.7	n.t.i.				
Mir376c 3p	Yes	No	+5.0	Cntn4	86	−2.1	Axon adhesion, toxicity/methylation	[46,47]
Mir654 3p	Yes	No	+4.9	Sema5a[d]	89	−1.7	Synaptic, migration	[42,43]
Mir382	No	No	+5.1	Kcnd2	99	−1.7	Synaptic, dendritic spines, epilepsy	[48–50]
Mir539	No	No	+2.0	n.t.i.				
Mir544	No	No	+4.1	Tenm4	89	−2.0	Axon guidance	[51]
Mir667 3p	No	No	+4.4	n.t.i				
Mir495 3p	No	No	+3.0	n.t.i				
Mir666 3p	No	No	+2.0	n.t.i				

n.t.i. = no target identified.

[a] Expression in fold change vs heterozygous control.

[b] See Table 2.

[c] See Mir654.

[d] See Mir369.

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Table 2

Hypermethylated miRNA genes.

Gene	Expression	Target	Mirbase score	Target expression	Functional note	Refs
Mir23a 3p	+2.3	n.t.i.				
Mir27a 3p[b]	+2.0	Kcnk2	97	−1.5	Multiple neurologic functions	[52,53]
Mir24–2 3p[b]	+2.1	Nefm	93	−1.8	Neurofilament	[54]
Mir301 3p	+2.0	Kcn4a[a]	99	−2.1		[44,45]
Mir487b 3p	+2.1	n.t.i.				
Mir1897 3p	+3.7	n.t.i.				
Mir22 5p	+6.9	n.t.i.				
mir150 5p	+3.6	n.t.i.				

n.t.i. = no target identified.

[a] See Table 1.

[b] mir27a and mir24–2 are not hypermethylated but adjacent to the hypermethylated mir23a.

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Table 3

Promoter hypermethylated genes in the PKU brain.

Gene	Expression fold	Functional note	Refs
Pde10a	−2.0	Enriched in spiny neurons	[55,56]
Itpr1	−2.1	Post-synaptic density, down regulated in As toxicity	[57,58]
Arhgap21	−1.9	Cdc42 system, cytoskeleton, synaptic	[59,60]
Cdc42bpa	−1.8	Cdc42 system, cytoskeleton, cell migration	[61]