RESEARCH ARTICLE

Coupling global methylation and gene expression profiles reveal key pathophysiological events in liver injury induced by a methyl-deficient diet

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Scope: A methyl-deficient diet induces liver injury similar to human nonalcoholic steatohepatitis, one of the main risk factors for the development of hepatocellular carcinoma. Previous studies have demonstrated that this diet perturbs DNA methylation by causing a profound loss of global cytosine methylation, predominantly at heavily methylated repetitive sequences. However, whether methyl deficiency affects the methylation status of gene promoters has not been explored.

Methods and results: Mouse gene expression and CpG island microarrays were used to characterize the gene expression and CpG island methylation profiles in the livers of C57BL/6J mice fed a methyl-deficient diet. We detected 164 genes that were differentially expressed and exhibited an inverse relationship between the gene expression and the extent of CpG island methylation. Furthermore, these genes were associated with altered lipid and glucose metabolism, DNA damage and repair, apoptosis, the development of fibrosis, and liver tissue remodeling. Although there were both increased and decreased levels of CpG island methylation, the number of hypomethylated genes was substantially greater than the number of hypermethylated genes.

Conclusion: The results this study demonstrate that pairing methylation profiles with gene expression profiles is a powerful approach to identify dysregulated high-priority fundamental pathophysiological pathways associated with disease development.

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1 Introduction

Cytosine DNA methylation, a heritable modification of DNA, is one of the best-known and most characterized epigenetic features in mammalian cells [1]. DNA methylation involves the addition of a methyl group onto the 5′ position of a cytosine residue and is an essential event for normal development and proper maintenance of cellular function. Current evidence has linked deregulation of DNA methylation, both decreases and increases, to the development of a wide range of human pathologies [2–4]. It is clear that disease by itself can disturb the normal DNA methylation status; however, the alterations in DNA methylation can also have an impact on the predisposition to pathological states and disease development.

The classic approach for identifying pathological states and the underlying mechanistic basis for their development has focused on histopathological, chemical, biochemical, and molecular examination of affected tissues [5]. In recent years,

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Abbreviations: Ahcy, *S*-adenosylhomocysteine hydrolase; **LINE,** long-interspersed nucleotide element; **MeDIP,** methylated DNA immunoprecipitation; **SAH,** *S*-adenosyl-L-homocysteine; **SAM,** *S*-adenosyl-L-methionine; **SINE,** short-interspersed nucleotide element; **qRT-PCR,** quantitative reverse transcription-PCR

this traditional diagnostic methodology has been complemented successfully by high-throughput and high-resolution microarray gene expression analysis. This approach has improved diagnostic power dramatically and has become a powerful tool for increasing the understanding of the molecular mechanisms of disease pathogenesis, resulting in discovery of novel disease biomarkers [6–8]. Additionally, emerging evidence has demonstrated the utility of genome-scale high-resolution DNA methylation analysis for the identification of new differentially methylated (mainly hypermethylated) candidate genes associated with the development of various human pathological states, including cancer and neuro-developmental disorders [9–11].

It has been suggested recently that combining gene expression profiles with methylation profiles may improve the identification of high-priority disease-specific candidate genes [11]. In light of these considerations, in this study, we paired differentially methylated genes with inversely expressed CpG island-containing genes to determine critical pathophysiological processes associated with liver injury induced by a methyl-deficient diet in mice.

2 Materials and methods

2.1 Animals, diets, and experimental design

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were housed in sterilized cages in a temperature-controlled (24°C) room, with a 12 h light/dark cycle, and given *ad libitum* access to purified water and NIH-31 pelleted diet (Purina Mills, Richmond, IN). At 8 wk of age, the mice were allocated randomly into two groups, one control and one experimental. Mice from the experimental group were maintained on a low methionine (0.18%) diet, lacking in choline and folic acid (Dyets, Bethlehem, PA), for 12 wk. Mice from the control group received control diet containing 0.4% methionine, 0.3% choline bitartrate, and 2 mg/kg folic acid. Diets were stored at 4°C and given *ad libitum*, with twice a week replacement. Five experimental and five control mice were sacrificed at 12 wk after diet initiation. The livers were excised and a slice of the medial lobe was fixed in 10% neutral buffered formalin for 48 h for histopathologic examination. The remaining liver was frozen immediately in liquid nitrogen and stored at −80°C for subsequent analyses. All animal experimental procedures were performed in accordance with an animal study protocol approved by the National Center for Toxicological Research Animal Care and Use Committee.

2.2 Methylated DNA immunoprecipitation (MeDIP) microarray analysis

Genomic DNA was isolated from rat liver and kidney tissues by standard digestion with proteinase K, followed by phenol–chloroform extraction and ethanol precipitation [12]. Methylated DNA immunoprecipitation (MeDIP) was performed as previously described by Weber *et al.* [13]. Briefly, 5 μg of genomic DNA was randomly sheared by sonication to an average length of 0.2–1.0 kb and divided into immunoprecipitated and input portions. DNA from the immunoprecipitated portions was incubated overnight at 4°C with a monoclonal antibody (5 μg) against 5-methylcytosine (Abcam, Cambridge, MA), followed by overnight incubation with Pan-mouse IgG Dyna magnetic beads (Invitrogen, Carlsbad, CA) at 4°C. The methylated DNA/antibody complexes were then digested with proteinase K, and DNA enriched in 5-methylcytosine was recovered by phenol–chloroform–isoamyl alcohol extraction followed by ethanol precipitation. The immunoprecipitated DNA and input DNA pellets were then dissolved in 30 μL water for labeling. Immunoprecipitated DNA and input DNA samples were labeled with cyanine 3-dUTP and cyanine 5-dUTP, respectively, using an Agilent Genomic DNA Labeling Kit Plus (Agilent Technologies, Santa Clara, CA). The labeled DNA samples were purified using Microcon YM-30 columns (Millipore, Billerica, MA) and eluted in 22 μL 10 mM Tris, 1 mM EDTA buffer, pH 8.0. The labeled immunoprecipitated DNA and input DNA were cohybridized to Agilent Mouse 2 × 105 K CpG Island Microarrays (Agilent Technologies), containing ~97 652 oligonucleotide probes covering 16 030 CpG islands (average probe spacing ~100 bp), according to the manufacturer's protocol. Microarrays were scanned on an Agilent DNA microarray scanner (Agilent Technologies) and the resulting images were analyzed with Agilent Feature Extraction Software (Version 10.5.1). The resulting data files were visualized with Agilent Genomic Workbench (Version 5.0). The data files were loaded into the Array Track database [14] for further statistical analyses.

2.3 RNA extraction and microarray gene expression analysis

Total RNA was extracted from liver tissue using mRNAeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The gene expression profile was determined utilizing Agilent whole genome 4 × 44 K mouse microarrays (Agilent Technologies). Sample labeling and microarray processing were performed as detailed in the "One-Color Microarray-Based Gene Expression Analysis" Version 1.0 (Agilent Technologies) protocol. The hybridized slides were scanned with an Agilent DNA Microarray scanner (Agilent Technologies) at 5 μm resolution. The resulting images were analyzed by determining the Cy3 fluorescence intensity of all gene spots (features) on each array using the Agilent Feature Extraction Software (Version 9.5). The raw data were then loaded into the ArrayTrack database [14]. The median fluorescence intensity of all the pixels within one feature was taken as the intensity value for that feature. The raw intensity values were then normalized using 75 percentile channel scaling normalization using ArrayTrack. The list of

differentially expressed genes was generated using a *t*-test at *p*-value <0.01 and a fold change at >1.5. The Ingenuity Pathway Analysis Software (Ingenuity Systems, Redwood City, CA) was used for pathway analyses.

2.4 Quantitative reverse transcription-PCR

The levels of *S*-adenosylhomocysteine hydrolase (*Ahcy*), growth arrest and DNA-damage-inducible protein 45 a (*Gadd45a*), and *Gadd45b* gene transcripts were determined by quantitative reverse transcription-PCR (qRT-PCR) using TagMan Gene Expression Assays (Applied Biosystems, Forrest City, CA) according to the manufacturer's protocol. Relative quantification of gene expression was performed by using glyceraldehyde-3-phosphate dehydrogenase as an internal control. The $2^{-\Delta\Delta Ct}$ method was used for calculating the relative amount of the target RNA [15]. The qRT-PCR was performed at least three times and always included a no-template sample as a negative control.

2.5 MeDIP-quantitative PCR assay

A MeDIP assay, combined with qPCR, was used to assess quantitatively the methylation status of long-interspersed nucleotide elements-1 (LINE-1) and short-interspersed nucleotide elements-B2 (SINE-B2) in the livers. MeDIP was performed as described above. Purified DNA from the immunoprecipitated DNA complexes and from input DNA was analyzed by qPCR on an Applied Biosystems 7900 Real-Time PCR System using the primer sets described by Martens *et al.* [16]. The relative changes in the extent of LINE-1 and SINE-B2 methylation were determined by measuring the amount of LINE-1 and SINE-B2 in immunoprecipitated DNA after normalization to the input DNA.

2.6 Determination of hepatic triglycerides content

Hepatic triglycerides were extracted by homogenizing 20 mg of liver tissue in 500 μL of isopropyl alcohol. A 4 μL aliquot of the extract was used for subsequent analysis. The level of triglycerides was determined by using a Wako L-Type TG-M Assay Kit (Wako Diagnostic, Richmond, VA) according to the manufacturer's instruction.

2.7 Immunohistochemistry

The extent of apoptosis in the liver sections was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling of DNA fragments using an ApopTag Peroxidase *In Situ* Apoptosis Detection Kit obtained from Serologicals (Norcross, GA) as described previously [17].

2.8 Determination of hepatic *S*-adenosyl-L-methionine, *S*-adenosyl-L-homocysteine contents

The determination of *S*-adenosyl-L-methionine (SAM) and *S*-adenosyl-L-homocysteine (SAH) content in liver tissue extracts was performed by a HPLC method, with coulometric electrochemical detection, as previously described by Melnyk *et al.* [18].

2.9 Statistical analyses

Results are presented as mean $\pm$ SD. Comparisons between control and methyl-deficient mice were made by Student's *t*-test. *p*-Values <0.05 were considered significant.

3 Results and discussion

3.1 Differential gene-specific methylation changes in the livers of mice fed a methyl-deficient diet

In our previous studies, we have demonstrated that feeding a methyl-deficient diet caused loss of global DNA methylation in the livers of rats and mice [19, 20]. Figure 1 shows a similar hypomethylation effect of a methyl-deficient diet on the status of hepatic LINE-1 and SINE-B2 repetitive elements. The levels of methylation of LINE-1 and SINE-B2 repetitive elements in the livers of mice fed the methyl-deficient diet decreased by 42 and 45%, respectively, as

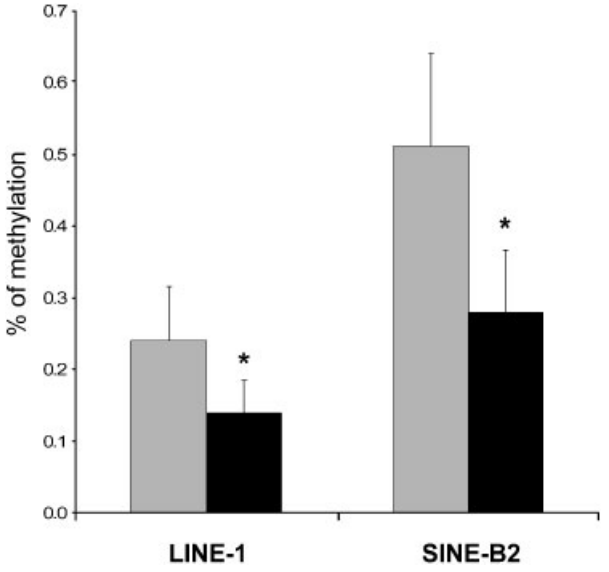

Figure 1. Methylation levels of LINE-1 and SINE-B2 repetitive elements in the livers of C57BL/6J mice fed a methyl-deficient diet for 12 wk and age-matched control mice. *Significantly different from control mice ($n=5$, mean $\pm$ SD). Gray bars – control mice; black bars – methyl-deficient mice.

compared with age-matched control mice. In contrast to the established effects of methyl deficiency on the level of global DNA methylation, the effect of methyl-deficient diets on the methylation status in regions other than repetitive DNA elements has not been explored. Through the use of immunoprecipitation-enriched methylated DNA coupled with CpG microarrays, we identified 1798 genes that were differentially methylated (760 hypermethylated and 1038 hypomethylated) in the livers of mice fed the methyl-deficient diet compared with the livers of control mice (Supporting Information Table 1).

3.2 Microarray analysis of gene expression in the livers of mice fed a methyl-deficient diet

Microarrays were also used to characterize the gene expression profiles in the livers of C57BL/6J mice fed a methyl-deficient diet as compared to age-matched control mice. The analyses indicate there were 1750 differentially expressed genes (344 downregulated and 1396 upregulated; Supporting Information Table 2) and that these genes were associated with diverse cellular processes, including cellular growth and differentiation, cell-to-cell signaling and interaction, lipid metabolism, small molecule biochemistry, molecular transport, and drug metabolism. Interestingly, the number of upregulated genes is substantially greater than the number of downregulated genes, which corresponds to the greater number of hypomethylated genes (Supporting Information Table 1).

3.3 Concordance of differentially methylated and differentially expressed genes

To identify and prioritize genes that may be involved in the liver injury induced by the methyl-deficient diet, we compared the differentially methylated genes (Supporting Information Table 1) to the differentially expressed genes (Supporting Information Table 2) in the livers of mice fed the methyl-deficient diet. We detected 164 genes that were differentially expressed and exhibited an inverse relationship between the gene expression and the extent of CpG island methylation (Table 1, Supporting Information Table 3). Table 1 summarizes that feeding a methyl-deficient diet caused both CpG island hypomethylation (114 genes) and hypermethylation (50 genes). Importantly, 139 of the 164 differentially methylated and inversely expressed genes had changes in methylation in CpG islands located in their promoter regions, with 41 genes being hypermethylated and 98 being hypomethylated (Table 1).

To examine further the relationship between these genes and biological processes, we used a network analysis approach (Table 2). The network analysis revealed that the major cellular and molecular processes impacted by the methyl-deficient diet were associated with altered lipid and glucose metabolism, DNA damage and repair, apoptosis, the development of fibrosis, and liver tissue remodeling (Table 2).

Table 1. Differentially expressed genes with associated changes in methylation of CpG islands in the livers of C57BL/6J mice fed control and a methyl-deficient diet for 12 wk

Location[a)]	Methylation	Number of genes
Total	Hypermethylated	50
	Hypomethylated	114
Promoter	Hypermethylated	41
	Hypomethylated	98
Inside	Hypermethylated	9
	Hypomethylated	16

a) Total: entire genome; Promoter: immediate 5′-promoter regions of genes including the transcription start site; Inside: within coding region of the gene, excluding first exon.

Table 2. Functions of differentially methylated and inversely expressed genes in the livers of C57BL/6J mice fed control and a methyl-deficient diet for 12 wk

Category function and number of genes	Genes
Impaired lipid metabolism; 9	Nr5a2, Zhx2, Nr3c1, Mtmr4, Flt1, Fads3, Fa2h, Plcd1, Prdm16
Impaired glucose metabolism; 9	Ero1lb, Ptprn, Pgd, Igf2bp2, Pdk3, Ptprs, Me2, Tkt, Pfkfb2
DNA damage and repair; 9	Tor1aip1, Ssbp4, Hmgn2, Eaf2, Ddit4l, Rad51, Rad23b, Gadd45b, Gpx3
Apoptosis; 8	Bat3, Syvn1, Stk11ip, Bcl2, Fgf12, Eaf2, Bambi, Fos
Liver development, remodeling, and liver homeostasis; 7	Onecut2, Smarca2, Pex12, Nr5a2, Itgb4, Slc3a2, Vegfa
Insulin resistance; 5	Ptprn, Igf2bp2, Me2, Slc16a1, Prkcb1
Gene transcription regulation; 6	Smarca2, Lhx6, Cnot8, Zhx2, Tcea2, Tcf21

The dysregulation of these processes was further confirmed by series of independent experiments. Specifically, histomorphological analyses indicated profound changes associated with altered lipid metabolism as evidenced by severe steatosis and triglyceride accumulation (Figs. 2A and C), necrotic and apoptotic cell death (Figs. 2A and B), and the upregulation of fibrosis-relevant genes (Fig. 2D) in the livers of C57BL/6J mice fed the methyl-deficient diet compared with control mice. Interestingly, it is well established that dysregulation of the exact same processes is associated with pathogenesis of nonalcoholic steatohepatitis [21–23].

The results of the previous studies demonstrated that diets lacking substrates or cofactors in one-carbon metabo-

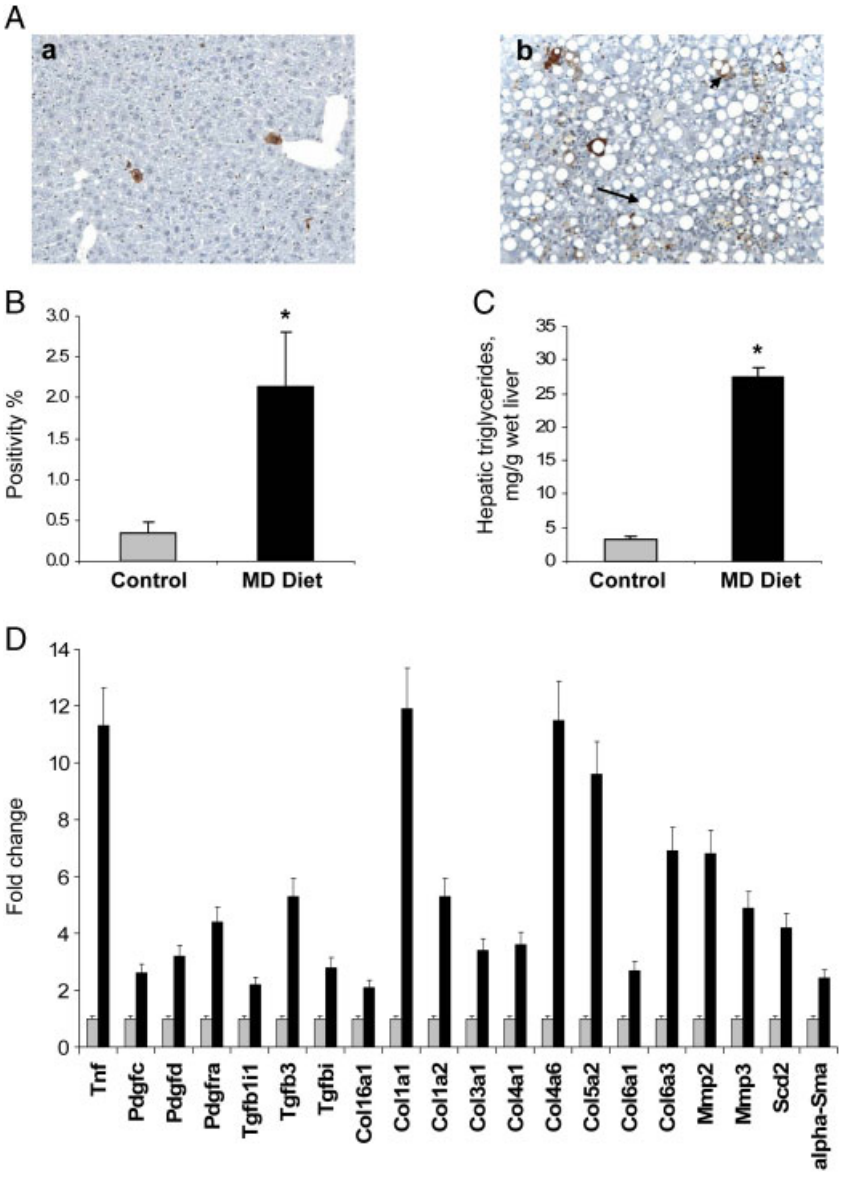

Figure 2. Histomorphological changes, triglyceride concentrations, and expression of fibrosis-related genes in the livers of C57BL/6J mice fed a methyl-deficient diet (MD) for 12 wk and age-matched control mice. (A) Apoptotic cell death and lipid accumulation in the livers of control mice and mice fed a methyl-deficient diet. (a) Liver from a control C57BL/6J mouse. (b) Liver from a methyl-deficient C57BL/6J mouse: apoptotic bodies as detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (brown color; short arrow) and diffuse marked macrovesicular steatosis (long arrow). (B) Quantitative analysis of apoptotic bodies in liver sections of control and methyl-deficient mice. (C) Hepatic triglycerides concentrations in the livers of control mice and mice fed a methyl-deficient diet. (D) Expression of fibrosis-related genes, as detected by qRT-PCR, in the livers of control mice and mice fed a methyl-deficient diet. Gray bars – control mice; black bars – methyl-deficient mice. Data are presented as mean $\pm$ SD ($n = 5$). *Significantly different from control mice.

lism perturbed DNA methylation by causing a profound loss of global cytosine methylation, predominantly at repetitive sequences [20, 24–26]. In this study, we demonstrated that feeding a methyl-deficient diet resulted in profound alterations in gene-specific methylation in addition to hypomethylation of repetitive elements. Interestingly, we detected a substantial number of genes with either an increased or decreased level of CpG island methylation, and the expression of many of these genes correlated inversely with the change in promoter methylation (Table 1). Although there were both increased and decreased levels of CpG island methylation, the number of hypomethylated genes was substantially greater than the number of hypermethylated genes (Table 1).

DNA hypomethylation induced by methyl-deficient lipogenic diets has been attributed to a lack of dietary methyl donors, which is a resultant decrease of intracellular levels of SAM [24, 27], the primary universal donor of methyl groups for all cellular methylation reactions [28]. The results of our and other researchers' studies suggest that impaired SAM metabolism [29, 30] and compromised integrity of DNA [31] are the main factors causing methyl deficiency-induced loss of DNA methylation. As outlined below, the results of this study provided additional evidence that support this suggestion.

One of the most downregulated and hypermethylated genes, as detected by microarray gene expression profiling and confirmed by qRT-PCR, was *Ahcy* (Supporting Information Table 3, Fig. 3), which encodes the only mammalian enzyme known to catalyze hydrolysis of SAH [28, 32]. The inhibition of Ahcy has been suggested to affect cellular methylation reactions, including DNA methylation [28], *via*

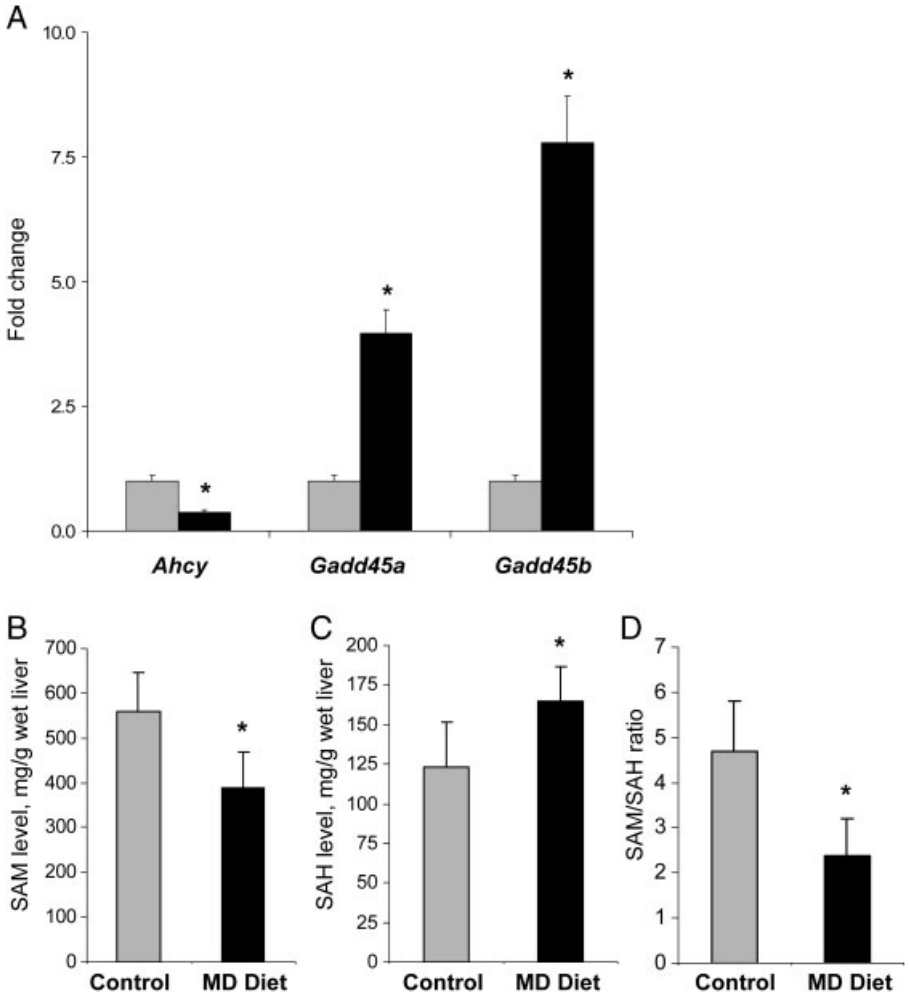

Figure 3. Expression of *Ahcy*, growth arrest and *Gadd45a*, and *Gadd45b* genes (A) and levels of SAM (B), SAH (C), and SAM/SAH ratio (D) in the livers of C57BL/6J mice fed a methyl-deficient (MD) diet for 12 wk and age-matched control mice. Data are presented as mean ± SD ($n = 5$). *Significantly different from control mice. Gray bars – control mice; black bars – methyl-deficient mice.

increased accumulation of SAH, a potent inhibitor of all cellular methyltransferases, including DNA methyltransferases [33, 34]. Figure 3 shows that down-regulation of *Ahcy* (Fig. 3A) was associated with a substantially increased intracellular level of SAH (Fig. 3C) in the livers of mice fed a methyl-deficient diet.

Gadd45b was also one of the most hypomethylated and upregulated genes in the livers of mice fed a methyl-deficient diet (Supporting Information Fig. 3 and Fig. 3). Recent reports have established that Gadd45a and Gadd45b function as key regulators of active DNA demethylation by promoting DNA repair, which erases DNA methylation marks at the promoters of functional genes [35, 36]. Interestingly, intense upregulation of the hepatic *Gadd45b* gene has been observed during phenobarbital-induced liver carcinogenesis [37], characterized by significant DNA methylation, mainly hypomethylation, changes [38, 39].

Interestingly, in this study, we detected 41 genes being hypermethylated in the livers of mice fed a methyl-deficient diet (Table 1). Previous studies have reported similar findings on gene-specific hypermetylation in the livers of methyl-deficient animals [40, 41]. Several possible explanations exist for the mechanism of gene hypermethylation induced by methyl-deficient diet. Particularly, upregulation of the maintenance DNA methyltransferase Dnmt1 and/or *de novo* DNA methyltransferases Dnmt3a and Dnmt3b, frequently found in methyl-deficient animals [20, 41, 42], may cause abnormal methylation of unmethylated CpG islands. Additionally, loss of histone H4 lysine 20 and histone H3 lysine 9 trimethylation associated with methyl deficiency [20, 43] and a consequent heterochromatin relaxation may facilitate the access of DNA methyltransferases to unmethylated DNA regions.

4 Concluding remarks

The results of this study demonstrate that pairing methylation profiles with gene expression profiles that exhibited an inverse relationship between the gene expression and the extent of CpG island methylation is a powerful approach to identify dysregulated high-priority fundamental pathophysiological pathways associated with disease development. Additionally, two genes, *Ahcy* and *Gadd45b*, which were

identified from coupling of methylation with gene expression data, shed light on the underlying mechanisms of cytosine demethylation under methyl-deficient conditions.

The authors have declared no conflict of interest.

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