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Difference in expression of hepatic microRNAs miR-29c, miR-34a, miR-155, and miR-200b is associated with strain-specific susceptibility to dietary nonalcoholic steatohepatitis in mice

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Abstract

The importance of dysregulation of microRNA (miRNA) expression in nonalcoholic steatohepatitis (NASH) has been increasingly recognized; however, the association between altered expression of miRNAs and pathophysiological features of NASH and whether or not there is a connection between susceptibility to NASH and altered expression of miRNAs are largely unknown. In the present study, male inbred C57BL/6J and DBA/2J mice were fed a lipogenic methyl-deficient diet that causes liver injury similar to human NASH, and the expression of miRNAs and the level of proteins targeted by these miRNAs in the livers were determined. The administration of the methyl-deficient diet triggered NASH-specific changes in the livers of C57BL/6J and DBA/2J mice with a magnitude being more severe in DBA/2J mice. This was evidenced by a greater extent of expression of fibrosis-related genes in the livers of methyl-deficient DBA/2J mice. The development of NASH was accompanied by prominent changes in the expression of miRNAs, including miR-29c, miR-34a, miR-155, and miR-200b. Interestingly, changes in the expression of these miRNAs and protein levels of their targets, including Cebp-β, Socs 1, Zeb-1, and E-cadherin, in the livers of DBA/2J mice fed a methyl-deficient diet were more pronounced as compared to the C57BL/6J mice. These results demonstrate that alterations in expression of miRNAs are a prominent event during development of NASH induced by methyl deficiency and strongly suggest that severity of NASH and susceptibility to NASH may be determined by variations in miRNA expression response. More importantly, our data provide a

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mechanistic link between alterations in miRNA expression and pathophysiological and pathomorphological features of NASH.

Keywords

Methyl-deficient diet; mice; microRNA; nonalcoholic steatohepatitis; strain-difference

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), a hepatic manifestation of the metabolic syndrome, and NASH, a progressive form of NAFLD, are two of the most common chronic liver diseases in the United States and worldwide.[1–3] These two pathological states have been shown to progress to cirrhosis and hepatocellular carcinoma.[3] The successful prevention and treatment of NASH relies on the ability to identify vulnerable subpopulations and detect the disease early and greatly depends on a better understanding of the underlying molecular mechanisms involved in NASH pathogenesis. It is generally accepted that dysregulation of several physiological processes, including lipid metabolism, insulin resistance, immune response, inflamation, and oxidative stress, is associated with NASH pathogenesis.[1,2,4] Additionally, the results of recent studies have established a critical role of miRNAs in NASH.[5–8] Specifically, expression profiling studies have identified several differentially expressed miRNAs, including miR-21, miR-34a, miR-122, miR-155, in human[5] and mouse NASH,[6–8] indicating their role in the pathogenesis of NASH. However, despite the current efforts and accomplishments in NASH experimental and clinical research and NASH clinical management, major gaps remain in understanding the etiology and progression of the disease at the basic level,[2,3] particularly, whether or not the different susceptibility to NASH is associated with an altered expression of miRNAs.

The differential susceptibility of mice to NASH has been attributed to strain variations in the expression of genes involved in hepatic lipid metabolism,[9] triglyceride levels,[10] oxidative stress,[11] and c-Myc and NFκB signaling networks.[8] Recently, we demonstrated that strain differences in the liver epigenomic status are associated with individual susceptibility to hepatic steatosis.[12] Specifically, feeding DBA/2J mice a lipogenic methyl-deficient diet resulted in more prominent epigenetic and pathomorphological changes in the livers as compared to C57BL/6J mice.[12]

The present study was undertaken to: (1) determine the association between altered expression of miRNAs and pathophysiological features of NASH in mice; and (2) to determine whether or not strain-specific susceptibility of mice to NASH is associated with differences in miRNA expression.

MATERIALS AND METHODS

Animals, diets, and experimental design

Male C57BL/6J and DBA/2J mice (Jackson Laboratory, Bar Harbor, ME) were housed in sterilized cages in a temperature-controlled (24°C) room, with a 12 hour light/dark cycle, and given *ad libitum* access to purified water and NIH-31 pelleted diet (Purina Mills,

Richmond, IN). At eight weeks of age, the mice from each strain were allocated randomly into two groups, one control and one experimental. The mice from the experimental group were maintained on a low methionine (0.18%) diet, lacking in choline and folic acid (Dyets, Inc, Bethlehem, PA), for 12 weeks. The mice from the control group received diet supplemented with 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 6 and 12 weeks after diet initiation. The livers were excised, frozen immediately in liquid nitrogen, and stored at −80°C for subsequent analyses. All animal experimental procedures were performed in accordance with the animal study protocol approved by the National Center for Toxicological Research Animal Care and Use Committee.

Necropsy, tissue processing, and histopathology

The liver tissue samples were processed, analyzed, and scored for steatosis, hepatocellular degeneration, inflammation, hepatocellular karyocytomegaly and oval cell proliferation as previously described.[12]

RNA extraction and microarray expression analysis

Total RNA was extracted from liver tissue using miRNAeasy Mini kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. The gene expression profile was determined utilizing Agilent whole genome 4×44K mouse microarrays (Agilent Technologies, Santa Clara, CA). Sample labeling and microarray processing was performed as detailed in the "One-Color Microarray-Based Gene Expression Analysis" version 1.0 (Agilent Technologies) protocol. The miRNA microarray analysis was performed by LC Sciences (Houston, TX), as reported previously in detail.[13,14]

miRNA expression analysis by quantitative reverse transcription real-time PCR (qRT-PCR)

Total RNA (200 ng) was used for qRT-PCRs of the miR-29c, miR-34a, miR-122, miR-155, miR-192, miR-200b, miR-203, and miR-221 utilizing TaqMan miRNA assays (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. snoRNA202 was used as an endogenous control. The relative amount of each miRNA was measured using the $2^{-\Delta\Delta Ct}$ method.[15] All qRT-PCR reactions were conducted in triplicate and repeated twice.[13,14]

Western blot analysis of protein expression

The levels of DNA methyltransferase 1 (Dnmt1), Dnmt3a, Dnmt3b, CCAAT enhancer binding protein beta (C/ebp-β), C/ebp-α, suppressor of cytokine signaling-1 (Socs1), E-cadherin (Cdh1), and β-actin proteins in the livers of control mice and mice fed the methyl-deficient diet were determined as described previously.[12]

Transfection of mouse primary hepatocytes with pre-miR-155 and pre-miR-200b

Mouse (CD-1) primary hepatocytes (Invitrogen, Carlsbad, CA) were transfected with 20 nM of either pre-miR-155 or pre-miR-200b (20 nM each; Applied Biosystems), in three independent replicates, using Lipofectamin™ 2000 transfection reagent (Invitrogen)

according to the manufacturer's instructions. Primary hepatocytes transfected with scrambled RNA oligonucleotide served as a control. At 48 hours post-transfection the media was changed and transfection was repeated. Forty eight hours after the second transfection, adherent cells were harvested by mild trypsinization, washed in phosphate-buffered saline (PBS), and the viability of cells was determined. The cells were then immediatelly frozen at −80°C for subsequent analyses.

Isolation of hepatic DNA

DNA was extracted by a slight modification of the method reported previously by Nakamura *et al*.[16]

Liquid chromatography combined with electrospray tandem mass spectrometry (LC-MS/MS) analyses of 8-oxodeoxyguanosine (8-oxodG)

The measurement of 8-oxodG by LC-MS/MS was adapted from the method described by Powel et al.[17] DNA samples (30–50 μg) were dissolved in 80 mM Tris-HCl buffer/20 mM $MgCl_2$ (pH 7.0) containing 2.75 pmol [$^{15}N_5$]8-OH-dG internal standard and enzymatically hydrolyzed to individual nucleosides by incubation with 40 U of DNase I, 2.7 mU of phosphodiesterase I, and 2 U of alkaline phosphatase. The released 8-oxodG was purified by reverse-phase high-performance liquid chromatography (HPLC) using a Beckman Ultrasphere ODS C18 column (5 μm, 4.6 × 250 mm, Beckman, Fullerton, CA). The isocratic mobile phase was 7% MeOH in 10 mM ammonium formate (pH 4.3) with a flow rate of 1 ml/min. Fractions were collected 2 min intervals preceding and following the elution of 8-oxodG. The quantitative analysis of 8-oxodG by LC-MS/MS was performed with a 1100 capillary high-performance liquid chromatograph (Agilent, Wilmington, DE) coupled to a TSQ-Quantum triple quad mass analyzer (ThermoFinnigan, San Jose, CA). A 3.5-μm Zorbax XDB-C18 column (0.3 × 150 mm; Agilent) was operated with a binary mobile phase of 2% 10 mM ammonium formate (pH 4.3) and 98% methanol followed by a linear gradient increase of methanol from 2% to 30% from 0 to 5 min, holding at 30% for 10 min, and immediately returned to initial conditions that were held for 15 min. Both 8-oxodG and [$^{15}N_5$]8-OH-dG internal standard were detected by single reaction monitoring of the transition of nucleoside to base adduct *m/z* 284.2 to 168.2 and *m/z* 289.2 to 173.2, respectively. MS conditions were as follows: spray voltage, 2200 V and heated capillary temperature, 350°C. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) and were of ACS grade or higher.

Determination of genomic and mitochondrial DNA damage

The extent of genomic DNA damage was determined by measuring the levels of histone H2AX phosphorylation and histone H4 lysine 20 dimethylation by Western immunoblotting.[12,18] The extent of mitochondrial DNA was determined by a quantitative PCR technique (qPCR).[19,20]

Statistical analyses

Results are presented as mean ± S.E.M. Statistical analyses were conducted by two-way ANOVA, using diet and strain as fixed factors. Pair-wise comparisons were conducted by the Student-Newman-Keuls test. P-values <0.05 were considered significant.

RESULTS

Expression of fibrosis-relevant genes in the livers of C57BL/6J and DBA/2J mice fed a methyl-deficient diet

The results of our previous study demonstrated that DBA/2J mice are more susceptible to NASH induced by methyl-deficiency than C57BL/6J.[12] In order to further confirm that finding, we analyzed the histomorphological changes and the expression level of several critical fibrosis-associated genes,[21] including tumor necrosis factor alpha (*Tnf*), procollagen (*Col*) genes, platelet-derived growth factor (*Pdgf*) C and D, platelet-derived growth factor receptor (*Pdgfr*) alpha, and matrix metallopeptidase (*Mmp*) 2 and 3, in the livers of C57BL/6J and DBA/2J mice fed the methyl-deficient diet for 12 weeks. Feeding a methyl-deficient diet resulted more pronounced pathological changes in the livers of DBA/2J mice as compared to C57BL/6J mice (Suppl. Fig. 1) and a greater up-regulation of *Col1a1*, *Col1a2*, *Col3a1*, *Col4a6*, *Col5a2*, *Col6a1*, *Col16a1*, *Pdgfc*, *Pdgfd*, *Pdgfr*α, *Mmp2*, and *Mmp3* genes in livers of methyl-deficient DBA/2J mice than in C57BL/6J mice (Suppl. Table 1).

Effects of methyl-deficient diet on the extent of genomic and mitochondrial DNA damage in the livers of C57BL/6J and DBA/2J mice

Another well-documented fundamental event in the development of NASH is mitochondrial dysfunction and induction of oxidative stress.[2,4,22] In view of this, we studied the extent of genomic and mitochondrial DNA damage in the livers of C57BL/6J and DBA/2J mice fed a methyl-deficient diet. Fig. 1A shows that in livers of control mice, the level of 8-oxodG did not change over the 12 week period. Administration of the methyl deficient diet to DBA/2J mice resulted in progressive accumulation of 8-oxodG in hepatic DNA with a difference being significant after 6 and 12 weeks of deficiency (Fig. 1A). In contrast, in the livers of C57BL/6J mice fed a methyl-deficient diet the levels of 8-oxodG slightly increased after 12 weeks only.

In addition to the increased levels of 8-oxodG in DNA, the livers from methyl-deficient C57BL/6J and DBA/2J mice were characterized by an increased level of histone H2AX phosphorylation (Fig. 1B) and histone H4 lysine 20 dimethylation (data not shown), dependable markers for DNA damage. However, the magnitude of DNA damage in the livers of DBA/2J mice was more pronounced compared to the C57BL/6J strain, with the difference being significant after 12 weeks on a diet (Fig. 1B). Feeding mice the methyl-deficient diet also resulted in mitochondrial DNA damage, with extent being greater in DBA/2J (Fig 1C).

Dysregulation of miRNAs in the livers of C57BL/6J and DBA/2J mice fed a methyl-deficient diet

miRNA microarrays were used to analyze the miRNA expression profiles in the livers of control C57BL/6J mice and C57BL/6J mice fed a methyl-deficient diet that causes a pathological state similar to human NASH.[6,23,24] It is believed that that this model is the best-established experimental model to study NASH.[25] We identified 74 miRNAs (40 up-regulated and 34 down-regulated) that were differentially expressed ($p<0.05$) after 12 weeks of methyl deficiency (Suppl. Fig. 2). The expression of the most altered miRNAs (miR-29c, miR-34a, miR-122, miR-155, miR-192, miR-200b, miR-203, and miR-221) in the livers of C57BL/6J mice fed the methyl-deficient diet was confirmed by qRT-PCR. Additionally, we analyzed the expression of these miRNAs in the livers of control DBA/2J mice and DBA/2J fed the methyl-deficient diet. Interestingly, we detected an increased expression of miR-34a, miR-155, and miR-200b in the livers of both C57BL/6J and DBA/2J mice fed the methyl deficient diet for 12 weeks, with a magnitude being significantly greater in DBA/2J mice (Table 1). Specifically, the expression of miR-34a, miR-155, and miR-200b in the livers of DBA/2J mice fed a methyl-deficient diet was 4.9, 5,9, and 2.9 times greater than in C57BL/6J mice. In contrast, the livers of C57BL/6J mice fed the methyl-deficient diet were characterized by pronounced down-regulation of miR-29c, whereas its expression in the livers of DBA/2J mice did not change.

Protein level of miRNA targets in the livers of C57BL/6J and DBA/2J mice fed a methyl-deficient diet

To establish the significance of miRNA dysregulation in the pathogenesis of NASH, specifically, whether or not the aberrant expression of miRNAs is associated with pathophysiological features of NASH-induced liver injury, we determined protein levels of the targets of the most differentially expressed miRNAs. The results of recent studies have demonstrated that the targets for miR-155 are C/ebp-β,[6,26] a liver-enriched transcription factor, and Socs1 tumor suppressor.[27] Fig. 2 shows that the increased expression of miR-155 in the livers of C57BL/6J and DBA/2J methyl-deficient mice (Fig. 2A) was associated with the decreased levels of C/ebp-β and Socs1 proteins (Fig 2B). Furthermore, the increase in miR-155 and decrease in C/ebp-β and Socs1 was more pronounced in the livers of DBA/2J mice (Fig. 2).

The experimentally confirmed targets for miR-29 family are *de novo* DNA methyltransferases Dnmt3a and Dnmt3b.[28] Fig. 3 shows that down-regulation of miR-29c (Fig. 3A) was associated with increased protein levels of Dnmt3a and Dnmt3b in the livers of C57BL/6J mice fed the methyl-deficient diet (Fig. 3B) as compared to age-matched control C57BL/6J mice. In contrast, the expression of miR-29 and the level of Dnmt3a and Dnmt3b proteins in the livers of DBA/2J mice fed the methyl-deficient diet did not change (Fig. 3).

Feeding C57BL/6J and DBA/2J mice a methyl-deficient diet for 12 weeks resulted in substantial up-regulation of miR-200b (Fig. 4A) and down-regulation of its Zeb1 target[29] (Fig. 4B), a well-known transcriptional repressor of E-cadherin, by 26% and 52%, respectively. The decreased Zeb1 levels in the livers of C57BL/6J and DBA/2J mice

exposed to methyl deficiency were accompanied consequently with 2.2 and 3.3 times up-regulation of E-cadherin (Cdh1) protein level, respectively, as compared to age-matched control mice (Fig 4B). Interestingly, a greater increase of E-cadherin protein in the livers of methyl-deficient DBA/2J mice corresponded to a greater expression of miR-200b. In contrast, the protein level of C/ebp-α, another predicted target for mouse miR-200b, did not change in the livers of C57BL/6J mice fed the methyl-deficient diet, and slightly decreased in DBA/2J mice (Fig. 4B).

Ectopic up-regulation of miR-155 and miR-200b in mouse primary hepatocytes

In order to confirm that altered levels of C/ebp-β, Socs1, and E-cadherin proteins in the livers of mice fed a methyl-deficient diet are associated with aberrant expression of miR-155 and miR-200b, we transfected mouse primary hepatocytes with these microRNAs. Fig. 5 shows that ectopic up-regulation of miR-155 in mouse primary hepatocytes was accompanied by a decreased level of C/ebp-β and Socs1 proteins, while the level of these proteins remained unchanged in hepatocytes transfected with miR-200b (Fig. 5). Similarly, transfection of hepatocytes with miR-200b up-regulated the level of E-cadherin (Cdh1) only.

DISCUSSION

The current view on the pathogenesis of NASH favors a model in which NASH is associated with altered hepatic lipid metabolism, inflammation, and oxidative stress; however, the underlying mechanisms of these pathophysiological processes are not fully understood. The results of the present study demonstrate that feeding of C57BL/6J and DBA/2J mice a methyl-deficient diet, which is known to induce NASH in mice,[6,23,24] results in aberrant expression of miRNAs, specifically miR-29c, miR-34a, miR-122, miR-155, miR-192, miR-200b, miR-203, and miR-221. Similar findings have been reported recently by Wang et al.[6] during NASH-induced hepatocarcinogenesis in mice fed a choline-deficient and amino acid-defined diet. Additionally, the present study demonstrates the association of aberrant expression of miRNAs with fundamental pathophysiological features of NASH. These data indicate that dysregulation of miRNA expression is a critical event in the pathogenesis of NASH. More importantly, the results of our study demonstrate that strain-specific differences in susceptibility of mice to NASH are associated with differences in miRNA expression. Specifically, DBA/2J mice, a strain that develops more prominent NASH-specific changes, evidenced by more severe pathomorphological changes[12] (Suppl. Fig. 1) and greater up-regulation of fibrosis-related genes (Suppl. Table 1) as compared to C57BL/6J mice, were characterized by a greater magnitude of miRNA expression changes in the livers.

The key pathophysiological features of NASH are an altered lipid metabolism and hepatocyte apoptosis.[30,31] The results of our study demonstrate a profound down-regulation of miR-122, a liver–specific miRNA, which is important for normal lipid metabolism,[32,33] and dramatic up-regulation of miR-34a, a critical regulator of apoptosis,[34] in the livers of mice fed a methyl-deficient diet (Table 1). These changes correspond to more pronounced triglyceride accumulation and more severe cell death in the DBA/2J mice fed a methyl-deficient diet as detected in our previous study[12] and present study. Several recent reports

have shown that miRNAs miR-122 and miR-34a are two of the most frequently dysregulated miRNAs in NASH.[5,35] Specifically, increased expression of miR-34a and decreased expression of miR-122 have been demonstrated recently in the livers of ob/ob mice, a well-established hyperglycemic model of NAFLD, and in streptozotocin-induced type 1 diabetic mice.[35] These are two main risk factors for the development of NAFLD and NASH in humans. Indeed, similar dysregulation in expression of miR-34a and miR-122 has been reported in human NASH.[5]

The results of a recent study have demonstrated the importance of miR-155 up-regulation in NASH-induced hepatocarcinogenesis.[6] miR-155 targets two critical tumor suppressors C/ebp-β and Socs1[26,27] that are frequently down-regulated in hepatocellular carcinomas and hepatoblastomas.[36–38] Moreover, in our previous study we demonstrated that inhibition of Socs1 expression is a critical event in hepatocarcinogenesis.[39] In addition to the crucial role of Socs1 as a negative regulator of cytokine-induced signal transduction pathway,[40] Socs1 acts as an important mediator of cellular oxidative stress.[41] It is well-known that several hepatic genes, including methionine adenosyltransferase 1a (*Mat1a*) and catalase, involved in regulation of oxidative stress and frequently down-regulated in NASH also,[42–44] are regulated by C/ebp-β.[45,46] This corresponds to the results of our study that demonstrate the induction of oxidative stress (Figure 5), another key event in the pathogenesis of NASH,[2,4,22] in the livers of mice fed the methyl-deficient diet.

In a previous study,[12] we demonstrated that feeding DBA/2J mice a lipogenic methyl-deficient diet resulted also in more prominent epigenetic changes in the livers of DBA/2J mice as compared to C57BL/6J mice. Specifically, administration of a methyl-deficient diet to C57BL/6J and DBA/2J mice resulted in a pronounced demethylation of genomic DNA and repetitive elements, with the magnitude being greater in DBA/2J mice. The results of our present study indicate that the greater degree of demethylation of genomic DNA and, particularly, repetitive elements in the livers of methyl-deficient DBA2J mice compared to methyl-deficient C57BL/6J mice may be associated with the miR-29-driven differences in DNMTs expression (Figure 3). It has been shown that cooperation between Dnmt1 and *de novo* DNA methyltransferases Dnmt3a and Dnmt3b is absolutely required for the maintenance of the methylation of repetitive elements and that Dnmt1 by itself is not capable of maintaining methylation of repetitive elements.[47] Therefore, the decreased miR-29 expression and consequent up-regulation of Dnmt3a and Dnmt3b may be a mechanism that protects the genome from excessive demethylation.

Differential expression of the miR-200 family, including miR-200b, miR-200c, and miR-429, in the livers of methyl-deficient C57BL/6J and DBA/2J mice is an important finding of the study. These miRNAs target transcriptional repressors of E-cadherin, ZEB1 and ZEB2,[29,48] resulting in increased expression of E-cadherin. Importantly, increased E-cadherin expression starting at preneoplastic stages has been associated with a more aggressive hepatocarcinogenic process in mice.[49]

The results of our *in vitro* experiments confirmed the mechanistic link between altered expression of miR-155 and miR-200b and their targets. Likewise, in our previous study we have demonstrated that transfection of rat hepatocytes with miR-34a induced apoptosis.[50]

In conclusion, these findings demonstrate that alterations in the expression of miRNAs are a prominent event in NASH induced by methyl deficiency in mice and strongly suggest that differences in the susceptibility to NASH and the severity of NASH may be determined by the variations in miRNA expression response. More importantly, our data provide a mechanistic link between alterations in miRNA expression and pathophysiological and pathomorphological features of NASH. Additional studies will be necessary to determine whether or not altered expression of miRNAs, by itself, may induce the development of NASH and if miRNA correction weakens NASH symptoms and decreases NASH progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

List of abbreviations

Cdh1	E-cadherin
C/ebp-β	CCAAT enhancer binding protein beta
Col	procollagen
Dnmt	DNA methyltransferase
MDD	methyl-deficient diet
microRNA	miRNA
Mmp	matrix metallopeptidase
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
Pdgf	platelet-derived growth factor
Pdgfr	platelet-derived growth factor receptor
8-oxodG	8-oxodeoxyguanosine
Socs1	suppressor of cytokine signaling-1
Tnf	tumor necrosis factor alpha

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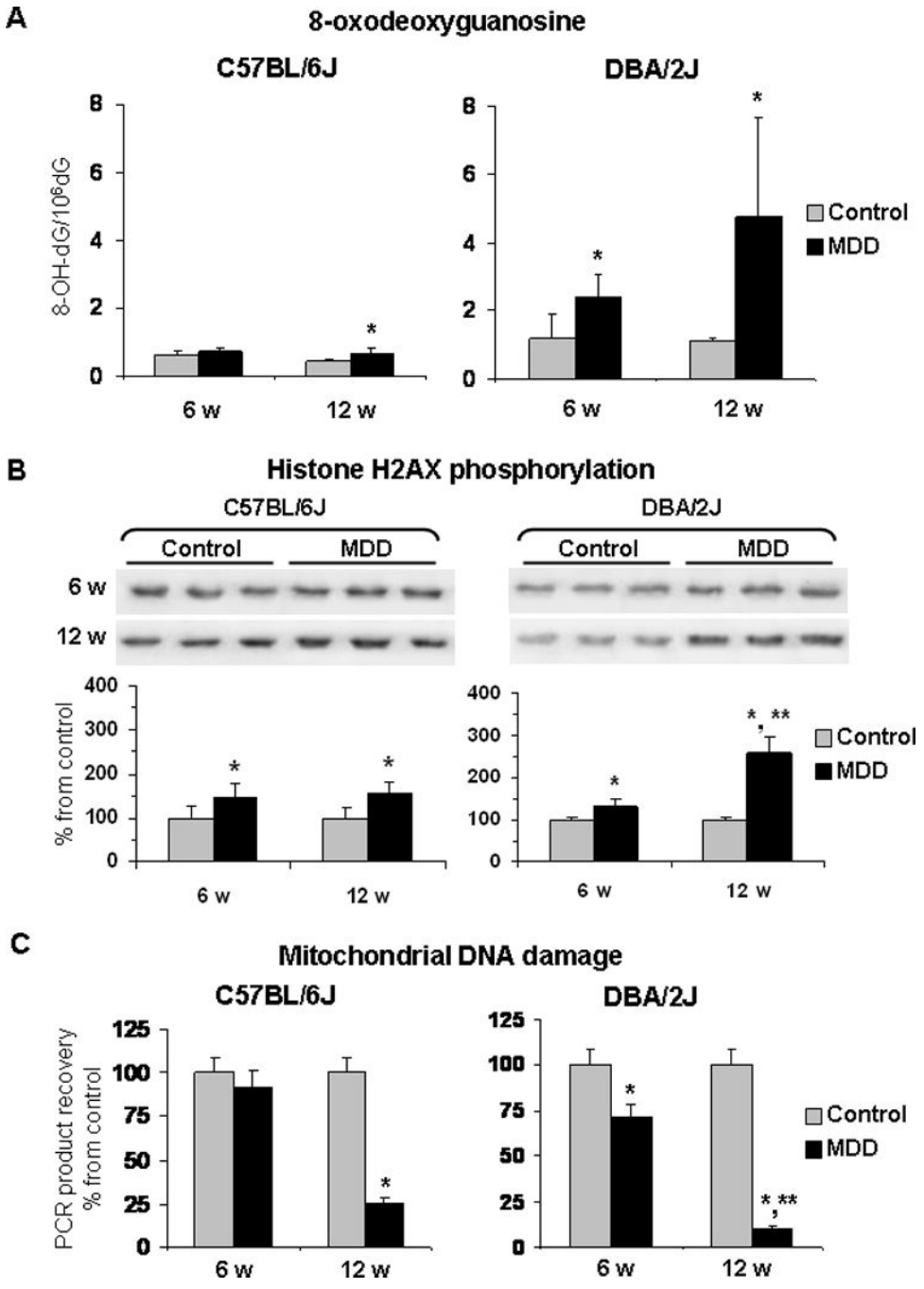

Figure 1. Genomic and mitochondrial DNA damage in the livers of C57BL/6J and DBA/2J mice fed control and methyl-deficient diet for 12 weeks

(A) Levels of 8-oxodG in DNA in the livers of C57BL/6J and DBA/2J mice fed control or a methyl-deficient diet for 12 weeks. **(B)** Western blot analysis of histone H2AX phosphorylation. **(C)** Level of mitochondrial DNA damage as detected by qPCR. Data are presented as mean ± S.D. (n=5).

* Significantly different from age-matched control mice. ** Significantly different from methyl-deficient C57BL/6J mice.

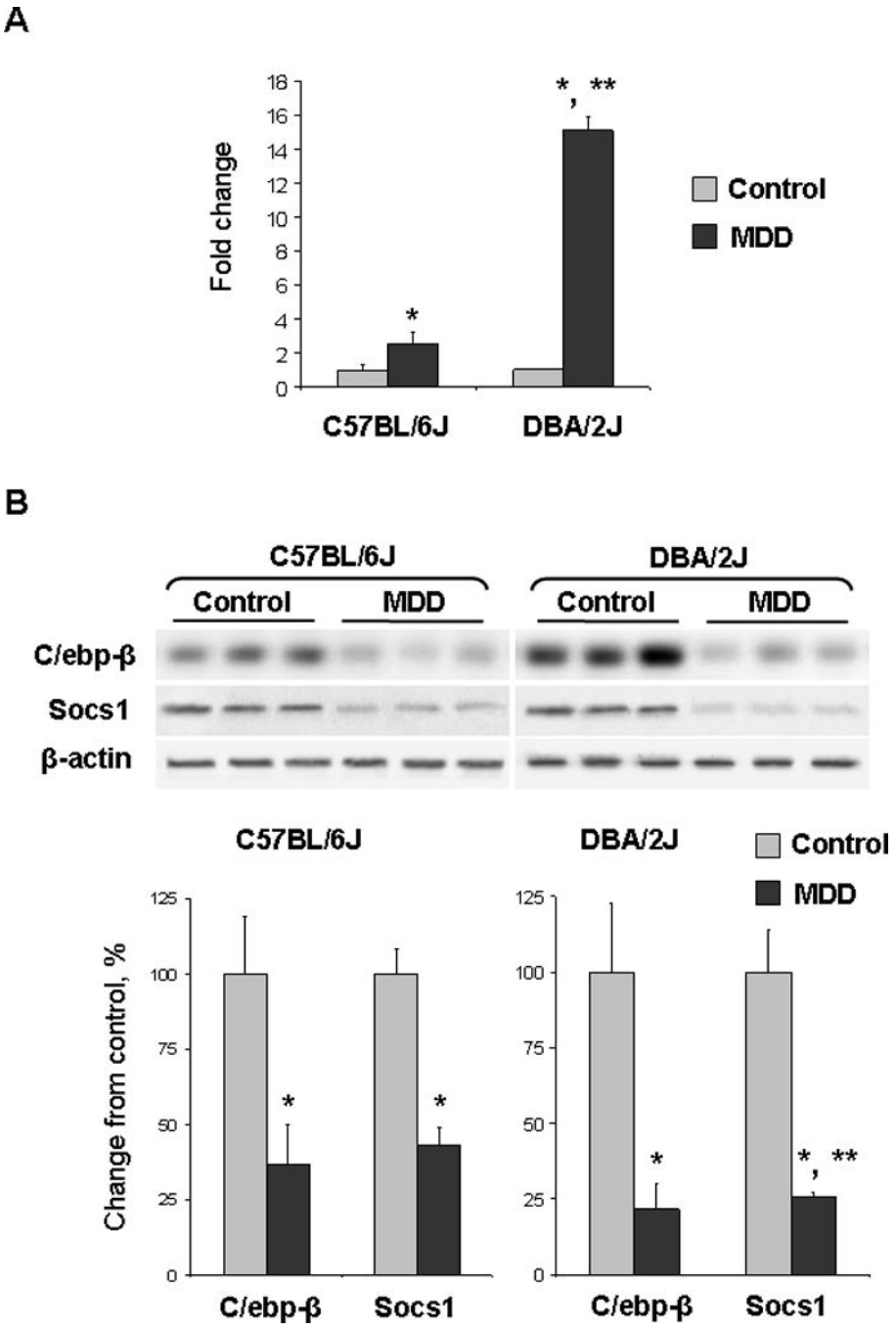

Figure 2. Expression changes of miR-155 and C/ebp-β and Socs1 proteins in the livers of C57BL/6J and DBA/2J mice fed control and methyl-deficient diet (MDD) for 12 weeks

(A) qRT-PCR analysis of miR-155 expression. The miRNA expression data are presented as average fold change normalized to snoRNA-202 in the livers of methyl-deficient mice compared to control mice. The level of miRNA expression was measured using the $2^{-\Delta\Delta Ct}$ method.[15] These results were reproduced in two independent experiments. **(B)** Western blot analysis of C/ebp-β and Socs1 proteins. Liver tissue lysates were separated by SDS-PAGE and subjected to Western immunoblotting using specific antibodies against C/ebp-β and

Socs1 proteins. Equal sample loading was confirmed by immunostaining against β-actin. These results were reproduced in two independent experiments. Representative Western immunoblot images are shown. Chemiluminescence detection was performed with the Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation) and measured directly by a BioSpectrum Imaging System (UVP). The signal intensity was analyzed by ImageQuant software (Molecular Dynamics). Data are presented as mean ± S.D. (n=5).

* Significantly different from age-matched control mice. ** Significantly different from methyl-deficient C57BL/6J mice.

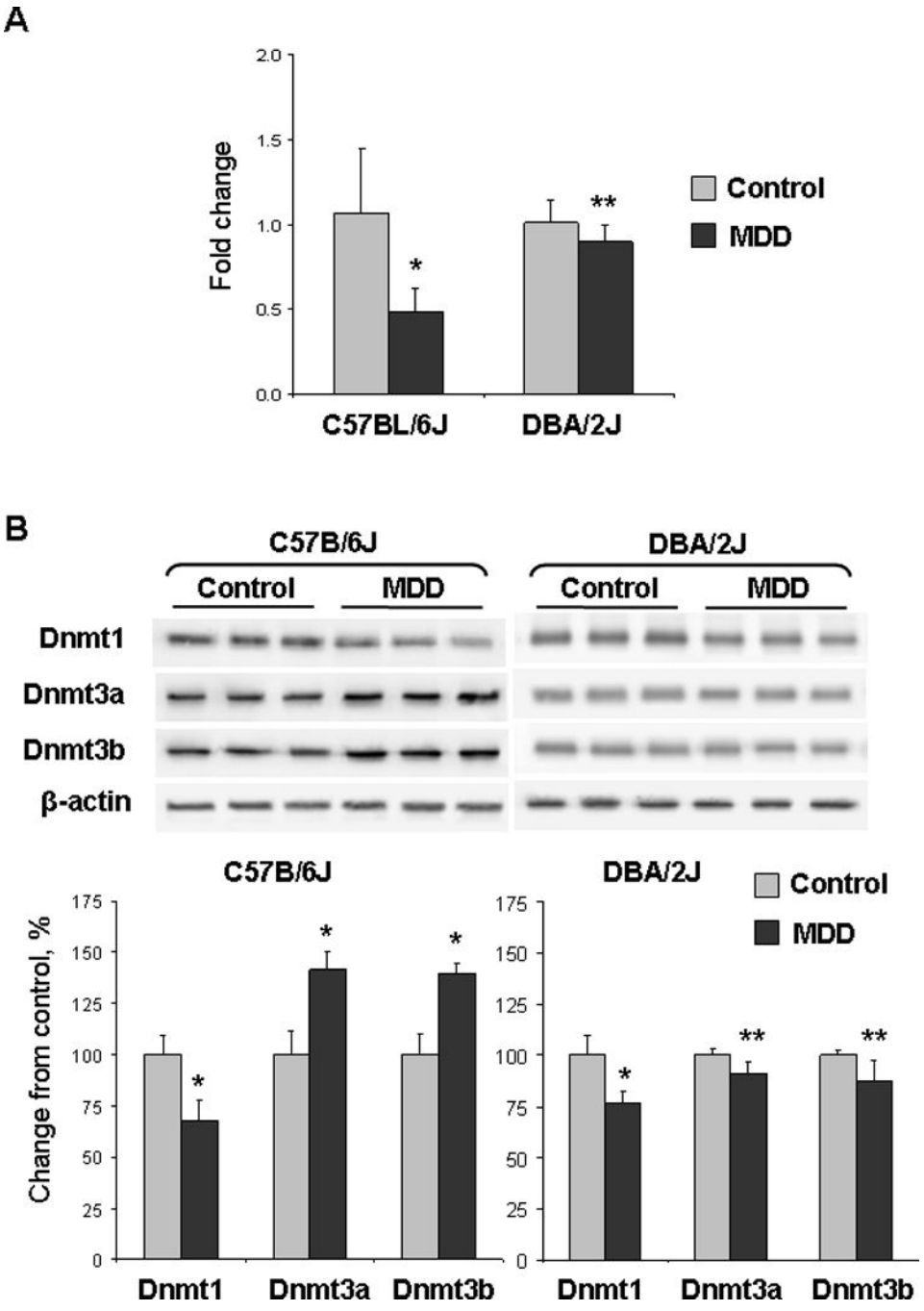

Figure 3. Expression changes of miR-29c and Dnmt1, Dnmt3a, and Dnmt3b proteins in the livers of C57BL/6J and DBA/2J mice fed control and methyl-deficient diet for 12 weeks

(A) qRT-PCR analysis of miR-29c expression. **(B)** Western blot analysis of Dnmt1, Dnmt3a, and Dnmt3b proteins. Data are presented as mean ± S.D. (n=5).

* Significantly different from age-matched control mice. ** Significantly different from methyl-deficient C57BL/6J mice.

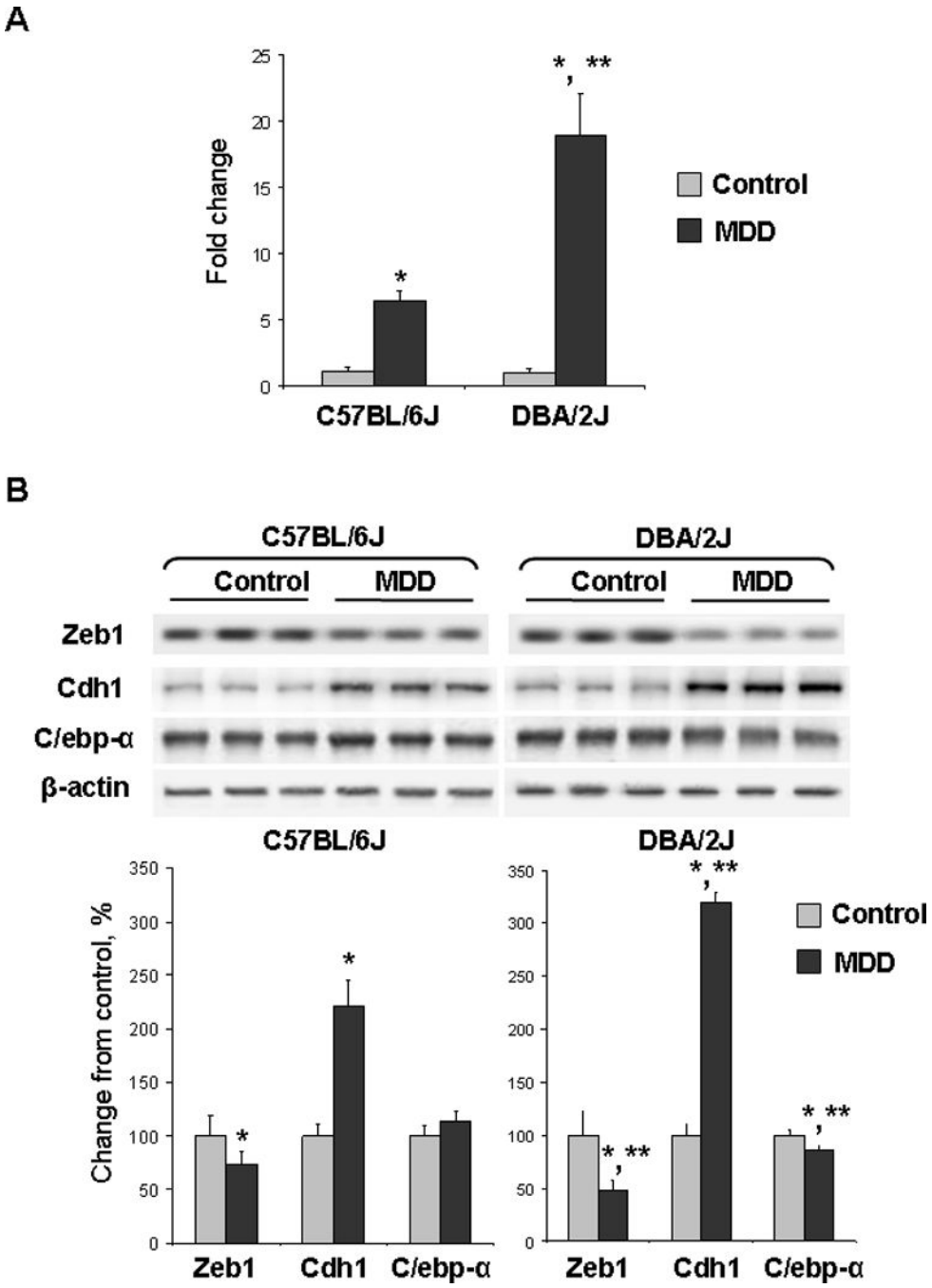

Figure 4. Expression changes of miR-200b and Cdh1 and C/ebp-α proteins in the livers of C57BL/6J and DBA/2J mice fed control and methyl-deficient diet for 12 weeks

(A) qRT-PCR analysis of miR-200b expression. **(B)** Western blot analysis of Cdh1 and C/ebp-α proteins. Data are presented as mean ± S.D. (n=5).

* Significantly different from age-matched control mice. ** Significantly different from methyl-deficient C57BL/6J mice.

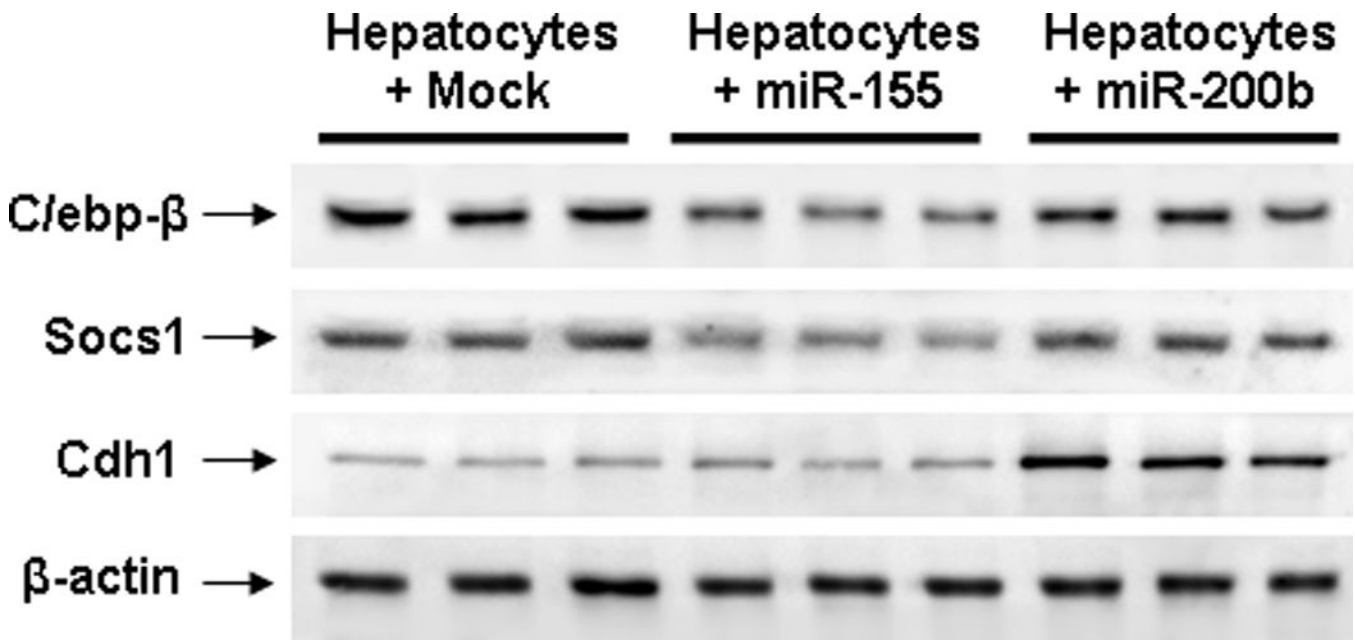

Figure 5. Western blot analysis of C/ebp-β, Socs1, and Cdh1 proteins in mouse primary hepatocytes transfected with pre-mir-155 or pre-miR-200b

Mouse primary hepatocytes were transfected with 20 nM of either pre-miR-155 or pre-miR-200b. Mouse primary hepatocytes transfected with scrambled RNA oligonucleotide (mock) served as a control.

Table 1

miRNAs expression changes in liver of C578BL/6J and DBA/2J mice fed a methyl deficient diet for 12 weeks.

miRNA	C57BL/6J		DBA/2J	
	Control	MD diet	Control	MD diet
Up-regulated				
miR-34a	1.00 ± 0.07	3.62 ± 1.18*	1.01 ± 0.15	17.57 ± 3.75*
miR-155	0.96 ± 0.31	2.56 ± 0.68*	1.00 ± 0.06	15.08 ± 0.80*
miR-200b	1.08 ± 0.35	6.41 ± 0.76*	1.02 ± 0.24	18.86 ± 3.26*
miR-221	1.33 ± 0.42	2.74 ± 0.58*	1.02 ± 0.22	2.42 ± 0.44*
Down-regulated				
miR-29c	1.06 ± 0.38	0.49 ± 0.13*	1.01 ± 0.13	0.90 ± 0.10
miR-122	1.03 ± 0.22	0.28 ± 0.06*	1.03 ± 0.28	0.39 ± 0.05*
miR-192	0.97 ± 0.09	0.33 ± 0.14*	1.00 ± 0.05	0.41 ± 0.04*
miR-203	1.15 ± 0.23	0.55 ± 0.15*	1.01 ± 0.17	0.56 ± 0.09*

* - Significantly different from the control, $p<0.05$.

Data presented as mean±S.D., n=5.