

Hyperhomocysteinemia-Induced Monocyte Chemoattractant Protein-1 Promoter DNA Methylation by Nuclear Factor- κ B/DNA Methyltransferase 1 in Apolipoprotein E-Deficient Mice

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Abstract

Hyperhomocysteinemia is considered to be a significant risk factor in atherosclerosis and plays an important role in it. The purpose of this study was to determine the molecular mechanism of blood monocyte chemoattractant protein-1 (MCP-1) promoter DNA hypomethylation in the formation of atherosclerosis induced by hyperhomocysteinemia, and to explore the effect of nuclear factor- κ B (NF- κ B)/DNA methyltransferase 1 (DNMT1) in this mechanism. The atherosclerotic effect of MCP-1 in apolipoprotein E-deficient (ApoE^{-/-}) and wild-type C57BL/6J mice was evaluated using atherosclerotic lesion area; serum NF- κ B, MCP-1, and DNMT1 levels; and MCP-1 promoter DNA methylation expression. *In vitro*, the mechanism responsible for the effect of NF- κ B/DNMT1 on foam cells was investigated by measuring NF- κ B and DNMT1 levels to determine whether NF- κ B/DNMT1 had an effect on gene expression. Compared with the control group, atherosclerotic lesions in ApoE^{-/-} mice fed a high methionine diet significantly increased, as did the expression of MCP-1. *In vitro* study showed that pyrrolidine dithiocarbamate treatment down-regulated levels of NF- κ B and raised DNMT1 concentrations, confirming the effect of NF- κ B/DNMT1 in the MCP-1 promoter DNA methylation process. In conclusion, our results suggest that through NF- κ B/DNMT1, MCP-1 promoter DNA hypomethylation may play a key role in formation of atherosclerosis under hyperhomocysteinemia.

Key words: atherosclerosis; DNA methylation; hyperhomocysteinemia; monocyte chemoattractant protein-1

Introduction

HOMOCYSTEINE (Hcy) IS AN INTERMEDIATE in methionine metabolism.¹ A large amount of epidemiological studies² have confirmed that an increased plasma Hcy concentration is an independent risk factor for atherosclerosis leading to cardiovascular diseases. Though mechanisms such as inflammation and oxidative stress³ of hyperhomocysteinemia-induced atherosclerosis have been reported in related research, the mechanism itself is not definitive so far.

Atherosclerosis has been established as a complex inflammatory disease characterized by the accumulation of lipids, fibrous materials, and cell debris in the arteries,⁴ and it is mediated by complicated molecular interactions, in which chemokines play a key role.⁵ A significant and the most well-characterized chemokine is monocyte chemoattractant protein-1 (MCP-1/CCL₂),

which is increased in atherosclerotic lesions but not in normal arteries, suggesting its potential value in stimulating the migration of monocytes into the intima of the arterial wall and taking part in most processes during atherosclerosis.^{6,7} The role of MCP-1 appears to be more evident, and research has simultaneously demonstrated that anti-MCP-1 gene therapy not only limits progression of established atheroma but also limits transformation from destabilized plaque to stable plaque.⁸ This strongly shows a major role of MCP-1 in the pathogenesis of atherosclerosis and suggests that MCP-1 may be a promising therapeutic target against atherosclerosis. However, it is not clear whether MCP-1 comes into play in hyperhomocysteinemia-induced atherosclerosis. Therefore, our study explored the molecular mechanism of MCP-1 in atherosclerosis.

DNA methylation is an important epigenetic mechanism and may explain the relationships between an individual's

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genetic background, environment, and disease.⁹ DNA methylation patterns are inhibited by S-adenosylhomocysteine (SAH), and pattern maintenance depends on DNA methyltransferase 1 (DNMT1) and intracellular S-adenosylmethionine (SAM) levels.^{10,11} Aberrant DNA methylation regulates gene expression¹² and is associated with advanced stages of disease, such as atherosclerosis. The presence of aberrant DNA methylation in an individual may indicate predisposition to atherosclerosis and related diseases.¹³ Pilot studies^{14–16} have suggested the relationship between atherosclerosis-related genes and DNA methylation in vascular smooth muscle cells, endothelial cells, and foam cells, however, there remain limitations in these *in vitro* experiments. Consequently, we chose to observe MCP-1 promoter DNA methylation of blood, which is closely related to an individual's atherosclerotic pathogenesis. DNA methylation takes part in transcription silencing, and hypomethylation activates the expression of genes via inhibition of the association of DNA promoter recognition site and special transcription factors such as nuclear factor- κ B (NF- κ B).¹⁷ Activated NF- κ B binds to specific DNA sequences of target genes and regulates transcription of genes involved in growth regulation and inflammation.¹⁸ Because of various levels of regulation, the NF- κ B signaling pathway can be potentially targeted at various levels including nuclear translocation, DNA binding, and methyltransferases.¹⁹ Contemporary research has pointed out the ability of NF- κ B (RelA/p65) to directly recruit DNMT1 to chromatin, resulting in promoter-specific methylation,²⁰ and suggested that bortezomib can result in down-regulation of DNMT1 via the SP1/NF- κ B pathway and induce genomic DNA hypomethylation in human leukemia cells.²¹ We postulated that disruption of the NF- κ B and DNMT1 interplay might in turn lead to DNMT1 down-regulation and DNA hypomethylation.

Marumo et al.²² showed that Hcy can induce elevated MCP-1 secretion via the NF- κ B in human umbilical vein endothelial cells and human vascular smooth muscle cells. However, the connections between Hcy, MCP-1 promoter DNA methylation, and atherosclerosis remain largely unknown. The present study explored whether blood MCP-1 promoter DNA hypomethylation may promote formation of atherosclerosis under hyperhomocysteinemia, as well as the effect of NF- κ B/DNMT1 in the pathogenesis.

Materials and Methods

Animal model and separation of mononuclear cells

To establish an animal model with atherosclerosis, 12 male wild-type mice and 36 male apolipoprotein E-deficient (ApoE^{-/-}) mice on a C57BL/6J genetic background were kept in our laboratory for 15 weeks. The animals were purchased from Jackson Laboratory (Bar Harbor, ME) and bred to 5 weeks of age in the Animal Center of Beijing University (Beijing, China). After 1 week of acclimatization, ApoE^{-/-} mice were randomly divided into three groups ($n=12$ each). Mice of the ApoE^{-/-} control group were fed with regular mouse diet for 15 weeks. The high-methionine group (HM) received the same diet but with a 1.7% methionine supplement. The high-methionine plus folate and vitamin B₁₂ group (HM+FA+VB) received regular mouse diet plus 1.7% methionine, 0.006% folate, and 0.0004% vitamin B₁₂. Wild-type C57BL/6J mice ($n=12$) were fed with a regular mouse diet as blank control group. After 15-week experimental

diets, mice were euthanized with 20% ethylcarbamate (2 mL/100 g) enterocoelia injection, and blood was immediately collected to measure serum Hcy, NF- κ B, MCP-1, and DNMT1 levels. The aortas were stored at -80°C until analysis.

Blood used for separation of mononuclear cells should be fresh and free of clots in preservative-free anticoagulant. Histopaque 1083 (Sigma, St. Louis, MO) was added to centrifuge tubes. Whole blood was carefully layered onto the Histopaque 1083 surface and centrifuged at 400 *g* for exactly 30 min at room temperature. After centrifugation, the upper layer was aspirated carefully with a plastic pipe until only 3 mm of the opaque interface containing the mononuclear cells remained. Isotonic phosphate-buffered saline (PBS) was then added, and this was centrifuged at 250 *g* for 10 min for three times to remove any remaining Histopaque 1083 from the mononuclear cells. The cells suspended in isotonic PBS were used for the following assays.

Cell culture

The TH-1 monocyte cell line was obtained from Sichuan University (Chengdu, China). THP-1 cells were cultivated in Roswell Park Memorial Institute (RPMI) 1640 with 15% fetal bovine serum (FBS), penicillin (100 U/mL)-streptomycin (100 $\mu\text{g}/\text{mL}$) at 37°C in a 5% CO₂ atmosphere, to a density of 10⁷ cells/mL. Then THP-1 cells were differentiated into macrophages by incubation for 24 h with 50 nmol/L phorbol myristate acetate (PMA, Sigma). THP-1 cell-derived macrophages (West China Hospital of Sichuan University) were treated with oxidized low density lipoprotein (50 mg/L, Sigma) and Hcy (0, 100 $\mu\text{mol}/\text{L}$, Sigma) for 24 h at 37°C and stained with oil red O. Foam-cell formation was observed under an inverted microscope (Olympus, Tokyo, Japan). Foam cells were divided into three groups: foam cell, foam cell intervened by Hcy (100 $\mu\text{mol}/\text{L}$), and foam cell intervened by Hcy (100 $\mu\text{mol}/\text{L}$) and pyrrolidine dithiocarbamate (PDTC, 50 $\mu\text{mol}/\text{L}$, Sigma).

Determination of serum Hcy, SAM, and SAH levels

After 30 min standing at room temperature, serum was obtained by centrifugation (3000 *g* for 10 min at 4°C), and serum Hcy concentrations were measured by automatic biochemistry analyzer. Serum SAM and SAH concentrations were measured by high-performance liquid chromatography (HPLC; D-2000 Elite HPLC, Hitachi High Technologies, Tokyo, Japan). The supernatant of each sample was filtered through a 0.22- μm filter (Millipore, Billerica, MA) and then loaded onto a C18 column (250 mm \times 4.6 mm internal diameter, 5- μm particle) fitted with a matched guard column, run by a Waters HPLC system (D-2000 Elite HPLC, Hitachi High Technologies), and connected to an ultraviolet detector. Absorption of eluted compounds was monitored at $\lambda_{\text{ex}}=254$ nm. Chromatograms were recorded by a D-2000 Elite HPLC integrator with its quantification accomplished by automatic peak area integration. SAM and SAH standards (Sigma) were used to identify the elution peaks.

Tissue preparation and atherosclerotic lesion examination

The analysis of paraffin histological sections was used for evaluating atherosclerotic lesions. The aortas were separated carefully and then put into 4% (w/v) formalin for 24 h. The samples subsequently underwent washing,

dehydration, clearing, embedding, slicing, coating, grilling, and hematoxylin-eosin (HE) staining. Finally, the sections were observed via microscope (Olympus).

Serum NF- κ B, MCP-1, and DNMT1 concentrations and NF- κ B and DNMT1 levels in foam cells measured by enzyme-linked immunosorbent assay

Serum NF- κ B, MCP-1, and DNMT1 concentrations and NF- κ B and DNMT1 levels in foam cells were determined by using commercially available enzyme-linked immunosorbent assay kit (ELISA; R&D Systems, Emeryville, CA) according to the manufacturer's instructions. All reagents were kept at room temperature for at least 30 min before assays began. All standards, controls, and samples were run in duplicate. Finally, absorbance was read on a microplate reader at 450 nm.

Nested methylation-specific polymerase chain reaction for MCP-1

Genomic DNA was isolated from the peripheral blood mononuclear cells using the Wizard[®] Genomic DNA Purification Kit (Promega, Madison, WI). DNA concentration and purity were detected by spectrophotometry at 260 and 280 nm. The A260/A280 absorbance ratio was consistent at approximately 1.8. It integrated DNA denaturation and bisulfite conversion processes into one-step by EZ DNA Methylation-Gold[™] Kit (ZYMO, Irvine, CA). Following bisulfite treatment of genomic DNA, unmethylated cytosine residues are converted into uracil. Their improved sequence specificity facilitates relative quantification of methylated and unmethylated alleles that are simultaneously amplified in a single tube. MCP-1 outer primers were 5'-TTG TTG AAA TGA ATT TTA AGG GTT T-3' and 5'-CCC AAA TAA CTC CAA CCT AAC TAT C-3'. The process of reaction was as follows: 94°C for 5 min, 94°C for 30 sec, 65°C for 30 sec, and 72°C for 1 min, repeating for 30 cycles, decreasing 0.5°C every cycle; 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min, repeating for 20 cycles; and 72°C for 7 min. MCP-1 methyl and unmethyl primer: left M primer, 5'-TTT AAG GGT TTT TAG ATT TTA TCG T-3'; right M primer, 5'-AAC TCT CTA CCC TAT TTC CTT CGT A-3'; left U primer, 5'-TTT AAG GGT TTT TAG ATT TTA TTG T-3'; right U primer, 5'-AAC TCT CTA CCC TAT TTC CTT CAT A-3'. The process of reaction was as follows: 94°C for 5 min, 94°C for 30 sec, 61.6°C for 30 sec, and 72°C for 1 min, repeating for 30 cycles, decreasing 0.5°C every cycle; 94°C for 30 sec, 46.6°C for 30 sec, and 72°C for 1 min, repeating for 20 cycles; and 72°C for 7 min. The PCR products were analyzed by 2% agarose gel electrophoresis.

Statistical analysis

The results were expressed as means plus or minus standard error (means \pm SEM). Differences among groups were analyzed by one-way analysis of variance and analysis of multiple comparisons within treatment groups was carried out using Student-Newman-Keuls's test; $p < 0.05$ was considered as statistically significant.

Results

Analysis of aortic pathological section with HE staining and levels of serum Hcy, SAM, and SAH in mice

We found that atherosclerotic lesion areas of ApoE^{-/-} control group, HM group, and HM+FA+VB group were in-

creased respectively compared with those of the blank control group (Fig. 1A). In the blank control group, the aorta walls were round, and smooth muscle cells were not seen underneath the endoderm (Fig. 2A). The aorta walls were rough, and the intima revealed hyperplasia and had few foam cells and atherosclerotic substances in the ApoE^{-/-} control group (Fig. 2B). In the HM group, a large amount of atherosclerotic substances, many foam cells, and a few inflammatory cells were seen (Fig. 2C). In the HM+FA+VB group, the thickness of aorta walls was uneven, the thickness ratio of the intima to media was decreased, and there were fewer foam cells under the fiber caps, fewer atheronecrotic substances, and fewer inflammatory cells compared with the HM group (Fig. 2D). In summary, the atherosclerosis mouse model was established successfully.

Hcy is a key intermediate of methionine metabolism. After 15 weeks, serum Hcy concentrations were confirmed in mice fed with different diets. The levels of serum Hcy were elevated 38.9% in the ApoE^{-/-} control group, ~2.5-fold in the HM group, and 96.0% in the HM+FA+VB group; a remarkable increase was observed in HM group compared with blank control group ($p < 0.01$). Apolipoprotein E (ApoE)

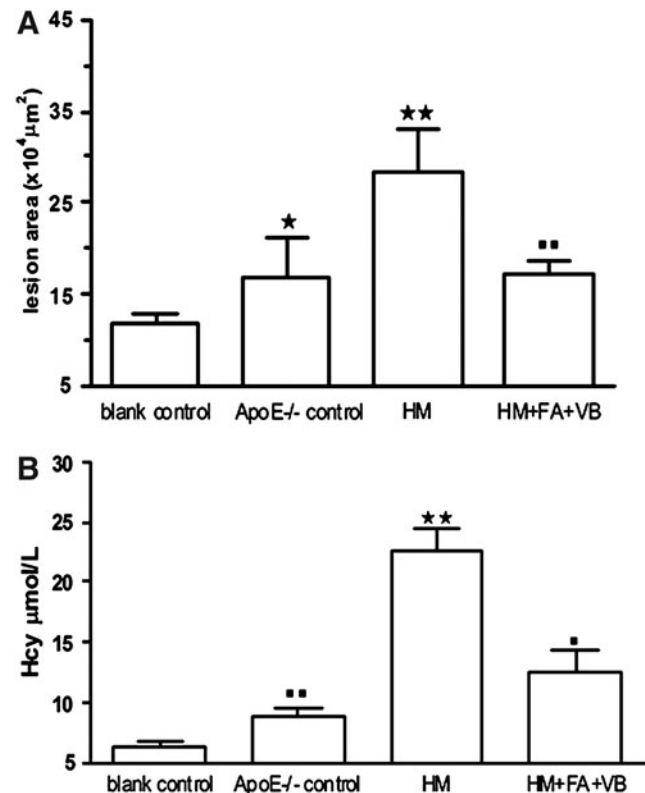


FIG. 1. Methionine is converted through SAM and SAH to Hcy, which is subsequently remethylated back to methionine. Many investigations have revealed several potential mechanisms that Hcy induced the formation of atherosclerosis. (A) Serum Hcy concentrations. (B) Statistical analysis of lesion area of atherosclerosis. Values were means \pm SEM. * $p < 0.05$, ** $p < 0.01$ vs. blank control; ■ $p < 0.05$, ■■ $p < 0.01$ vs. HM, $n = 10$. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; Hcy, homocysteine; HM, high-methionine.

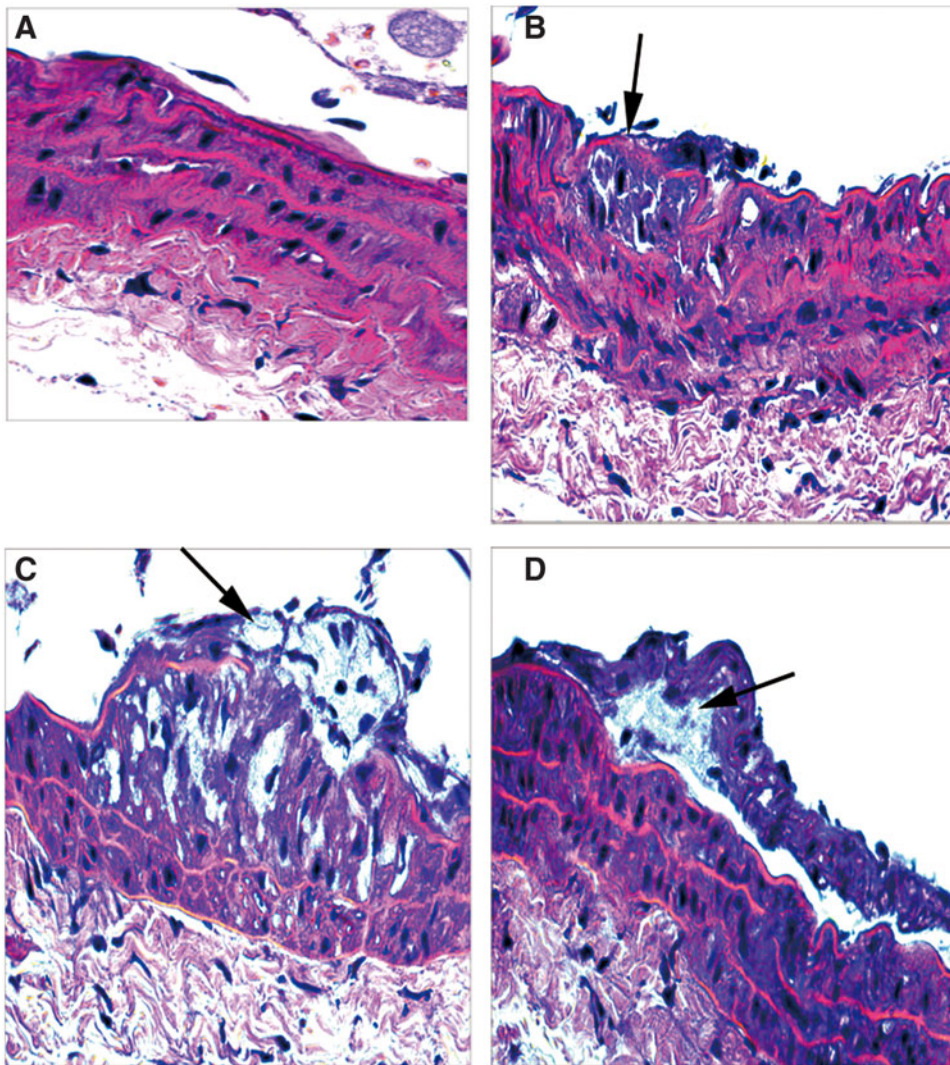


FIG. 2. Atherosclerotic lesion (HE, $\times 400$ magnification) on the aortas in mice fed with diets varying in methionine for 15 weeks. The cross-sections were from aortas from each group, and these were chosen from mice whose average lesion area approximated the mean value for that group. The arrows indicate atherosclerotic plaques. (A) blank control group; (B) ApoE^{-/-} control group; (C) HM group; (D) HM+FA+VB group. HE, hematoxylin and eosin; ApoE^{-/-}, apolipoprotein E-deficient; FA, folic acid; VB, vitamin B₁₂.

plays a central role in lipid metabolism and is polymorphic with three common alleles—e2, e3, and e4. ApoE4 carrier may promote greater binding of Hcy to lipoproteins and a faster clearance of the lipoprotein-bound Hcy fraction.²³ Therefore serum Hcy levels were higher in mice of the ApoE^{-/-} control group with the regular mouse diet. In comparison with the HM group, serum Hcy concentrations were slightly lower: ~ 1.6 -fold in the ApoE^{-/-} control group ($p < 0.01$), and 80.6% in the HM+FA+VB group ($p < 0.05$, Fig. 1B). Taken together, our data suggested that Hcy may induce the formation of atherosclerosis in ApoE^{-/-} mice, and folate and vitamin B₁₂ could reduce the effect of Hcy-induced atherosclerosis.

There are other metabolic intermediates of methionine, including SAM, which provides a methyl group for DNA methylation, and SAH, which can inhibit DNMT1 activation. After 15-week experimental diets, serum SAM concentrations were elevated by 3-, 3.6-, and 2.45-fold in the ApoE^{-/-} control, HM, and HM+FA+VB groups, respectively, compared with the blank control group, and serum SAH concentrations of the HM group were obviously higher in comparison with the blank control group (Fig. 3, Table 1).

Analysis of MCP-1 promoter DNA methylation and serum MCP-1 concentrations in mice

DNA methylation has emerged as an important epigenetic modification and a means to regulate gene transcription. After 15 weeks, MCP-1 promoter DNA methylation was determined in the blank control, ApoE^{-/-} control, HM, and HM+FA+VB groups (Fig. 4A, B). Our data suggested that in the HM group, the change of MCP-1 promoter DNA hypomethylation was the most obvious, with levels $\sim 6.2\%$ higher than those in the blank control group ($p < 0.05$). In the HM+FA+VB group, antagonistic effects could be observed on the hypomethylation change (Fig. 4A, B). In general, DNA methylation inhibited transcription and hypomethylation was associated with gene activation. As a result, serum MCP-1 levels were the highest in the HM group ($p < 0.05$; Fig. 4C). MCP-1 played a lead role in leukocyte infiltration into the vessel and activation during the inflammatory process of atherosclerosis. The atherosclerotic lesions in the HM group were the most severe and widespread, and MCP-1 promoter DNA hypomethylation and atherosclerotic area were positively correlated ($\gamma = 0.5175$, $p < 0.05$; Fig. 4D).

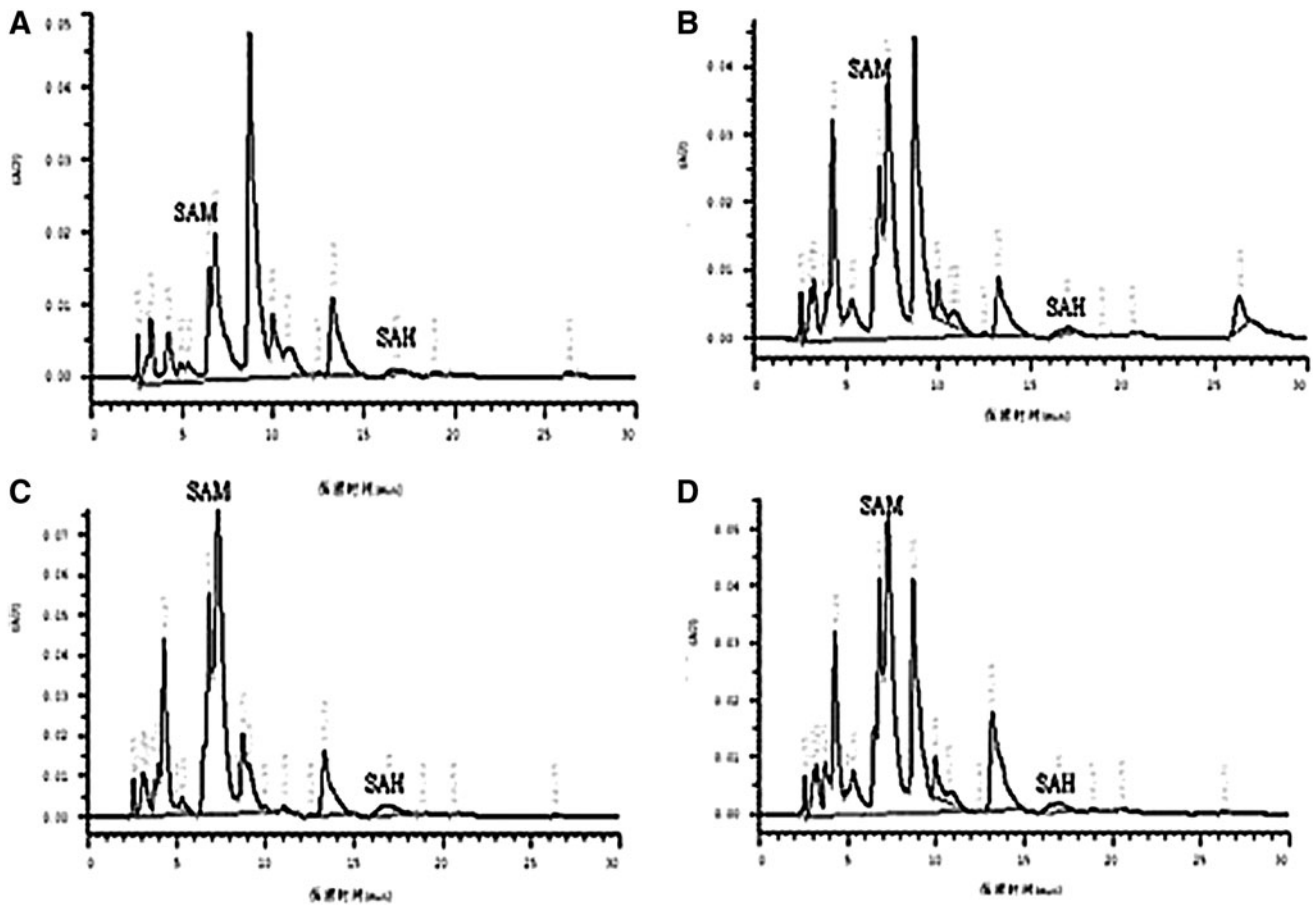


FIG. 3. A representative chromatograms showing elution profiles for SAM and SAH by high-performance liquid chromatography (HPLC) in all groups. SAM and SAH standards were used to identify the elution peaks, chromatograms were recorded by an integrator. (A) blank control group; (B) ApoE^{-/-} control group; (C) HM group; (D) HM+FA+VB group.

Serum NF- κ B and DNMT1 levels by ELISA analysis in mice and foam cells

Mice were fed with different experimental diets for 15 weeks, after which the amounts of NF- κ B and DNMT1 were estimated by ELISA (Table 2). Hcy could induce NF- κ B activation in the early stage of atherosclerosis, and serum NF- κ B levels were the highest in the HM group ($p < 0.05$). In the HM+FA+VB group, serum NF- κ B levels were 2.9-fold lower than in the HM group ($p < 0.01$). Serum DNMT1 concentrations were decreased by 20.8% in the HM group in compar-

ison with the blank group ($p < 0.01$) and slightly higher in the HM+FA+VB group compared with the HM group ($p < 0.05$). Serum NF- κ B and DNMT1 concentrations were negatively correlated ($\gamma = 0.3394$, $p < 0.05$; Fig. 5).

Our results might indicate a negative correlation between NF- κ B and DNMT1 in wild-type and ApoE^{-/-} mice. In order to further confirm the interaction between NF- κ B and DNMT1, we studied foam cells (Fig. 6) to explore the relationship of NF- κ B and DNMT1. NF- κ B is a transcription factor involved in cell proliferation, adhesion, invasion, and metastasis, and PDTC is an antioxidant and potent NF- κ B inhibitor that has been used to suppress the activity of NF- κ B in other cell types. DNMTs are also responsible for genomic DNA methylation, which is then maintained by DNMT1. Our results appear to confirm that NF- κ B concentration was decreased by PDTC intervention ($p < 0.01$; Fig. 7A) and DNMT1 level was increased in the foam cell+Hcy+PDTC group in comparison with the HM group ($p < 0.05$; Fig. 7B). Taken together, these findings showed that NF- κ B might suppress the activity of DNMT1 with the involvement of DNA hypomethylation.

TABLE 1. LEVELS OF SERUM S-ADENOSYLMETHIONINE AND S-ADENOSYLHOMOCYSTEINE

Group	SAM (nmol/L)	SAH (nmol/L)
Blank control	4.533 ± 1.56	12.42 ± 0.04
ApoE ^{-/-} control	18.22 ± 3.73**	12.72 ± 0.14
HM	20.82 ± 0.82**	13.12 ± 0.17**
HM+FA+VB	15.68 ± 1.14**	12.97 ± 0.19*

Values are means ± SEM.

* $p < 0.05$, ** $p < 0.01$ vs. blank control, $n = 10$.

SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; ApoE^{-/-}, apolipoprotein E-deficient; HM, high-methionine; HM+FA+VB, HM plus folate and vitamin B₁₂.

Discussion

Over the past decade, an accumulating body of evidence has indicated that Hcy is an independent risk factor for

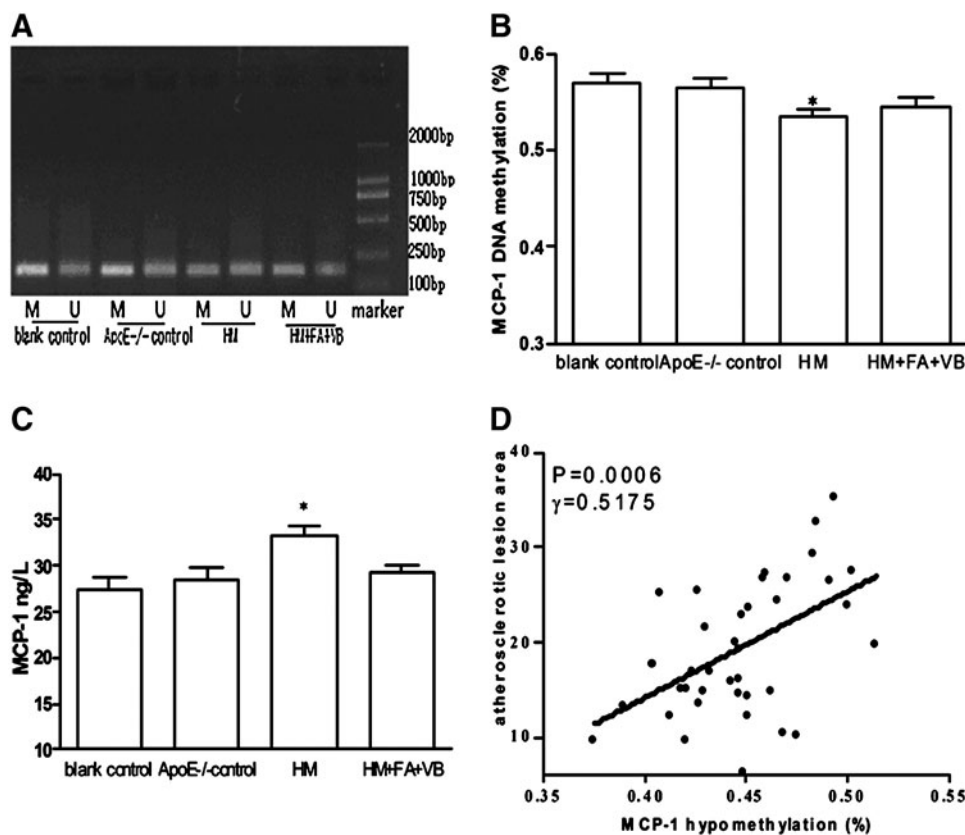


FIG. 4. The monocyte chemoattractant protein-1 (MCP-1) promoter was amplified from bisulfite-converted DNA and analyzed by nested methylation-specific polymerase chain reaction (nMS-PCR). MCP-1 promoter DNA hypomethylation in mice (A), statistical analysis of MCP-1 promoter DNA hypomethylation (B), serum MCP-1 levels in mice (C), correlation of MCP-1 hypomethylation and atherosclerosis area (D) ($r=0.5175$, $p=0.0006$, $n=10$). M, amplified band by methylation-specific primer; U, amplified band by unmethylation-specific primer. Values were means \pm SEM. * $p < 0.05$ vs. blank control, $n=10$.

atherosclerosis, and an obvious association between Hcy and atherosclerosis has been reported in many retrospective and prospective studies.^{24,25} However, the mechanisms of hyperhomocysteinemia-induced atherosclerosis is not yet well understood. We chose wild-type and ApoE^{-/-} mice fed with a high-methionine diet to confirm the role of MCP-1 in the formation of hyperhomocysteinemia-induced atherosclerosis. Our data provided the first evidence that atherosclerosis models were established successfully by analyzing pathological sections with HE staining and serum Hcy concentrations.

A related study suggested that MCP-1 is a part of inflammation and acts as a basic mediator in a biological system that plays a significant role in the early stages of atherosclerosis formation.²⁶ In another study, ApoE^{-/-} mice with increased MCP-1 secretion were at an increased risk of atherosclerosis.²⁷ The structure of MCP-1/CCL₂ [location:

11C-E1; 1146.5 cM; sequence: chromosome: 11; NC-000077.6 (82035577...82037452)] is illustrated in Figure 8. Simultaneously, MCP-1 SNP-2518 may be a valuable genetic marker for assessing the risk of developing distant metastasis after radiation therapy in nasopharyngeal carcinoma patients.²⁸ There is research showing that MCP-1 promoter hypomethylation

TABLE 2. LEVELS OF SERUM NUCLEAR FACTOR-κB AND DNA METHYLTRANSFERASE 1

Group	NF-κB (μmol/L)	DNMT1 (U/L)
Blank control	190.40 \pm 11.17	17.42 \pm 0.56
ApoE ^{-/-} control	200.40 \pm 18.22	15.92 \pm 0.63
HM	261.50 \pm 6.45*	14.42 \pm 0.36**
HM+FA+VB	67.31 \pm 4.22 [†]	16.37 \pm 0.42 [‡]

Values are means \pm SEM.

* $p < 0.05$, ** $p < 0.01$ vs. blank control.

[†] $p < 0.05$, [‡] $p < 0.01$ vs. HM.

NF-κB, nuclear factor-κB; DNMT1, DNA methyltransferase 1.

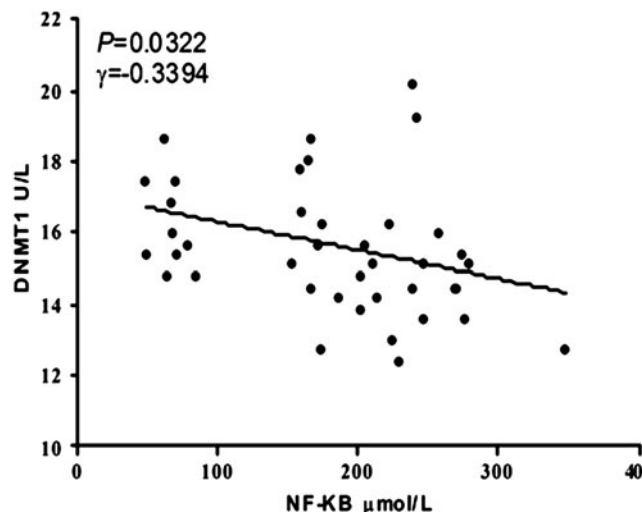


FIG. 5. Correlation between serum nuclear factor-κB (NF-κB) and DNA methyltransferase 1 (DNMT1) concentrations in wild-type and ApoE^{-/-} mice ($r=-0.3394$, $p=0.0322$, $n=10$).

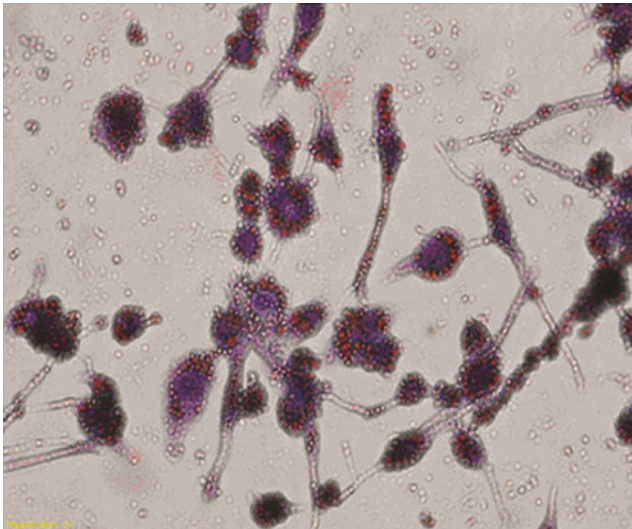


FIG. 6. Foam cell formation assay. Phorbol myristate acetate (PMA)-induced THP-1 monocytes to transformate for macrophages with pseudopodium for 48h and macrophages were incubated with oxidized low density lipoprotein (ox-LDL; 50 $\mu\text{g}/\text{mL}$). Foam cells were irregular shapes and bubble-like change with oil-red O staining (magnification $\times 400$). Red particles in the cytoplasm represent lipid droplets.

may be affected by blood glucose and triglyceride.²⁹ However, the interaction of hyperhomocysteinemia and MCP-1 promoter DNA methylation has not been demonstrated by much experimental evidence. In our study, the MCP-1 promoter was hypomethylated and the level of MCP-1 hypomethylation was the highest in the HM group. Recently, small interfering RNAs (siRNAs) have been found to participate in gene regulation together with methylation and can induce the methylation of the promoter, which thus silences the gene.³⁰ As a result of siRNA participation, serum Hcy levels were increased nearly 2.5 times and a concomitant increase of lesion area also occurred in ApoE^{-/-} mice fed with the high methionine diet, but there was only a slight reduction in MCP-1 DNA methylation and a modest increase in serum MCP-1. The current prevailing explanation for Hcy-induced DNA hypomethylation is that DNA methylation modification needs SAM as the methyl donor. SAM is converted into SAH, which potentially inhibits transmethylation reactions.³¹ The accumulation of SAH can affect the DNA methylation pattern by causing a feedback inhibition of SAM-dependent methyltransferases.³² In this study, our results suggested that SAM and SAH could also explain MCP-1 promoter DNA hypomethylation. After the transmethylation reaction, SAM converts to SAH, undergoing hydrolysis to form Hcy; Hcy can be remethylated to methionine.³³ An increased level of Hcy would interfere with this methionine cycle, and an increased level of Hcy is associated with DNA hypomethylation.³⁴ Therefore we hypothesized that Hcy could also take part in MCP-1 promoter DNA hypomethylation by the methionine cycle. The correlation of MCP-1 hypomethylation and atherosclerotic lesion area proved that MCP-1 promoter DNA hypomethylation might promote the formation of atherosclerosis.

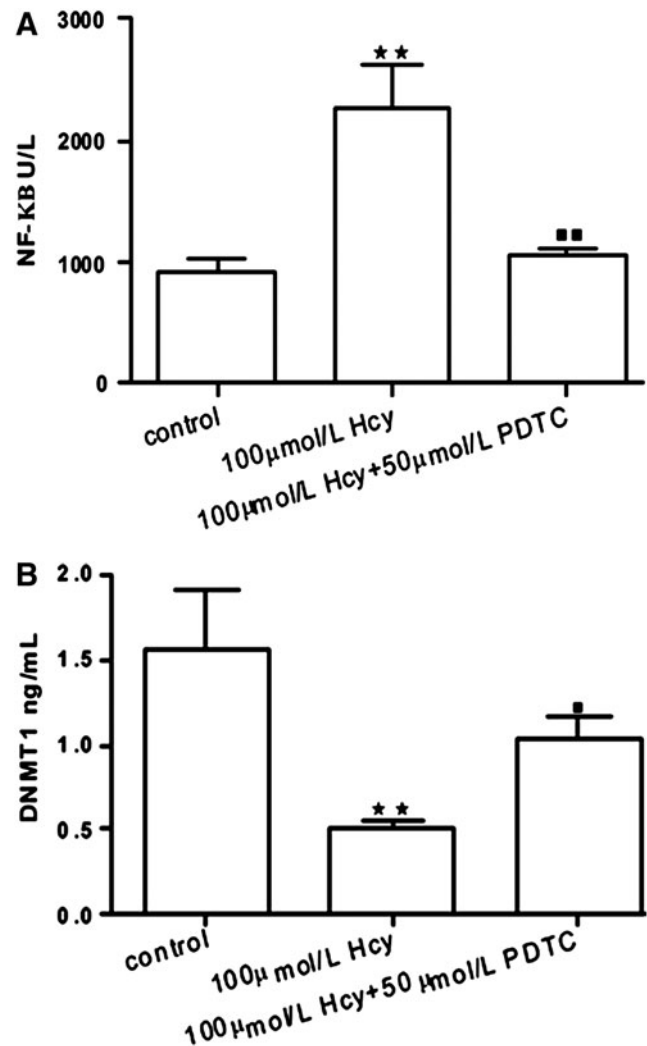


FIG. 7. NF- κ B and DNMT1 levels in foam cells. Values are expressed as means with their standard errors depicted by vertical bars. ** $p < 0.01$ vs. foam cell, ■ $p < 0.05$, ■■ $p < 0.01$ vs. 100 $\mu\text{mol}/\text{L}$ Hcy.

DNA methylation is maintained by DNMT1 and serves as a key mechanism that controls gene expression in a variety of chronic diseases such as atherosclerosis.³⁵ DNA methylation affects the expression of genes via inhibiting the association of DNA promoter recognition site and special transcription factor like NF- κ B. Hcy activates NF- κ B in endothelial cells via oxidative stress.³⁶ Consistent with those results, our research also suggested that serum NF- κ B concentrations in the HM group were higher than those in the blank control group, and we found that serum NF- κ B and DNMT1 levels were negatively correlated in ApoE^{-/-} mice. Hcy up-regulates the expression of Fas, a death receptor, via NF- κ B activation and promotes apoptosis in endothelial cells through activation of the extrinsic cell death pathway.^{37,38} Thus, intracellular substances are released into the blood. Then we tested NF- κ B and DNMT1 concentrations in serum as indicators of intracellular levels. So in the *in vitro* experiment, foam cells were chosen as the object, and PDTC was supplemented to inhibit the effect of NF- κ B in the hope of further verifying the

A *Ccl2*

chemokine (C-C motif) ligand 2 [Gene Summary](#)

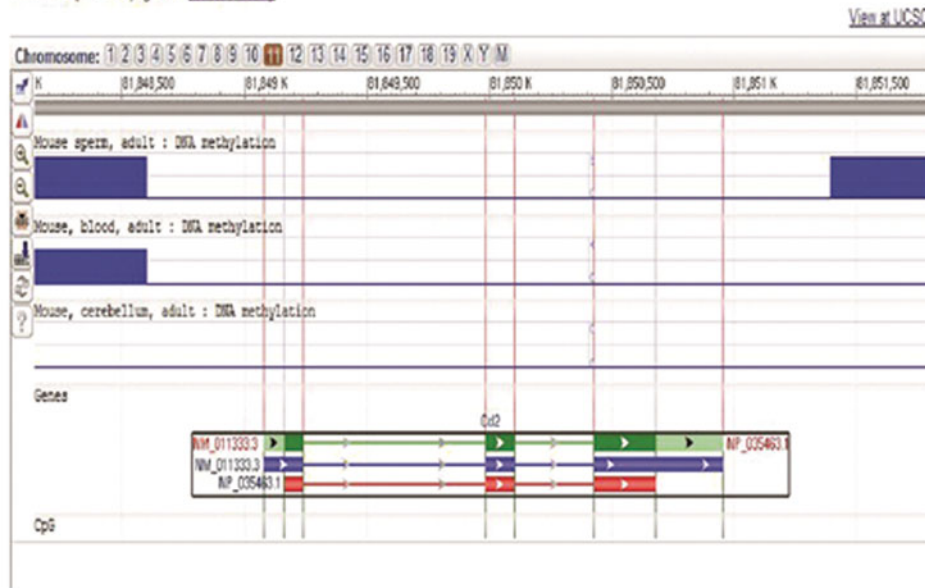
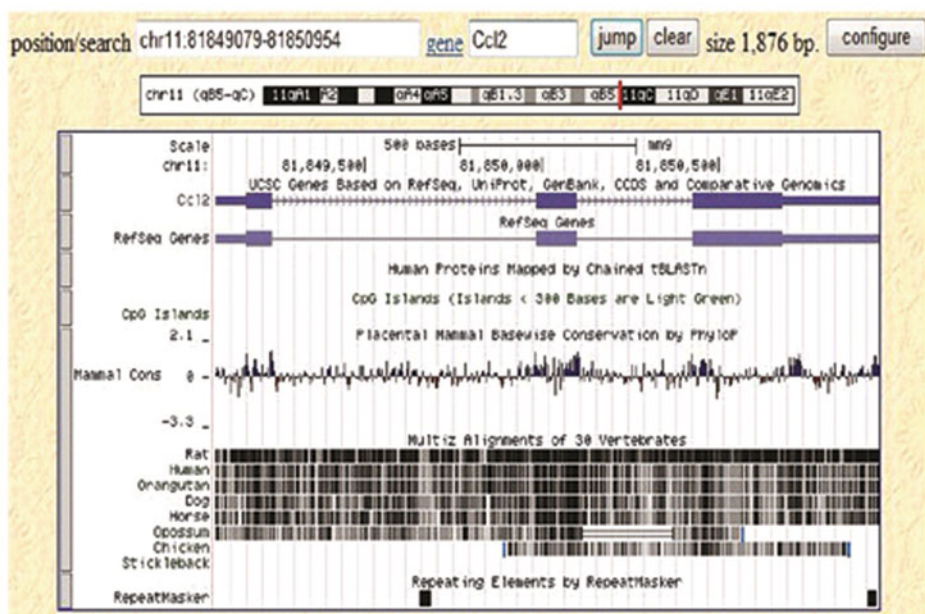


FIG. 8. The epigenomics and structure of MCP-1/CCL₂ are suggested by NCBI (A, B).

B



relationship between NF-κB and DNMT1. Once NF-κB binds to target gene promoters, the target gene transcription can be activated, which in turn leads to DNMT1 down-regulation and DNA hypomethylation. The results confirmed that the DNMT1 levels were increased concomitant with the decreased concentration of NF-κB in the foam cell + Hcy + PDTC group. In *in vivo* and *in vitro* experiments, the results showed that Hcy might play a role in DNA methylation of atherosclerosis by NF-κB/DNMT1.

In conclusion, accumulating evidence suggests MCP-1 promoter DNA hypomethylation may promote the formation of atherosclerotic plaque under hyperhomocysteinemia through NF-κB/DNMT1. This fact may be significant for therapy of atherosclerosis that is associated with gene expression due to hypomethylation of a gene's regulatory regions. The induc-

tion of MCP-1 promoter DNA hypomethylation by hyperhomocysteinemia is a new element in their mechanisms. In all, our findings reveal a novel role of Hcy in the pathogenesis of atherosclerosis.

Acknowledgment

This work was supported by the Specialized Research Fund for the National Natural Science Foundation of China (30960124, 81160044) and by the New Century Excellent Talents in University (NCET-10-0916) awarded to Y.J.

Disclosure Statement

The authors declare that no competing financial interests exist.

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