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Hyperhomocysteinemia induced endothelial progenitor cells dysfunction through hyper-methylation of CBS promoter

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Abstract

Bone marrow (BM)-derived endothelial progenitor cells (EPCs) are the key players in angiogenesis and vascular function. Cystathionine- β -synthase (CBS), an H₂S-generating enzyme in methionine metabolism, regulates the function of these EPCs. This study aims to examine whether CBS hyper-methylation contributes to the bone marrow endothelial progenitor cell (BM-EPCs) function and subsequent bone blood flow in mice fed with a high methionine diet (HMD). Bone marrow (BM) cells were collected from HMD and control mice, differentiated into BM-EPCs, and were characterized by acLDL-DiI labeling. CBS mRNA expression was analyzed by real-time PCR, and the global methylation status and methylation of the CBS promoter were detected by nuclear 5-mC assay and methylation-specific PCR (qMSP) respectively. The result reveals that CBS promoter in BM-EPCs from HMD mice was hyper-methylated and the methylation level was, indeed, negatively correlated with CBS mRNA and angiogenic function of BM-EPCs. In addition, global methylation (5-mC) and DNA methyltransferase-1 (DNMT1) expression were increased in HMD condition. *In vitro* study also shows that HMD induced hyperhomocysteinemia (HHcy) impaired both adhesion and angiogenesis properties of BM-EPCs, accompanied by higher methylation level of CBS promoter that compared to control. Furthermore, bone blood flow was found to be decreased in HMD mice as compared to wild-type mice. To dissect the epigenetic mechanism, we also administrated DNMT inhibitor, 5-azacytidine (5-Aza) to HMD mice. The administration of 5-Aza in HMD mice restored the CBS expression, EPC mediated angiogenesis and blood flow by reducing abnormal DNA hyper-methylation. In conclusion, HHcy dismantles BM-EPCs function and bone blood flow through the hyper-methylation of the CBS promoter in HMD fed mice.

Keywords

DNA hyper-methylation; CpG islands; DNMTs; Blood flow; BM-EPCs; Angiogenesis

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Disclosure of interest

The authors declare that there is no conflict of interest.

Appendix A. Supplementary data

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Transparency document

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1. Introduction

Increasing evidence suggests that endothelial repair/regeneration depends on the bioavailability of endothelial progenitor cells (EPCs) in the circulatory system [1]. These EPCs are primarily derived from the bone marrow environment, which is the precursor to endothelial differentiation. These circulating EPCs in the blood may contribute to the vascular repair process in response to tissue ischemia and vascular trauma [2]. However, its level was disrupted by different pathophysiological settings such as cardiovascular disease (hypertension, hypercholesterolemia and coronary artery disease) and diabetes [3–5]. The recent study also suggested that HHcy is a critical factor that mediates endothelial damage and vascular dysfunction associated with oxidative damage [6]. The absence of circulating EPCs may lead to the development of different pathological afflictions. The recent study also indicates that number and migratory activity of EPCs were ablated during excess accumulation of plasma homocysteine (Hcy) in patients [7]. However, the precise epigenetic mechanism by which Hcy mediated EPC dysfunction and regulation of bone blood flow is yet to be explained.

Methionine is an essential amino acid and is converted into homocysteine during methionine metabolism [8]. Increased level of circulating Hcy, a byproduct of methionine metabolism, has been implicated in the endothelial dysfunction [8]. There are two different enzymes, Cystathionine β -synthase (CBS) and cystathionine γ -lyase (CSE), in the transsulfuration pathway, which responsible for the catabolism of Hcy. Thereby basal level of H₂S production is maintained in the tissue. Previously, we reported that these enzymes are the mediator of osteoblast lineage and bone mineralization during bone development, [9,10] and impairment in CBS enzyme activity leads to osteoclastic bone loss through elevation of plasma Hcy and decreased the level of H₂S. The recent work of Sen et al. described the potential protective role of CBS gene therapy in renovascular function during HHcy.

DNA methylation is important for the regulation of gene transcription and ultimately protein expression [11]. Therefore, alternation in DNA methylation during pathological condition like HMD induced HHcy may disturb this function. However, the precise mechanism of the epigenetic role is not well defined yet in HMD induced HHcy. DNMTs are classified into DNA methyltransferases 3a (DNMT-3a) and DNA methyltransferase 3b (DNMT-3b; regulate *de novo* methylation), and DNA methyltransferases 1 (DNMT-1; maintain methylation) [12,13]. Therefore, these enzymes are involved in the regulation of physiological and pathophysiological process under Hcy-mediated vascular damage.

Taking into account, the current study was undertaken to study the epigenetic mechanism of bone marrow-derived EPCs (BM-EPCs) dysfunction and subsequent inhibition of bone blood flow in high methionine diet (HMD) induced HHcy mice model and ameliorating role of the epigenetic DNMT inhibitor; 5-Aza if any. In the current study, we found that hyper-methylation of the CBS promoter was associated with BM-EPCs dysfunction and subsequent bone blood flow under high methionine insult in mice model. Further, DNMT inhibitor; 5-Aza administration to HMD mice has restored the BM-EPCs function and blood flow.

2. Materials and methods

2.1. Animals and experimental procedures

All experiments were conducted in female C57BL/6J (wild-type, WT) mice starting at 12 weeks old. The animal procedures were carefully reviewed and approved by the Institutional Animal Care and Use Committee of The University of Louisville. Female WT mice were fed a high methionine-rich (HMD) diet (methionine enriched (1.2%), low folate (0.08 mg/kg), low vitamin B6 (0.01 mg/kg) and B12 (10.4 µg/kg), Harlan Laboratories, Cat No.TD. 97345) for 8 weeks. Otherwise, control mice were fed standard chow. All mice were allowed water *ad libitum*. To study the epigenetic effect on BM-EPCs function and bone blood flow in HMD mice, 5-azacytidine was intraperitoneally (i.p.) injected (0.5 mg/kg body weight) for the 8 weeks period. Animals of the control group received normal saline through i.p. injections. The mouse groups (n = 5–7) were: 1. Wild-type C57BL/6 mice (WT), 2. Wild-type mice fed with HMD (WT + HMD, or HHcy), 3. 5-Azacytidine-supplemented HMD mice (WT+HMD + 5-Aza).

2.2. Isolation and culture of primary bone marrow (BM) culture

Following sacrifice of experimental animals, both femurs and tibias were harvested into ice cold phosphate buffered saline (PBS). Mouse bone marrow (BM) cells were flushed out from the bone cavities of femurs and tibias and kept in the 2% heat-inactivated fetal bovine serum (ATCC) in PBS. The cell solution was then centrifuged for 2 min at 1500 rpm. The cells were then loaded on the top of a histopaque solution (3:1; Sigma-Aldrich Germany, Munich, Germany) and centrifuged at 2200 rcf for 15 min. The buffy coat mononuclear cells (MNCs) were collected following centrifugation. The MNCs were seeded in 12-well plates pre-coated with mouse fibronectin and cultured in Endothelial Cell Growth Medium-2 (EGM2, Lonza, Germany). After 2-days of culture, non-adherent cells were washed away, washed with PBS and the adherent cells were cultured again under EGM2.

2.3. Characterization of BM-derived EPC by flow cytometry

BM-derived MNCs (BM-MNC) were cultured for 7-days to obtain the differentiated population of BM-EPC in the culture plate. To characterize the differentiated BM-EPCs, cells were stained with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine- labeled acetylated low-density lipoprotein (acLDL-DiI) at 37 °C for 2 h and imaged under confocal microscopy. To further characterize the BM-EPCs, cells were stained with the following antibodies (PE-conjugated mouse anti-CD34 antibody, and APC-conjugated mouse monoclonal anti-CD31 antibodies) and performed flow cytometry analysis for detection of the EPC markers expression. Mouse IgG was also substituted with these antibodies as isotype control. Then samples were analyzed by BD Accuri™ C6 Cytometer BD Biosciences.

2.4. Tube matrigel assay

Briefly, 100 µl Matrigel was coated on the sterile 24-well plates and incubated at 37 °C for 30 min to allow the gel formation. Following polymerization of the gels, BM-EPCs were seeded on the matrigel in each well and incubated with EGM2 media for 24 h. 2-

dimensional organization of vessel network was photographed using an inverted phase contrast photomicroscope (DP70, Olympus, Tokyo, Japan). The length of the tubes and branching point of the vessel network was measured using AngioQuant image analysis software.

2.5. Bone blood flow by Laser Doppler flowmetry

Laser Doppler hind-limb blood flow was measured according to our previously published protocol [9]. Hind-limb blood flow was analyzed in experimental HMD mice model in post-HMD diet fed animals. Following blood flow measurement, the average flow values for the post-HMD fed mice were calculated using Moor LDI software and presented in form of a flux unit trace for all the experimental group.

2.6. Western blot analysis

Protein western blot analysis was performed as our previously published protocol [12]. Following electrophoresis, protein gels were transferred to polyvinylidene difluoride (PVDF) membranes using an electrotransfer apparatus (Bio-Rad) overnight. For non-specific blocking, membranes were blocked with 5% non-fat dry milk in TBS-T solution for 1 h and followed by desired primary antibody and secondary antibody [horseradish peroxidase-conjugated] at room temperature. The membranes were developed with Pierce ECL Western Blotting Substrate and imaged in the ChemiDoc XRS+ system (Bio-Rad). Band density was normalized with housekeeping control GAPDH.

2.7. DNA methylation by qMSP-PCR

We performed a bioinformatics analysis to identify potential CpG islands in the CBS genes, by using the USCS Human Genome Browser public database (<http://genome.ucsc.edu>). CBS-methylated (M) and unmethylated (U) alleles were amplified by using primer sets (designed with MethPrimer: designing primers for methylation PCRs (available from the Li Lab)) to cover different CpG sites in - 1049 to end 1269 bp, Table 1). The genomic DNA was isolated using Quick-DNA™ Plus Kit (Zymo Research) and followed by bisulfite conversion using Zymo EZ DNA Methylation-direct kit (Zymo Research) as per manufacturer's instruction. Then the qMSP-PCR reaction was performed to amplify the bisulfite DNA. The PCR product of each sample was loaded onto 1.5% agarose gel, and band intensities were normalized to unmethylated PCR product.

2.8. Statistical analysis

Data analyses and graphical presentations were performed with GraphPad Prism, version 8.00 (GraphPad Software, Inc., La Jolla, CA). Data are represented as mean values $\pm$ standard error mean (SEM). The experimental groups were compared by one-way analysis of variance (ANOVA) in combination with Tukey's multiple comparison tests. The significance of differences between groups was determined using Two-tailed, unpaired Student's t-test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Isolation and characterization of BM-EPCs

Bone marrow (BM) derived mononuclear cells (BM-MNCs) were isolated from experimental mice. After 7 days in culture, BM-MNCs showed a change in phenotypic characteristics from a round to a spindle-shaped morphology and formed colonies on the 7th day of culture, called BM-EPCs (Fig. 1A). These BM-EPCs were characterized by the uptake of Dil-Ac-LDLs (Fig. 1B). Flow cytometry analysis of BM-EPCs further demonstrated the presence of endothelial cell marker CD34 and of CD31. 76.9% of BM-EPCs stained positive for both CD31 and CD34 (Fig. 1C), suggesting that we have isolated BM-EPCs (Fig. 1).

3.2. Effect of high methionine on Hcy and H₂S production

Next, we investigated targeted metabolite changes by determining the concentration of Hcy and H₂S in BM-EPCs. The data demonstrate that Hcy concentration was higher in both BM plasma and cultured BM-EPCs of HMD group compared to WT respectively (Fig. 2A and B). In contrast, the concentration of H₂S was lower in both BM plasma and cultured BM-EPCs of HMD group compared to WT respectively (Fig. 2C, D).

3.3. Effect of high methionine on CBS protein and enzymatic function

It is well known that CBS is an essential enzyme of methionine metabolism, which metabolizes the Hcy to H₂S in the cellular system. The data suggested that both CBS protein and mRNA transcript levels were decreased in the HMD group compared to WT respectively (Fig. 2E–F and G). Further, CBS enzyme activity was, indeed, lower in the HMD group compared to WT (Fig. 2H). These data suggest that HMD alters the normal Hcy metabolism by decreasing CBS protein expression and activity.

3.4. HMD induces hyper-methylation of CBS promoter

The DNA methylation is a part of the epigenetic regulation that regulates gene expression. Previously, we have shown that c-Jun/ JNK mediated signaling is essential in transcriptional activation of DNMT1 expression during HHcy condition [12]. In the current study, we tested the potential involvement of DNMTs expression that directs for the global DNA methylation in the BM-EPCs during HMD condition. The data suggest that DNMT1 expression was upregulated in BM-EPCs culture under HMD condition as compared to WT, as assessed by both protein western blot analysis and qPCR analysis (Fig. 3A, B and C). No significant change in DNMT-3a protein and mRNA levels was observed in the HMD condition as compared to WT. In addition, we also found increased DNMT activity and global DNA methylation (% 5-mC levels) in HMD mice derived BM-EPCs as compared to WT respectively (Fig. 3D and E). To study DNMT1 mediated active DNA methylation within CBS promoter that may be involved in BM-EPCs dysfunction, we investigated HMD regulated 5 mC enrichment within the CBS locus. The data found that 5 mC enrichment was elevated within the CBS promoter in BM-EPCs of HMD condition, as assessed by MeDIP-qPCR analysis (Fig. 3F). Further, using ChIP assay analysis, the data demonstrate that DNMT1 binding to the CBS promoter was indeed increased in HMD condition compared to

the WT group (Fig. 3G). However, treatment of DNMTs inhibitor, 5-Aza reverses the above changes.

3.5. Administration of 5-aza reverses the hyper-methylation of CBS promoter in BM-EPCs

Using a CpG Island Search engine, we found that the CBS promoter contains a potential CpG island spanning a 221-bp region (Fig. 3H). Using qMSP-PCR analysis, we found that DNA hypermethylation was observed at CpG region of the CBS gene promoter of BM-EPCs from HMD condition compared to WT control (Fig. 3I). Interestingly, the administration of 5-Aza, an inhibitor of DNMTs, to HMD mice, inhibited the hyper-methylation of the CBS gene promoter. Real-time PCR analysis showed that the treatment of 5-aza increased the CBS mRNA expression in BM-EPCs of HMD+5-Aza group compared with HMD control (Fig. 3J). Similarly, CBS activity was also improved in the HMD+5-Aza group compared to the HMD group (Fig. 3K).

3.6. Administration of 5-aza treatment restores BM-EPCs function and hind limb blood flow in HMD fed mice

Further, we would like to investigate the CBS function in BM-EPCs by inhibiting the methylation potential of DNMTs by DNA methylation inhibitor 5-AZA in the HMD condition. The data demonstrated that both cell proliferation and migration capacity of BM-EPCs was, indeed, improved in the HMD group treated with 5-Aza (HMD+5-Aza) in comparison with HMD alone, as assessed by MTT assay and Trans-well migration assay (Fig. 4A, C). qPCR analysis further confirmed that the mRNA transcript of Ki67; a marker of cell proliferation, was upregulated upon administration of 5-Aza in HMD (HMD+5-Aza) mice compared to HMD mice alone (Fig. 4B). Similarly, the cell adhesion capacity of BM-EPCs in a fibronectin coated plate was better recovered in HHcy+5-Aza group compared to HMD mice, as indicated in typical nuclear staining of BM-EPCs by DAPI image (Fig. 4D). Furthermore, tube formation abilities of BM-EPCs was also significantly recovered *in vitro* using a 3D-matrigel capillary tube formation assay in the HMD+5-Aza group, in comparison with HMD alone (Fig. 4E). To determine whether the decrease in CBS function in the BM-EPCs of HMD mice may cause the overall decrease in blood flow to the bone, we examined blood flow response by using a Laser Doppler imaging flow meter. The data demonstrate that administration of 5-Aza in HMD mice resulted in improvement of hind limb blood flow in comparison with HMD mice alone (Fig. 4F). These data suggested that 5-Aza administration causes demethylation of CBS, leading to restored BM-EPCs function and recovery of bone blood flow.

4. Discussion

In the current study, we demonstrated that the angiogenic function of BM-EPCs was significantly impaired in HMD-fed mouse models, accompanied by hyper-methylation in the CpG island of the CBS promoter, which leads to downregulation of CBS expression. We also showed CBS promoter hyper-methylation was negatively associated with the angiogenic function of BM-EPCs from HMD mice. Further, we also demonstrated that hind limb blood flow of HMD mice was severely affected as compared to WT alone, accompanied by aberrant hyper-methylation of the CBS promoter and downregulation of

CBS expression. However, administration of 5-Aza, an inhibitor of DNMT to HMD mice, reversed these phenotypes. Taking this into account, our data suggested for the first time that aberrant hyper-methylation of the CBS promoter may play a critical role in attenuating the function of BM-EPCs and bone blood flow in mice.

To date, there are no reliable surface expression markers that have been reported to define the characterization of BM-EPCs. In the present study, we characterized the BM-EPCs by both flow cytometry analysis of surface markers (CD34⁺, CD31) and uptake of Dil-Ac-LDLs staining (Fig. 1). Therefore, we chose the combination of these two markers to detect BM-EPCs populations in this study. The presence of BM-EPC in bone milieu contributes to bone marrow angiogenesis [2,14]. In regenerative medicine, BM-derived EPCs could potentially be used as therapeutic strategies to combat several diseases, including wound healing, myocardial infarction, and chronic obstructive pulmonary disease [15–18]. Both hypercholesteremia and HHcy are accepted as common risk factors for the development of vascular disease, thus limiting EPC-mediated vascular repair [19,20]. In the present study, we also provided evidence that the angiogenic ability of BM-derived EPCs and its involvement in hind-limb blood flow was significantly lower in HMD mice compared with WT mice (Fig. 4).

It is well established that Hcy is detrimental to EPCs function and differentiation [1,21, and 21]. CBS is an important catabolic enzyme of Hcy metabolic pathway, and is responsible for the maintenance of cellular homeostasis via production of H₂S [23,24]. Additionally, it is expressed at relatively high levels in different tissues, including tissues of the bone, brain and vascular system [10,12,24–26]. In the current study, we found that both CBS expression and intracellular H₂S production were downregulated in BM-EPCs isolated from HMD mice compared with WT control. In corroboration with cellular CBS activity, we found that increased BM plasma level of tHcy, in HMD mice (Figs. 2 and 4). However, the molecular mechanism responsible for downregulation of CBS expression under HMD remains unclear.

DNA methylation is the process of transfer of a methyl (–CH₃) group to the cytosine residue of the nucleotide region of the gene from an activated donor (s-adenosylmethionine, SAM) by the enzyme DNA methyltransferases (DNMTs) [27,28]. DNMTs play an important role in DNA methylation [28]. Studies have suggested that abnormal methylation is linked to aging, inflammation, obesity, and osteoporosis [1,10,28,29]. Previously, we reported that HMD fed mice develop HHcy phenotype, leading to osteoclast activation and bone loss [10,12]. The study also suggests that Hcy is induced to cause apoptosis of EPCs via oxidative stress-dependent Nox4 and p38MAPK signaling [30]. However, the epigenetic mechanism of excessive Hcy accumulation by reducing the CBS activity and subsequently inhibition of BM-EPCs function remain to be determined. Taking this into account, we examined that the CpG island of the CBS promoter was hyper-methylated in BM-EPCs of HMD mice, as assessed by qMSP-PCR. Further, this aberrant CBS hyper-methylation was reversed in HMD+5-Aza mice by specific DNA methylation inhibitor; 5-Aza (Fig. 3). Furthermore, we also demonstrated that hyper-methylation of CBS was found to decrease the angiogenic function of BM-EPCs (Fig. 4).

Collectively, our data provided evidence for the first time that aberrant hyper-methylation in the CBS promoter is associated with the impaired angiogenic function of BM-EPCs and subsequent reduction of hind limb blood flow of HMD mice (Fig. 4G). Further study is warranted for development of a new epigenetic target in the treatment of HHcy mediated BM-EPCs dysfunction.

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Fig. 1. Characterization of differentiated BM-EPCs from BM-MNCs.

A. Representative images of BM-MNCs culture on the 0th day and differentiation of BM-EPCs on the 7th day (magnification 200 $\times$), as indicated with yellow arrows. B. Fluorescent imaging indicated Dil-acetylated LDL incorporation by differentiated BM-EPCs on the 7th day, as represented with white arrows. The typical nuclear staining of BM-EPCs by DAPI image (magnification 200 $\times$). C. Flow cytometry analysis of BM-EPCs that express both stem cell marker CD34, and endothelial marker CD31 in BM-EPCs at 7th day. n = 5 mice per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 2. Effect of high methionine diet (HMD) on homocysteine metabolism.

(A–B) Biochemical assay of tHcy level in BM plasma *in vivo* and cultured BM-EPCs *in vitro*. (C–D) H_2S levels were measured in BM plasma *in vivo* and cultured BM-EPCs *in vitro*. (E–F) Western blot analysis of BM-EPCs expressed CBS. (G) mRNA transcript of CBS in BM-EPCs, as assessed by qPCR. (H) CBS activity in whole BM-EPCs homogenate, as assessed by CBS activity fluorometric assay kit. Data are expressed as mean $\pm$ SEM. $n = 5$ mice per group. * $p < 0.05$ compared with the WT mice.

Fig. 3. Administration of 5-aza treatment reverses the hyper-methylation of CBS promoter in BM-EPCs.

(A–B). Western blot analysis of DNMT1 and DNMT3a protein expression in BM-EPCs homogenate. Bar graphs showing the quantitative estimation of DNMT1 and DNMT3a proteins after normalization with GAPDH. (C). mRNA transcript of DNMT1 and DNMT3a by qPCR. (D–E). DNMT activity and 5-mC levels were studied in nuclear extract of BM-EPCs. (F) Enrichments of 5 mC in CBS promoter region is assessed by MeDIP-qPCR analysis. (G) ChIP-PCR analyzes DNMT1 binding in the promoter regions of CBS in BM-EPCs. IgG is used as a control. (H). In silico analysis of CpG island (1049–1269 region) prediction in CBS promoter. (I) MSP-PCR was performed to show the CBS promoter CpG-island DNA methylation in the experimental condition. (J) mRNA transcript of CBS by qPCR. (K). CBS activity was measured in whole BM-EPCs homogenate, using CBS activity fluorometric assay kit. Data are expressed as mean $\pm$ SEM. $n = 5$ mice per group. * $p < 0.05$ compared with the WT mice, # $p < 0.05$ compared with the WT+HMD mice.

Fig. 4. Administration of 5-aza treatment restores BM-EPCs function and hind limb blood flow during hyperhomocysteinemia.

(A) Cell proliferation under 5-Aza treatment by MTT assay. (B) mRNA transcript of Ki67 by qPCR analysis. (C) Representative nuclear staining of DAPI images of the BM-EPCs migration, as assessed by Trans-well migration. (D) Representative nuclear staining of DAPI images (left) of BM-EPCs cell adhesion on the fibronectin-coated plate. (E) Representative images of the endothelial tube formation by 3D-matrigel tube assay, as indicated with yellow arrows (left) and quantitation of percentage of tube formation in the endothelial network (right) of BM-EPCs. (F) Effect of 5-Aza treatment on HMD-mediated bone blood flow was monitored by laser Doppler perfusion imaging after HMD fed mice (8 weeks) and quantitative evaluation of blood flow were expressed as arbitrary flux unit. (G) A proposed epigenetic mechanism of HMD induced BM-EPCs dysfunction and subsequent ablation of bone blood flow in mice. Data are expressed as mean $\pm$ SEM. $n = 5$ mice per group. * $p < 0.05$ compared with the WT mice, # $p < 0.05$ compared with the WT+HMD mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1

Sequences of PCR primers used for real time quantitative PCR.

Gene	Primer Sequences (5'→3')
Mouse CBS	FP:AGGGCTATCGCTGCATTATCTGA RP:AGCTTCCACCACATAGCAGTCCTT
Mouse DNMT1	FP: AGGGAAAAGGGAAGGGCAAG RP: CCAGAAAACACATCCAGGGTCC
Mouse DNMT-3a	FP: CCATAAAGCAGGGCAAAGACC RP: AGTGGACTGGGAAACCAAATACC
Mouse GAPDH	FP: TGCACCACCAACTGCTTGC RP: GGCATGGACTGTAGTCAGAG
Gene	qMSP primer sequences
Mouse CBS	Left M primer: GGAGTTCGAGGAGTTGAATTAGAC Right M primer: TACGATAAACATACGAACAAAAACG Left U primer: GGAGTTTGAGGAGTTGAATTAGATG Right U primer: CAATAAACATACAAACAAAAACAAA
Gene	ChIP primer sequences
Mouse CBS	FP:TCGCTGGGGTTCGGTAAC RP:GGAAGTGGGCAACGGATG