

Research Article

RAGE-mediated functional DNA methylated modification contributes to cigarette smoke-induced airway inflammation in mice

Ping Li^{1,*}, Tao Wang^{1,*}, Mei Chen^{2,*}, Jun Chen¹,  Yongchun Shen¹ and  Lei Chen¹

¹Laboratory of Pulmonary Diseases and Department of Respiratory and Critical Care Medicine, West China Hospital, West China School of Medicine, Sichuan University, Chengdu, Sichuan 610041, P.R. China; ²Department of Respiratory and Critical Care Medicine, Chengdu Fifth People's Hospital, Chengdu, Sichuan 611130, P.R. China

Correspondence: Lei Chen (lchens@126.com) or Yongchun Shen (shen_yongchun@126.com)



Our previous study indicated knockout of receptor for advanced glycation end-products (RAGE) significantly attenuated cigarette smoke (CS)-induced airway inflammation in mice. In the present study, we aim to further detect the mediatory effects of RAGE in DNA methylated modification in CS-induced airway inflammation. Lung tissues from the CS-exposed mouse model of airway inflammation were collected for profiling of DNA methylation by liquid hybridization capture-based bisulfite sequencing, which were used for conjoint analysis with our previous data of gene expression by cDNA microarray to identify functional methylated genes, as well as hub genes selected by protein-protein interaction (PPI) network analysis, and functional enrichment analyses were then performed. After RAGE knockout, 90 genes were identified by intersection of the differentially methylated genes and differentially expressed genes. According to the reversed effects of methylation in promoters on gene transcription, 14 genes with functional methylated modification were further identified, among which chemokine (C-X-C motif) ligand 1 (CXCL1), Toll-like receptor 6 (TLR6) and oncostatin M (OSM) with hypomethylation in promoters, were selected as the hub genes by PPI network analysis. Moreover, functional enrichment analyses showed the 14 functional methylated genes, including the 3 hub genes, were mainly enriched in immune-inflammatory responses, especially mitogen-activated protein kinase, tumor necrosis factor, TLRs, interleukin (IL)-6 and IL-17 pathways. The present study suggests that RAGE mediates functional DNA methylated modification in a cluster of 14 targeted genes, particularly hypomethylation in promoters of CXCL1, TLR6 and OSM, which might significantly contribute to CS-induced airway inflammation via a network of signaling pathways.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by persistent respiratory symptoms and airflow limitation that are associated with persistent airway inflammation induced by cigarette smoke (CS), a major causative factor for COPD [1].

Receptor for advanced glycation end-products (RAGE), a membrane protein from the immunoglobulin superfamily, has been implicated in the pathogenesis of COPD [2]. Overexpression of RAGE contributed to airway inflammation in CS-associated COPD [3]. Our previous study further indicated knockout (KO) of RAGE gene significantly attenuated CS-induced airway inflammation in a mouse model [4]. However, the mechanisms regarding the effects of RAGE on airway inflammation in COPD remain not clear. Recently, some evidences implied DNA methylation, an epigenetic regulation, played important

*These authors contributed equally to this work.

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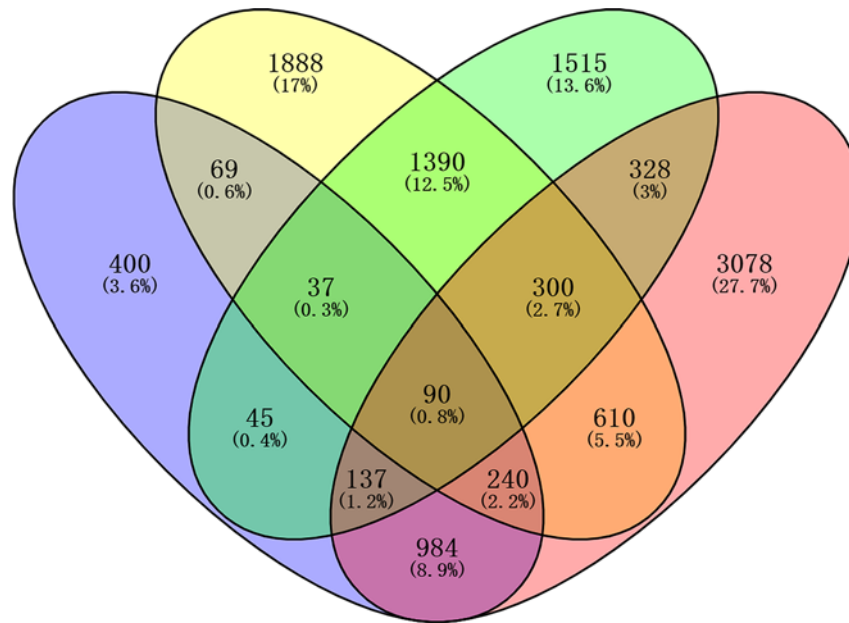


Figure 1. Venn diagram of the intersection model

The colorful oval shapes represent CS-associated DEGs (yellow), CS-associated DMGs (blue), RAGE-associated DEGs (green) and RAGE-associated DMGs (purple).

roles in RAGE-mediated inflammatory responses in various diseases [5–7], although the role in DNA methylated modification mediated by RAGE in airway inflammation in COPD was not reported.

In consequence, we did the present study, using the established mouse model of CS-induced airway inflammation, to explore the underlying mechanisms of RAGE-mediated DNA methylated modification in airway inflammation in COPD.

Materials and methods

Animal model

The animal model of CS-induced airway inflammation has been established in our former study [4]. C57BL/6 mice (7–9 weeks old, 20–22 g weight) were used to generate RAGE KO mice through CRISPR/Cas9 gene targeting technology by Bioray Biotechnology (Shanghai, China). The four experimental groups ($n=3$ mice per group) were included in the present study, as follows: (i) wildtype (WT) group, (ii) CS+WT group, (iii) CS+KO group, (iv) WT+KO group. All mice were specific pathogen-free and kept on a 12-h light/12-h dark cycle, at a room temperature of $22 \pm 2^\circ\text{C}$, with free access to food and water. WT and RAGE KO mice were exposed to mainstream CS or room air for 2 h twice daily, 6 days per week for consecutive 4 weeks. After 4-week CS exposure, the mice were anesthetized intraperitoneally with pentobarbital sodium and killed by femoral artery transection. The animal study was approved by the Panel on Laboratory Animal Care of West China Hospital of Sichuan University and took place at the Experimental Animal Center of West China Hospital of Sichuan University.

DNA extraction

The total DNA of lung tissues was extracted and purified by DNeasy Blood Tissue Kit (Qiagen), according to the manufacturer's instructions. Purified DNA was then quantified by NanoDrop2000 Spectrophotometer (Thermo) and agarose gel electrophoresis. Only DNA samples with $A_{260/280}$ ratio between 1.8 and 2.0 were used for further experiments.

Liquid hybridization capture-based bisulfite sequencing

Genomic DNA (1 μg per sample) was randomly fragmented into approximately 200–300 bp by sonication. After purification, the DNA fragments were repaired in the blunt and phosphorylated ends, which were subsequently 3'

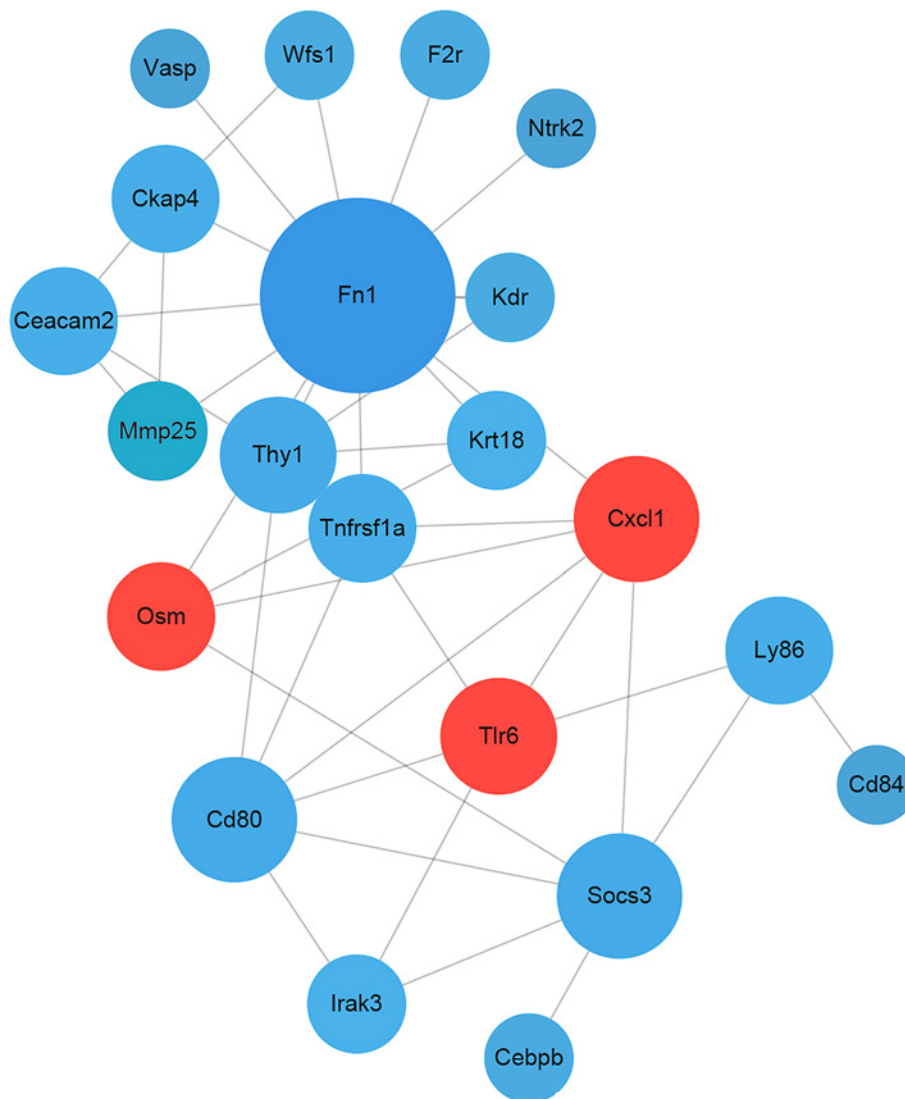


Figure 2. PPI network analysis of the candidate genes

Edges indicate correlations between two genes. The bigger circles indicate more related genes and red circles indicate the hub genes.

adenylated and then ligated to the methylated adapter using the SureSelect^{XT} Mouse methyl-seq Library Prep Kit (Agilent). After that, the DNA hybridization was performed using the SureSelectTM Methyl-SeQ Hybridization Kit (Agilent), which covered 100 Mb of mouse genomic regions, including CpG islands, Gencode promoters, tissue-specific differential methylated regions (DMRs) and DNase I hypersensitive sites. The hybridized DNA was subsequently bisulfite-treated using the EZ DNA Methylation-GoldTM Kit (Zymo Research) to convert unmethylated cytosine into uracil according to the manufacturer's instructions. Finally, the treated DNA was amplified by polymerase chain reaction (PCR) and sequenced on Illumina Novaseq PE150.

DNA methylation data analyses

The Fastp software (version 1.2.1) was used to process methylated raw data and remove the low quality reads, including (i) contaminated sequences; (ii) the Q-value of 3' end is less than 20; (iii) reads with less than 15 bp; (iv) reads with 40% base Q-value less than 15; (v) reads containing N bases greater than 5 ($Q = -10 \times \log_{10}(P)$, P is the probability of error) [8]. The clean reads were aligned to the reference genome using Bismark software (version 0.19.0) with bowtie2 (version 2.3.4.2) to attain the methylated type, status and proportion [9]. DMRs were subsequently analyzed using the R package methylKit (version 1.6.1) [10] and eDMR [11], with an adjusted P -value < 0.05 and absolute differential

methylation levels (absolute meth. diff > 5%). Finally, related differential methylated genes (DMGs) were located and annotated in the DMRs by the ChIPseeker software.

cDNA microarray

The data of gene expression by cDNA microarray have been obtained in our former study [4]. In the present study, differentially expressed genes (DEGs) were identified by fold-change, and only genes that at least 1.2-fold up-regulated or down-regulated were analyzed.

Candidate genes selection

To identify the candidate genes with methylated modification mediated by RAGE, a novel intersection model was performed (Figure 1). Briefly, CS-associated (CS+WT vs WT) and RAGE-associated (CS+KO vs CS+WT plus WT+KO vs WT) DMGs intersected to get the overlapped DMGs, and so did CS-associated and RAGE-associated DEGs (overlapped DEGs). Then, the overlapped DMGs and DEGs intersected again to select the candidate genes.

Functional methylated genes and hub genes selection

The candidate genes with functional methylated modification, also called functional methylated genes, were identified according to the reversed effects of methylation in promoters on gene transcription. To further identify the hub genes that were most correlated with other genes, protein–protein interaction (PPI) network analysis among the functional methylated genes was performed using the online database STRING [12] and displayed using Cytoscape [13].

Functional enrichment analyses

Functional enrichment analyses on the functional methylated genes, as well as the hub genes, using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were performed with the clusterProfiler package using *P*-value <0.05 was set as the threshold.

Results

Data production

Based on the liquid hybridization capture-based bisulfite sequencing (LHC-BS) method, 15 Gbp raw sequence data were generated on average for each sample. More than 79% were mapped to at least one genomic position, covering ~77% of the target regions, with an average of 57× sequencing depth per CpG and 12.5% duplication data, and the duplicated sequence reads were filtered for the subsequent analyses.

RAGE-mediated functional DNA methylated modification in targeted genes in CS-induced airway inflammation

After RAGE KO, as reported in our previous study, CS-induced airway inflammation was significantly improved [4]. Meanwhile, 90 overlapping candidate genes were identified via the intersection of DMGs with DEGs (Supplementary Table S1). It is well-known that DNA methylation occurs almost in CpG islands that are primarily located in the regions of promoter and are negatively correlated with gene expression [14]. As a result, 14 genes from the 90 candidate genes, with functional methylated modification in promoters were identified (Table 1), among which, three genes, including chemokine (C–X–C motif) ligand 1 (CXCL1), Toll-like receptor 6 (TLR6) and oncostatin M (OSM) with hypomethylation in promoters were finally selected as the hub genes by PPI network analysis (Figure 2). Furthermore, GO and KEGG analyses indicated the 14 functional methylated genes, including the 3 hub genes, were significantly enriched in immune-inflammatory responses, especially mitogen-activated protein kinase (MAPK), tumor necrosis factor (TNF), TLRs, interleukin (IL)-6 and IL-17 signaling pathways (Supplementary Tables S2 and S3).

Discussion

DNA methylation is closely associated with COPD susceptibility, exacerbation and lung function decline [15,16]. In this process, RAGE may play a regulatory role in DNA methylated modification in COPD, whereas the mechanisms remain unexplained. Consequently, in the present study, the 90 DMGs, regarding RAGE-mediated airway inflammation induced by CS exposure, were initially selected using a novel intersection model, and the functional DNA methylated modification in 14 targeted genes were subsequently identified, especially hypomethylation in CXCL1, TLR6 and OSM promoters, which might significantly contribute to RAGE-mediated airway inflammation in COPD via a network of signaling pathways, such as MAPK, TNF, TLRs, ILs, etc.

Table 1 The 14 functional methylated genes

Gene	Description	Genomic localization	Methylation change*	Fold change
<i>Alox5ap</i>	Aachidonate 5-lipoxygenase activating protein	Promoter Distal intergenic 3' UTR	Hyper (15.6131412) Hypo (-12.032621795) Hypo (-13.27280036)	-1.2
<i>Arhgap22</i>	Rho GTPase activating protein 22	Promoter Promoter	Hyper (10.29366302) Hypo (-6.476953839)	-1.3
<i>Cxcl1</i>	Chemokine (C-X-C motif) ligand 1	Promoter	Hyper (10.2010746)	-1.2
<i>Dmc1</i>	DNA meiotic recombinase 1	Promoter	Hypo (9.450360901)	1.2
<i>Inf2</i>	Inverted formin, FH2 and WH2 domain containing	Promoter Distal intergenic	Hyper (13.93768033) Hyper (13.60386215)	-1.2
<i>Mir1981</i>	MicroRNA 1981	Promoter	Hypo (-6.417671883)	1.2
<i>Mmp25</i>	Matrix metalloproteinase 25	Promoter Promoter	Hyper (15.40303433) Hypo (-13.06629416)	-1.4
<i>Osm</i>	Oncostatin M	Promoter	Hyper (15.05770235)	-1.4
<i>Psmb9</i>	Proteasome 20S subunit β 9	Promoter	Hyper (10.74327874)	-1.3
<i>Rnaseh2b</i>	Ribonuclease H2, subunit B	Promoter	Hyper (11.78927527)	-1.3
<i>Slc16a6</i>	Solute carrier family 16 member 6	Promoter Intron	Hyper (8.126762879) Hyper (8.360891276)	-1.2
<i>Srgn</i>	Srglycin	Promoter	Hyper (9.932392771)	-1.3
<i>Tlr6</i>	Toll-like receptor 6	Promoter	Hyper (17.51844104)	-1.4
<i>Tmod2</i>	Tropomodulin 2	Promoter	Hypo (-7.597567585)	1.3

*Methylation changes (%) in gene expression by CS+KO vs CS+WT. Abbreviations: Hyper, hypermethylation; Hypo, hypomethylated; Inc, increased variance.

According to the GO and KEGG analyses, the majority of the candidate genes, especially the 14 functional methylated genes widely participated in CS-associated inflammatory-immune responses. In particular, the three hub genes were documented to play important roles in the inflammatory process in COPD. CXCL1 is a member of chemokine subfamily of CXC [17] and the increased level of CXCL1 was detected in the lungs of COPD [18]. CXCL1 served as a chemoattractant for neutrophils migrating from circulation to respiratory tracts, which contributed to neutrophilic inflammation of COPD [19]. TLR6 belongs to the Toll-like receptor family [20], which initiates innate immune responses in airway epithelial cells and triggers inflammatory responses [21]. Furthermore, TLRs activation could increase CXCL1 production in human pulmonary macrophages [22]. OSM, a member of IL-6 subfamily [23], participates in a variety of inflammatory diseases with a high level [24–26]. In COPD, the inflammatory mechanisms for OSM may be stimulating IL-6 production and IL-6 related inflammation, which is positively correlated with pulmonary function decline [23,27]. Noticeably, as suggested in our study, CXCL1, TLR6 and OSM have been documented to be targeted genes of DNA methylated modification in other diseases, such as schizophrenia [28], type 1 diabetes [29] and Richter syndrome [30].

However, some limitations in the present study should be considered. First, the sample size of mice in each group was relatively small, although the minimum requirement for biological repeat was reached. Second, the novel intersection model might be theoretically imperfect. Third, validation was thus needed in future studies.

In summary, the present study performed intersections of DMGs with DEGs in a CS-exposed mouse model and indicated RAGE could mediate functional methylated modification in multiple targeted genes, especially CXCL1, TLR6 and OSM, which might significantly contribute to airway inflammation in COPD.

Data Availability

The data presented in this manuscript are available from the corresponding author (Lei Chen) on reasonable request.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Ping Li: Software, Investigation, Visualization, Methodology, Writing—original draft. **Tao Wang:** Software, Investigation, Visualization, Methodology. **Mei Chen:** Software, Investigation, Visualization, Methodology. **Jun Chen:** Software, Investigation. **Yongchun Shen:** Data curation, Supervision, Validation, Writing—review and editing. **Lei Chen:** Conceptualization, Formal Analysis, Writing—review and editing, Funding acquisition.

Abbreviations

COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; CXCL1, chemokine (C–X–C motif) ligand 1; DEG, differentially expressed gene; DMG, differential methylated gene; DMR, differential methylated region; GO, Gene Ontology; IL, interleukin; KEGG, Kyoto Encyclopedia of Genes and Genomes; KO, knockout; MAPK, mitogen-activated protein kinase; OSM, oncostatin M; PPI, protein–protein interaction; RAGE, receptor for advanced glycation end-product; TLR, Toll-like receptor; TNF, tumor necrosis factor; WT, wildtype.

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Table S1. The 90 candidate genes selected according to the methylation and gene expression

Gene	Description	Genomic localization	Methylation change*	Fold Change
1600014C10Rik	RIKEN cDNA 1600014C10 gene	Promoter/Intron	Hypo/Hyper	-1.3
2610035D17Rik	RIKEN cDNA 2610035D17 gene	Intron	Inc	1.3
2610528J11Rik	RIKEN cDNA 2610528J11 gene	Promoter/Distal Intergenic	Hypo/Hypo	-1.3
Agpat4	1-acylglycerol-3-phosphate O-acyltransferase 4	Intron	Inc	-1.3
Alox5ap	arachidonate 5-lipoxygenase activating protein	Promoter/Distal Intergenic/3' UTR	Hyper/Hypo/Hypo	-1.2
Arhgap22	Rho GTPase activating protein 22	Promoter	Inc	-1.3
Atoh8	atonal bHLH transcription factor 8	Intron /Distal Intergenic	Hypo/Hypo	-1.7
Best2	bestrophin 2	3' UTR	Hypo	1.2
Blm	Bloom syndrome, RecQ like helicase	Exon/3' UTR	Inc/Hypo	-1.2
Btg3	B cell translocation gene 3	Intron/Distal Intergenic	Hyper/Hyper	-1.6
Cd80	CD80 antigen	Intron/Exon	Hypo/Hypo	-1.3
Cd84	CD84 antigen	Promoter /Intron/Distal Intergenic/3' UTR	Hyper/Hyper/Hyper/Hypo	1.3
Cdc6	cell division cycle 6	3' UTR	Hypo	-1.3
Ceacam2	carcinoembryonic antigen-related cell adhesion molecule 2	Intron	Hyper	1.3
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	Promoter/Intron	Hypo/Hyper	-1.3
Ckap4	cytoskeleton-associated protein 4	Distal Intergenic	Hyper	-1.4
Cxcl1	chemokine (C-X-C motif) ligand 1	Promoter	Hyper	-1.2
Dmc1	DNA meiotic recombinase 1	Promoter	Hypo	1.2
Dusp6	dual specificity phosphatase 6	Exon/Distal Intergenic	Hyper/Hyper	-1.7
Ear1	eosinophil-associated, ribonuclease A family, member 1	Distal Intergenic	Hyper	1.7
Emilin2	elastin microfibril interfacier 2	Exon/Intron/Downstream	Hyper/Inc/Hyper	-1.5
F2r	coagulation factor II (thrombin) receptor	Exon/Distal Intergenic/Downstream	Hyper/Hyper/Hyper	-1.3

Fn1	fibronectin 1	Exon/Distal Intergenic	Hyper/Hypo	-1.7
Galnt15	polypeptide N-acetylgalactosaminyltransferase 15	Promoter	Hypo	-1.3
Galnt2	polypeptide N-acetylgalactosaminyltransferase 2	Intron	Hyper	-1.3
Galnt9	polypeptide N-acetylgalactosaminyltransferase 9	Intron	Inc	-1.3
Gclc	glutamate-cysteine ligase, catalytic subunit	Distal Intergenic	Hypo	1.6
Gdf10	growth differentiation factor 10	Intron	Hyper	-1.3
Gm3696	predicted gene 3696	Intron	Hyper	1.6
Gm5148	predicted gene 5148	Distal Intergenic	Inc	-1.2
Gm6548	eukaryotic translation elongation factor 1 alpha 1 pseudogene	Promoter	Hyper	1.3
Gm8300	predicted gene 8300	Distal Intergenic	Hyper	1.6
Gp6	glycoprotein 6 (platelet)	Distal Intergenic	Hyper	-1.2
Gsg11	GSG1-like	Promoter	Hyper	1.2
Ifitm2	interferon induced transmembrane protein 2	Promoter	Hypo	-1.5
Inf2	inverted formin, FH2 and WH2 domain containing	Promoter/Distal Intergenic	Hyper/Hyper	-1.2
Irak3	interleukin-1 receptor-associated kinase 3	Intron	Inc	-1.3
Kcnj8	potassium inwardly-rectifying channel, subfamily J, member 8	Distal Intergenic	Inc	-1.3
Kdr	kinase insert domain protein receptor	Exon/Distal Intergenic	Hyper/Hypo	1.4
Krt18	keratin 18	Promoter/Distal Intergenic	Hypo/Hypo	-1.5
Ldb2	LIM domain binding 2	Intron	Hyper	-1.2
Lgi2	leucine-rich repeat LGI family, member 2	Distal Intergenic	Hypo	-1.2
Lmb1	lamin B1	Distal Intergenic	Hyper	-1.2
Ly86	lymphocyte antigen 86	Exon/Distal Intergenic	Hyper/Hypo	-1.2
Map3k7c1	Map3k7 C-terminal like	Distal Intergenic	Hyper	-1.3
Mctp1	multiple C2 domains, transmembrane 1	Intron	Hyper	-1.5

Mir1981	microRNA 1981	Promoter	Hypo	1.2
Mmp25	matrix metalloproteinase 25	Promoter	Inc	-1.4
Mthfd11	methylenetetrahydrofolate dehydrogenase (NADP+ Dependent) 1 Like	Exon	Hypo	-1.4
Myo18b	myosin XVIIIb	Intron/Exon	Hypo/Hypo	1.2
Nav2	neuron navigator 2	Intron	Inc	1.2
Ncf1	neutrophil cytosolic factor 1	Intron/3' UTR	Hypo/Hypo	-1.3
Ntrk2	neurotrophic tyrosine kinase, receptor, type 2	Intron	Hypo	-1.6
Nup12	nucleoporin like 2	Intron	Hyper	1.3
Osm	oncostatin M	Promoter	Hyper	-1.4
Padi4	peptidyl arginine deiminase, type IV	Exon/Downstream	Hyper/Hypo	-1.6
Pgam2	phosphoglycerate mutase 2	Promoter	Hyper	1.3
Pgm1	phosphoglucomutase 1	Exon/Distal Intergenic	Hyper/Hyper	-1.4
Pln	phospholamban	Distal Intergenic	Hyper	1.5
Ppargc1a	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	Distal Intergenic	Hypo	-1.3
Prkar2b	protein kinase, cAMP dependent regulatory, type II beta	Intron/Exon/Distal Intergenic	Hyper/Hyper/Hyper	1.4
Psmb9	proteasome 20S subunit beta 9	Promoter	Hyper	-1.3
Pttg1	pituitary tumor-transforming gene 1	Promoter	Hyper	1.3
Rcsd1	RCS domain containing 1	Distal Intergenic	Hypo	-1.2
Rerg	RAS-like, estrogen-regulated, growth-inhibitor	Promoter/Intron	Hypo/Hyper	-1.2
Rfc5	replication factor C (activator 1) 5	Distal Intergenic	Hyper	-1.3
Rnaseh2b	ribonuclease H2, subunit B	Promoter	Hyper	-1.3
Rps9	ribosomal protein S9	Promoter /Distal Intergenic	Hyper/Hypo	1.3
Rrm2	ribonucleotide reductase M2	Exon/Distal Intergenic	Hyper/Hyper	-1.3
Slc16a6	Solute Carrier Family 16 Member 6	Promoter/Intron	Hyper/Hyper	-1.2

Slc1a5	solute carrier family 1 member 5	Exon/Distal Intergenic	Inc/Hyper	1.3
Slc7a5	solute carrier family 7 member 5	Distal Intergenic	Hyper	-1.5
Slc7a8	solute carrier family 7 member 8	Intron/Distal Intergenic	Hyper/Hyper	-1.3
Soes3	suppressor of cytokine signaling 3	Distal Intergenic	Hyper	-1.6
Spata18	spermatogenesis associated 18	Distal Intergenic	Inc	1.5
Srgn	serglycin	Promoter	Hyper	-1.3
Srp9	signal recognition particle 9	Intron/Distal Intergenic	Hyper/Inc	-1.2
St3gal1	ST3 beta-galactoside alpha-2,3-sialyltransferase 1	Intron /Distal Intergenic	Hyper/Hyper	-1.2
Tbc1d30	TBC1 domain family, member 30	Promoter/Exon/3' UTR	Hyper/Hypo/Hyper	1.2
Thy1	thymus cell antigen 1, theta	Promoter	Hypo	-1.3
Timm22	translocase of inner mitochondrial membrane 22	Intron/Exon	Hypo/Hypo	-1.4
Tlr6	toll-like receptor 6	Promoter	Hyper	-1.4
Tmem45b	transmembrane protein 45b	Distal Intergenic	Inc	1.5
Tmod2	tropomodulin 2	Promoter	Hypo	1.3
Tnfrsf1a	tumor necrosis factor receptor superfamily, member 1a	3' UTR	Hypo	-1.2
Vasp	vasodilator-stimulated phosphoprotein	Downstream	Hyper	-1.3
Vmn2r33	vomeronal 2, receptor 33	Distal Intergenic	Hyper	1.6
Wfs1	wolframin ER transmembrane glycoprotein	Intron	Hyper	-1.3
Zbp1	Z-DNA binding protein 1	Intron	Hypo	-1.3
Zbtb20	zinc finger and BTB domain containing 20	Intron	Hypo	1.3

*Methylation changes with fold changes in gene expression by CS+KO vs CS+WT. Hyper, hypermethylation; Hypo, hypomethylated; Inc, increased variance.

Table S2. GO enrichment analysis (the functional methylated genes marked red)

Cellular component/Biologic process/Molecular function	Genes	Count	P value
regulation of MAPK cascade	Osm, Tlr6 , Irak3, F2r, Fn1, Gdf10, Ncf1, Lmnb1, Map3k7cl, Kdr, Dusp6, Ntrk2	12	1.40729E-05
regulation of interleukin-6 production	Tlr6 , F2r, Zbtb20, Cebpb, Irak3	5	2.34084E-05
MAPK cascade	Osm, Tlr6 , Irak3, F2r, Map3k7cl, Fn1, Ncf1, Kdr, Gdf10, Dusp6, Ntrk2, Lmnb1	12	3.43092E-05
inflammatory response	Cxcl1, Alox5ap, Mmp25, Tlr6 , F2r, Tnfrsf1a, Ly86, Fn1, Ncf1, Socs3	10	9.48037E-05
defense response	Tlr6, Mmp25, Cxcl1, Alox5ap , Padi4, Kcnj8, F2r, Zbp1, Socs3, Tnfrsf1a, Ly86, Fn1, Ncf1, Cebpb, rak3, Ifitm2	16	0.000251799
positive regulation of MAPK cascade	Osm, Tlr6 , F2r, Lmnb1, Map3k7cl, Kdr, Ncf1, Ntrk2	8	0.000312195
response to cytokine	Cxcl1, Osm , Ppargc1a, Ifitm2, Zbp1, Irak3, Cebpb, Krt18, Tnfrsf1a, Gclc, Socs3	11	0.000511441
negative regulation of cytokine production	Tlr6, Srgn , Irak3, Cd84, Fn1	5	0.000563016
cytokine-mediated signaling pathway	Cxcl1, Osm , Irak3, Zbp1, Socs3, Tnfrsf1a, Krt18	7	0.000723893
regulation of cytokine production	Srgn, Tlr6 , F2r, Irak3, Cd84, Fn1, Cebpb, Zbtb20	8	0.001398345
regulation of cytokine secretion	Srgn, Tlr6 , Fn1, F2r	4	0.001544646
cellular response to cytokine stimulus	Cxcl1, Osm , Socs3, Krt18, Tnfrsf1a, Cebpb, Ppargc1a, Zbp1, Irak3	9	0.002115125
cytokine secretion	Tlr6, Srgn , Fn1, F2r	4	0.002629006
cytokine production	Tlr6, Srgn , Irak3, Cd84, F2r, Cebpb, Fn1,	8	0.002833485

	Zbtb20			
regulation of MAP kinase activity	Tlr6, F2r, Irak3, Map3k7cl, Dusp6	5	0.002862216	
immune system process	Psmb9, Tlr6, Cxcl1, Osm, Galnt2, Kcnj8, Zbp1, Tnfrsf1a, Ncf1, Padi4, Cd80, Cd84, Thy1, Blm, Irak3, Ifitm2, Ly86, Cebpb	18	0.008472928	
negative regulation of immune system process	Tlr6, Cd84, Irak, Thy1, Cebpb	5	0.015585279	
positive regulation of immune system process	Tlr6, Cxcl1, Cd80, Blm, Cd84, Irak3, Zbp1, Thy1	8	0.024008534	
immune response	Osm, Tlr6, Cxcl1, Tnfrsf1a, Ly86, Ifitm2, Irak3, Padi4, Thy1, Zbp1, Cd84	11	0.031630554	

Table S3 KEGG enrichment analysis

Pathways	Count	Genes	P value
TNF signaling pathway	4	Cxcl1 , Socs3, Tnfrsf1a, Cebpb	0.00164
DNA replication	2	Rnaseh2b , Rfc5	0.00333
IL-17 signaling pathway	2	Cxcl1 , Cebpb	0.04311
Toll-like receptor signaling pathway	2	Tlr6 , Cd80	0.05491

(the functional methylated genes marked red)