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Alterations of the Lung Methylome in Allergic Airway Hyper-Responsiveness

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Abstract

Asthma is a chronic airway disorder characterized by recurrent attacks of breathlessness and wheezing, affecting 300 million people around the world (available at: www.who.int). To date, genetic factors associated with asthma susceptibility have been unable to explain the full etiology of asthma. Recent studies have demonstrated that the epigenetic disruption of gene expression plays an equally important role in the development of asthma through interaction with our environment. We sensitized 6-week-old C57BL/6J mice with house-dust-mite (HDM) extracts intraperitoneally followed by 5 weeks of exposure to HDM challenges (three times a week) intratracheally. HDM-exposed mice showed an increase in airway hyper-responsiveness (AHR) and inflammation together with structural remodeling of the airways. We applied methylated DNA immunoprecipitation-next generation sequencing (MeDIP-seq) for profiling of DNA methylation changes in the lungs in response to HDM. We observed about 20 million reads by a single-run of massive parallel sequencing. We performed bioinformatics and pathway analysis on the raw sequencing data to identify differentially methylated candidate genes in HDM-exposed mice. Specifically, we have revealed that the transforming growth factor beta signaling pathway is epigenetically modulated by chronic exposure to HDM. Here, we demonstrated that a specific allergen may play a role in AHR through an epigenetic mechanism by disrupting the expression of

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AUTHOR CONTRIBUTIONS

Drs. Cheng, Shang, Mitzner, and Tang designed the study. Dr. Das, Mr. Limjunyawong, and Mr. Rabold performed the lung physiological study. Dr. Shang and Ms. Dao performed the gene expression and methylation study with advice of Dr. Tang. Drs. Cheng and Tang analyzed and interpreted the results. Drs. Cheng, Sham, Mitzner, and Tang prepared the article. All authors reviewed and approved the manuscript prior submission.

Additional Supporting Information may be found in the online version of this article.

genes in lungs that might be involved in airway inflammation and remodeling. Our findings provide new insights into the potential mechanisms by which environmental allergens induce allergic asthma and such insights may assist in the development of novel preventive and therapeutic options for this debilitative disease.

Keywords

DNA methylation; house dust mite; airway hyperresponsiveness; asthma; next generation sequencing

INTRODUCTION

Epigenetics provides a promising approach for understanding complex diseases like asthma. The epigenetic disruption of gene expression is now known to play an equally important role as genetic predispositions (such as polymorphism, mutation, deletion, and insertion) in the development of disease [Jiang et al., 2004; Godfrey et al., 2007]. Exposures to environmental stimuli, such as hypoxia, hypo-or hyper-nourishment, infection, hormones, drugs, or allergens, allow epigenetic reprogramming in cells, resulting in permanent alterations in the structure or function of specific organ systems [Tang et al., 2007]. This phenomenon is linked to gene activation, gene silencing, and chromosomal instability without altering DNA sequences. To date, there are three known epigenetic mechanisms: DNA methylation, histone modification, and noncoding RNAs involved in gene regulation.

Recent evidence has shown that epigenetic modifications via DNA methylation can provide a possible mechanistic explanation for the link between exposure to environmental pollutants and asthma [Perera et al., 2009; Kabesch et al., 2010]. DNA methylation refers to the covalent addition of a methyl group derived from *S*-adenosyl-L-methionine to the fifth carbon of the cytosine ring to form the fifth base, 5-methyl cytosine (5-mC) [Costello et al., 2001]. DNA methylation at the 5' CpG island (CGI; CG-rich region with >60% GC content and an obs/exp ratio >0.6 in the 5' flanking region of the gene promoter) alters gene transcription machinery and eventually leads to a decrease in gene expression. DNA methylation patterns have been shown to be affected by postnatal or adult exposure to diesel exhaust [Liu et al., 2008, Perera et al., 2009; Tang et al., 2012], black carbon [Belinsky et al., 2002], particulate matter [Tarantini et al., 2009], benzene [Bollati et al., 2007], and cigarette smoke [Pulling et al., 2004]. Our group employed an innovative epigenetic profiling approach to investigate allergen-induced airway hyper-responsiveness (AHR). We showed that acute exposure to house-dust-mite (HDM) extracts induced AHR that was associated with epigenetic modulation of genes related to airway smooth muscle (ASM) cell proliferation and contraction [Shang et al., 2013]. We speculated that these epigenetic changes in the lungs could persist over longer time spans with repeated exposure to HDM and eventually lead to airway remodeling and AHR. Advanced sequencing technology now enables the demonstration of the epigenetic changes that reprogram the lungs in response to environmental allergens in a high throughput manner. In the present study, we utilized methylated DNA immunoprecipitation-next generation sequencing (MeDIP-seq), which is a technique utilizing methylated DNA enrichment with the aid of methylation-binding domain

(MBD) proteins followed by a single-run of massive parallel sequencing (also called next generation sequencing, NGS) on lung tissue DNA from saline- and HDM-exposed mice. Using bioinformatics analysis, we demonstrated a set of specific genes that are epigenetically reprogrammed by HDM and associated with an increase in AHR.

MATERIALS AND METHODS

Animals and Allergy Challenge

All experimental protocols conducted on mice were performed in accordance with the standards established by the US Animal Welfare Acts, as set forth in National Institutes of Health (NIH) guideline and the Policy and Procedures Manual of the Johns Hopkins University Animal Care and Use Committee. C57BL/6J male mice, 6 weeks old, were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were first sensitized with 100 μg HDM (D. pteronyssinus) extract (Greer Laboratories, Lenoir, NC), or saline (as control) intraperitoneally (i.p.) on day 1. Starting from day 14, mice were challenged with 100 μg HDM or saline intratracheally (i.t.) three times a week for a total of 5 weeks. Two days after the last challenge six saline-exposed mice and six HDM-exposed mice were subjected to airway responsiveness measurement and sample collection (bronchoalveolar lavage (BAL), serum and lung tissues). Another set of four mice from each treatment group were harvested for isolation of ASM cells from the lung trachea.

Isolation of Mouse ASM Cells (MASMCs)

Lung tracheas from saline- or HDM-exposed mice were collected as previously described [Liu et al., 2007]. Cell purity was assessed by immunofluorescence staining using antibodies against cytokeratin, vimentin, and α-tropomyosin (data not shown) (Santa Cruz Biotechnology, Dallas, TX). RNA and DNA were isolated from MASMCs 3 days after cell preparation.

Measurement of Airway Responsiveness

Two days after the last challenge with HDM, mice were anesthetized, paralyzed, and then ventilated with a Flexivent as described [Ewart et al., 1995]. Methacholine (Mch) (Sigma-Aldrich, St. Louis, MO), a broncho-constrictive agent, was administered to the animal at increasing half-log doses (from 0.01 to 30 mg/mL) by aerosol inhalation. Airway responsiveness was assessed as the change in pulmonary resistance (R, $cm/H_2O/mL/s$) compared to baseline after the challenge.

Differential Cell Counts From BAL

BAL cells were washed once in saline and counted using a hemocytometer (Paul Marienfeld, LaudaKoenigshofen). Differential cell counts were obtained under light microscope after staining with Diff-Quick stain (Dade Behring, Deerfield, IL).

Assessment of IL-4, IL-5, IgE, and Ifnc Protein Levels From Serum

The protein levels of IL-4, IL-5, IgE, and Ifng were measured in serum using enzyme-linked immunosorbent assay (ELISA) kits obtained from eBiosciences (San Diego, CA).

Histopathological Analysis

The left lung was inflated to lung capacity with warmed agarose, cooled, and immersed in 10% normal buffered formalin before being embedded in paraffin. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) for the assessment of peribronchial inflammation. Presence of goblet cells in the epithelium of the bronchus was demonstrated by staining the paraffin-embedded section with periodic acid-Schiff (PAS). Subepithelial fibrosis and deposition of collagen was assessed by Masson's trichrome staining on sections. Increased ASM layer was assessed by immunohistochemistry (IHC) staining of alpha-smooth muscle actin (Sma) (Santa Cruz Biotechnology, Dallas, TX).

Global DNA Methylation and Hydroxymethylation Quantification Assay

Two hundred nanograms of genomic DNA from mouse lung tissues or isolated MASMCs was used to measure the level of 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) in DNA by ELISA kits (Zymo Research, Irvine, CA).

Methylated DNA Immunoprecipitation Coupled With Next Generation Sequencing (MeDIP-seq)

Immunoprecipitated DNA was prepared from both saline and HDM-exposed lung tissues. Sample DNA concentrations were quantified with the Quanti-iTM Picogreen dsDNA assay kit (Invitrogen, CA). A second quality check was done on Agilent 2100 HS DNA chip after DNAs were fragmented. 1 lg of DNA from each sample was fragmented on Covaris S2 and had an average length of 200 bp. Methylated DNA was captured using the MethylCap kit (Diagenode, NJ) and DNA was eluted from the protein complex. Two hundred fifty nanograms fragmented DNAs were amplified, ligated with linkers, and subjected to flow cells using the HiSeq2000 platform according to the manufacturer's NGS protocol (Illumina, CA). About 20 million reads obtained by a single-run of massive parallel sequencing with 51 bp paired-end reads were found. Bioinformatics analysis on the raw NGS data was performed with use of CLC Genomic Workbench 6.0.3 (CLC Bios, MA) and followed manufacturer's standard data import protocol. Reads (peaks) were mapped to the Mouse Reference Genome Build NCBI38/mm10. Significant peaks by threshold profiles at a height equivalent to an estimated false discovery rate (FDR) ≥0.001 were identified. Differentially methylated regions between HDM-exposed and saline-exposed mice were annotated to their chromosomal loci and Gene Ontology (GO) categories. To better understand the biological meaning behind the differentially methylated genome, the list of methylated candidates was exported to the Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Redwood City, CA).

Methylation-Specific PCR (MSPCR)

Genomic DNA (200 ng) from lung tissues or MASMCs was modified with sodium bisulfite with the EZ DNA Methylation Kit (Zymo Research, Irvine, CA) before real-time PCR. Real-time PCR was done on bisulfite-treated DNA samples using primers specific for methylated DNA. Primers were listed in Supporting Information Table I. Control DNAs (fully methylated and fully unmethylated, Zymo Research, Irvine, CA) were mixed in various concentrations and served as quantification standards (0, 20, 40, 60, and 100%

methylated DNA) when determining methylation degrees (%) of samples from real-time PCR.

Real-Time Reverse-Transcriptase-PCR (RTPCR)

Total RNA (1 μg) was isolated from lung tissues and MASMCs and reverse transcribed with iScript Reverse Transcriptase (BIO-RAD, Hercules, CA). mRNA levels of the genes were quantified by TaqMan-based or SYBR Green-based real-time PCR. Primers were listed in Supporting Information Table I and described in Shang et al. [2013]. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of transcripts normalized to *Rpl19*.

Statistical Analysis

Data on airway responsiveness, cell counts, cytokines levels, relative gene expression, and methylation status were expressed as the mean- ± standard deviation (SD) with six mice per treatment group (saline-exposed or HDM-exposed). The Spearman's rank correlation coefficient (ρ) was calculated to measure the strength of the relationship between the percent of methylation of a gene's promoter and its transcript expression level in mouse lung tissues. Ninety-five percent confidence intervals and two-tailed *P* values were obtained to determine the expected range and significance of observed rho values. Moreover, we obtained data from MASMCs from four additional mice per treatment group (saline-exposed or HDM-exposed with repeated HDM challenges). Technical triplicates were performed in each assay. Bonferroni's multiple comparisons were applied to determine if data between treatment groups was statistically significant. $*P < 0.05$ in comparison with control meant results were statistically significant. All data were analyzed and plotted with Prism6 (GraphPad, La Jolla, CA).

RESULTS

Chronic Exposure to HDMInduced Airway Inflammation, Remodeling, and Hyper-Responsiveness

HDM-exposed mice showed increases in airway reactivity and inflammation together with structural remodeling of the airways consistent with that found in other studies [Cates et al., 2004; Johnson et al., 2004; Kohl et al., 2006]. HDM-exposed mice showed an increase in airway resistance in response to the contractile agonist, Mch (Fig. 1). We found increased numbers of macrophages, lymphocytes, and eosinophils in BAL from HDM-exposed mice (Table I). We also observed an increase in interleukin 4, 5 (IL-4 and -5) and IgE protein levels in the serum of HDM-exposed mice (Table I). Increased numbers of lymphocytes were also recruited to the airways, as revealed by H&E staining (Fig. 2A). Lung sections were also stained with Masson's trichrome and PAS. An increase in subepithelial fibrosis and collagen deposition around the airway was demonstrated in the HDM-exposed group by Masson's trichrome staining (Fig. 2B). Results from the PAS staining of lung sections showed there was an increase in goblet cells in HDM-exposed mice (Fig. 2C), suggesting that prolonged inflammation may induce structural changes in the airway epithelium. By histological assessment of lung sections with alpha-smooth muscle actin, HDM-exposed mice showed an increase in the thickness of their airways and peribronchial muscle layer (Fig. 2D). Furthermore, there were increases in the mRNA levels of genes involved in cell

proliferation (*Pcna*), cell mass (*Sma*), and cell contraction (*Camk2d*) in the lung tissues of HDM-exposed mice (Table II). Taken together, our chronic HDM-induced AHR mouse model showed several similar features consistent with human asthma pathophysiology beyond airway inflammation and remodeling.

Differential DNA Methylation in Lung Associated With HDM-Induced AHR

We sought to determine whether HDM-induced AHR is associated with epigenetic alterations via DNA methylation in the mouse lung. Previously, we identified global and gene-specific methylation in lung tissues and isolated ASM cells from mice with acute HDM exposure [Shang et al., 2013]; 5-hmC has been recently called the "sixth" base and is the product of DNA hydroxymethylation of cytosine. Hydroxymethylation contributes to gene transcription by influencing DNA demethylation and the recruitment of transcription complexes [Wu et al., 2011]. In the present study, adult mice that received repeated HDM exposures were shown to have global decreases in 5-mC and increases in 5-hmC levels in lung DNA compared to saline-exposed mice (Fig. 3). Our results suggested that HDM exposure induces epigenetic changes in the lung genome via DNA methylation and/or hydroxy-methylaton. We next employed MeDIP coupled with NGS to identify genes with aberrant DNA methylation status in HDM-exposed mice. Raw NGS data were imported into CLC Genomic Workbench 6.0.3. Approximately 20 million reads were obtained per sample. After combining the paired-end data and identifying overlapped pairs, there were approximately 20 million paired-end reads. We next mapped the reads to mouse reference genome mm10 (NCBI38/GRCm38). A total of 70 to 75% of the reads eventually mapped to the mouse reference genome. We ran the quality control for raw, paired-end, and mapped reads to ensure high-quality data for subsequent data mining. Using the CLC Genomic Workbench Software we applied the "ChIP-Seq Analysis" algorithm with default settings on the mapped reads. It allowed us to identify differentially methylated regions among our samples and reveal the genes nearby or encompassing the differentially methylated regions. A total of 213 genes were identified as differentially methylated candidates, and 83 of them are known genes mapped to the Reference Sequence Database (listed in Supporting Information Table II). We clustered these 213 genes according to their biological functions by IPA. IPA software revealed a total of 27 networks (by canonical pathways analysis). Ranking of the networks was based on the connectivity between genes (according to their biological functions) and the number of "connected" genes.

The most "connected" network was identified around the *Tgfb2* and *Smad3* genes (Fig. 4). The IPA analysis provides us the "highly connected" network, transforming growth factor beta (Tgfb) signaling pathway. We validated the significance of this network by measuring the gene expression and promoter methylation status in the lung tissues. A total of 19 genes (Fig. 4, capitalized and bold) in the Tgfb signaling network were chosen for RTPCR and quantitative MSPCR assays. Among 19 genes, 10 genes (indicated by red circles in Fig. 4) were found to be differentially methylated and expressed ($P < 0.05$) (Table III). *Tgfβ2*, *Smad2*, *Smad3*, *Il6r*, *Trpm2*, *Slc22a15*, *Cartpt*, and *Bmp3* were hypomethylated and up-regulated in HDM-exposed mice compared to saline-exposed mice. Hypermethylation and down-regulation of *Ski* and *Gal3st1* was seen in HDM-exposed mice. In addition, methylation changes of these genes (except Ski and Gal3st1) were significantly correlated to

the decreases in their mRNA expression levels, as indicated by the Spearman's rank coefficient (ρ, ρ). No significant changes in promoter methylation and gene expression of *Crebzf, Fam198b Il36b, Msr1, Pde10a, Plcl2, Prkg2, P4ha2 and Unc93b1* were found between saline- and HDM-exposed mice.

In addition, we determined if repeated exposures to HDM induced changes in the mRNA expression of genes encoding proteins involved in regulating cytosine DNA methylation/hydroxmethylation/demethylation and chromatin remodeling: DNA methyltransferases (*Dnmt1, Dnmt3a, and Dnmt3b*), methyl-CpG binding domain proteins (*Mecp2, Mbd1, Mbd2, Mbd3, and Mbd4*), ten-eleven-translocation proteins (*Tet1, Tet2, and Tet3*) and DNA repair enzymes (*Aid, Smug1 and Tdg*). We observed reduced expression of *Dnmt3a, Mbd2 and Mbd3* and increased expression of *Tet1, Aid,* and *Smug1* in HDM-exposed mice compared to saline-exposed mice (Table IV).

Gene-Specific Promoter Methylation in MASMCs

We examined whether the global DNA methylation patterns and/or the gene–specific methylation of genes involved in Tgfb signaling are aberrantly altered in MASMCs as a result of HDM exposures. First, we observed increases in 5-hmC levels in DNA from the tracheal MASMCs isolated from HDM-exposed mice compared to those of saline-exposed mice (Fig. 5). Second, compared to the MASMCs of saline-exposed mice, we showed promoter hypomethylation and up-regulation of *Tgfb2, Smad2, Smad3, Il6ra,* and *Trpm2* in the MASMCs of HDM-exposed mice. *Cartpt* was over-expressed but not hypomethylated in the MASMCs of HDM-exposed mice. In contrast, hypermethylation and down-regulation of *Ski* was seen in HDM-exposed mice (Table V). *Gal3st1* was down-regulated but not hypermethylated in the MASMCs of HDM-exposed mice. No significant difference was noted in gene expression or promoter methylation of *Slc22a15* and *Bmp3* between MASMCs isolated from HDM- or saline-exposed mice. Results demonstrated that HDM exposure can alter gene-specific methylation in MASMCs, which might alter gene expression, and in turn, affect gene function causing the structural remodeling of ASM and the development of AHR.

DISCUSSION

In urban areas, HDM is one of the most common allergens. It has been postulated that chronic exposure to HDM extracts induces airway remodeling and AHR via immune mechanisms. For example, HDM extracts can stimulate dendritic cells to secret Il6 and induce T-helper cell (Th)2 and Th17 differentiation, which contribute to AHR [Krishnamoorthy et al., 2008]. It has been proposed that HDM extracts can destroy airway epithelial and ASM cells by its proteolytic activity. Furthermore, HDM has been found to induce changes in ASM responsiveness via ERK1/2 signaling [Grunstein et al., 2005]. In our experimental asthma model, we found an increase in Th2 cytokine levels and the numbers of inflammatory cells in response to HDM. Furthermore, we observed thickening of the bronchial smooth muscle and increased levels of *Pcna, Sma,* and *Camk2d* gene expression in HDM-exposed mice. These findings confirm that repeated exposures to HDM can induce airway inflammation and ASM remodeling, resulting in increased AHR. There is

an agreement that epigenetic regulation interacts with environmental exposure and its dysregulation leads to asthma pathogenesis [Perera et al., 2009; Kabesch et al., 2010; Niedzwiecki et al., 2012; Shang et al., 2013]. DNA methylation at 5' CGIs alters gene transcription machinery and eventually leads to decreases in gene expression. Gene dysregulation further affects cellular functions. Global DNA methylation levels assayed by methylation sensitive pyrosequencing or PCR of the repetitive elements *LINE* and *Alu* have been found to be decreased in response to prenatal tobacco smoke [Breton et al., 2009] and polya-crylic aromatic hydrocarbons (PAHs) [Herbstman et al., 2012]. In these studies, reduced global DNA methylation was associated with an increased risk of childhood asthma. Our results support these studies by showing a decrease in global DNA methylation in mice with AHR. Furthermore, we demonstrated that there was an increase in 5-hmC levels in the lung DNA of HDM-exposed mice, indicating that hydroxymethylation at CpGs may occur concurrently with the removal of DNA methylation.

A number of studies show epigenetic modulation of B cell and Th2 phenotypes in both allergic and non-allergic asthma [Kwon et al., 2008; Pascual et al., 2011; Brand et al., 2012; Tang et al., 2012]. Few studies have demonstrated genome-wide DNA methylation changes in the lungs and their link to structural remodeling and hyper-responsiveness of the airway. In the present study, we demonstrated that repeated allergen exposure induced epigenetic changes in the lung genome, associated with increased AHR. By using MeDIP-seq and pathway analysis, we identified sets of genes differentially methylated in mice with repeated HDM challenges. Methylome research coupled with NGS could be an innovative and promising approach for understanding the pathogenesis of complex diseases with proper bioinformatics tools and data mining analysis [Robertson et al., 2007; Brunner et al., 2009; Grimm et al., 2013]. A genome-wide methylation profiling of the bronchial mucosa of asthmatics identified 48 genes that were hypomethylated in the bronchial mucosa of atopic asthmatics, and six genes that were hypermethylated compared to those of nonatopic asthmatics [Kim et al., 2013]. With GO analysis, these hypomethylated genes are assigned to hormone metabolic processes and growth factor receptor binding. The authors suggested the use of their selected genes for future asthma research. Our genome-wide methylation study has revealed that key pathways could be epigenetically regulated via DNA methylation in response to HDM exposure. We further demonstrated the epigenetic changes in well-defined airway cells, MASMCs. Phenotypic changes of ASM, including ASM hypertrophy, hyperplasia, and/or migration, were demonstrated in the thickening of the airway walls and increased ASM mass [Bentley et al., 2008; Hirota et al., 2009; Al Heialy et al., 2011]. These effects contribute to further narrowing of the airway and reduced airflow, leading to AHR [Siddiqui et al., 2008; Janson et al., 2010; Hamid et al., 2012]. Epigenetic mechanisms, including DNA methylation, histone modifications, and miRNA, have been shown to regulate the gene transcription of specific signaling pathways to maintain and alter ASM cell phenotypes that may contribute to asthma pathogenesis [Clifford et al., 2013]. Previously, we utilized one of the methylation profiling techniques, methylation sensitive restriction fingerprinting, and demonstrated that acute exposure to HDM induced methylation changes in *Pde4d*, *Slc8a3*, and *Pom121l2*. Dysregulation of these genes have been found to contribute in ASM proliferation and contraction, which may lead to increased ASM hyper-responsiveness [Shang et al., 2013]. In the present study, we demonstrated

aberrant methylation of Tgfb signaling genes in lung tissues and specifically in MASMCs from HDM-exposed mice.

Tgfß2, a member of the Tgfb family of cytokines, regulates cell proliferation, differentiation, adhesion, and migration by transducing its signal through combinations of transmembrane type I and type II receptors (Tgfbr1 and Tgfbr2) and their downstream effectors, the Smad proteins. In airway cells, Smad2/3 proteins act as transcriptional activators, which transduce Tgfb-regulated gene transcription intra-cellularly. *Tgfß2* has been shown to play a role in the regulation of airway inflammation and remodeling in both human and murine models of asthma [Chu et al., 2004; Balzar et al., 2005; Hatsushika et al., 2007; Bottoms et al., 2010; Lopez-Guisa et al., 2012]. In bronchial biopsies from asthmatic subjects, there was an increase in *Smad2* expression [Sagara et al., 2002]. Inhibition of *Smad3* has been reported to suppress ovalbumin-induced airway remodeling [Le et al., 2007]. Royce et al. also suggested that *Tgfb2, Smad2,* and *Smad3* are key regulators of fibrosis in airway remodeling [Royce et al., 2012]. *Ski*, which is co-repressor of *Smad2/3* complexes, represses Tgfb signaling [Groneberg et al., 2004]. *Ski* expression has been shown to inhibit vascular smooth muscle cell proliferation by suppressing *Tgfb/Smad3* signaling but up-regulating *p38* signaling [Li et al., 2013]. Activation of Tgfb signaling in OVA-sensitized and -challenged rats has been found to contribute to the pathogenesis of airway remodeling through the reduction of Ski proteins [Li et al., 2010]. We found CpG site-specific hypomethylation at *Tgfß2, Smad2* and *Smad3*, and *Ski* hypermethylation in lung and MASMCs, which may be associated with HDM-induced AHR. Other indirect interactions with Tgfb signaling are suggested to be epigenetically regulated and associated with increased AHR. *Il6r* functions as a regulator of inflammation and has been shown to be associated with asthma risk factors [Ferreira et al., 2011]. In our model, HDM induced *Il6r* demethylation in murine lungs and MASMCs, suggesting the epigenetic effect of HDM on Il6r-related AHR. On the other hand, calcium signaling is one of the key mediators in airway contraction, remodeling, and inflammation [Jude et al., 2008]. Hypomethylation of *Trpm2, Slc22a15* and *Cartpt* (calcium signaling gene) was found in HDM-exposed mice. In addition, *Trpm2*, but not *Slc22a15* or *Cartpt*, was hypomethylated in MASMCs. Although *Trpm2* has been shown not to be involved in airway inflammation in OVA-induced allergic asthma in mice [Sumoza-Toledo et al., 2013], how *Trpm2* contributes to ASM functions or airway structural remodeling has not been studied. In summary, we demonstrated that the most common allergen, HDM, induced the dysregulation of a specific set of genes via epigenetic modifications in the lungs. Specifically, the differentially methylated genes are highly enriched in the Tgfb signaling pathway, and these epigenetic changes may modulate the ASM cell functions and lead to AHR.

There are sets of functional components that modulate the epigenetic information embedded in the chromatin of gene promoters in response to environmental stimuli. Down-regulation of *Mbd2, Mbd3*, and *Dnmt3a* was observed in HDM-exposed mice. Mbd2 and Mbd3 can bind to methylated DNA and recruit enzymes, like Dnmt3a, to catalyze DNA methylation [Guibert et al., 2013]. We observed a significant decrease in global DNA methylation in HDM-exposed mice. Reductions in levels of *Mbd2, Mbd3*, and *Dnmt3a* may explain this phenomenon, and it is also possible that Mbd2, Mbd3, and Dnmt3a may act on specific gene

promoters or affect DNA demethylation machinery. HDM-exposed mice showed increases in *Tet1, Aid,* and *Smug1* expression and these proteins might contribute to global and gene-specific demethylation. Tet proteins convert 5-mC into 5-hmC; 5-hmC has been recently demonstrated to be a stable epigenetic mark and contributes to the differentiation of embryotic stem cells during development [Tahiliani et al., 2009; Wu et al., 2011]. Hydroxymethylation at 5' CpGs contributes to gene transcription by influencing DNA demethylation and/or the recruitment of transcription complexes. Both Aid and Smug1 are also involved in demethylation. Aid activates the deamination of 5-mC, while Smug1 catalyzes base-excision repair to exchange cytosine [Krokan et al., 2002; Zhu, 2009; Gavin et al., 2013]. Taken together, our results suggested that HDM might trigger global and gene-specific DNA methylation machinery either thru the inhibition of DNA methylation, hydroxymethylation, or the passive/active demethylation via DNA repair, resulting in increased gene transcription. How these epigenetic machineries contribute to gene transcription and its functions in the airways remains unanswered and requires further study.

Advanced epigenetic technology, bioinformatics support, and proper validation assays will likely increase our ability to reveal markers for the prevention, diagnosis, and prognosis of asthma. Herein, we demonstrated the epigenetic changes associated with allergic AHR in an environmentally and clinically relevant experimental asthma model in a step-by-step manner. We first utilized a high-throughput and unbiased DNA methylation profiling approach to explore the genome-wide methylation changes in mouse lungs in response to the environmental allergen, HDM. Next, we chose two commercially available databases (CLC Genomic Workbench and IPA) to analyze the raw NGS data and map out biological networks that can be validated in subsequent studies. Then, we validated the gene expression and promoter methylation of 19 genes from the top "connected" network determined by IPA (Tgfb signaling pathway) by RTPCR and quantitative MSPCR respectively. The primers used in the MSPCR are designed to target the specific CpG sites showing the highest peak signal from NGS data for the potential candidate genes determined from the MeDIP-seq data. Furthermore, we correlated the gene expression levels of the candidate genes with their methylation status (ρ value) in order to associate DNA methylation changes with gene expression changes in the lungs from mice with allergic AHR (Table III). While bisulfite genomic sequencing may be the gold standard approach to examine the DNA methylation status of individual CpG sites, our current approach allows us to generate a candidate gene list by MeDIP-seq and validate the potential candidates in another set of HDM-exposed mice or well-defined airway cells like MASM cells (Table V) in a time- and cost-effective manner.

In the present study, we utilized lung tissues for the methylome analysis in order to get a sufficient amount of genomic DNA for MeDIP-seq. However, we do acknowledge the heterogeneity of cells (including MASMCs, airway epithelial cells, fibroblasts, goblet cells and immune cells) in the lung tissues may have influenced our results. Epigenetic changes induced by HDM exposure in the lung or airway may involve gene dysregulation in all or a subset of these cell types. We validated the gene expression and promoter methylation status of potential MeDIP-seq candidates in both lung tissues and MASMCs cells. We examined the epigenetic changes in MASMCs for test of concept because ASM is one of the key

components in AHR. We clearly demonstrated that many of the candidate genes identified using the lung tissue also undergo DNA methylation and expression changes in MASMCs from exposed mice, suggesting that the methylation changes demonstrated are occurring in a relevant cell type to AHR. However, further validation of methylation status in different cell types from the mouse lung is recommended allowing for an even clearer understanding of the pathogenesis of asthma. Future experiments like gene knock-in or knock-out assays in mouse lung are needed to demonstrate the underlying mechanisms of how epigenetic changes of potential candidates alter ASM functions and AHR. Herein, we presented differentially methylated genes, which are highly enriched in the Tgfb signaling pathway, in HDM-exposed lung and MASMCs as compared to that of saline-control. A better understanding of the regulation of Tgfb signaling in transcription would provide more specific targets for therapeutic intervention of asthma [Howell et al., 2006; Halwani et al., 2011]. All together, our current findings have provided insight into experimental designs needed to demonstrate the epigenetic changes involved in asthma pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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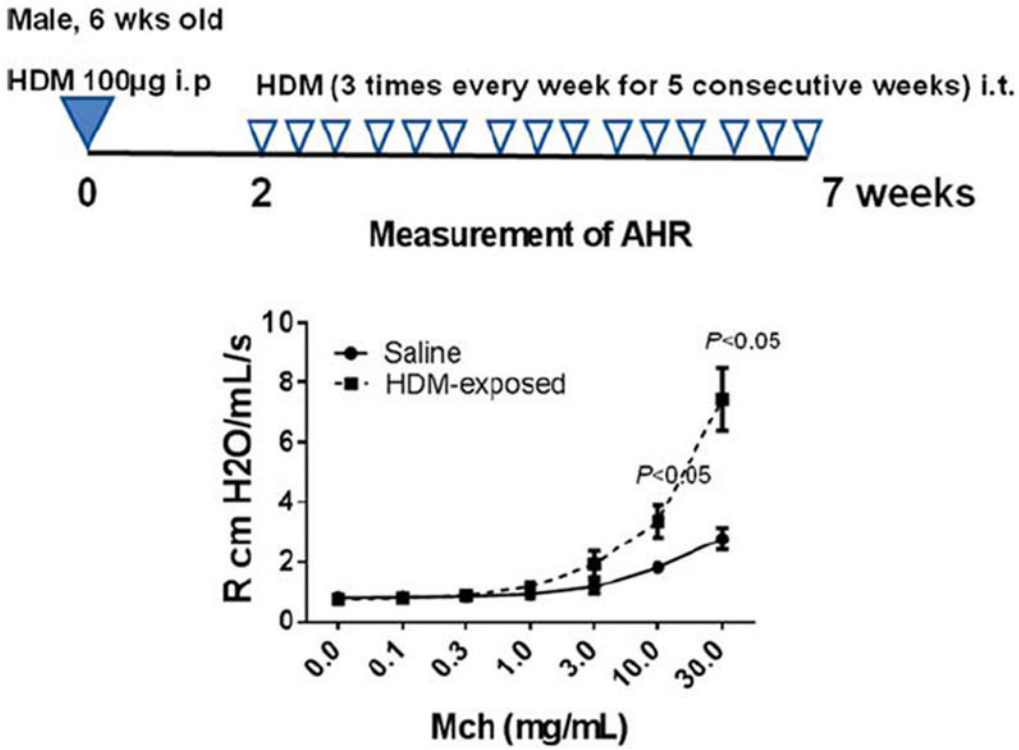

Fig. 1.

Increased airway responsiveness in HDM-exposed mice. Upper panel: schematic picture depicting the HDM sensitization and challenges used in this study. Lower panel: Methacholine (MCh) was administered to the animals at increasing doses (0.1, 0.3, 1, 3, 10, and 30 mg/mL) by a 10 s aerosol inhalation. AHR was assessed as the change in pulmonary resistance (R, cm H_2O/mL/s) compared to baseline after the challenge to MCh. Circle: saline-exposed mice; square: HDM-exposed mice. Results were expressed as mean values ± SD, with six mice per treatment group (saline-exposed or HDM-exposed). Technical triplicates were performed in each measurement. *P< 0.05 in comparison with saline-exposed mice meant results were statistically significant.

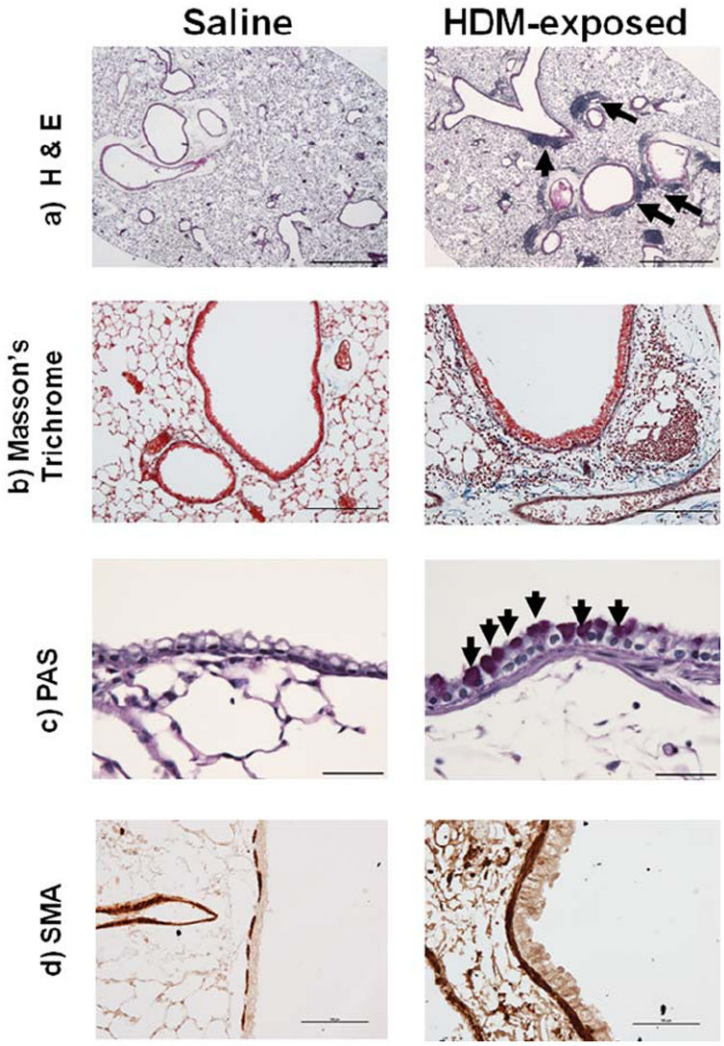

Fig. 2.

Increased airway inflammation and remodeling in HDM-exposed mice. The lung sections were stained with (**A**) H&E (scale bar = 1,000 μm), (**B**) Masson's Trichrome (scale bar = 100 μm), (**C**) Periodic acid-Schiff (PAS) (scale bar = 50 μm), and (**D**) Smooth Muscle Actin (SMA) (scale bar 100 = μm). Representative images of lungs following saline or chronic exposure to HDM are presented. (a) Inflammatory cells were recruited close to the airway in HDM-exposed mice (shown by arrow). (b) Masson's trichrome revealed subepithelial fibrosis and deposition of collagen in HDM-exposed mice. (c) Increase in number of goblet cells (arrow) in HDM-exposed mice. (d) Staining with SMA indicated the increase in smooth muscle layer in HDM-exposed mice.

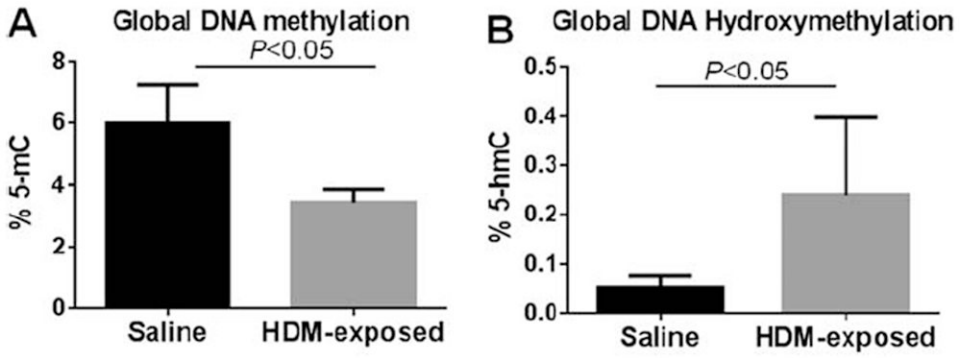

Fig. 3.

HDM-exposed mice demonstrated decreased 5-mC and increased 5-hmC content in DNA from lungs; 5-mC and 5-hmC levels in lung DNA were detected using capture and detection antibodies followed by colorimetric quantification. Black bar: saline-exposed mice; gray bar: HDM-exposed mice. *y*-Axis represents percentage of 5-mC (**A**) or 5-hmC (**B**) levels in DNA from saline- and HDM-exposed mice. Results were expressed as mean values ± SD, with six mice per treatment group (saline-exposed or HDM-exposed). Technical triplicates were performed in each measurement. **P* < 0.05 in comparison with saline-exposed mice meant results were statistically significant.

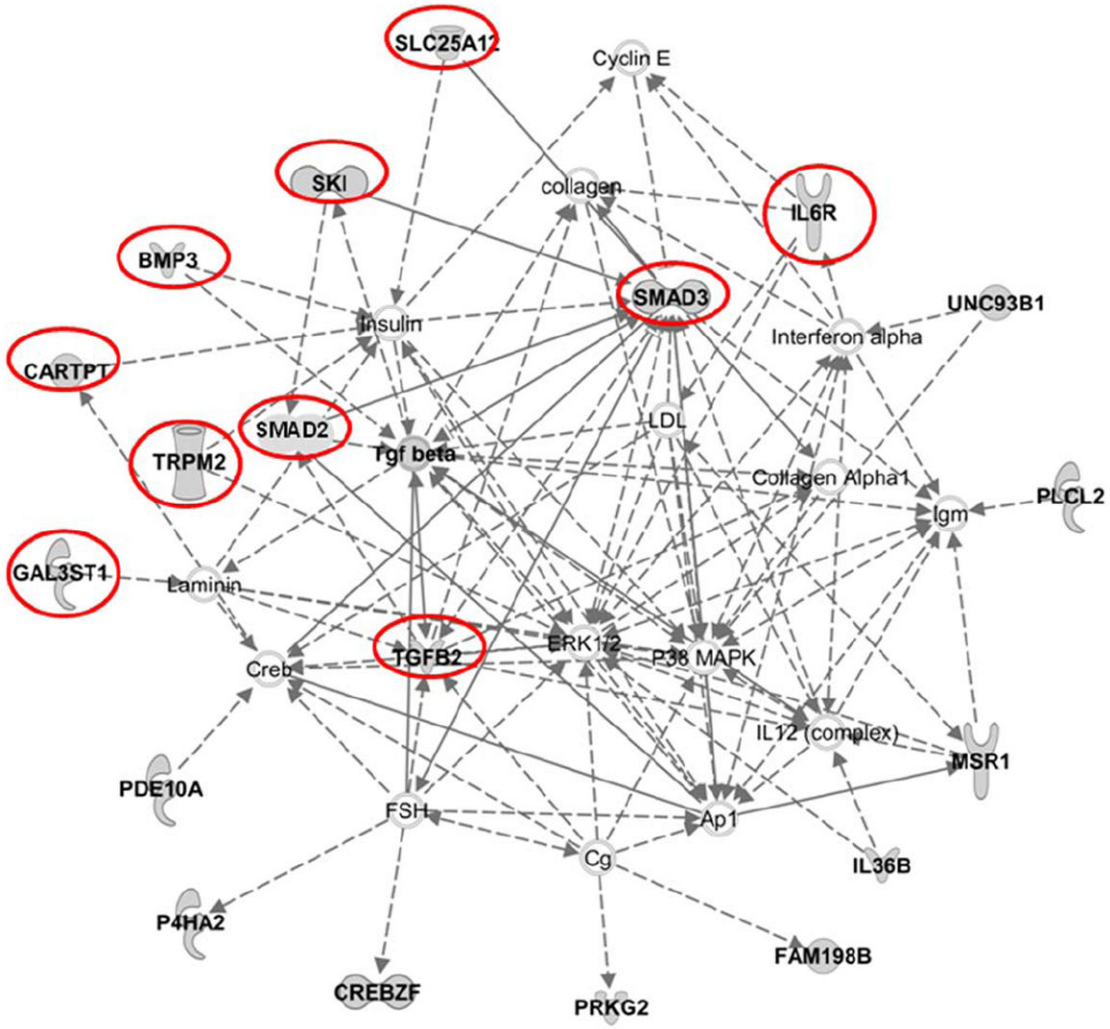

Fig. 4.

Ingenuity pathway analysis (IPA) revealed TGF-beta signaling as the top "connected" network of differentially methylated candidates in HDM-exposed group. Candidates identified from MeDIP-seq are capitalized and in bold. The shapes of candidates in a picture represent the classifications of gene functions. PLCL2, P4HA2, PDE10A, and GAL3ST1 are classified as Enzymes. TGFB2, BMP2a, and IL36B are classified as Cytokine/Growth Factors. TRPM2 are Ion Channels. PRKG2 is a Kinase. CREBZF, SMAD2, and SKI are classified as Transcription Regulator. MSR1 and IL6R belong to Transmembrane Receptor. SLC25A12 belongs to Transporter. UNC93B1, FAM198B and CARTPT are classified as unknown functions. Solid line: direct interaction; broken line: indirect interaction. Candidates indicated by red circle were found to be significantly methylated and expressed (*$P < 0.05$ in comparison with saline-exposed mice) as assayed by quantitative MSPCR and RTPCR. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

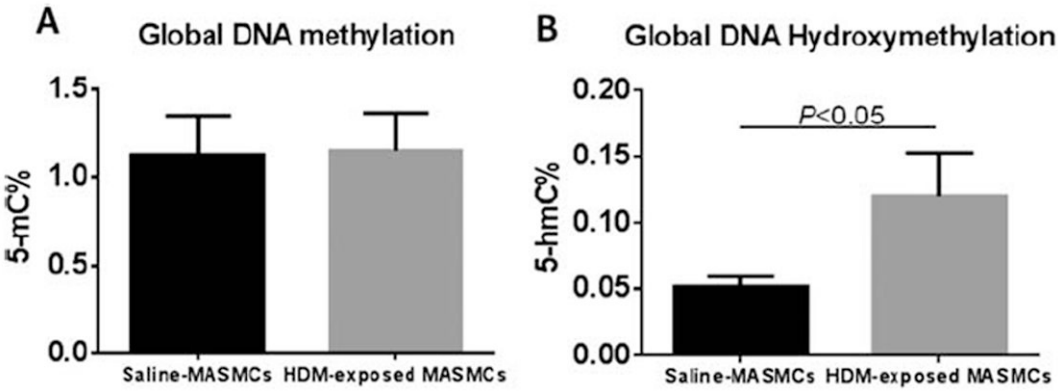

Fig. 5.

HDM-exposed mice had increased 5-hmC in DNA from mouse airway smooth muscle cells (MASMCs). (**A**) 5-mC and (**B**) 5-hmC levels in DNA of MASMCs were detected using capture and detection antibodies followed by colorimetric quantification. Black bar: isolated MASMCs from saline-exposed mice; gray bar: isolated MASMCs from HDM-exposed mice. *y*-Axis represents percentage of 5-mC or 5-hmC levels in DNA from MASMCs. Results were expressed as mean values ± SD, with four mice per treatment group (MASMCs from saline-exposed or HDM-exposed mice). Technical triplicates were performed in each measurement. **P* < 0.05 in comparison with MASMCs of saline-exposed mice meant results were statistically significant.

TABLE I

Inflammation Response Assayed by Measurement of the Number of Inflammatory Cells in BAL, and Protein Levels of Cytokines (IL-4, IL-5, and Ifnγ) and IgE in Serum

Type of immune cells[a]		No. cells (1×10^4)[b]	SD[b]	P[b]
Total	Saline	9.22	2.95	<0.01*
	HDM	182.29	63.51	
Macrophages	Saline	9.22	2.94	<0.01*
	HDM	30.47	8.98	
Neutrophils	Saline	0	0	0.13
	HDM	1.71	3.24	
Lymphocytes	Saline	0.11	0.33	<0.01*
	HDM	21.91	3.20	
Eosinophils	Saline	0.01	0.04	<0.01*
	HDM	128.06	64.83	
Inflammatory response[c]		Protein levels in serum[b]	SD[b]	P[b]
IL-4	Saline	33.93	23.58	<0.01*
	HDM	165.24	85.05	
IL-5	Saline	140.22	19.28	<0.01*
	HDM	192.72	30.34	
Ifnγ	Saline	379.38	223.31	0.10
	HDM	623.13	326.08	
IgE	Saline	133.75	30.33	<0.01*
	HDM	650.63	354.65	

[a] Differential cell counts from BAL were obtained under light microscope after staining with Diff-Quick stain.

[b] Results were expressed as mean values ± standard deviation (SD), with six mice per treatment group. Technical triplicates were performed in each assay.

[c] Protein levels of IL-4, IL-5, Ifnγ, and IgE (pg/mL) in serum were determined by ELISA.

* $P < 0.05$ in comparison with saline-exposed control mice meant results were statistically significant.

TABLE II

mRNA Levels of Genes Related to Smooth Muscle Mass, Cell Proliferation, and Smooth Muscle Contraction in Mouse Lung Tissues

		mRNA level (RER) in lung[a]	SD[b]	*P*[b]
Smooth muscle mass				
α-Smooth muscle actin (*Sma*)	Saline	1.90	0.63	0.03*
	HDM	10.28	5.86	
Tropomyosin (*Tpm*)	Saline	1.56	0.58	0.32
	HDM	2.54	1.72	
Cell proliferation				
Cyclin D1 (*Ccd1*)	Saline	1.92	0.72	0.90
	HDM	1.89	0.75	
Proliferation cell nuclear antigen (*Pcna*)	Saline	1.20	0.17	0.02*
	HDM	2.13	0.51	
Smooth muscle contraction				
Camodulin kinase II delta (*Camk2d*)	Saline	0.88	0.13	0.03*
	HDM	2.19	0.95	
Myosin light chain kinase (*Mlck*)	Saline	0.89	0.23	0.76
	HDM	0.99	0.50	

[a] mRNA levels of samples were determined by RTPCR and normalized to *Rpl19* transcript in the same sample. The relative level of gene transcript in saline-exposed control samples was arbitrarily assigned a value of 1.0. The relative level of gene transcript [relative expression ratio, RER] of HDM-exposed mice was normalized to the mean value of the saline group.

[b] Results were expressed as mean values ± standard deviation (SD), with six mice per treatment group. Technical triplicates were used in the assay

* $P < 0.05$ in comparison with control mice meant results were statistically significant.

TABLE III

Promoter Methylation and Gene Expression of MeDIP-seq Candidates in Mouse Lung Tissues

	Promoter methylation (%, SD)[a]			mRNA levels (RER, SD)[b]			
Gene	**Saline**	**HDM**	***P* value vs. saline[a]**	**Saline**	**HDM**	***P* value vs. saline[b]**	**ρ, *P* value[c]**
Tgfb2	85.15, 4.00	34.06, 5.84	<0.01*	1.02, 0.25	1.99, 0.51	0.01*	−0.84, <0.01*
Smad2	67.83, 4.46	10.61, 3.46	<0.01*	0.75, 0.20	1.94, 0.21	0.01*	−0.76, <0.01*
Smad3	90.50, 2.92	35.55, 11.00	0.01*	3.53, 2.12	27.02, 12.11	0.05*	−0.81, 0.06
Il6r	62.29, 2.49	24.97,4.10	0.01*	1.30, 0.43	3.11, 1.15	0.02*	−0.84, <0.01*
Trpm2	54.09, 6.52	5.52, 4.04	0.01*	1.96, 0.88	4.46, 0.68	0.01*	−0.77, <0.01*
Slc22a15	78.95, 4.13	23.02, 2.10	0.01*	3.07, 1.48	23.74, 6.35	0.04*	−0.69, 0.01*
Cartpt	90.71, 2.83	16.47, 4.21	0.01*	4.05, 2.94	14.84, 5.18	0.02*	−0.78, <0.01*
Bmp3	91.31, 8.63	66.74, 4.31	0.01*	0.74, 0.10	1.12, 0.28	0.04*	−0.86, <0.01*
Ski	21.24, 1.16	66.74, 4.31	0.01*	1.29, 0.29	1.00, 0.09	0.05*	−0.31, 0.27
Gal3st1	65.40, 2.61	95.23, 3.90	0.01*	0.76, 0.15	0.43, 0.21	0.04*	−0.38, 0.19

[a] Percent of gene promoter methylation from lung tissue samples of saline- or HDM-exposed mice was assayed by quantitative MSPCR. Results were expressed as mean values ± standard deviation (SD), with six mice per treatment group. Technical triplicates were used in the assay.

[b] mRNA levels of samples were determined by RTPCR and normalized to *Rpl19* transcript in the same sample. The relative level of gene transcript in saline-exposed control samples was arbitrarily assigned a value of 1.0. The relative level of gene transcript [relative expression ratio, RER] of HDM-exposed mice was normalized to the mean value of the saline group. Results were expressed as mean values ± standard deviation (SD), with six mice per treatment group. Technical triplicates were performed in the assay.

[c] The Spearman's rank correlation coefficient (ρ) was calculated to measure the strength of the relationship between the percent of methylation of a gene's promoter and its transcript expression level in mouse lung tissues (saline- and HDM-exposed mice). Negative sign indicated inverse relationship between % promoter methylation and RER. The larger the negative value meant greater negative correlation and statistical significance was determined by a two-tailed test with $P < 0.05$.

* $P < 0.05$ in comparison with saline-exposed mice meant results were statistically significant.

TABLE IV
Gene Expression of Epigenetic Components in Mouse Lung Tissues

	mRNA levels (RER, SD)[a]		
Gene	**Saline**	**HDM**	***P* value vs. saline[a]**
Dnmt1	1.28, 0.17	1.37, 0.09	0.10
Dnmt3a	1.35, 0.51	0.96, 0.42	0.03*
Dnmt3b	1.52, 0.25	1.54, 0.36	0.15
Mbd1	1.09, 0.41	1.39, 0.48	0.41
Mbd2	1.06, 0.12	0.87, 0.13	0.03*
Mbd3	1.32, 0.34	0.92, 0.22	0.03*
Mbd4	1.24, 0.31	1.57, 0.35	0.27
Mecp2	1.35, 0.31	1.34, 0.28	0.32
Tet1	1.10, 0.23	2.25, 0.46	0.01*
Tet2	1.05, 0.21	1.39, 0.21	0.36
Tet3	0.61, 0.34	0.63, 0.49	0.94
Aid	0.78, 0.18	14.37, 11.86	0.05*
Smug1	8.35, 7.67	48.75, 16.64	0.02*
Tdg	0.73, 0.27	0.76, 0.20	0.81

[a] mRNA levels of samples were determined by RTPCR and normalized to Rpl19 transcript in the same sample. The relative level of gene transcript in saline-exposed control samples was arbitrarily assigned a value of 1.0. The relative level of gene transcript [relative expression ratio, RER] of HDM-exposed mice was normalized to the mean value of the saline group. Results were expressed as mean values ± standard deviation (SD), with six mice per treatment group. Technical triplicates were used in the assay.

* $P < 0.05$ in comparison with saline-exposed mice meant results were statistically significant.

TABLE V

Promoter Methylation and Gene Expression in Mouse Airway Smooth Muscle Cells (MASMCs)[a]

	Promoter methylation (%, SD)[b]			mRNA level (RER, SD)[c]			
Gene	**Saline-MASMCs**	**HDM-MASMCs**	***P* value vs. saline[b]**	**Saline-MASMCs**	**HDM-MASMCs**	***P* value vs. saline[c]**	**ρ, *P* value vs. saline[d]**
Tgfb2	88.09, 7.49	29.14, 2.33	<0.01*	0.85, 0.15	1.49, 0.23	0.02*	−0.80, <0.01*
Smad2	43.70, 4.80	19.60, 6.71	<0.01*	0.85, 0.15	1.78, 0.07	0.01*	−0.74, 0.01*
Smad3	41.19, 2.95	11.16,4.32	<0.01*	0.97, 0.03	3.96, 0.01	0.01*	−0.83, <0.01*
Il6r	56.16, 22.31	15.64, 6.68	<0.01*	0.85, 0.15	3.79, 0.32	0.02*	−0.79, <0.01*
Trpm2	77.25, 7.49	19.68, 5.29	<0.01*	0.82, 0.18	2.94, 1.47	0.01*	−0.72, 0.01*
Slc22a15	5.56, 5.23	10.45, 2.83	0.07	0.71, 0.29	1.278, 0.09	0.06	−0.38, 0.22
Cartpt	7.75, 2.25	6.53, 2.45	0.39	1.30, 0.30	2.74, 1.03	0.02*	−0.38, 0.20
Bmp3	6.01, 3.74	10.79, 4.21	0.06	0.69, 0.02	1.73, 0.36	0.44	0.69, 0.02
Ski	24.79, 9.64	76.62, 12.41	<0.01*	0.82, 0.18	0.41, 0.09	0.03*	−0.77, <0.01*
Gal3st1	12.37, 6.14	9.33, 5.45	0.38	0.79, 0.21	0.03, 0.002	0.02*	0.24, 0.45

[a] Airway smooth muscle cells were isolated from trachea of saline- ($N = 4$) and HDM-exposed mice ($N = 4$). B6 male mice were subjected to the protocol of HDM sensitization and challenges that was described as Figure 1.

[b] Percent of promoter methylation from MASMCs isolated from saline- or HDM-exposed mice was assayed by quantitative MSPCR. Results were expressed as mean values ± standard deviation (SD), with four mice per treatment group. Technical triplicates were used in the assay.

[c] mRNA levels of samples were determined by RTPCR and normalized to *Rpl19* transcript in the same sample. The relative level of gene transcript in saline-exposed MASMCs samples was arbitrarily assigned a value of 1.0. The relative level of gene transcript [relative expression ratio, RER] of HDM-exposed MASMCs was normalized to the mean value of the saline group. Results were expressed as mean values ± standard deviation (SD), with four mice per treatment group. Technical triplicates were used in the assay.

[d] The Spearman's rank correlation coefficient (ρ) was calculated to measure the strength of the relationship between the percent of methylation of a gene's promoter and its transcript expression level in MASMCs (saline- and HDM-exposed mice). Negative sign indicated inverse relationship between % promoter methylation and RER. The larger the negative value meant greater negative correlation and statistical significance was determined by a two-tailed test with $P < 0.05$.

* $P < 0.05$ in comparison with saline-exposed MASMCs meant results were statistically significant.

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