Epigenetic Alterations by DNA Methylation in House Dust Mite–Induced Airway Hyperresponsiveness

Yan Shang[1,2,3], Sandhya Das[2], Richard Rabold[2], James S. K. Sham[4], Wayne Mitzner[2], and Wan-yee Tang[2,3]

[1]Department of Respiratory Diseases, Changhai Hospital, Second Military Medical University, Shanghai, China; [2]Division of Physiology, Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland; [3]Division of Molecular Translational Toxicology, Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland; and [4]Division of Pulmonary and Critical Care Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland

Asthma is one of the most prevalent chronic lung diseases, affecting 235 million individuals around the world, with its related morbidity and mortality increasing steadily over the last 20 years. Exposure to the environmental allergen, house dust mite (HDM), results in airway inflammation with a variable degree of airway obstruction. Although there has been much experimental work in the past using HDM challenge models to understand mechanistic details in allergic inflammation and airway hyperresponsiveness (AHR), there has been no study on reprogramming of lung or airways mediated through epigenetic mechanisms in response to an acute HDM exposure. Male mice, 6 weeks of age, were administrated HDM extracts or saline at Days 1, 14, and 21. Exposure of mice to HDM extracts caused significant airway inflammation and increased AHR. These HDM-challenged mice also exhibited a change in global DNA methylation as compared with saline-exposed (control) mice. Next, by employing methylation-sensitive restriction fingerprinting, we identified a set of genes, showing aberrant methylation status, associated with the HDM-induced AHR. These candidate genes are known to be involved in cAMP signaling (*pde4 d*), Akt-signaling (*akt1 s1*), ion transport (*tm6 sf1*, *pom121l2*, and *slc8a3*), and fatty acid metabolism (*acsl3*). *Slc8a3* and *acsl3* were down-regulated, whereas *pde4 d, akt1 s1, tm6 sf1*, and *pom121l2* were up-regulated in the mice exposed to HDM. Hence, our results suggest that HDM exposure induces a series of aberrant methylated genes that are potentially important for the development of allergic AHR.

Keywords: airway hyperresponsiveness; DNA methylation; epigenetics; house dust mite

CLINICAL RELEVANCE

In this study, we show that acute exposure to house dust mite induces airway hyperresponsiveness (AHR) that is associated with epigenetic alterations in the mouse lung. The specific epigenetic changes in a number of genes related to AHR in this model may be directly relevant to the allergen-induced mechanisms that underpin allergic asthma in humans.

Asthma is one of the most prevalent chronic lung diseases, affecting 235 million individuals around the world (www.who.int). Individuals with asthma experience great difficulties in breathing and have reduced quality of life, with repeated visits to clinics or emergency rooms. It is well recognized that the development of asthma is related to a combination of genetic and environmental factors, but the precise mechanisms of pathogenesis in asthma are still unclear (1, 2). There are many triggers of asthmatic attacks; most of them are associated with environmental exposure to allergens. In the inner city environment, house dust mite (HDM) allergy is reported to be the most prevalent cause of allergic sensitization in individuals with asthma (3). Exposure to HDM results in airway inflammation with a variable degree of airway obstruction. In animal studies, chronic exposure of mice to HDM leads to significant airway inflammation, increased airway hyperresponsiveness (AHR), and remodeling of bronchi (4–6). Even though much work has been done in the past using this HDM model to probe the immunologic pathways, there is no study addressing the epigenetic basis of how this environmental allergen "reprograms" the airways, thereby predisposing the animals to asthma.

Epigenetics provide a promising approach for improving our understanding of complex diseases like asthma, because epigenetic disruption of gene expression is known to play an equally important role as that of genetic predisposition (such as polymorphism, mutation, deletion, and insertion) in the development of chronic, complex diseases (7, 8). A variety of molecular mechanisms are involved in epigenetic regulation, including DNA methylation, histone modifications, and noncoding RNA. This phenomenon is linked to gene activation, gene silencing, and chromosomal instability without alterations in DNA sequences. Compared to histone modifications or noncoding RNA silencing, DNA methylation studies are commonly demonstrated in both clinical studies and experimental models of lung diseases (9–11, 17–19). 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 (12). Clusters of 5′-cytosine-guanine-3′ (CpG) dinucleotides (CGs), known as CpG islands (CGIs), are preserved in 1–2% of the mammalian genome. Typically, they range in length from 200 bp to 3 kb. Most are unmethylated under normal circumstances except those associated with imprinted genes, genes subjected to X chromosome inactivation, and transposable elements. In addition, roughly 70% of CGIs are located in the promoter, the first and second exons, and the first intron regions of all genes (5′ CGIs). Promoter methylation alters gene transcription machinery and possibly leads to decreases in gene expression (12). Recent evidence has shown that epigenetic modifications can provide a possible mechanistic explanation for the link between exposure to environmental pollutants and asthma. DNA methylation patterns have been shown to be affected by postnatal or adult exposure to diesel exhaust (13), black carbon (14), particulate matter (15), benzene (16), and cigarette smoke (17). Moreover,

(*Received in original form October 8, 2012 and in final form March 22, 2013*)

This work was supported by National Institute of Environmental Health Sciences grants ES016887 (W.-y.T.) and P30ES03819, National Institutes of Health grant HL10342 (W.M.), and National Natural Science Foundation of China grant 81000006 (Y.S.).

Correspondence and requests for reprints should be addressed to Wan-yee Tang, Ph.D., 615 North Wolfe Street, Room E7545, Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205. E-mail: wtang@jhsph.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 49, Iss. 2, pp 279–287, Aug 2013

Originally Published in Press as DOI: 10.1165/rcmb.2012-0403OC on March 22, 2013
Internet address: www.atsjournals.org

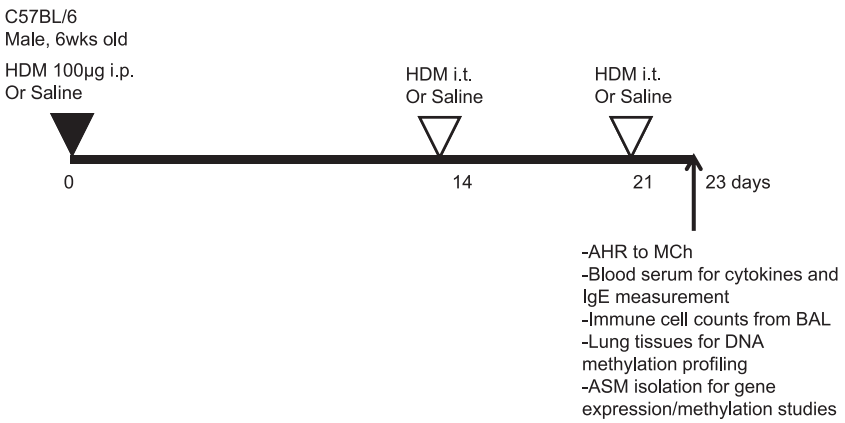

Figure 1. Experimental protocol for acute house dust mite (HDM) exposure induced airway hyperresponsiveness (AHR) in mouse model. C57BL/6J male mice, 6 weeks old, were sensitized with 100 μg HDM or saline (as control) intraperitoneally (i.p.) on Day 1. On Days 14 and 21, mice were challenged with 100 μg HDM or saline intratracheally (i.t.). On Day 23, after airway responsiveness in response to methacholine (MCh) was assessed, lung tissue, blood serum, and bronchoalveolar lavage (BAL) fluid were collected for methylation assay, cytokine measurement, and immune cell counting.

we have reported that promoter methylation status of acyl-coA synthetase long chain member 3 (*acsl3*) (18) and/or *ifnγ* (19) may be the possible biomarker linking maternal exposure to polyacrylic aromatic hydrocarbons (PAHs) to childhood asthma.

In the present study, we show that HDM-induced AHR is associated with epigenetic alterations in the mice lung. The specific epigenetic changes in a number of genes related to AHR in this model may be directly relevant to the allergen-induced mechanisms that underpin allergic asthma in humans.

MATERIALS AND METHODS

Animal and Allergy Challenge

C57BL/6J male mice (6 wk old) were obtained from Jackson Laboratory (Bar Harbor, ME). The experimental protocol for allergy challenge in mice is illustrated in Figure 1. Mice were first sensitized with 100 μg HDM (Greer Laboratories, Lenoir, NC) or saline (as control) intraperitoneally on Day 1. On Days 14 and 21, mice were challenged intratracheally with 100 μg HDM or saline.

Differential Cell Counts from Bronchoalveolar Lavage

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

Assessment of IL4, IL5, IFN-γ, and IgE from Serum

The concentration of IL4, IL5, IFN-γ and IgE was measured in serum using ELISA kits obtained from eBiosciences (San Diego, CA).

Measurement of Airway Responsiveness

On Day 23, mice were anesthetized, paralyzed, and then ventilated with a Flexivent as previously described (20). Airway responsiveness was assessed as the change in pulmonary resistance compared with baseline after the challenge with methacholine (MCh; Sigma-Aldrich, St. Louis, MO).

Global DNA Methylation and Hydroxymethylation Quantification Assay

Genomic DNA (200 ng) isolated from lung tissues was used to measure the level of 5-methyl-cytosine (5-mC) and 5-hydroxy-methyl-cytosine (5-hmC) (Epigentek, Farmingdale, NY).

Methylation-Sensitive Restriction Fingerprinting

Methylation-sensitive restriction fingerprinting (MSRF) was performed as previously described (18) on lung tissues. Candidate sequences displaying differential methylation status between HDM-challenged mice and control mice were isolated, extracted from the gel, cloned, identified, and aligned with RefSeq and BLAT databases.

Bisulfite Genomic Sequencing

Genomic DNA (200 ng) was modified with sodium bisulfite with the EZ DNA Methylation Kit (Zymo Research, Irvine, CA) before PCR. Primers were designed at MethPrimer (21) to amplify fragments encompassing the 5′-CGI(s) of a candidate gene from bisulfite-modified DNA. Amplicons generated from individual sample were subcloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) and sequenced (MacrogenUSA, Rockville, MD). DNA methylation status from sequencing data was analyzed with the BiQ analyzer (22).

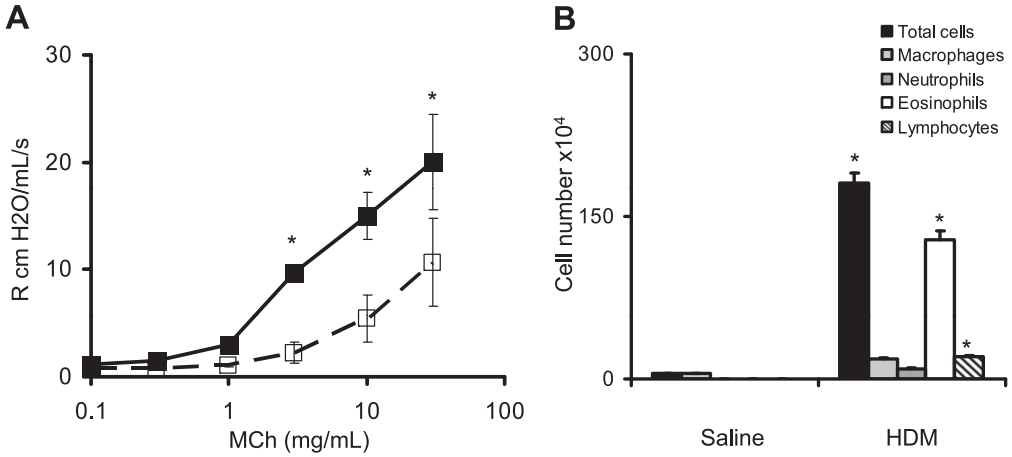

Figure 2. HDM-challenged mice exhibited increased AHR and lung inflammation. (*A*) A bronchoconstrictive agent, MCh, was administered to the animal at increasing doses (0.1, 0.3, 1, 3, 10, and 30 mg/ml) by a 10-second aerosol inhalation. AHR was assessed as the change in pulmonary resistance (R) compared with baseline after the challenge with MCh. *Open square*, control mice; *solid square*, HDM-challenged mice. (*B*) Differential cell counts were performed after staining of BAL cells with Diff-Quick Stain. Morphologically differentiated cells were observed under a light microscope. Numbers ($\times 10^4$) of alveolar macrophages, neutrophils, eosinophils, and lymphocytes were counted. Results are expressed as means (±SD), with $n = 5$–7 mice per treatment group (saline-exposed or HDM-challenged). Technical triplicates were performed in each measurement. *$P < 0.05$ in comparison to control mice means results were statistically significant.

TABLE 1. MEASUREMENT OF IL-4, IL-5, IgE, AND IFN-γ IN BLOOD SERUM

Inflammatory Response	Mean Expression Level in Serum (SD) (*pg/ml*)	*P* Value
IL-4		
Saline	91.43 (38.62)	0.02*
HDM	151.43 (79.40)	
IL-5		
Saline	203.50 (52.37)	0.97
HDM	218.81 (40.74)	
IgE		
Saline	113.75 (27.50)	0.01*
HDM	387.50 (70.41)	
IFN-γ		
Saline	457.50 (131.05)	0.87
HDM	619.38 (189.87)	

Definition of abbreviation: HDM, house dust mite.

Expression levels of IL-4, IL-5, IgE, and IFN-γ were determined by ELISA measuring the concentration of IL-4, IL-5, IgE and IFN-γ present in the serum with use of a range of protein standards. Results are expressed as means (SD), with $n = 5$–7 mice per treatment group. Technical triplicates were performed in the assay.

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

Real-Time RT-PCR

Total RNA (1 μg) was isolated from lung tissues 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 listed in Table E1 in the online supplement). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of transcripts normalized to *rpl19*.

Isolation of Airway Smooth Muscle Cells

Airway smooth muscle (ASM) cells were isolated from trachea, as described previously (23). Cell purity (>90%) was assessed by immunofluorescence staining using antibodies against cytokeratin, vimentin (Vector Lab, Burlingame, CA), and α-smooth muscle actin (α-SMA; Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical Analysis

Data on relative gene expression and average percentage of methylated CGs were expressed as the mean (±SD) (with $n = 5$–7 mice per treatment group, saline-exposed control or HDM-challenged). Technical triplicate (three repeats for each sample) or quadruplicate (four repeats for each sample) were performed in each assay. All data groups were analyzed by one-way ANOVA, followed by *post hoc* Bonferroni tests with two-tailed distribution, and significant differences between groups were accepted at a P value less than 0.05.

RESULTS

To verify successful establishment of the HDM-challenged mouse model, we first examined the airway reactivity by determining the airway resistance during MCh challenge and quantifying the immune cells in BAL. Figure 2 summarizes the effect of acute exposure to HDM on airway reactivity and BAL cell profile. Allergen exposure significantly increased airway reactivity to MCh challenge at concentrations of 3–30 mg/ml in HDM-challenged mice, whereas it had only minimal effect in the control animals (Figure 2A). Differential cell count showed that the total number of immune cells, including eosinophils and lymphocytes, were significantly increased in BAL harvested from the HDM-challenged mice (Figure 2B). These findings are consistent with those normally observed with allergic sensitization (6). In addition, there were significant increases in IL4 and IgE levels in serum obtained from HDM-challenged mice, indicating, serologically, an inflammatory response (Table 1). Moreover, exposure to HDM was found to induce gene expression of α-SMA (*α-sma*), camodulin kinase II delta (*camk2 d*), and myosin light chain kinase (*mlck*), but had no effect on the cell proliferation genes, cyclin D1 (*ccnd1*) and proliferation cell nuclear antigen (*pcna*) (Table 2), suggesting significant alterations in the airway contractile machinery in our mouse model.

The epigenetic influences of HDM exposure on lung was first probed by determining the global methylation and hydroxymethylation. Figure 3 shows the levels of 5-mC and 5-hmC in DNA isolated from mouse lungs. Levels of 5-mC reflect the amount of mC on the 5′ DNA transcripts, and indicate the occurrence of global DNA methylation in the genome. 5-hmC has been identified as the intermediate in DNA demethylation, which contributes to the dynamics of DNA methylation and demethylation (24). HDM-challenged mice showed significant increases in both 5-mC and 5-hmC levels, indicating an alteration in DNA methylation in the lung.

A breakdown of the gene-specific DNA methylation in the lung in response to HDM was identified from MSRF. MSRF is one of the methylation profiling techniques, and uses methylation-sensitive restriction enzymes and PCR to reveal methylated and unmethylated DNA fragments on a denaturing polyacrylamide gel. More than 30 DNA sequences (distinct bands in the gels) were identified as potential leads, with repeatable methylation alterations across multiple samples and treatment groups (saline exposed versus HDM challenged). These bands were cut, amplified, cloned, sequenced, and aligned with the RefSeq database using Basic Local Alignment Search Tool (National Center for Biotechnology Information) and BLAT search (University of California San Francisco Genome Center). Redundant sequences and sequences that aligned to expressed sequence tags were eliminated. Remaining sequences were mapped to six unique, known genes with more than 95% homology, and were harbored in CGIs located

TABLE 2. MEASUREMENT OF EXPRESSION LEVELS OF GENES RELATED TO CELL PROLIFERATION, SMOOTH MUSCLE PHYSIOLOGY AND SMOOTH MUSCLE CONTRACTION IN MOUSE LUNG TISSUES

	Expression Ratio in Lung (SD)	*P* Value
Cell proliferation		
Cyclin D1		
Saline	1.35 (0.04)	0.33
HDM	1.01 (0.59)	
Proliferation cell nuclear antigen		
Saline	1.25 (0.18)	0.92
HDM	1.24 (0.41)	
Smooth muscle contraction		
α-Smooth muscle actin		
Saline	1.06 (0.17)	0.05*
HDM	4.32 (2.77)	
Tropomyosin		
Saline	1.96 (1.29)	0.60
HDM	2.23 (1.76)	
Camodulin kinase II delta		
Saline	1.18 (0.12)	0.001*
HDM	1.92 (0.14)	
Myosin light chain kinase		
Saline	0.90 (0.25)	0.03*
HDM	1.46 (0.41)	

For definition of abbreviation, *see* Table 1.

Expression ratios were determined by real-time PCR 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, and values from HDM-challenged mice were normalized to the mean value of this group. Results are expressed as means ± SD, with $n = 5$–7 mice per treatment group. Technical triplicates were used in the assay.

*$P < 0.05$ in comparison to control mice means results were statistically significant.

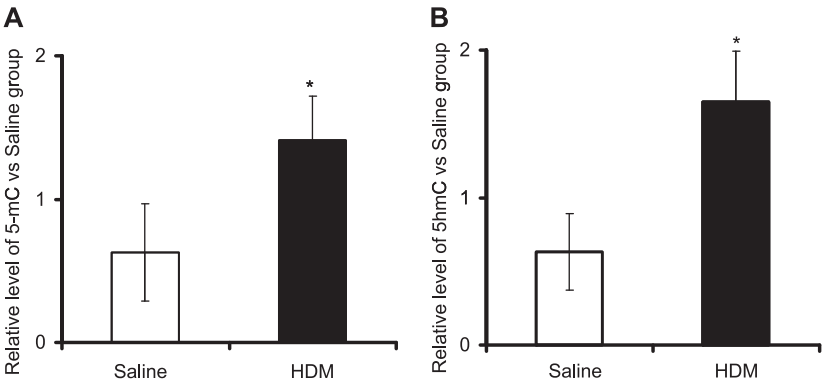

Figure 3. HDM-challenged mice showed increase in level of both 5-methyl-cytosine (5-mC) DNA and 5-hydroxy-methyl-cytosine (5-hmC) DNA in lung. (*A*) 5-mC and (*B*) 5-hmC DNA was detected using capture and detection antibodies, followed by colorimetric quantification. *Open bar*, control (saline-exposed) mice; *solid bar*, HDM-challenged mice. The *y axis* represents relative ratio of 5-mC/5-hmC levels compared with that of control mice. Results were expressed as means (±SD), with $n = 5$–7 mice per treatment group (saline-exposed or HDM-challenged). Technical triplicates were performed in each measurement. *$P < 0.05$ in comparison to control mice means results were statistically significant.

in their proximal promoter region, exon 1 and/or intron 1 (Table 3 and Figure 4). Methylation at CGIs located in 5′ flanking region are more likely involved in transcription regulation (12), we next decided to choose these six candidates with 5′ CGI for further study. These genes have been reported to be involved in cAMP signaling (phosphodiesterase 4D [*pde4 d*]), ion transport (transmembrane 6 superfamily member 1 [*tm6 sf1*], POM121 membrane glycoprotein-like 2, [*pom121l2*], and solute carrier family 8 [sodium/calcium exchanger] member 3 [*slc8a3*]), Akt signaling (AKT1 substrate 1 [*akt1 s1*]), and fatty acid metabolism (*acsl3*).

Promoter methylation and gene expression levels of these MSRF candidates in mouse lungs (Table 4) were validated by bisulfite genomic sequencing and real-time PCR, respectively. *Acsl3* and *slc8a3* were down-regulated, whereas *akt1 s1*, *pde4 d*, *pom121l2*, and *tm6 sf* were up-regulated in the HDM-challenged mice. By measuring the average methylation percentage of all CGs, HDM exposure decreased promoter methylation of *pde4 d* and *pom121l2*, whereas it increased *slc8a3* promoter methylation. No statistically significant changes were found in average methylation percent of all CGs among the *akt1 s1*, *acsl3*, and *tm6 sf* promoters. It is known that DNA methylation of a single CG (or very few) can alter gene transcription (19). Therefore, it was necessary to assess the methylation status at individual CG sites. As shown in Figure 5, HDM exposure decreased promoter methylation at specific CGs of the 5′ promoter regions of *akt1 s1* (CG6 and 8), *pde4 d* (CG 17–18, 25, 43, and 50–52), and *pom121l2* (30–32, 34, 36–39, 43, 44, 46), whereas it increased *slc8a3* promoter hypermethylation at CG 9–13. Results indicate that HDM exposure may alter differential methylation at these CG sites that may affect gene transcription. To confirm it, we will carry out mutations in these specific CpG sites of their promoters and compare the mutated promoter activity with normal promoter activity.

Epigenetic alterations in the expression of these specific genes may have important impacts on AHR. We hypothesized that dysregulation of ASM functions via epigenetic modifications of gene expression may contribute to AHR because of the ASM's proliferative, contractile, and secretory properties (25, 26). As a test of concept, we used the ASM cells isolated from trachea of control animals and HDM-challenged mice, and tested for the methylation status and expression of six potential candidate genes. Among six genes, *pde4 d* and *pom121l2* were up-regulated (hypomethylated), whereas *slc8a3* was down-regulated (hypermethylated) in ASM cells isolated from HDM-challenged mice (Table 5). Results were not exactly the same as those observed in lung tissues. They indicated that aberrant methylation patterns appeared to be cell-type specific. Moreover, we demonstrated that DNA demethylating agent, AZA, up-regulated (demethylated) *pde4 d* and *pom121l2*, which were normally down-regulated (methylated) in ASM cells of control mice (Table 6). In ASM cells of HDM-challenged mice, *slc8a3* promoter was demethylated by AZA, and resulted in an increase in gene expression (Table 6). Our findings further suggest that acute exposure to HDM in mice may induce epigenetic regulation of *pde4 d*, *pom121l2*, and *slc8a3* in ASM, at least partly mediated by DNA methylation.

We chose *pde4 d*, *pom121l2*, and *slc8a3* for further study of their functions in ASM by employing small interfering RNA

TABLE 3. DIFFERENTIALLY METHYLATED CANDIDATE GENES IDENTIFIED WITH METHYLATION-SENSITIVE RESTRICTION FINGERPRINTING

Clone ID	Primer 1	Primer 2	Gene Homology	RefSeq	Chr Location	CpG Island at 5′	Gene Ontology
4p10p11	Bs10	Bs11	POM121 membrane glycoprotein-like 2 (Pom121l2)	NM_001162928.1	13 A3.1	<0.5 kb downstream from TSS	Transmembrane transport (35)
4p10p23	Bs10	Bs23	AKT1 substrate 1 (Akt1s1)	NM_026270.3	7 B2	<1 kb upstream from TSS and < 1 kb downstream from TSS	Signal transduction (31, 34)
7p10p23	Bs10	Bs23	Transmembrane 6 superfamily member 1 (Tm6sf1)	NM_145375.3	7 D1	<0.5 kb downstream from TSS	Transmembrane transport
4p11p11	Bs11	Bs11	Phosphodiesterase 4D, cAMP specific (Pde4d)	NM_011056.3	13 D2.1-2.1	<1 kb upstream from TSS	cAMP signaling (37-40)
1p22p23	Bs22	Bs23	Solute carrier family 8 (sodium/calcium exchanger) member 3 (Slc8a3)	NM_001167920.1	12 D1	<1 kb upstream from TSS	Ion transport (44)
2p22p23	Bs22	Bs23	Acyl-coA synthetase long chain member 3 (Acsl3)	NM_028817.3	1 C4	<0.5 kb upstream rom TSS	Fatty acid metabolism (30)

Definition of abbreviations: Chr, chromosome CpG, 5′-cytosine-guanine-3′; HDM, house dust mite; RefSeq, National Center for Biotechnology Information database identification; TSS, transcription start site.

Sequences were identified based on BLAT (University of California, Santa Cruz genome center database) and RefSeq search. Only candidates locating at 5′ regulatory region of gene are shown.

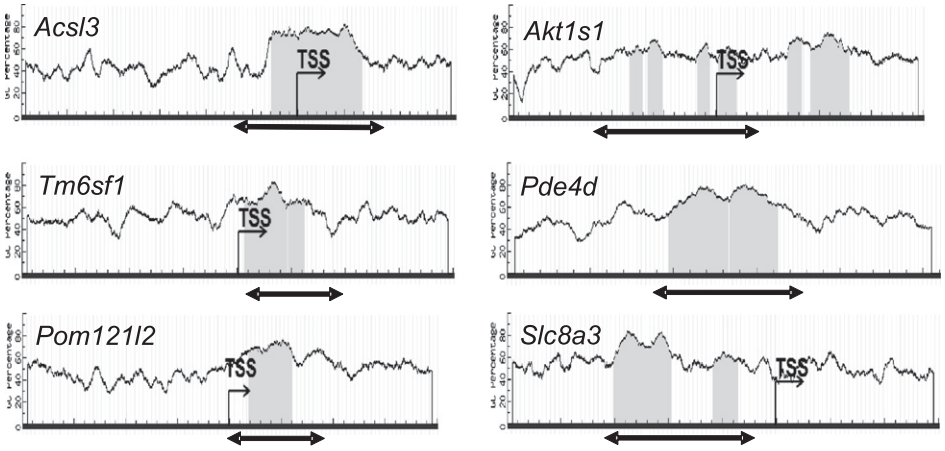

Figure 4. Schematic diagram of CpG dinucleotide (CG) content (%) in the 5′ flanking region of methylation-sensitive restriction fingerprinting (MSRF) targets, *acsl3*, *akt1 s1*, *tm6 sf1*, *pde4 d*, *pom121l2*, and *slc8a3*. The CGI(s) (*shaded in gray* in the genomic DNA sequence) were identified *in silico* based on CG content, 60%, with an observed:expected ratio of 0.6 according to instructions from MethPrimer. TSS, transcription start site. The PCR-amplified region is indicated by a *line with double arrow heads* and methylation status of this region was determined by bisulfite genomic sequencing.

(siRNA)-mediated gene knockdown on ASM. We demonstrated that siRNA-mediated knockdown of *pde4 d* or *pom121l2* decreased cell viability (Figure E1) and gene expression of *α-sma* and *ccnd1* (Table E2) in ASM cells of HDM-challenged mice. Moreover, *siPde4 d* decreased *camk2 d* and *siPom121l2* downregulated *mlck*. *siSlc8a3* increased expression of *camk2 d* and *mlck*, but exerted no effect on *α-sma* or *ccnd1* in ASM cells of control mice. Results indicate that *pde4 d*, *pom121l2*, and *slc8a3* might be involved in alteration in expression of genes related to cell proliferation, migration, and contraction and might, in addition, affect ASM phenotypes.

DISCUSSION

In this study, we employed the mouse model of HDM-induced AHR to demonstrate that the reprogramming of lung or airways by allergens can be mediated through epigenetic mechanisms. Aberrant DNA methylation of *pom121l2*, *pde4 d*, and *slc8a3* appears to be related to HDM exposure and associated with allergen-induced AHR. The model we studied leads to AHR after an acute allergen sensitization and challenge, and we hypothesize that this occurs by activating a set of genes responsible for ASM contraction, proliferation, and remodeling. ASM is postulated to play an important role in asthma pathophysiology because of its proliferative and migration phenotypes, together with its contractile and secretory properties (25, 26). Airway remodeling involves thickening of the airway wall and increase in ASM mass (27). The increase in ASM mass may by itself narrow the airway, but it can also amplify ASM contraction (48), leading to AHR. Even though the acute models generally do not show airway remodeling as observed in chronic allergen exposure models with repeated allergen exposures (5, 28), the increased gene expression of *α-sma*, *camk2 d*, and *mlck* that we observed after acute exposure are consistent with their potential involvement in chronic airway remodeling. Our results clearly show that exposure to HDM induced alterations in global DNA methylation and hydroxymethylation in lung tissue of HDM-challenged mice. This supports the notion that a set of methylated or unmethylated genes specifically linked to AHR development can be reprogrammed via epigenetic modifications. Specifically, we identified genes with differential DNA methylation status in HDM-challenged mice by MSRF. MSRF allows for screening of novel CpG-rich sequences, the methylation status of which undergoes alterations after exposures, and has been used in proof-of-principle epigenetic studies in childhood asthma (18).

Gene silencing of ACSL3 has been reported previously in humans associated with maternal exposure to airborne PAH and PAH-associated asthma (18). *Acsl3* is located in 2q36.1, which is a region associated with the asthma susceptibility loci in specific human populations (29). Moreover, *acsl3* belongs to the ACSL family of genes, which encode key enzymes in fatty acid metabolism (30). Silencing of *acsl3* in lung tissue is expected to influence membrane phospholipid composition and generation

TABLE 4. HOUSE DUST MITE–CHALLENGED MICE ABERRANTLY METHYLATED AND DIFFERENTIALLY EXPRESSED *AKT1S1*, *ACSL3*, *PDE4D*, *POM121L2*, *SLC8A3*, AND *TM6SF* IDENTIFIED FROM METHYLATION-SENSITIVE RESTRICTION FINGERPRINTING

	Average Methylation % of all CG sites		Gene Expression (RER)	
Gene	Mean (SD)	*P* Value	Mean (SD)	*P* Value
Acsl3				
Saline	2.08 (4.46)		1.06 (0.12)	
HDM	2.34 (4.74)	0.93	0.60 (0.14)	0.04*
Akt1s1				
Saline	0.51 (1.39)		0.85 (0.15)	
HDM	0.00 (0.39)	0.27	5.51 (1.17)	0.02*
Pde4d				
Saline	3.58 (1.62)		0.82 (0.27)	
HDM	0.24 (0.39)	0.01*	14.63 (7.59)	0.01*
Pom121l2				
Saline	21.50 (12.37)		5.61 (0.34)	
HDM	2.14 (0.40)	0.03*	19.26 (10.20)	0.05*
Slc8a3				
Saline	0.83 (1.39)		0.86 (0.20)	
HDM	18.75 (5.61)	0.002*	0.48 (0.03)	0.05*
Tm6sf				
Saline	0.35 (0.29)		0.38 (0.08)	
HDM	0.27 (0.32)	0.75	0.96 (0.28)	0.03*

Definition of abbreviations: CG, CpG dinucleotide; HDM, house dust mite; RER, relative expression ratio.

Average methylation % of all CG sites obtained from the average percentage of methylated CGs from lung samples of saline-exposed ($n = 7$) or HDM-challenged ($n = 5$) mice. Six individual clones from each mouse sample for each group were sequenced. A total of 42 clones from saline control group and 30 clones from HDM-challenged mice were sequenced. BiQAnalyzer was used to visualize the methylated/unmethylated CpG sites and export the methylation status to Microsoft Excel (Microsoft Corp., Redmond, WA) for calculation of percentage of CpGs at each promoter. Results are expressed as means (SD), with $n = 5$–7 mice per treatment group. Technical triplicates were performed in the assay. SD was determined by 42 clones from the saline group or 30 clones from the HDM-challenged group. One-way ANOVA was applied to determine if the difference in percentage of CpG promoter methylation between HDM-exposed and saline-exposed mice was statistically significant. Relative levels of gene transcript were determined by real-time PCR and normalized to *rpl19* transcript in the same sample. The relative level of gene transcript (RER) in control mice exposed to saline was arbitrarily assigned a value of 1.0, and values from HDM-challenged mice were normalized to the mean value of this group.

*$P < 0.05$ in comparison to control mice means results were statistically significant.

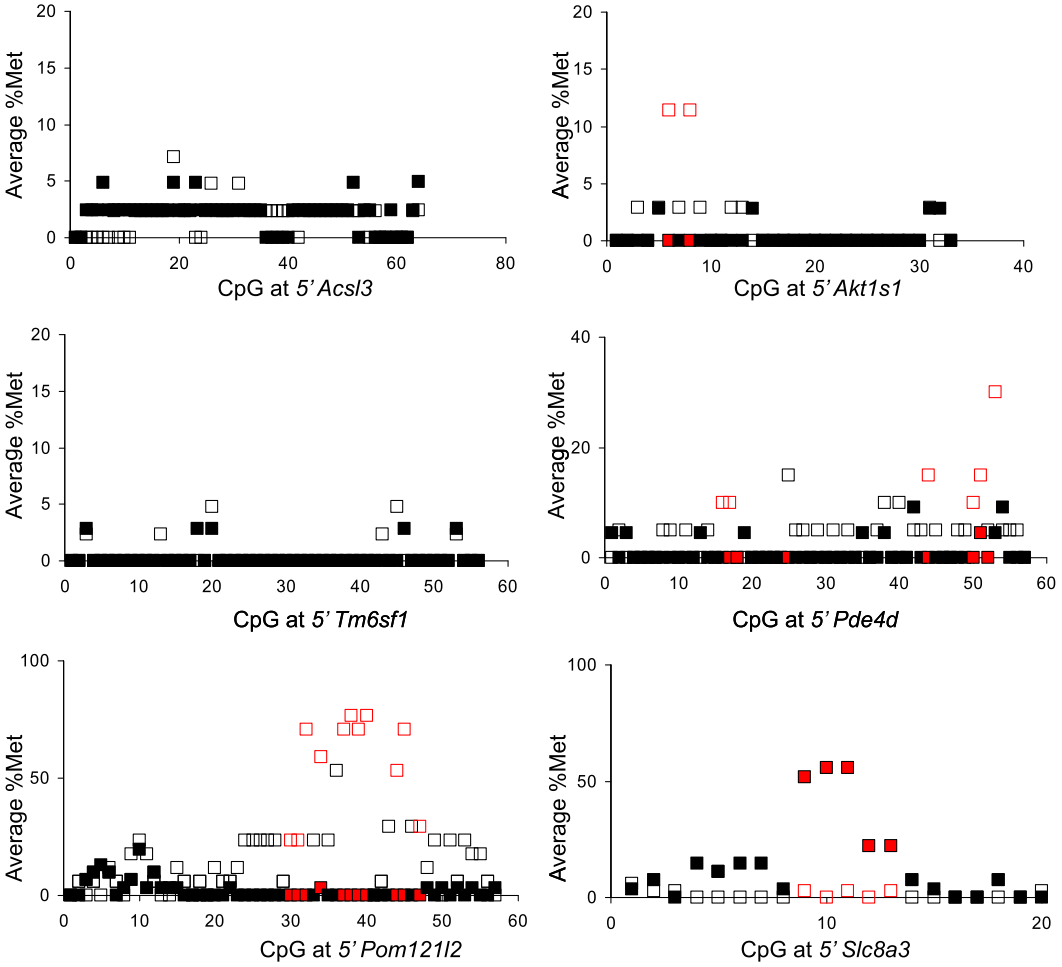

Figure 5. Site-specific DNA methylation at *acsl3*, *akt1 s1*, *tm6 sf1*, *pde4 d*, *pom121l2*, and *slc8a3* promoters. Methylation status at each CG site of the promoter is indicated as average % methylation (Met) from all clones of each sample. *Open squares*, control (saline-exposed) mice; *solid squares*, HDM-challenged mice. Bisulfite-treated DNA was amplified for region of CGIs of the MSRF candidates by PCR (indicated as Figure 4) and PCR product was subcloned into pCR2.1 vector. Six clones were chosen from each mouse DNA sample. Results are expressed as mean values from $n = 5$–7 mice per treatment group (saline-exposed or HDM-challenged). Differentially methylated CG sites ($P < 0.05$ in comparison of methylation % to that of control mice) are shown in *red*.

of inflammatory mediators. Our data show that HDM induced *acsl3* gene expression, but not hypermethylation of its promoter, suggesting that *acsl3* promoter methylation may be specific to PAH exposure only. Alternatively, down-regulation of *acsl3* by HDM may be mediated by other gene regulation mechanisms. This phenomenon might apply in the case of *tm6 sf*, as up-regulation of *tm6 sf* was also not associated with *tm6 sf* promoter hypomethylation.

Akt1 s1 was overexpressed and hypomethylated in lung tissue of HDM-challenged mice. AKT1S1 (also known as proline-rich Akt substrate of 40 kD) is a proline-rich substrate of AKT that binds 14-3-3 protein when phosphorylated. Upon binding, it can activate both the AKT- and mammalian target of rapamycin (mTOR)–mediated signaling pathways. Activation of mTOR pathways is involved in the progression of diseases, such as cancer and type 2 diabetes (31). In the lung, mTOR may modulate immune responses and cell proliferation in the airway. Fredriksson and coworkers (32) and Mushaben and coworkers (33) demonstrated that inhibition of mTOR with rapamycin reduces HDM-induced AHR and immune responses. Furthermore, mTOR-activated p70S6K results in an increased proliferative and contractile phenotype of ASM cells (34). In our case, there was no change in gene expression or promoter methylation of *akt1 s1* in ASM cells of control and HDM-challenged mice. Aberrant gene expression of *akt1 s1* may be cell type, time, or allergen specific. The function of *akt1 s1* in the pathogenesis of asthma may require further investigation.

We speculate that dysregulation of *pom121l2* may affect transport gateways and chromatin organization in response to HDM. POM121 was identified as an integral membrane protein specifically localized in the pore-membrane domain of the nuclear envelope. It may play a role in assembly of nuclear pore complexes that mediate transport between nucleus and cytoplasm and organize genome architecture (35). However, the function of *pom121l2* in airway or immune cells has not been investigated. We found that down-regulation of *pom121l2* in ASM cells decreased cell proliferation and gene expression of *α-sma* and *mlck*, suggesting that *pom121l2* appears to be involved in ASM cell structure and contraction.

Our observation of a greater than 90% reduction in *pde4 d* promoter methylation, and the corresponding increase in *pde4 d* transcript expression in HDM-challenged mouse lung and ASM, has important implications for HDM-induced AHR. PDE4 is shown to be the major PDE subtype in human ASM cells (36). It hydrolyzes cAMP, which is known to activate protein kinase A (PKA)- and exchange protein directly activated by cAMP (Epac)-dependent pathways to regulate ASM functions, such as inhibition of ASM cell proliferation and airway constriction (37–39). The dramatic increase in *pde4 d* expression in lungs of HDM-challenged mice, therefore, could cause a significant reduction in cAMP and enhance airway reactivity and remodeling. This notion is congruent with the reported reduction in cAMP level in asthmatic ASM (40), and the use of PDE4 inhibitors, such as roflumilast and β2-agonists for the treatment of asthma (41). *Pde4 d* was shown to decrease airway reactivity in a genetic knockout mouse model (42), where antigen-induced AHR to cholinergic stimulation was abolished in *pde4* $d^{-/-}$ mice, whereas inflammatory responses were undisturbed. In a preliminary study, we found that *pde4* $d^{-/-}$ Agouti mice with acute HDM challenge showed decreased

TABLE 5. METHYLATION STATUS AND GENE EXPRESSION OF METHYLATION-SENSITIVE RESTRICTION FINGERPRINTING CANDIDATES IN MOUSE AIRWAY SMOOTH MUSCLE CELLS

	Average Methylation % of all CG Sites		Gene Expression (RER)	
Gene	Mean (SD)	*P* Value	Mean (SD)	*P* Value
Acsl3				
Saline	1.17 (0.67)		1.17 (0.20)	
HDM	1.30 (0.90)	0.4	1.47 (0.17)	0.19
Akt1s1				
Saline	0.51 (0.87)		1.56 (0.49)	
HDM	0.76 (0.44)	0.7	1.69 (1.00)	0.73
Pde4d				
Saline	29.82 (9.12)		1.47 (0.47)	
HDM	2.63 (1.51)	0.04*	5.17 (2.48)	0.05*
Pom121l2				
Saline	26.02 (5.57)		1.27 (0.23)	
HDM	6.14 (1.10)	0.04*	10.35 (4.73)	0.03*
Slc8a3				
Saline	12.50 (5.46)		0.87 (0.09)	
HDM	55.00 (2.50)	0.01*	0.19 (0.11)	0.03*
Tm6sf				
Saline	1.48 (1.03)		1.44 (0.43)	
HDM	1.79 (1.28)	0.6	2.87 (1.31)	0.17

For definition of abbreviations, *see* Table 4.

Airway smooth muscle (ASM) cells were isolated from trachea of saline-exposed ($n = 4$) or HDM-challenged ($n = 5$) mice. Average methylation % of all CG sites obtained from the average percentage of methylated CGs from ASM cells. Six individual clones from each mouse sample for each group were sequenced. A total of 24 clones from the saline control group and 30 clones from the HDM-challenged group were sequenced. Results are expressed as means (SD), with $n = 4$–5 mice per group (saline-exposed or HDM-challenged). Technical triplicates were performed in the assay. SD was determined by 24 clones from the saline group or 30 clones from the HDM-challenged group. One-way ANOVA was applied to determine if the difference in percentage of CpG promoter methylation between HDM-exposed and saline-exposed mice was statistically significant. Relative levels of gene transcript were determined by real-time PCR and normalized to *rpl19* transcript in the same sample. The relative level of gene transcript (RER) in ASM cells of saline-exposed mice was arbitrarily assigned a value of 1.0, and values from ASM cells from HDM-challenged mice were normalized to the mean value of this group.

*$P < 0.05$ in comparison to ASM cells of saline-exposed mice means results were statistically significant.

TABLE 6. EFFECT OF 5-AZA-DEOXYCYTIDINE ON PROMOTER METHYLATION AND GENE EXPRESSION OF PDE4D, POM121L2, AND SLC8A3 IN MOUSE AIRWAY SMOOTH MUSCLE CELLS

	Average Methylation % of all CG sites		Gene Expression (RER)	
Gene	Mean (SD)	*P* Value	Mean (SD)	*P* Value
Pde4d				
Control	29.82 (9.12)		1.00 (0.00)	
Aza0.5	2.34 (2.03)	0.04*	1.43 (0.55)	0.03*
Aza1.0	0.58 (1.01)	0.04*	1.25 (0.33)	0.03*
Pom121l2				
Control	26.02 (5.57)		1.00 (0.00)	
Aza0.5	8.12 (1.01)	0.04*	3.35 (2.01)	0.03*
Aza1.0	6.43 (1.01)	0.04*	2.61 (1.64)	0.08
Slc8a3				
Control	55.0 (2.5)		1.00 (0.00)	
Aza0.5	20.83 (8.03)	0.02*	3.23 (1.70)	0.08
Aza1.0	9.17 (2.88)	0.01*	2.04 (0.55)	0.04*

Definition of abbreviations: Aza, 5-aza-2′-deoxycytidine; CG, CpG dinucleotide; RER, relative expression ratio.

Airway smooth muscle (ASM) cells were isolated from trachea of saline-exposed or HDM-challenged mice. ASM cells were treated with 0.5, 1.0 μM Aza or 0.1% dimethyl sulfoxide (DMSO) as vehicle control for a total of 8 days. Media with Aza or DMSO were replenished every 2 days. ASM cells from saline-exposed mice were used for *pde4d* and *pom121l2* studies. ASM cells from HDM-challenged mice were employed for *slc8a3* study. Three independent experiments with Aza treatment were employed. Average methylation % of all CG sites values were obtained from the average percentage of methylated CGs from ASM cells. Six individual clones from each sample for each Aza concentration were sequenced. A total of 18 clones from each treatment group were sequenced. SD was determined by 18 clones from each treatment group. One-way ANOVA was applied to determine if the difference in percentage of CpG promoter methylation between control and Aza-treated samples was statistically significant. Relative levels of gene transcript were determined by real-time PCR and normalized to *rpl19* transcript in the same sample. The relative level of gene transcript (RER) in ASM cells with DMSO control was arbitrarily assigned a value of 1.0, and values from ASM cells with each Aza concentration were normalized to the mean value of the DMSO control. Technical triplicates were performed in the assay.

*$P < 0.05$ in comparison to ASM cells with DMSO or Aza treatment means results were statistically significant.

AHR in response to MCh challenge (unpublished data). siRNA-mediated knockdown of *pde4 d* caused significant suppression in ASM cell proliferation, as well as *α-sma*, *ccnd1*, and *camk2 d* expression. Recently, *pde4 d* has been shown to be epigenetically modified and associated with cell proliferation in the rat prostate (42). Hence, we suggest that long-term reduction of cAMP due to *pde4 d* up-regulation in ASM cells of allergen-challenged animals could contribute to airway remodeling and eventually lead to AHR.

Slc8a3 (also known as *Ncx3*) promoter hypermethylation observed in HDM-challenged mice suggests that down-regulation of this gene may lead to HDM-induced AHR by altering the Na^+–Ca^{2+} transport in the mouse lung/airway. Sodium chloride exchanger (NCX) has long been investigated in cardiovascular, neurological, and digestive physiology (44), but rarely in the lung. Under normal physiological conditions, NCX operates in the forward mode to extrude Ca^{2+} by exchanging three Na^+ ions. Paradoxically, under special conditions where Na^+ accumulates inside the cell, NCX is activated in a reverse mode to facilitate Ca^{2+} entry. The concept that altered Ca^{2+} signaling in ASM leads to asthmatic phenotypes with airway remodeling and AHR is increasingly being recognized (45). In ASM of humans without asthma, the reverse mode of NCX1 contributed to Ca^{2+} influx after histamine stimulation (46). Rahman and colleagues (47) recently suggested that the reverse mode of NCX1 altered Ca^{2+} mobility in mouse ASM. They also described increased gene expression of NCX1 in a chronic model with repeated HDM exposure that was not present in the acute model. However, there is little additional information regarding the mechanism(s) by which NCX1 participates in AHR and its development with cell proliferation in this chronic model. It is also not clear if the reverse mode of NCX1 on Ca^{2+} influx in ASM stimulated by short-term exposure of agonists (46) persists during the chronic progression of AHR. Down-regulation of NCX1 by siRNA has been recently shown to decrease both NCX forward and reverse exchange mode and alter intracellular Ca^{2+} levels and oscillations in human ASM cells (48). No study on the other forms of NCX (e.g., NCX3) has been investigated in the lung. Our data show that siRNA suppression of *slc8a3* expression led to significant up-regulation of *mlck* and *camk2 d* expression, which plays a major role in ASM contraction. Our data thus support the hypothesis that allergen-induced aberrant expression of *slc8a3* via promoter hypermethylation may participate in AHR.

We hypothesize that epigenetic regulation of gene expression in lung or airway influences the development of AHR. It may involve gene dysregulation in certain types of cells, but is not limited just to ASM cells. In this study, we identified the methylated candidates from whole lung tissue because of the need to obtain sufficient amounts of genomic DNA to be used in MSRF, although we acknowledge the heterogeneity of cells in the lung tissues. Our gene expression and promoter methylation studies in both lung tissues and ASM cells clearly demonstrate that there is cell type–specific methylation of certain genes. Recently,

epigenetic changes in T cells and B cells have been reported in both clinical and experimental animal studies of asthma. DNA methylation at T helper type 1/2 cytokine genes, *il4* and *ifnγ*, were demonstrated by Kwon and coworkers (49) in patients with bronchial asthma. In this study, the authors measured the gene expression and promoter methylation of *il4* and *ifnγ* in human $CD4^+$ T lymphocytes. Brand and coworkers (10) also reported increases in *ifnγ* promoter methylation in $CD4^+$ T cells after allergen sensitization and challenge in mice. Hypermethylation of *foxp3* locus in peripheral blood T regulatory cells was found to be associated with ambient air pollution exposure and asthma risk (50). Pascual and colleagues (9) revealed differential methylation patterns and gene expression of *cyp26a1* in $CD19^+$ B cells between HDM-allergic subjects, aspirin-intolerant subjects with asthma, and control subjects. Our finding in ASM cells proves the principle of epigenetic regulation of ASM and its association with AHR. It will be interesting to discover the methylation patterns of the MSRF candidates on other cell types in the future. Taken together, we recommend measuring gene methylation status on different cell types because this result will improve our understanding of the cellular and molecular mechanisms underlying the pathogenesis of asthma.

In summary, we employed an innovative epigenetic approach to investigate allergen-induced AHR. We have demonstrated that acute exposure to HDM induced AHR that was associated with epigenetic modulations of genes related to ASM cell proliferation and contraction. Although we did not observe chronic changes, such as of airway wall thickening or ASM hyperplasia, in this acute model, a set of genes potentially involved in airway inflammation and AHR were epigenetically "reprogrammed" in response to HDM exposure. Further studies will be needed to determine if these modifications persist over longer time spans with chronic exposure to HDM. Both *in vitro* and *in vivo* gene knockin or knockout models, together with lung function assays, will be required in the future to characterize the function of our chosen MSRF candidates related to allergen-induced AHR and ASM cell proliferation and contraction.

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors acknowledge Stephanie Chan, Juliana Wu, and Xiao Xiao for sample preparation.

References

1. Ho SM. Environmental epigenetics of asthma: an update. *J Allergy Clin Immunol* 2010;126:453–465.
2. Kabesch M, Michel S, Tost J. Epigenetic mechanisms and the relationship to childhood asthma. *Eur Respir J* 2010;36:950–961.
3. Thomas WR, Hales BJ, Smith WA. House dust mite allergens in asthma and allergy. *Trends Mol Med* 2010;16:321–328.
4. Cates EC, Fattouh R, Wattie J, Inman MD, Goncharova S, Coyle AJ, Gutierrez-Ramos JC, Jordana M. Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF–mediated mechanism. *J Immunol* 2004;173:6384–6392.
5. Johnson JR, Wiley RE, Fattouh R, Swirski FK, Gajewska BU, Coyle AJ, Gutierrez-Ramos JC, Ellis R, Inman MD, Jordana M. Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. *Am J Respir Crit Care Med* 2004;169:378–385.
6. Zhang X, Lewkowich IP, Kohl G, Clark JR, Wills-Karp M, Kohl J. A protective role for C5a in the development of allergic asthma associated with altered levels of B7-H1 and B7-DC plasmacytoid dendritic cells. *J Immunol* 2009;8:5123–5130.
7. Jiang YH, Bressler J, Beaudet AL. Epigenetics and human disease. *Annu Rev Genomics Hum Genet* 2004;5:479–510.
8. Godfrey KM, Lillycrop KA, Burdge GC, Gluckman PD, Hanson MA. Epigenetic mechanisms and the mismatch concept of the developmental origins of health and disease. *Pediatr Res* 2007;61:5R–10R.
9. Pascual M, Suzuki M, Isidoro-Garcia M, Padrón J, Turner T, Lorente F, Dávila I, Greally JM. Epigenetic changes in B lymphocytes associated with house dust mite allergic asthma. *Epigenetics* 2011;6:1131–1137.
10. Brand S, Kesper DA, Teich R, Kilic-Niebergall E, Pinkenburg O, Bothur E, Lohoff M, Garn H, Pfefferle PI, Renz H. DNA methylation of Th1/Th2 cytokine genes affects sensitization and progress of experimental asthma. *J Allergy Clin Immunol* 2012;129:1602–1610.
11. Kim EG, Shin HJ, Lee CG, Park HY, Kim YK, Park HW, Cho SH, Min KU, Cho ML, Park SH, *et al.* DNA methylation and not allelic variation regulates STAT6 expression in human T cells. *Clin Exp Med* 2010;10:143–152.
12. Costello JF, Plass C. Methylation matters. *J Med Genet* 2001;38:285–303.
13. Liu J, Ballaney M, Al-alem U, Quan C, Jin X, Perera F. Combined inhaled diesel exhaust particles and allergen exposure alter methylation of T helper genes and IgE production *in vivo*. *Toxicol Sci* 2008;102:76–81.
14. Baccarelli A, Wright RO, Bollati V, Tarantini L, Litonjua AA, Suh HH, Zanobetti A, Sparrow D, Vokonas PS, Schwartz J. Rapid DNA methylation changes after exposure to traffic particles. *Am J Respir Crit Care Med* 2009;179:572–578.
15. Tarantini L, Bonzini M, Apostoli P, Pegoraro V, Bollati V, Marinelli B, Cantone L, Rizzo G, Hou L, Schwartz J, *et al*. Effects of particulate matter on genomic DNA methylation content and iNOS promoter methylation. *Environ Health Perspect* 2009;117:217–222.
16. Bollati V, Baccarelli A, Hou L, Bonzini M, Fustinoni S, Cavallo D, Byun HM, Jiang J, Marinelli B, Pesatori AC, *et al*. Changes in DNA methylation patterns in subjects exposed to low-dose benzene. *Cancer Res* 2007;67:876–880.
17. Pulling LC, Vuillemenot BR, Hutt JA, Devereux TR, Belinsky SA. Aberrant promoter hypermethylation of the death-associated protein kinase gene is early and frequent in murine lung tumors induced by cigarette smoke and tobacco carcinogens. *Cancer Res* 2004;64:3844–3848.
18. Perera F, Tang WY, Herbstman J, Tang D, Levin L, Miller R, Ho SM. Relation of DNA methylation of 5′-CpG island of ACSL3 to transplacental exposure to airborne polycyclic aromatic hydrocarbons and childhood asthma. *PLoS One* 2009;4:e4488.
19. Tang WY, Levin L, Talaska G, Cheung YY, Herbstman JB, Miller R, Perera F, Ho S. Maternal exposure to polycyclic aromatic hydrocarbons is associated with methylation at a 5′-CpG island of interferon-γ in cord white blood cells. *Environ Health Perspect* 2012;120:1195–1200.
20. Ewart S, Levitt R, Mitzner W. Respiratory system mechanics in mice measured by end-inflation occlusion. *J Appl Physiol* 1995;79:560–566.
21. Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. *Bioinformatics* 2002;18:1427–1431.
22. Bock C, Reither S, Mikeska T, Paulsen M, Walter J, Lengauer T. BiQ Analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing. *Bioinformatics* 2005;21:4067–4068.
23. McGraw DW, Forbes SL, Kramer LA, Witte DP, Fortner CN, Paul RJ, Liggett SB. Transgenic overexpression of beta(2)-adrenergic receptors in airway smooth muscle alters myocyte function and ablates bronchial hyperreactivity. *J Biol Chem* 1999;274:32241–32247.
24. Dahl C, Grønbæk K, Guldberg P. Advances in DNA methylation: 5-hydroxymethylcytosine revisited. *Clin Chim Acta* 2011;412:831–836.
25. Bentley JK, Hershenson MB. Airway smooth muscle growth in asthma: proliferation, hypertrophy, and migration. *Proc Am Thorac Soc* 2008;5:89–96. (Review).
26. Mahn K, Ojo OO, Chadwick G, Aaronson PI, Ward JP, Lee TH. Ca(2+) homeostasis and structural and functional remodelling of airway smooth muscle in asthma. *Thorax* 2010 65:547–552.
27. Al Heialy S, McGovern TK, Martin JG. Insights into asthmatic airway remodelling through murine models. *Respirology* 2011;16:589–597.
28. Tomlinson KL, Davies GC, Sutton DJ, Palframan RT. Neutralisation of interleukin-13 in mice prevents airway pathology caused by chronic exposure to house dust mite. *PLoS One* 2010;5: pii: e13136.
29. Choudhry S, Taub M, Mei R, Rodriguez-Santana J, Rodriguez-Cintron W, Shriver MD, Ziv E, Risch NJ, Burchard EG. Genome-wide screen for asthma in Puerto Ricans: evidence for association with 5q23 region. *Hum Genet* 2008;123:455–468.
30. Poppelreuther M, Rudolph B, Du C, Großmann R, Becker M, Thiele C, Ehehalt R, Füllekrug J. The N-terminal region of acyl-CoA synthetase 3 is essential for both the localization on lipid droplets and the function in fatty acid uptake. *J Lipid Res* 2012;53:888–900.
31. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* 2011;12:21–35.

32. Fredriksson K, Fielhaber JA, Lam JK, Yao X, Meyer KS, Keeran KJ, Zywicke GJ, Qu X, Yu ZX, Moss J, *et al*. Paradoxical effects of rapamycin on experimental house dust mite–induced asthma. *PLoS One* 2012;7:e33984.
33. Mushaben EM, Kramer EL, Brandt EB, Khurana Hershey GK, Le Cras TD. Rapamycin attenuates airway hyperreactivity, goblet cells, and IgE in experimental allergic asthma. *J Immunol* 2011;187:5756–5763.
34. Halayko AJ, Kartha S, Stelmack GL, McConville J, Tam J, Camoretti-Mercado B, Forsythe SM, Hershenson MB, Solway J. Phophatidylinositol-3 kinase/mammalian target of rapamycin/p70S6K regulates contractile protein accumulation in airway myocyte differentiation. *Am J Respir Cell Mol Biol* 2004;31:266–275.
35. Hallberg E, Wozniak RW, Blobel G. An integral membrane protein of the pore membrane domain of the nuclear envelope contains a nucleoporin-like region. *J Cell Biol* 1993;122:513–521.
36. Nino G, Hu A, Grunstein JS, Grunstein MM. Mechanism regulating proasthmatic effects of prolonged homologous beta2-adrenergic receptor desensitization in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L746–L757.
37. Stewart AG, Tomlinson PR, Wilson JW. Beta 2-adrenoceptor agonist–mediated inhibition of human airway smooth muscle cell proliferation: importance of the duration of beta 2-adrenoceptor stimulation. *Br J Pharmacol* 1997;121:361–368.
38. Trian T, Burgess JK, Niimi K, Moir LM, Ge Q, Berger P, Liggett SB, Black JL, Oliver BG. β2-Agonist induced cAMP is decreased in asthmatic airway smooth muscle due to increased PDE4D. *PLoS One* 2011;6:e20000.
39. Janssen LJ, Tazzeo T, Zuo J. Enhanced myosin phosphatase and Ca (2+)-uptake mediate adrenergic relaxation of airway smooth muscle. *Am J Respir Cell Mol Biol* 2004;30:548–554.
40. Rinard GA, Rubinfeld AR, Brunton LL, Mayer SE. Depressed cyclic AMP levels in airways smooth muscle from asthmatic dogs. *Proc Natl Acad Sci USA* 1979;76:1472–1476.
41. Kobayashi M, Kubo S, Hirano Y, Kobayashi S, Takahashi K, Shimizu Y. Anti-asthmatic effect of ASP3258, a novel phosphodiesterase 4 inhibitor. *Int Immunopharmacol* 2012;12:50–58.
42. Hansen G, Jin S, Umetsu DT, Conti M. Absence of muscarinic cholinergic airway responses in mice deficient in the cyclic nucleotide phosphodiesterase PDE4D. *Proc Natl Acad Sci USA* 2000;97:6751–6756.
43. Ho SM, Tang WY, Belmonte de Frausto J, Prins GS. Developmental exposure to estradiol and bisphenol A increases susceptibility to prostate carcinogenesis and epigenetically regulates phosphodiesterase type 4 variant 4. *Cancer Res* 2006;66:5624–5632.
44. Lytton J. Na^+/Ca^{2+} exchangers: three mammalian gene families control Ca^{2+} transport. *Biochem J* 2007;406:365–382.
45. Sanderson MJ, Delmotte P, Bai Y, Perez-Zogbhi JF. Regulation of airway smooth muscle cell contractility by Ca^{2+} signaling and sensitivity. *Proc Am Thorac Soc* 2008;5:23–31.
46. Liu B, Peel SE, Fox J, Hall IP. Reverse mode Na^+/Ca^{2+} exchange mediated by STIM1 contributes to Ca^{2+} influx in airway smooth muscle following agonist stimulation. *Respir Res* 2010;11:168.
47. Rahman M, Inman M, Kiss L, Janssen LJ. Reverse-mode NCX current in mouse airway smooth muscle: Na(+) and voltage dependence, contributions to Ca(2+) influx and contraction, and altered expression in a model of allergen-induced hyperresponsiveness. *Acta Physiol (Oxf)* 2012;205:279–291.
48. Sathish V, Delmotte PF, Thompson MA, Pabelick CM, Sieck GC, Prakash YS. Sodium-calcium exchange in intracellular calcium handling of human airway smooth muscle. *PLoS One* 2011;6:e23662.
49. Kwon NH, Kim JS, Lee JY, Oh MJ, Choi DC. DNA methylation and the expression of IL-4 and IFN-gamma promoter genes in patients with bronchial asthma. *J Clin Immunol* 2008;28:139–146.
50. Nadeau K, McDonald-Hyman C, Noth EM, Pratt B, Hammond SK, Balmes J, Tager I. Ambient air pollution impairs regulatory T-cell function in asthma. *J Allergy Clin Immunol* 2010;126:845–852.