ORIGINAL RESEARCH

Aberrant DNA Methylation of Phosphodiestarase 4D Alters Airway Smooth Muscle Cell Phenotypes

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Abstract

Airway hyperresponsiveness (AHR) is a hallmark feature in asthma characterized by exaggerated airway contractile response to stimuli due to increased airway sensitivity and chronic airway remodeling. We have previously shown that allergen-induced AHR in mice is associated with aberrant DNA methylation in the lung genome, suggesting that AHR could be epigenetically regulated, and these changes might predispose the animals to asthma. Previous studies demonstrated that overexpression of phosphodiesterase 4D (*PDE4D*) is associated with increased AHR. However, epigenetic regulation of this gene in asthmatic airway smooth muscle cells (ASMCs) has not been examined. In this study, we aimed to examine the relationship between epigenetic regulation of *PDE4D* and ASMC phenotypes. We identified CpG site–specific hypomethylation at *PDE4D* promoter in human asthmatic ASMCs. We next used methylated oligonucleotides to introduce CpG site–specific methylation at *PDE4D* promoter and examined its effect on ASMCs. We showed that *PDE4D* methylation decreased cell proliferation and migration of asthmatic ASMCs. We further elucidated that methylated *PDE4D* decreased *PDE4D* expression in asthmatic ASMCs, increased cAMP level, and inhibited the aberrant increase in Ca^{2+} level. Moreover, *PDE4D* methylation reduced the phosphorylation level of downstream effectors of Ca^{2+} signaling, including myosin light chain kinase and p38. Taken together, our findings demonstrate that gene-specific epigenetic changes may predispose ASMCs to asthma through alterations in cell phenotypes. Modulation of ASMC phenotypes by methylated *PDE4D* oligonucleotides can reverse the aberrant ASMC functions to normal phenotypes. This has provided new insight to the development of novel therapeutic options for this debilitative disease.

Keywords: airway hyperresponsiveness; airway smooth muscle cell; DNA methylation; methylated DNA oligonucleotides; phosphodiesterase 4D

Chronic asthma is characterized by persistent airway hyperresponsiveness (AHR), an exaggerated narrowing of the airway in response to a variety of physical and chemical stimuli due to increased airway sensitivity, inflammation, and remodeling (1, 2). Antiinflammatory therapy is currently the primary medication for asthma, and studies of AHR have been prevalently focused on inflammatory cells, mediators, and immune responses (3). However, accumulating evidence shows that AHR may also present in patients with asthma without inflammation, suggesting that airway inflammation cannot fully explain the mechanisms underlying AHR (4–6). Therefore, noninflammatory AHR mechanisms should not be overlooked, and require additional in-depth investigations.

Airway remodeling characterized by airway smooth muscle (ASM) hypertrophy and hyperplasia is a salient feature of asthma, and is linked to AHR (7, 8). *In vitro* studies have consistently shown that ASM cells (ASMCs) of subjects with asthma are hypercontractile and proliferative

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Clinical Relevance

Prior epigenetic work has focused primarily on epigenetic regulation of pathways involved in the initiation of allergen sensitization in immune cells. However, few studies have investigated lung cell–specific epigenetic changes, which are directly linked to altered lung function. In the present study, we demonstrate that application of methylated DNA oligonucleotides can reverse the aberrant epigenetic changes in asthmatic airway smooth muscle cells and thus modify the airway smooth muscle responsiveness. Our findings may provide insight for the use of epigenetic modifiers in treating asthma through the reversible nature of epigenetic modulations.

compared with nonasthmatic ASMCs (9, 10), indicating that the phenotypic changes persisted in cultured asthmatic ASMCs. Epigenetic modification via DNA methylation, histone modifications, or noncoding RNA is a possible mechanism for the persistent phenotypic changes in cells, tissues, and organs (11). Aberrant changes in histone modifications and microRNAs have been implicated in phenotypic switch in ASMCs (reviewed in Ref. 12). Specific histone modifications at inflammatory or growth factor genes may contribute to the increased production that modulates the ASMC phenotypes (13, 14). Asthmatic ASMCs showed increased histone H3K18 acetylation, and enhanced binding of p300 at the *CXCL8* promoter as compared with nonasthmatic ASMCs. On the other hand, ASMCs showed alteration in expression of specific microRNAs in proinflammatory milieu, resulting in aberrant expression of genes modulating ASMC proliferation, hypertrophy, and contractility (15–18). Furthermore, DNA methylation is the best-studied epigenetic mechanisms in asthma studies. DNA methylation changes are associated with asthma (19, 20) and linked to specific triggers of asthma, such as pollutant exposures (21–24). We previously reported that acute exposure to house dust mite (HDM) induced AHR in a mouse model, and the AHR induction was associated with epigenetic modulations of genes related to ASMC proliferation and contraction (25, 26). Specifically, we found promoter demethylation of phosphodiesterase 4D (*Pde4d*) gene in tracheal ASMCs isolated from HDM-exposed mice. PDE4D is a cAMP-specific phosphodiesterase, which regulates intracellular cAMP level. Knockout of *Pde4d* abolishes the airway reactivity toward cholinergic stimulation and ovalbumin sensitization in mice (27). PDE4D, through regulation of intracellular cAMP level, modulates a variety of ASM functions, including cell death, cell proliferation, contraction, and migration (28–30). In fact, a genome-wide association analysis study has identified *PDE4D* as an asthma-susceptibility gene (31). Herein, we hypothesize that epigenetic regulation of PDE4D promoter methylation alters the expression of PDE4D, which leads to aberrant phenotypic changes of human asthmatic ASMCs. In this study, we aimed to: (*1*) examine the epigenetic alteration of *PDE4D* promoter occurring at human asthmatic ASMCs; (*2*) compare the phenotypes, including proliferation, migration, and agonist-induced Ca^{2+} response, of nonasthmatic and asthmatic ASMCs; and (*3*) investigate if modulation of *PDE4D* promoter methylation reverses the aberrant ASMC phenotypes in subjects with asthma. This study provides important information on the epigenetic regulation of *PDE4D*/cAMP signaling in asthmatic ASMCs, and may lead to a novel modality of treatment for this devastating disease.

Material and Methods

Detailed methods are described in the online supplement.

Cell Culture

Human ASMCs from subjects with and without asthma were either purchased (LONZA Inc., Walkersville, MD) or isolated from deceased donors using methods described previously (32).

Real-Time Quantitative PCR

Total RNA was extracted and reverse transcribed. mRNA levels were quantified by quantitative PCR (qPCR). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of transcripts normalized to *RPL19*.

Bisulfite Genomic Sequencing

Bisulfite conversion of genomic DNA extracted from ASMCs was performed before PCR. PCR amplicon was subcloned into pCR2.1 vector. Three to four individual clones from each donor were sequenced.

Methylation-Specific PCR

CpG site–specific methylation of *PDE4D* was assayed by qPCR. Control DNAs (fully methylated and fully unmethylated) were mixed in various concentrations and served as quantification standards when determining the percentage of DNA methylation of samples from qPCR.

Transfection with Methylated Oligonucleotides

ASMCs were transfected with nontargeting control or DNA oligonucleotides specific for methylated *PDE4D* for 3 days. The phosphorothioate oligonucleotides were designed to replace the cytosines in CpG dinucleotides with methylated cytosine (33–35).

Measurement of the Intracellular cAMP Level

Transfected cells were incubated with new medium with 5% FBS for 24 hours before measurement. cAMP levels in cell supernatants were measured with a cAMP high-throughput screening Immunoassay (EMD Millipore, Billerica, MA).

alamarBlue Cell Viability Assay

Cell viability at 3 days after transfection was measured by alamarBlue assay (Invitrogen, Carlsbad, CA).

Cell Migration Assay

Cell migration was measured by QCM Chemotaxis assay (EMD Millipore, Billerica, MA).

Cytosolic Calcium Measurement

Transfected ASMCs were seeded onto glass coverslips overnight in growth medium and were then serum deprived for 48 hours before calcium measurements. Intracellular calcium concentration ($[Ca^{2+}]_i$) was

monitored as described previously (36).

Western Blot Analysis

Transfected ASMCs were lysed, resolved by SDS-PAGE and electrotransferred onto a polyvinyl difluoride (PVDF) membrane. Standard Western blot procedures were followed.

Results

PDE4D mRNA was abundantly expressed in asthmatic ASMCs with a level fourfold higher than that in nonasthmatic ASMCs (Figure 1A). *In silico* analysis revealed that CpG islands (GC% >60%) at 5′ *PDE4D* encompassed the transcription and translation start sites (Figure 1B). The methylation status of a total of 99 CpG sites at the 5′ promoter region of *PDE4D* was examined by bisulfite sequencing, and the methylation status of individual CpG site (CpG sites 26–99) at the 5′ *PDE4D* (−98 to +608, including the 5′ untranslated exon and first exon) is shown in Figure 1C. There was no significant difference in methylation status of CpG sites 1–25 between nonasthmatic and asthmatic ASMCs (data not shown). By contrast, *PDE4D* promoter was demethylated significantly in asthmatic ASMCs comparing the average percent methylation of CpG sites 26–99 with nonasthmatic ASMC ($P = 0.02$). Furthermore, a cluster of CpG sites (boxed region, CpG sites 87–90; Figure 1C) showing differential methylation ($P < 0.01$) was identified in asthmatic ASMCs. These results indicate that *PDE4D* expression was increased in asthmatic ASMCs, and that the increased mRNA transcription is associated with demethylation in

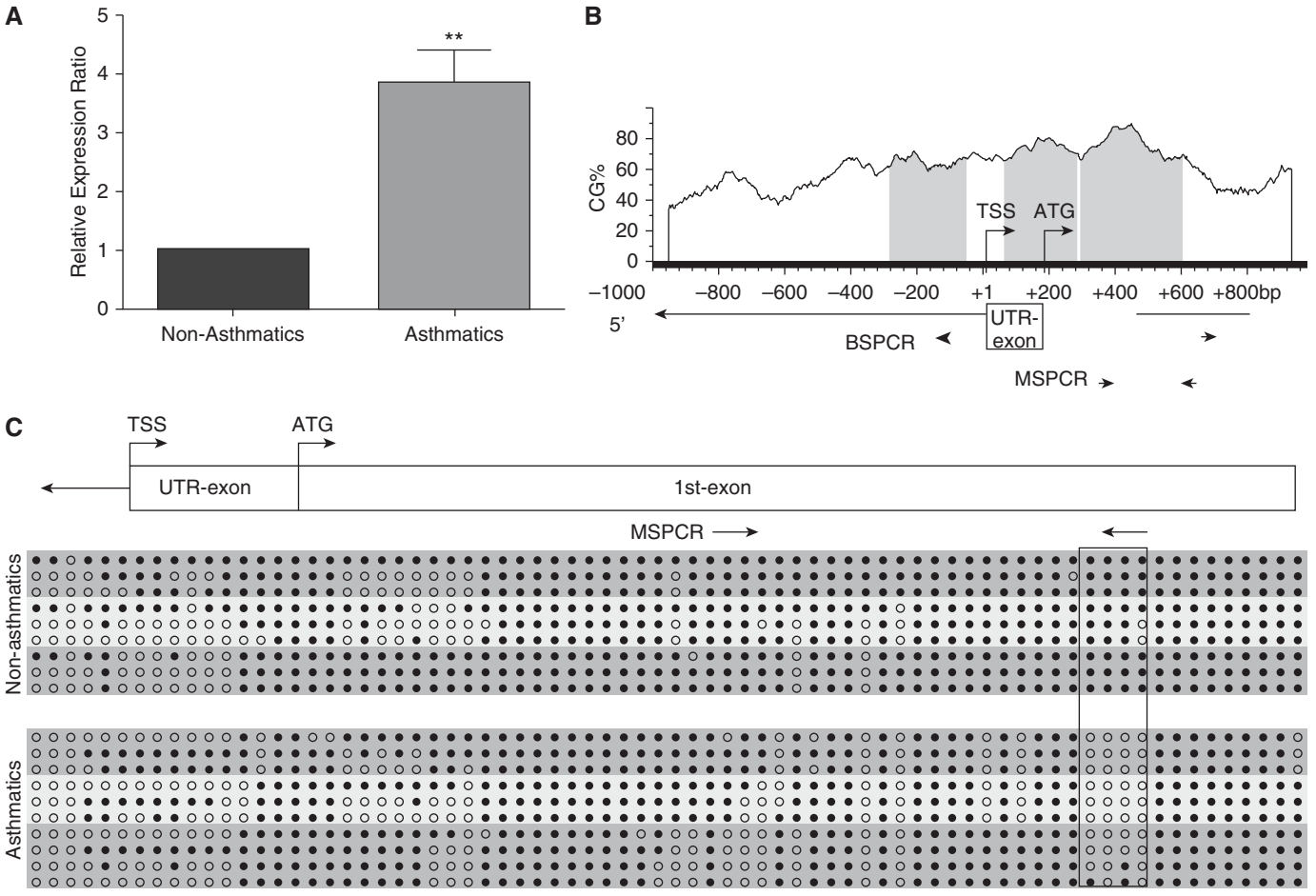

Figure 1. Difference in gene expression and promoter methylation level of phosphodiesterase 4D (*PDE4D*) between nonasthmatic and asthmatic airway smooth muscle cells (ASMCs). (*A*) mRNA expression level of *PDE4D* in asthmatic ASMCs (five donors) relative to nonasthmatic ASMCs (five donors) assayed by quantitative polymerase chain reaction (qPCR). Values are mean ± SD. $^{**}P < 0.01$. (*B*) Schematic diagram of CpG dinucleotide (CG) content (%) in the 5′ promoter region of *PDE4D*. *In silico* analysis identified the CpG islands (*shaded in gray* in the genomic DNA sequence) based on the CG content greater than 60% with an observed:expected ratio of 0.6 (MethPrimer). ATG, translational start site; TSS, transcription start site; UTR, untranslated region. PCR amplicon generated by bisulfite sequencing PCR (BSPCR) and methylation-specific qPCR (MSPCR) indicated by the regions bounded by *arrows*. (*C*) Methylation status of individual CpG site at the *PDE4D* promoter in nonasthmatic (three donors) and asthmatic ASMCs (three donors) was assayed by bisulfite sequencing. Unmethylated (*open circles*) or methylated (*solid circles*) CpGs are indicated. Each *row* of *circles* represents an individual clone sequenced. Three to four individual clones from each donor were picked for sequencing. *Boxed area* illustrates the specific CpG sites showing differential methylation in asthmatic ASMCs ($P < 0.01$).

a specific CpG cluster of *PDE4D* promoter.

Methylated DNA oligonucleotides were designed to introduce DNA methylation at the specific CpG sites on *PDE4D* promoter (boxed region). Synthetic methylated oligonucleotides can modify targeted cytosine residue to 5-methyl-cytosine and bind to one strand of the gene to generate a hemimethylated DNA. This hemimethylated DNA allows binding of DNA methyltransferase 1, which catalyzes methylation at the complementary strand, resulting in fully methylated target CpG sites in both DNA strands (33). Asthmatic ASMCs transfected with methylated *PDE4D* oligonucleotides (*M-PDE4D*) showed an eightfold reduction in mRNA level of *PDE4D* compared with those treated with nontargeting methylated DNA oligonucleotides (*M-CTL*) and lipofectamine (*LF*) control, whereas *PDE4D* expression in nonasthmatic ASMCs was unaffected (Figure 2A). In concord with the increased *PDE4D* expression, the percentage of promoter methylation of *PDE4D* was significantly lower in the asthmatic ASMCs (Figure 2B). Treatment of asthmatic ASMCs with *M-PDE4D* completely reversed the reduction of CpG site–specific methylation, as assayed by methylation-specific PCR (Figure 2B). *M-PDE4D* increased the percentage methylation of PDE4D by approximately 30% (to fully methylated) and a corresponding eightfold reduction in *PDE4D* mRNA level in asthmatic ASMCs. This suggests that *PDE4D* was silenced successfully in asthmatic ASMCs by *M-PDE4D*. The absence of additional silencing effect of the methylated oligonucleotides on *PDE4D* expression in nonasthmatic ASMCs suggests that their respective CpG sites were already fully methylated.

The effect of epigenetic modulation of *PDE4D* on cell phenotypes was gauged by determining growth and migration of ASMCs transfected with *M-PDE4D*, *M-CTL*, or *LF*. The number of viable asthmatic ASMCs in the *M-CTL* or *LF* control was noticeably higher than that of the corresponding nonasthmatic ASMCs, suggesting enhanced proliferation in asthmatic ASMCs. Transfection of *M-PDE4D* normalized the increase in the viable asthmatic ASMCs, but had no effect on the nonasthmatic ASMCs (Figure 3A).

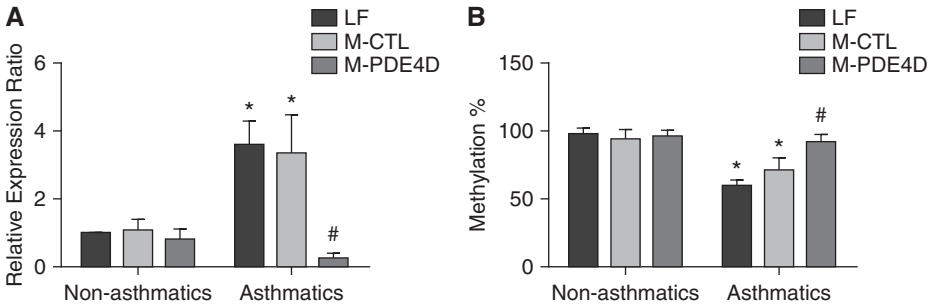

Figure 2. Effect of methylated oligonucleotides on gene expression and promoter methylation of *PDE4D* in nonasthmatic and asthmatic ASMCs. (*A*) mRNA expression level of *PDE4D* and (*B*) CpG site–specific methylation (%) of *PDE4D* (+366 to +592) assayed by MSPCR in nonasthmatic (five donors) and asthmatic (five donors) ASMCs transfected with *PDE4D*-targeting (*M-PDE4D*) or nontargeting methylated oligonucleotide control (*M-CTL*). β-actin was used as the reference gene to normalize the amount of bisulfite-treated DNA template. Methylated DNA standards (0, 25, 50, 75, and 100% methylated DNA) were used when determining percentage of DNA methylation of samples from qPCR. $^{*}P < 0.05$ in comparison to lipofectamine (LF) control of nonasthmatic ASMCs; $^{\#}P < 0.05$ in comparison to *M-CTL* of asthmatic ASMCs.

Chemotaxis assay showed that asthmatic ASMCs had a higher migration activity, which was inhibited by *M-PDE4D* (Figure 3B), but *M-PDE4D* had no effect on the migration of nonasthmatic ASMCs. These data demonstrate that epigenetic modulation of *PDE4D* regulates the cell proliferation and migration of asthmatic ASMCs.

Increase in intracellular cAMP concentration ($[cAMP]_i$) alters ASMC proliferation, migration, and contraction via modulation of the $[Ca^{2+}]_i$ (37). To investigate the mechanism underlying the epigenetic effect of *PDE4D* on the ASMC phenotypes, the change in $[cAMP]_i$ and $[Ca^{2+}]_i$ in *M-PDE4D*–transfected ASMCs was measured. There was no significant difference in basal $[cAMP]_i$ between nonasthmatic and asthmatic ASMCs, but $[cAMP]_i$ was elevated significantly in asthmatic ASMCs after *M-PDE4D* transfection (Figure 4A). The Ca^{2+} response elicited by 3 μM histamine was significantly higher in asthmatic ASMCs, compared with the nonasthmatic ASMCs (Figure 4B). *M-PDE4D* transfection decreased the histamine-induced Ca^{2+} response by 40% in asthmatic ASMCs to the same level of the nonasthmatic ASMCs. This inhibitory effect of *M-PDE4D* was not observed in nonasthmatic ASMCs. Our findings

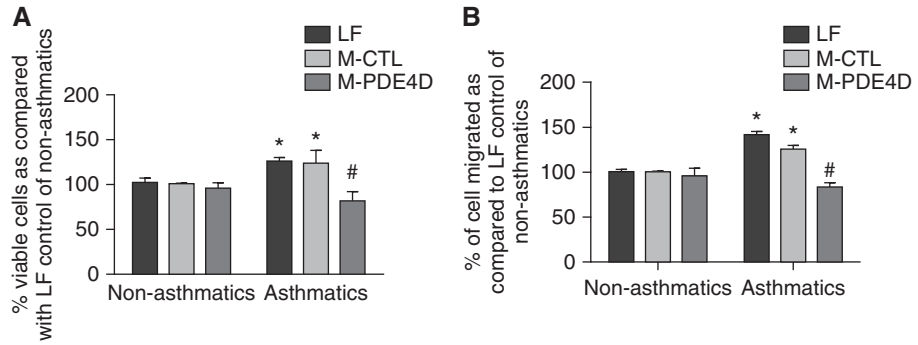

Figure 3. Methylated *PDE4D* inhibited cell proliferation and migration of asthmatic ASMCs. (*A*) Cell viability assayed by alamarBlue and (*B*) cell migration examined by chemotaxis assay in nonasthmatic (three donors) and asthmatic (three donors) ASMCs treated with LF with or without *PDE4D*-targeting (*M-PDE4D*) or nontargeting methylated oligonucleotide control (*M-CTL*). $^{*}P < 0.05$ in comparison to LF control of nonasthmatic ASMCs; $^{\#}P < 0.05$ in comparison to *M-CTL* of asthmatic ASMCs.

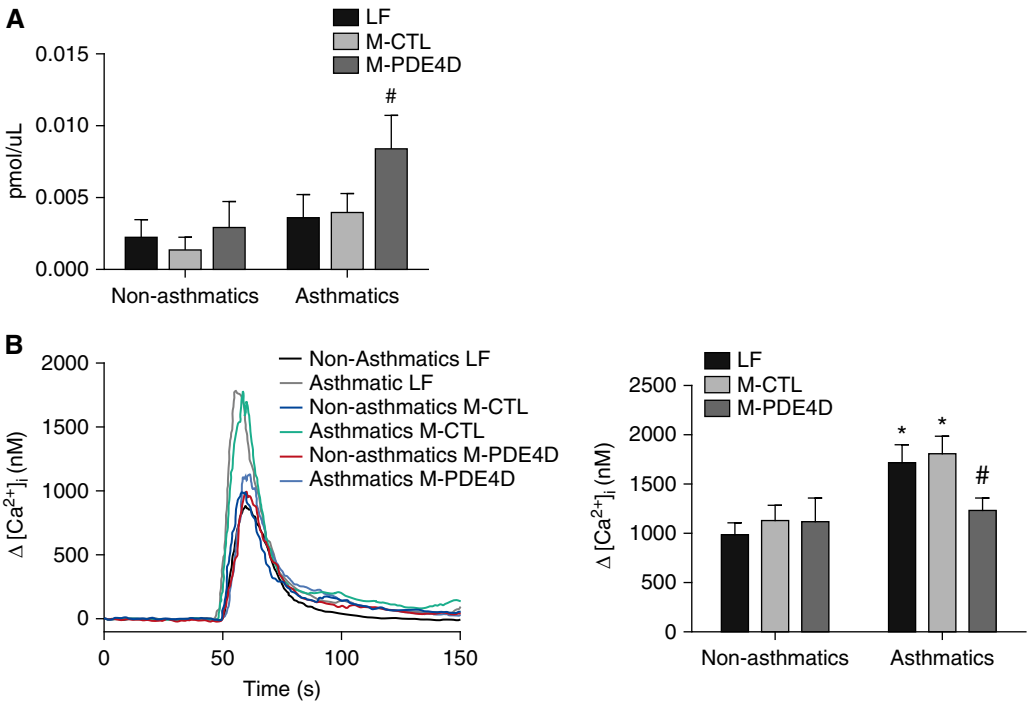

Figure 4. Methylated *PDE4D* altered intracellular cAMP and Ca^{2+} level in asthmatic ASMCs. (*A*) cAMP level (pmol/μl) was measured by cAMP high-throughput screening immunoassay in nonasthmatic (five donors) and asthmatic (five donors) ASMCs transfected with *PDE4D*-targeting (*M-PDE4D*) or nontargeting methylated oligonucleotide (*M-CTL*), or transfection control (LF). (*B*) Averaged Ca^{2+} transient traces showing histamine-induced Ca^{2+} response in LF, *M-CTL*, or *M-PDE4D*–transfected nonasthmatic (five donors) and asthmatic (five donors) ASMCs. Peak Ca^{2+} responses were quantified as means ± SEM (*right panel*; a total of 8–18 measurements per treatment group). $^{*}P < 0.05$ in comparison to LF control of nonasthmatic ASMCs; $^{\#}P < 0.05$ in comparison to *M-CTL* of asthmatic ASMCs. cAMP, cyclic adenosine monophosphate.

indicate that the enhanced Ca^{2+} response to histamine in asthmatic ASMCs could be related to the epigenetic alteration of *PDE4D*.

To further investigate if alterations in Ca^{2+} mobilization via gene silencing of *PDE4D* leads to aberrant asthmatic ASMC phenotypes, we examined the effect of *M-PDE4D* on the phosphorylation of myosin light chain kinase (MLCK), p38, and extracellular signal–regulated kinase (ERK) 1/2, which are the downstream effectors of Ca^{2+}-induced signaling (Figure 5). Asthmatic ASMCs showed increased levels of phosphorylated MLCK, p38, and ERK2 proteins as compared with that of nonasthmatic ASMCs. Furthermore, *M-PDE4D* caused a 120 and 50% decrease in phosphorylation of MLCK and p38, respectively, in asthmatic ASMCs, but had no effect on nonasthmatic ASMCs (Figures 5A and 5B). The expression levels of phosphorylated ERK1/2 in both nonasthmatic and asthmatic cells treated with *M-PDE4D* were unchanged.

Taken together, we provide evidence that epigenetic alteration of *PDE4D* can modulate the Ca^{2+} homeostasis and activation of MLCK and p38 signaling, which may contribute to the aberrant ASMC phenotypes observed in subjects with asthma.

Discussion

The present study demonstrates that *PDE4D* promoter demethylation contributes to the increased *PDE4D* gene expression in asthmatic ASMCs. This is in agreement with our previous study showing that HDM-induced AHR was associated with the epigenetic alterations of *Pde4d* (25). Furthermore, we showed that the epigenetic alteration of *PDE4D* was associated with the abnormal increase in cell proliferation, migration, and histamine-induced Ca^{2+} response in asthmatic ASMCs. By introducing *PDE4D* promoter methylation by methylated oligonucleotides, the aberrant phenotypes in asthmatic ASMCs could be reversed. Our results, hence, suggest that the CpG site–specific demethylation of *PDE4D* may account, at least in part, for the altered phenotypes of asthmatic ASMCs that are responsible for the airway remodeling and AHR in patients with asthma. Strikingly, a moderate change in CpG site–specific methylation at the *PDE4D* promoter by methylated oligonucleotides caused a dramatic decrease in *PDE4D* expression. By *in silico* search of the TRANSFAC database (38), we identified transcriptional factors (TFs), Sp1, Sp3, E2f-1, and p300, may potentially bind to the specific CpG sites (*boxed area*, Figure 1C) at the *PDE4D* promoter. *De novo* methylation has been shown to be associated with the recognition site of Sp1 and E2f-1 (39, 40). Sp3 is shown to interact with histone deacetylases (41), whereas p300 histone acetyltransferase modulates gene transcription via chromatin remodeling (42). Given the fact that DNA methylation occurring at recognition

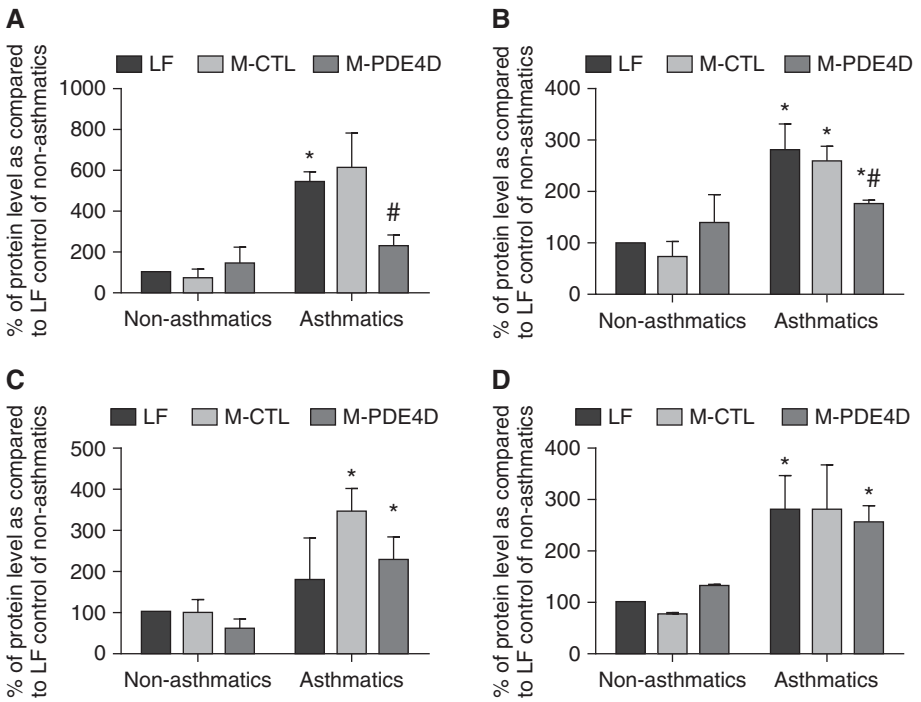

Figure 5. Methylated *PDE4D* altered phosphorylation of myosin light chain kinase (MLCK) and p38, but not extracellular signal–regulated kinase (ERK) 1/2 in asthmatic ASMCs. Expression of the phosphorylated (*A*) MLCK, (*B*) p38, (*C*) ERK1, and (*D*) ERK2 proteins in nonasthmatic (three donors) and asthmatic (three donors) ASMCs transfected with *PDE4D*-targeting (*M-PDE4D*) or nontargeting methylated oligonucleotide (*M-CTL*), or transfection control (LF) was quantitated as percent of band intensity as compared with LF control of nonasthmatic ASMCs. $^{*}P < 0.05$ in comparison to LF control of nonasthmatic ASMCs; $^{\#}P < 0.05$ in comparison to *M-CTL* of asthmatic ASMCs.

sites of TFs may suppress the gene transcription by either inhibiting the binding of TFs to the promoter and/or hindering the chromatin stability by further recruiting DNA methyltransferases and histone modification enzymes to the promoter, it is possible that moderate or slight changes in methylation pattern at specific CpGs may contribute to a larger change in gene expression level. Although future studies are needed to examine the regulation of the *PDE4D* transcriptional activity, our data provide an insight in to the development of efficient and specific inhibitors for PDE4D.

PDE4D belongs to the *PDE4* family, and is the major *PDE* subtype in human ASMCs. It is a cAMP-specific phosphodiesterase for the degradation of cAMP for lowering $[cAMP]_i$. cAMP plays important roles in various physiological functions of ASMCs. β2-Agonist inhibits ASMC proliferation and contraction through cAMP production mediated by G protein–coupled receptors and adenylyl cyclase (43, 44). Trian and colleagues (28) reported that β2 adrenergic receptor-mediated cAMP generation is dysregulated in asthmatic ASMCs by the increased cAMP degradation by PDE4, may be partly due to the increased PDE4D expression. They further propose the intrinsic abnormality seen in asthmatic ASMCs in the absence of proinflammatory milieu may be due to the genetic and/or epigenetic predisposition. Herein, we provide the evidence that *PDE4D* is overexpressed via promoter demethylation in asthmatic ASMCs. Increased *PDE4D* expression enhances hydrolysis of cAMP that inhibits activity of protein kinase A. Reduction in protein kinase A activity inhibits the sarcoplasmic reticulum (SR) Ca^{2+} release from inositol trisphoshate receptor and extracellular Ca^{2+} entry, and lessens the amelioration of SR Ca^{2+} uptake via sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) (45). Eventually, the intracellular Ca^{2+} level is increased, which may up-regulate MLCK and p38, leading to enhanced ASMC contraction, proliferation, and migration (46–49). Hence, *PDE4D* up-regulation can suppress the cAMP-dependent signaling pathways, leading to aberrant ASMC phenotypes. Our data support the hypothesis that the aberrant cAMP production is "programmed" via epigenetic regulation of PDE4D, and it may predispose the ASMCs to be hyperresponsive. On the other hand, gene silencing of *PDE4D* via DNA methylation contributes to the decreased Ca^{2+}-dependent activities through cAMP signaling to inhibit cell growth and migration, suppress ASM contraction, and shift ASM tone to relaxation. We summarized the possible mechanisms underlying epigenetic regulation of ASMCs via PDE4D methylation in Figure 6.

The important role of *PDE4D* in asthma has been implicated in a genome-wide association analysis study that identified *PDE4D* as an asthma-susceptibility gene. Multiple *PDE4D* single-nucleotide polymorphisms (SNPs) were strongly associated with patients with asthma of different ethnicities. However, to date, there has been no direct study on the effect of *PDE4D* SNPs on PDE4D function. Our present study indicates that, in addition to the SNPs, *PDE4D* is epigenetically modulated in asthmatic ASMCs. However, how the persistent epigenetic alteration of *PDE4D* is established in asthmatic ASMCs remains unknown. We previously demonstrated the changes in mRNA level of the DNA methylation modulators, including *Dnmt3A*, methyl-CpG–binding domain proteins (*Mbd2* and *Mbd3*), and ten-eleven translocation proteins (*Tet1*) in mouse chronically exposed to house allergen (26). It will be informative to further examine how *PDE4D* is epigenetically regulated by these DNA methylation modulators, and how these modifications persist in the presence or absence of stimuli as in our cultured asthmatic ASMCs. AHR is often considered an epiphenomenon of airway inflammation. However, recent studies revealed that ASMCs showed asthmatic phenotypes, even in the absence of the inflammation (4, 5, 6). Our findings show that asthmatic ASMCs are epigenetically modulated, and such epigenetic alterations

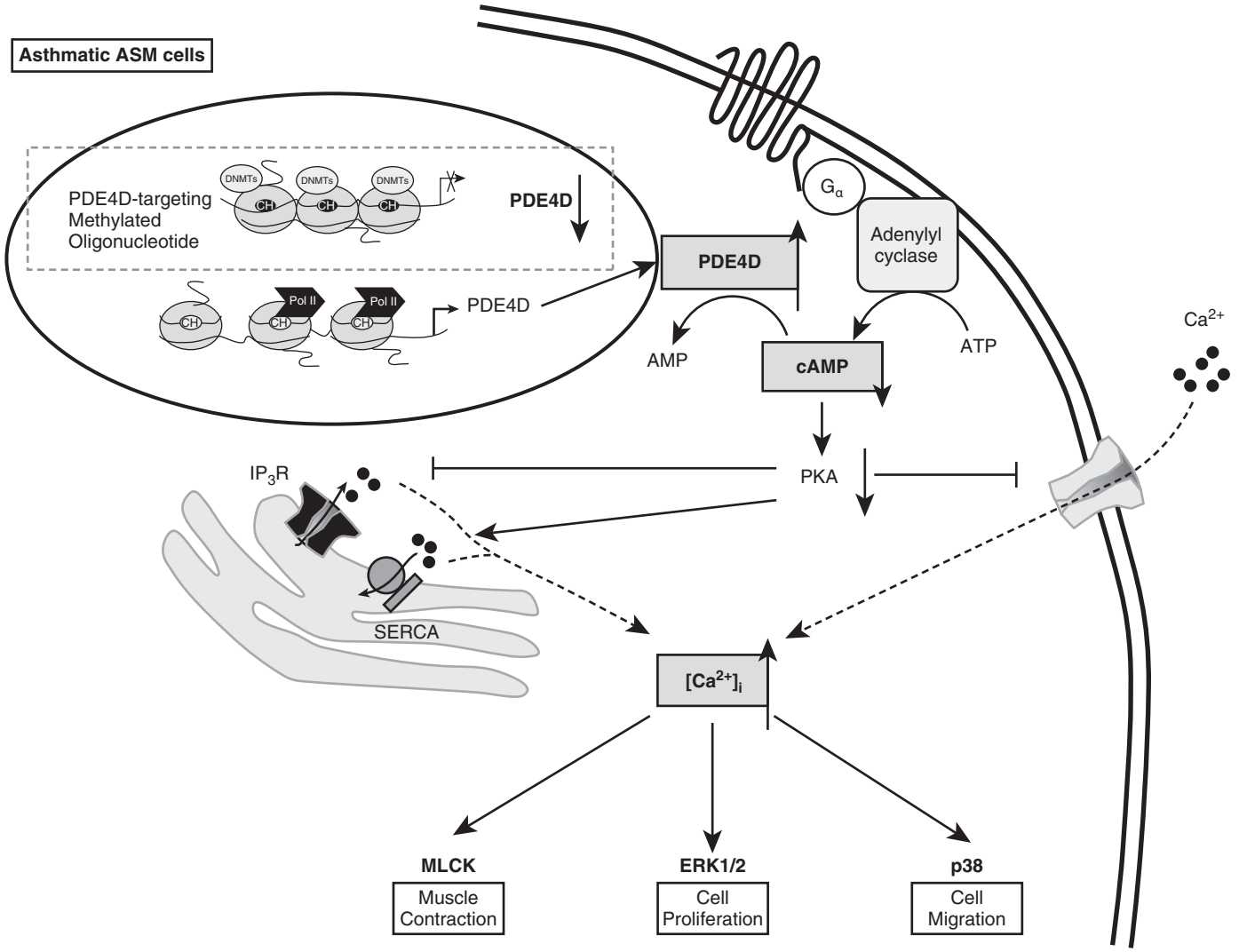

Figure 6. Proposed mechanisms of how epigenetic regulation of *PDE4D* via promoter methylation alters ASMC phenotypes. In asthmatic ASMCs, *PDE4D* promoter is aberrantly unmethylated, and hence promotes *PDE4D* gene expression. Overexpression of PDE4D enhances the hydrolysis of cAMP and lowers the intracellular level of cAMP that could lead to decreased activity of protein kinase A (PKA). Reduction in PKA activity inhibits the sarcoplasmic reticulum (SR) Ca^{2+} release from inositol trisphoshate receptor (IP_3R) and extracellular Ca^{2+} entry, and lessens the amelioration of SR Ca^{2+} uptake via sacro/endoplasmic reticulum Ca^{2+} ATPase (SERCA). Eventually, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is increased, which may up-regulate MLCK, p38, and/or ERK1/2, leading to enhanced cell contraction, proliferation, and migration. Aberrant ASMC phenotypes may predispose the ASMCs to be hyperresponsive. Introduction of CpG site–specific methylation at *PDE4D* promoter by methylated oligonucleotide results in suppression of PDE4D expression to the level of the nonasthmatic ASMCs and ultimately reverses the aberrant cell phenotypes seen in asthmatic ASMCs. CH, methyl group at 5' cytosine; DNMT, DNA methyltransferase; G_α, G-protein alpha; Pol II, RNA polymerase II.

could be the consequence of prior allergen exposures, inflammation, or some as-yet unidentified mechanisms that predispose the ASMC to being highly proliferative and contractile.

Pharmacological PDE inhibitors have been recently developed for the treatment of asthma because of their bronchodilator and antiinflammatory effects (50). Although the causal relationship between epigenetic regulation of *PDE4D* and airway inflammation has not yet been studied, we here demonstrate the successful modification of the CpG site–specific methylation of *PDE4D* in asthmatic ASMCs using methylated DNA oligonucleotides, which may provide an alternative method of epigenetic therapy for asthma. It is exciting to speculate as to the possibility of reversing the predisposed asthmatic phenotype in ASMCs to normal status with gene-specific methylated DNA oligonucleotides. ■

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

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