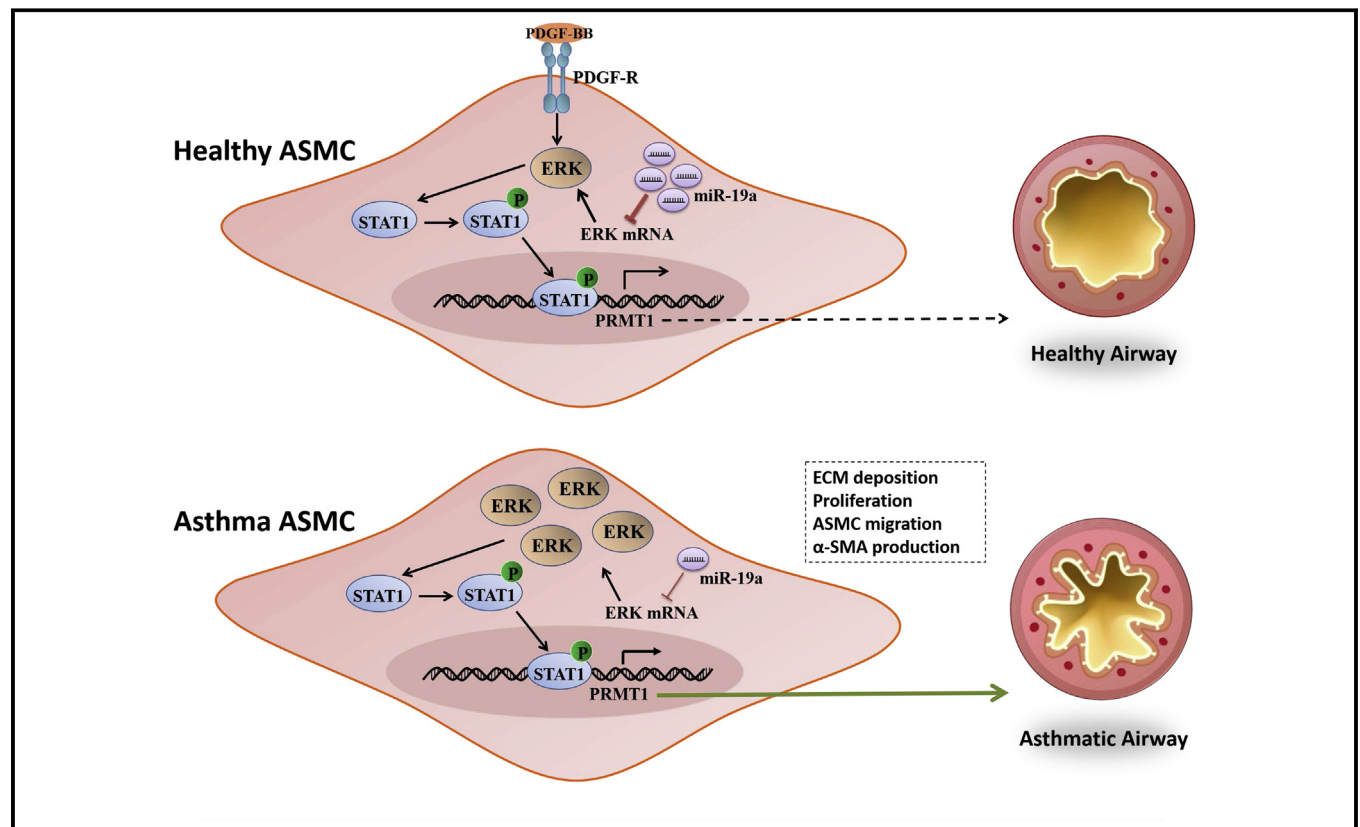


Constitutive high expression of protein arginine methyltransferase 1 in asthmatic airway smooth muscle cells is caused by reduced microRNA-19a expression and leads to enhanced remodeling

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GRAPHICAL ABSTRACT



Background: In asthma remodeling airway smooth muscle cells (ASMCs) contribute to airway wall thickness through increased proliferation, migration, and extracellular matrix deposition. Previously, we described that protein arginine

methyltransferase 1 (PRMT1) participates in airway remodeling in pulmonary inflammation in E3 rats. **Objective:** We sought to define the asthma-specific regulatory mechanism of PRMT1 in human ASMCs.

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Methods: ASMCs from healthy subjects and asthmatic patients were activated with platelet-derived growth factor (PDGF)–BB. PRMT1 was localized by means of immunohistochemistry in human lung tissue sections and by means of immunofluorescence in isolated ASMCs. PRMT1 activity was suppressed by the pan-PRMT inhibitor AMI-1, signal transducer and activator of transcription 1 (STAT1) was suppressed by small interfering RNA, and extracellular signal-regulated kinase (ERK) 1/2 mitogen-activated protein kinase (MAPK) was suppressed by PD98059. MicroRNAs (miRs) were assessed by using real-time quantitative PCR and regulated by miR mimics or inhibitors.

Results: PRMT1 expression was significantly increased in lung tissue sections and in isolated ASMCs of patients with severe asthma. PDGF-BB significantly increased PRMT1 expression through ERK1/2 MAPK and STAT1 signaling in control ASMCs, whereas in ASMCs from asthmatic patients, these proteins were constitutively expressed. ASMCs from asthmatic patients had reduced miR-19a expression, causing upregulation of ERK1/2 MAPK, STAT1, and PRMT1. Inhibition of PRMT1 abrogated collagen type I and fibronectin deposition, cell proliferation, and migration of ASMCs from asthmatic patients. **Conclusions:** PRMT1 is a central regulator of tissue remodeling in ASMCs from asthmatic patients through the pathway: PDGF-BB–miR-19a–ERK1/2 MAPK and STAT1. Low miR-19a expression in ASMCs from asthmatic patients is the key event that results in constitutive increased PRMT1 expression and remodeling. Therefore PRMT1 is an attractive target to limit airway wall remodeling in asthmatic patients. (J Allergy Clin Immunol 2016;■■■:■■■–■■■.)

Key words: Airway wall remodeling, primary airway smooth muscle cell, protein arginine methyltransferase 1, miR-19a, extracellular signal-regulated kinase, STAT1

Asthma is a respiratory disease characterized by chronic inflammation and remodeling of airway tissues. Airway wall remodeling in asthmatic patients describes the structural changes of the tissue composition, including smooth muscle hypertrophy, hyperplasia, basement membrane thickening, and subepithelial fibrosis.¹ It also includes goblet cell hyperplasia and derangement of the airway epithelium. It is currently discussed whether airway wall remodeling and inflammation are 2 independent events or whether they are causally linked in the pathogenesis of asthma.^{2,3} In the pathologically changed airway wall structure, airway smooth muscle cells (ASMCs) play a central role through their increased capacity to proliferate, migrate, and deposit proinflammatory extracellular matrix (ECM) components, such as collagen type I or fibronectin.^{4,5} These ASMC-specific cellular pathologies are maintained in isolated cells and are considered major contributing factors to the pathogenesis of asthma.¹

Increased ASMC mass in the airway wall in asthmatic patients also increases the contractility of the airways, which further reduces the lumen during asthma attacks.^{2,3} The mechanisms that upregulate ASMC mass and change the ASMC phenotype toward increased secretion of proinflammatory cytokines and mediators are incompletely understood. Several studies indicated that platelet-derived growth factor (PDGF)–BB, which is secreted by activated platelets, as well as by endothelial, epithelial, glial, or inflammatory cells, is a major stimulus for ASMC proliferation and airway tissue remodeling in patients with chronic lung

Abbreviations used

ASMC:	Airway smooth muscle cell
COPD:	Chronic obstructive pulmonary disease
ECM:	Extracellular matrix
ERK:	Extracellular signal-regulated kinase
MAPK:	Mitogen-activated protein kinase
miR:	MicroRNA
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PDGF:	Platelet-derived growth factor
PRMT1:	Protein arginine methyltransferase 1
RT-qPCR:	Real-time quantitative PCR
siRNA:	Small interfering RNA
SMA:	Smooth muscle actin
STAT:	Signal transducer and activator of transcription
UTR:	Untranslated region

inflammation.^{6,7} Increased levels of PDGF-BB have been reported in the lungs of asthmatic patients and might contribute to the increased ASMC hyperplasia. Both *in vivo* and *in vitro* studies elucidated that PDGF-BB also drives the shift of human ASMCs from the contractile phenotype toward a proliferative, migratory, and synthetic phenotype (preremodeling phenotype).^{8,9} The molecular mechanisms that regulate these changes of ASMC biology are not completely understood.

The causative role of posttranscriptional events in ASMCs and their contribution to asthma pathologies is an emerging field of investigation. Different epigenetic mechanisms have been reported to modulate ASMC contractility, proliferation, and chemokine secretion, and they are critical to identify novel potential therapeutic targets for asthma, especially to limit or reverse airway wall remodeling.¹⁰ Arginine methylation of histones and other proteins catalyzed by protein arginine methyltransferase 1 (PRMT1) was suggested to play an important role in lung cancer, pulmonary fibrosis, pulmonary hypertension, chronic obstructive pulmonary disease (COPD), and asthma.¹¹ In regard to lung inflammation, our previous studies revealed that PRMT1 is upregulated in both the acute and chronic stages of inflammation in an E3 rat asthma model.^{12,13} Furthermore, in primary human fibroblasts we showed that PDGF-BB stimulated PRMT1 expression through the extracellular signal-regulated kinase (ERK) 1/2 mitogen-activated protein kinase (MAPK) signaling pathway,¹⁴ highlighting its potential role in asthma-associated airway wall remodeling. However, the regulatory mechanisms underlying the expression and activation of PRMT1 remained unclear.

Functional studies identified the critical role or roles of microRNAs (miRs) in the regulation of airway inflammation, remodeling, and hyperresponsiveness, and thus miRs have to be considered novel regulators of PRMT1 expression and airway wall remodeling.^{15,16} Specifically, the members of the miR cluster 17-92 have been suggested to participate in the pathogenesis of lung diseases by regulating protein expression at the posttranscriptional level.¹⁷ In tumor cells the expression of the miR cluster 17-92 was linked to upregulated ERK1/2 MAPK activity.¹⁸ Although miRs are important regulators of MAPK1 expression, there are no functional data in asthmatic patients. Therefore it is imperative to hypothesize that changes in miR expression modulate MAPK1 expression and function and thereby contribute to the pathogenesis of asthma.

Based on our previous studies, we aimed to identify the asthma-specific regulatory mechanism or mechanisms controlling PRMT1 expression and airway wall remodeling in ASMCs from asthmatic patients and prove the involvement of miRs in this process.

METHODS

Primary human lung ASMCs

Lung tissue specimens were obtained from the Clinic of Pneumology (University Hospital Basel, Basel, Switzerland) with approval of the local ethics committee (EK: 05/06) and with written consent of each tissue donor (control subjects, $n = 8$; asthmatic patients, $n = 9$; patients with COPD, $n = 9$) who underwent biopsy or partial therapeutic lung tissue reduction for other reasons.

Details for age, sex, diagnosis, and therapy are provided in Table I. The control group consisted of patients who underwent lung biopsy for diagnosis of diseases other than asthma and COPD, mainly for lung metastases of tumors in other organs.

Primary human lung ASMCs were isolated from tissues of lung biopsy specimens, as previously described, and propagated under standard cell-culture conditions (37°C, 100% humidity, and 5% CO₂).¹⁹ ASMC growth medium consisted of Dulbecco modified Eagle medium supplemented with 5% FBS, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES, and 8 mmol/L L-glutamine (all from GIBCO Laboratories, Grand Island, NY). All experiments were performed in ASMCs between passages 2 and 8.

ASMC isolation and stimulation

ASMCs were seeded into 6-well plates, allowed to adhere, serum deprived overnight, and stimulated with PDGF-BB (10 ng/mL) for 1, 3, 6, 12, 24, and 48 hours. ASMCs were treated with different signal protein inhibitors to investigate PDGF-BB-induced PRMT1 expression: ERK1/2 MAPK was inhibited by PD98059 (10 μ mol/L), which was added 2 hours before PDGF-BB stimulation. For signal transducer and activator of transcription (STAT) 1 experiments, ASMCs were pretreated with small interfering RNAs (siRNAs), according to the manufacturer's protocol (Santa Cruz Biotechnology, Santa Cruz, Calif) for 48 hours. The siRNA and the corresponding control sequence were used at a final concentration of 50 nmol/L and transfected into ASMCs with Lipofectamine 2000 (Invitrogen, Carlsbad, Calif) for 48 hours. PRMT1 activity was inhibited after 2 hours of preincubating ASMCs with AMI-1 (10 μ mol/L).

Lung immunohistochemistry and immunofluorescence

Tissue sections from healthy control lungs ($n = 4$) and lungs of asthmatic patients ($n = 5$) were obtained from the Department of Respiratory Diseases, University of Naples, Naples, Italy, after the consent of the local ethical committee. Healthy control lungs were defined as lungs obtained from patients without known lung diseases.

After deparaffinization and rehydration, lung tissue sections were incubated with 1:100 diluted anti-PRMT1 antibody (Abcam, Cambridge, United Kingdom) in blocking solution (2% BSA in PBS) at 4°C overnight, followed by visualization with the 2-step plus Poly-HRP anti-goat IgG detection kit (Abcam). The intensity of the brown color was determined by using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, Md) to estimate protein expression in lung tissues.

For immunofluorescence analysis, ASMCs were seeded on 10-mm sterile glass slides in 24-well plates. After 24 hours of serum deprivation, ASMCs were stimulated with PDGF-BB with or without pretreatment with the ERK1/2 MAPK inhibitor. ASMCs were fixed in 4% paraformaldehyde in PBS for 2 $\times$ 5 minutes. Immunofluorescence staining was performed, as described previously.¹⁴ Briefly, anti-PRMT1 antibody, anti- α -smooth muscle actin (SMA) antibody, anti-collagen type I antibody, or anti-fibronectin

antibody (all from Abcam) was diluted 100-fold in 2% BSA-PBS and incubated at 4°C overnight, followed by 3 washes and subsequent staining with a phycoerythrin-labeled goat anti-rabbit IgG secondary antibody for PRMT1, fibronectin, and COL1A1 or fluorescein isothiocyanate-labeled goat anti-mouse IgG secondary antibody (Santa Cruz Biotechnology) for α -SMA. Nuclei were stained with 4'-6-diamidino-2-phenylindole dihydrochloride. Images were captured with an Olympus BX61 fluorescence Microscope (Olympus Optical, Tokyo, Japan).

Western blotting

Cells were lysed in RIPA buffer, lysates were centrifuged at 12,000 rpm (15 minutes), and the protein concentration of the supernatant was quantified by using BCA (Thermo Scientific, Waltham, Mass). Equal amounts of denatured proteins (20 μ g) were separated in 8-16% SDS-PAGE (Thermo Scientific) and subsequently electrotransferred onto polyvinylidene difluoride membranes. The proteins of interest were detected with antibodies specific to PRMT1 (Abcam), phosphorylated (phospho)-ERK, total ERK, α -tubulin (all from Cell Signaling Technology, Danvers, Mass), phospho-STAT1, collagen type I (COL1A1), or fibronectin (all from Santa Cruz Biotechnology). Protein bands were visualized after binding of species-specific secondary horseradish peroxidase-conjugated antibodies (Abcam) by using chemiluminescent substrate (Thermo Scientific).

miR expression and function

Growing ASMCs were transfected with either mimics or inhibitors of miR-19a or miR-101 by using Transfection Reagent from Qiagen (Hombrechtikon, Switzerland). Detailed information of mimics and inhibitors is as follows: has-miR-19a-3p mimic (catalog no. MSY0000073); anti-has-miR-19a-3p miR inhibitor (catalog no. MIN0000073); has-miR-101-3p mimic (catalog no. MSY0000099); and anti-has-miR-101-3p miR inhibitor (catalog no. MIN0000099). ASMCs were seeded in 6-well plates, and for each well, 0.6 μ L of miR mimic (20 μ mol/L stock) or 6 μ L of miR inhibitor (20 μ mol/L stock) was diluted in 400 μ L of culture medium without serum (final concentration: miR mimic, 5 nmol/L; miR inhibitor, 50 nmol/L). HiPerFect Transfection Reagent (10 μ L) was added to the diluted miR mimic/inhibitor and mixed by means of vortexing. After incubating the samples for 5 minutes at room temperature, the complex was added dropwise onto the cells. Proteins and total RNA were collected after incubating the cells with the transfection complex under normal growth conditions for 24 hours.

Real-time quantitative PCR

For detection of miRs, 500 ng of total RNA was isolated with the Quick-RNA Mini Prep Kit (Zymo Research, Los Angeles, Calif), and the miR-specific stem loop reverse transcription reaction was used to synthesize cDNA by using the Mir-X miRNA First-Strand Synthesis Kit (Clontech, Takara Shiga, Japan). For detection of normal genes, a total of 2 μ g of RNA were used for each reverse transcription reaction in the presence of oligo d(T) primers. cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit (Applied Biosystems, Foster City, Calif).

Real-time quantitative PCR (RT-qPCR) was performed by using the Applied Biosystems 7300/7500 Real-time System (Applied Biosystems) with Power SYBR Green PCR Master Mix (Applied Biosystems) for quantification. Gene or miR expression was normalized to β -actin or to U6 small nuclear RNA, respectively. The purity of PCR products was confirmed by using melting curve analysis, and all data were reanalyzed with the 2- $\Delta\Delta$ Ct (relative quantification) method. The information for all primers is shown in Table II.

ASMC activity, proliferation, and migration

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used to determine the overall activity of ASMCs, and direct cell count after

TABLE I. Clinical and medical characteristics of healthy control subjects, asthmatic patients, or patients with COPD

Diagnosis	Sex	Age (y)	FEV ₁	FVC	Medication
Asthmatic patients	Male	81	91	128	Oxis, Alvesco, Spiriva, Pantozol, Xolair, Singulair
	Female	64	64	87	Prednisone, Remicade, Alvesco, Foradil, Berotec
	Male	45	45	68	Seretide, Spiriva, Ventolin, Singulair, prednisone
	Female	55	63	102	Xolair
	Male	52	36	50	Prednisone, Alvesco, Spiriva, Onbrez, Symbicort, Singulair
	Female	64	64	87	Prednisone, Remicade, Alvesco, Foradil, Berotec
	Female	69	48	99	Avamys, Flutiform, Pantozol,
	Female	61	52	109	Symbicort, Ventolin
	Female	45	99	117	Seretide, Singulair
	Mean $\pm$ SEM	59.6 $\pm$ 3.2	62.4 $\pm$ 5.4	94.1 $\pm$ 6.6	
Patients with COPD	Female	76	51	79	Spiricort, Symbicort, Spiriva, Berodual, Liviol, Zandip
	Female	54	20	58	Spiriva, Relvar, aspirin, Spiricort
	Male	67	39	90	Ventolin, BecloSpin, Tarka
	Female	77	51	116	Ultibro, Flutiform, azithromycin, prednisone
	Male	71	41	82	Oxis, Spiriva, Pulmicort, Zitromax, metformin
	Male	69	63	88	Spiriva, azithromycin
	Male	68	40	82	Oxis, Spiriva, Pulmicort, Zitromax, metformin
	Male	70	40	82	Oxis, Spiriva, Pulmicort, Zithromax, metformin
	Male	62	27	65	Symbicort, Spiriva
	Mean $\pm$ SEM	68.2 $\pm$ 1.7	41.4 $\pm$ 3.6	82.5 $\pm$ 4.1	
Control subjects	Male	55	ND	ND	None
	Female	62	ND	ND	None
	Male	55	ND	ND	None
	Male	66	ND	ND	None
	Female	63	ND	ND	None
	Female	49	ND	ND	None
	Male	63	ND	ND	None
	Female	61	ND	ND	None
	Mean $\pm$ SEM	59.3 $\pm$ 2.6			

FVC, Forced vital capacity; ND, not determined.

48 hours was used as an indicator of proliferation.²⁰ Cells (1×10^4 cells/well) were seeded into 96-well plates, and proliferation was determined 48 hours after PDGF-BB stimulation with or without inhibitors of signaling proteins or PRMT1. Cells were incubated with 50 μ L of MTT reagent for 4 hours at 37°C and then lysed with dimethyl sulfoxide to determine the amount of formazan at 560 nm with a microplate reader (Synergy H1 Hybrid Microplate Reader; BioTek, Winooski, Vt).

For migration assays, ASMCs were seeded onto 6-well plates (0.25×10^6 cells/well) and allowed to adhere overnight, and then serum was deprived overnight. Scratches into the confluent cell layer were made with a sterile 10- μ L pipette tip. The migration of ASMCs into the wounded area was determined at different time points (6 and 24 hours) after treatment with or without PDGF-BB or AMI-1. All images were captured by a blinded observer.

Collagen type I and fibronectin ELISA

Levels of deposited collagen type I and fibronectin were measured by using an in-house ELISA, as described earlier.²⁰ ASMCs (1×10^4 cells/well) were seeded into 96-well plates and stimulated with PDGF-BB in the presence or absence of AMI-1 for up to 48 hours. Cells were fixed with 4% paraformaldehyde in PBS (2×5 minutes at room temperature), and plates were washed 3 times with PBS before being blocked by 2% BSA in PBS for 1 hour at room temperature. Afterward, 50 μ L of either anti-collagen 1A1 (Abcam) or anti-fibronectin antibody (1:200; Santa Cruz Biotechnology) was added and incubated overnight at 4°C. After 3 washes with PBS, 50 μ L of horseradish peroxidase-conjugated secondary antibody was added (1:1000; Santa Cruz Biotechnology) and incubated at room temperature for 1 hour. After 3 washes with PBS, TMB substrate was added and incubated at 37°C for 20 minutes. The reaction was terminated by adding 2 mol/L H₂SO₄, and the absorption was read at 450 nm with an ELISA reader (Synergy H1 Hybrid Microplate Reader; BioTek).

Prediction of candidate miRs binding to MAPK1 3' untranslated region

The MiRanda (<http://www.microna.org/microna/getGeneForm.do>) and TargetScan (http://www.targetscan.org/vert_50/) databases were used to predict the candidate miRs binding to the MAPK1 3' untranslated region (UTR) after online instructions.

Statistical analysis

The null hypothesis was that no treatment had any effect on signaling or PRMT1 expression, and a *P* value of less than .05 was regarded as significant. All data are expressed as means $\pm$ SEMs. The statistical analysis was performed by using the Mann-Whitney *U* test for comparison between groups. The effects of inhibitors on PRMT1 and remodeling were analyzed by using 1-way ANOVA.

RESULTS

PRMT1 is upregulated in lung sections from patients with severe asthma and in isolated ASMCs

As summarized in Table I, patients with asthma, patients with COPD, and control subjects had a comparable age range and sex distribution, which was not significantly different between groups (Mann-Whitney *U* test). Increased PRMT1 expression correlates with neither age nor sex but with the diagnosis of asthma, as described below.

Based on our earlier animal studies, we compared the expression level of PRMT1 in human airway tissue sections by means of immunohistochemical staining. In healthy and asthmatic tissue PRMT1 staining was found in epithelial cells

TABLE II. Primer information for RT-qPCR

Gene	Species	Sequence(5'-3')	Size (bp)	Annealing temperature (°C)
<i>PRMT1</i>	<i>Homo sapiens</i>	Forward TTGACTCCTATGCCCACT Reverse CCACATCCAGCACCACC	126	62
<i>STAT1</i>	<i>Homo sapiens</i>	Forward TGCGCGCAGAAAAGTTTCATT Reverse AGACATCCTGCCACCTTGTG	171	60
<i>GAPDH</i>	<i>Homo sapiens</i>	Forward CGGCAAGTTCAACGGCACAG Reverse GAAGACGCCAGTAGACTCCACGAC	148	60
<i>miR-101-3p</i>	<i>Homo sapiens</i>	TACAGTACTGTGATAACTGAA	21	60
<i>miR-19a-3p</i>	<i>Homo sapiens</i>	TGTGCAAATCTATGCAAACTGA	23	60
<i>miR-130a-3p</i>	<i>Homo sapiens</i>	CAGTGCAATGTTAAAAGGGCAT	22	60
<i>miR-200a-3p</i>	<i>Homo sapiens</i>	TAACACTGTCTGGTAACGATGT	22	60
<i>miR-1-3p</i>	<i>Homo sapiens</i>	TGGAATGTAAAGAAGTATGTAT	22	60
<i>miR-181c-5p</i>	<i>Homo sapiens</i>	AACATTCAACCTGTCGGTGAGT	22	60
<i>miR-21-5p</i>	<i>Homo sapiens</i>	TAGCTTATCAGACTGATGTTGA	22	60

and subepithelial ASMCs, with more PRMT1-positive cells in tissues from asthmatic patients (Fig 1, A, lower panels). Furthermore, the subepithelial layer of mesenchymal cells was significantly increased in tissue sections from asthmatic patients (Fig 1, A, lower right panel). The overall staining for PRMT1 in lung tissue sections was analyzed by means of optical densitometry, showing a significant increase ($P = .0244$) in tissue from asthmatic patients, with a mean of 272.3 ± 8.242 versus 44.07 ± 8.242 arbitrary units in control sections.

In isolated ASMCs PRMT1 expression was detected by means of Western blotting (Fig 1, B and C) and RT-qPCR (Fig 1, D). As in the tissue sections, we observed a significantly higher expression of PRMT1 in nonstimulated, nongrowing, serum-deprived ASMCs of asthmatic patients on both the protein and mRNA levels when compared with control ASMCs under the same conditions.

PRMT1 is upregulated by PDGF-BB in control ASMCs but not ASMCs from asthmatic patients

On the basis of our earlier studies, we selected PDGF-BB as the most potent mitogenic stimulus, which also induces ASMC remodeling through activation of TGF- β and also stimulates wound repair.¹²⁻¹⁴ For PRMT1 expression, we performed kinetic studies: control ASMCs were stimulated with 10 ng/mL PDGF-BB for various time periods over 48 hours, and we observed a time-dependent significant upregulation of PRMT1 starting at 1 hour and lasting for 48 hours (Fig 2, A). Based on our earlier studies, we assessed the role of ERK1/2 MAPK in PRMT1 regulation and show in Fig 2, A (lower panel), that the ERK1/2 MAPK inhibitor PD98059 significantly reduced the PDGF-BB-induced PRMT1 expression in control ASMCs. The densitometric analysis of all Western blotting is summarized in Fig 2, B.

Interestingly, in ASMCs from asthmatic patients, the baseline expression of PRMT1 was higher when compared with that in control ASMCs, and PRMT1 expression was not further increased by PDGF-BB (Fig 2, C). As in control ASMCs, preincubation with the ERK1/2 MAPK inhibitor significantly reduced the expression of PRMT1 in ASMCs from asthmatic patients (Fig 2, C), and the densitometric analysis of all Western blotting is depicted in Fig 2, D.

The immunofluorescence analysis of PRMT1 expression in ASMC cellular compartments showed that in unstimulated control

ASMCs, PRMT1 was faintly expressed in the cytosol (Fig 2, E, first panel), whereas after 24 hours of stimulation with PDGF-BB, it accumulated in the nuclei (Fig 2, E, second panel). In ASMCs from asthmatic patients, PRMT1 showed a consistently high expression in the nuclei (Fig 2, E, third panel), and this was not further increased by PDGF-BB (Fig 2, E, fourth panel).

PDGF-BB regulates PRMT1 through ERK1/2 MAPK and STAT1 signaling

Cross-talk of ERK1/2 MAPK with STAT signaling had been suggested earlier in lung fibroblasts.¹⁴ The expression of PRMT1, phospho-ERK1/2 MAPK, total ERK1/2 MAPK and phospho-STAT1 was detected by using Western blotting in 3 primary control ASMC lines and 3 primary ASMC lines from asthmatic patients. Compared on the basis of equal protein concentration, PRMT1, phospho-ERK1/2 MAPK, total ERK1/2 MAPK, and phospho-STAT1 levels were significantly increased in ASMCs from asthmatic patients (Fig 3, A, lanes 1-3 vs lanes 4-6). The densitometric analysis of the protein expression was determined in all Western blotting and is summarized in the corresponding bar charts (Fig 3, A).

In control ASMCs ERK1/2 MAPK was phosphorylated within 5 minutes by PDGF-BB and peaked at 15 minutes (Fig 3, B). PDGF-BB also induced phosphorylation of STAT1 within 5 minutes, which lasted more than 1 hour (Fig 3, B). In ASMCs from asthmatic patients, PDGF-BB further increased the pre-existing phosphorylation of ERK1/2 MAPK within 5 minutes, which lasted for an extended period compared with that seen in control ASMCs (Fig 3, C). However, PDGF-BB did not have any significant effect on the phosphorylation of STAT1 (Fig 3, C). The densitometric analysis of all Western blotting is provided as bar charts.

ASMCs were transfected with STAT1 siRNA or pretreated with the ERK1/2 inhibitor PD98059 and stimulated with PDGF-BB, and PRMT1 expression was assessed after 24 hours to confirm the role of ERK1/2 MAPK and STAT1 in PRMT1 expression (Fig 3, E). In control ASMCs PDGF-BB increased PRMT1 expression at 24 hours, which was prevented in ASMCs treated with either STAT1 siRNA or the ERK1/2 MAPK inhibitor PD (Fig 3, E). Similarly, in ASMCs from asthmatic patients, pretreatment with STAT1 siRNA, as well as with 10 μ mol/L PD, completely suppressed PRMT1 (Fig 3, E).

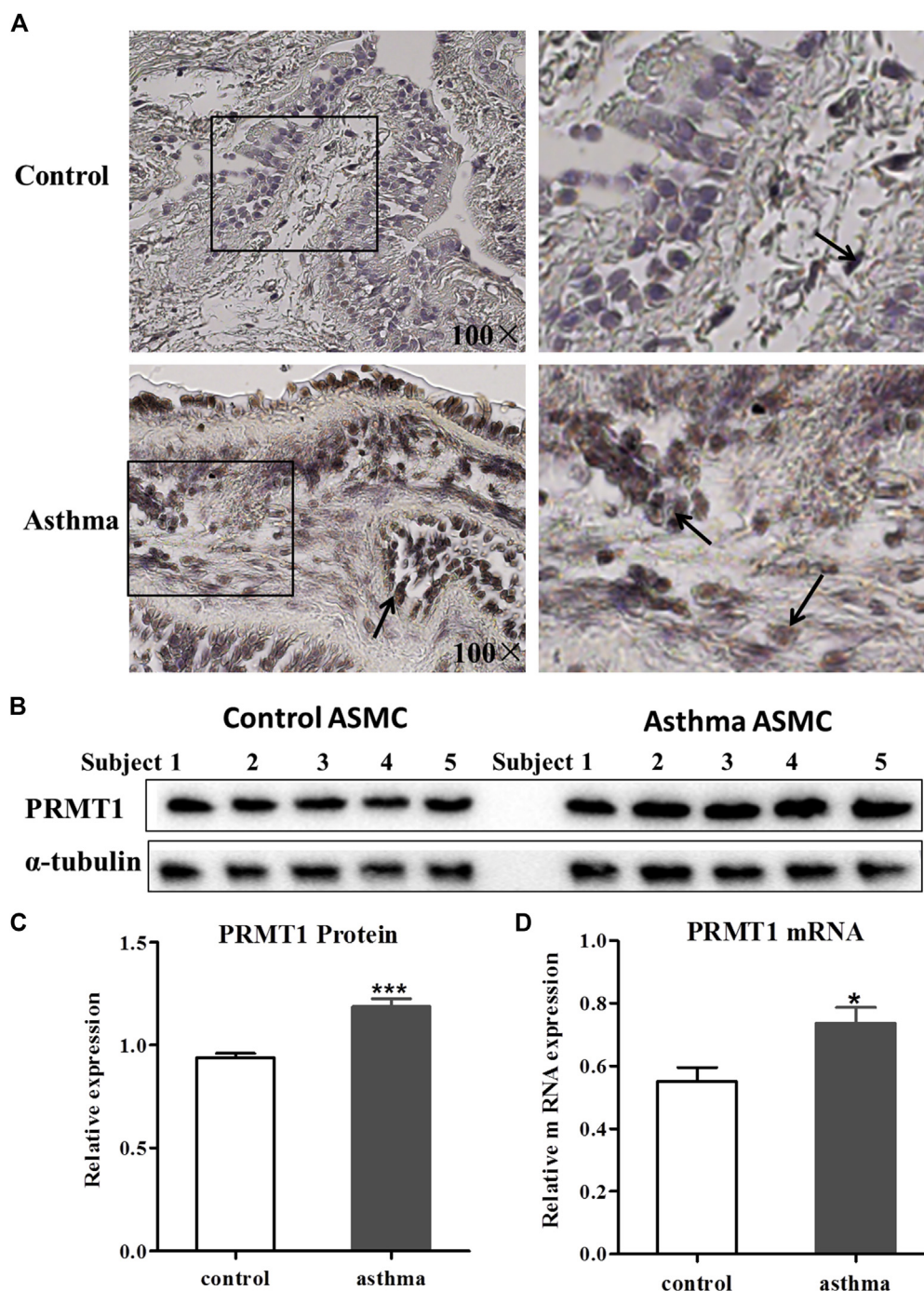


FIG 1. Expression of PRMT1 is upregulated in asthmatic patients. **A**, Representative PRMT1 (arrows) immunohistology in human lung sections ($n = 4$). **B** and **C**, PRMT1 was quantified by means of Western blotting in ASMCS of control subjects (Fig 1, **B**; $n = 5$) and asthmatic patients (Fig 1, **C**; $n = 5$). **D**, PRMT1 mRNA was normalized to actin. Bars = means $\pm$ SEMs. Mann-Whitney U test for control subjects versus asthmatic patients: * $P < .05$ and *** $P < .001$.

Inhibition of ERK1/2 MAPK activation decreases PRMT1 expression in ASMCS from asthmatic patients

Next, we confirmed the role of ERK1/2 MAPK in the increased expression of PRMT1 in ASMCS. In 3 primary ASMC lines from asthmatic patients, ERK1/2 MAPK inhibition significantly reduced phosphorylation of ERK1/2 MAPK and phospho-STAT1 and the expression of PRMT1 within 24 hours (Fig 4,

A). The densitometric analysis of all Western blotting is shown in Fig 4, **B**, for the ratio of phospho-ERK1/2 MAPK/total ERK1/2 MAPK and for PRMT1 in Fig 4, **C**.

miR-101 and miR-19a are downregulated in ASMCS from asthmatic patients

To identify miRs that regulate the expression of *MAPK1*, we searched for putative miR binding sites within the 3' UTR of

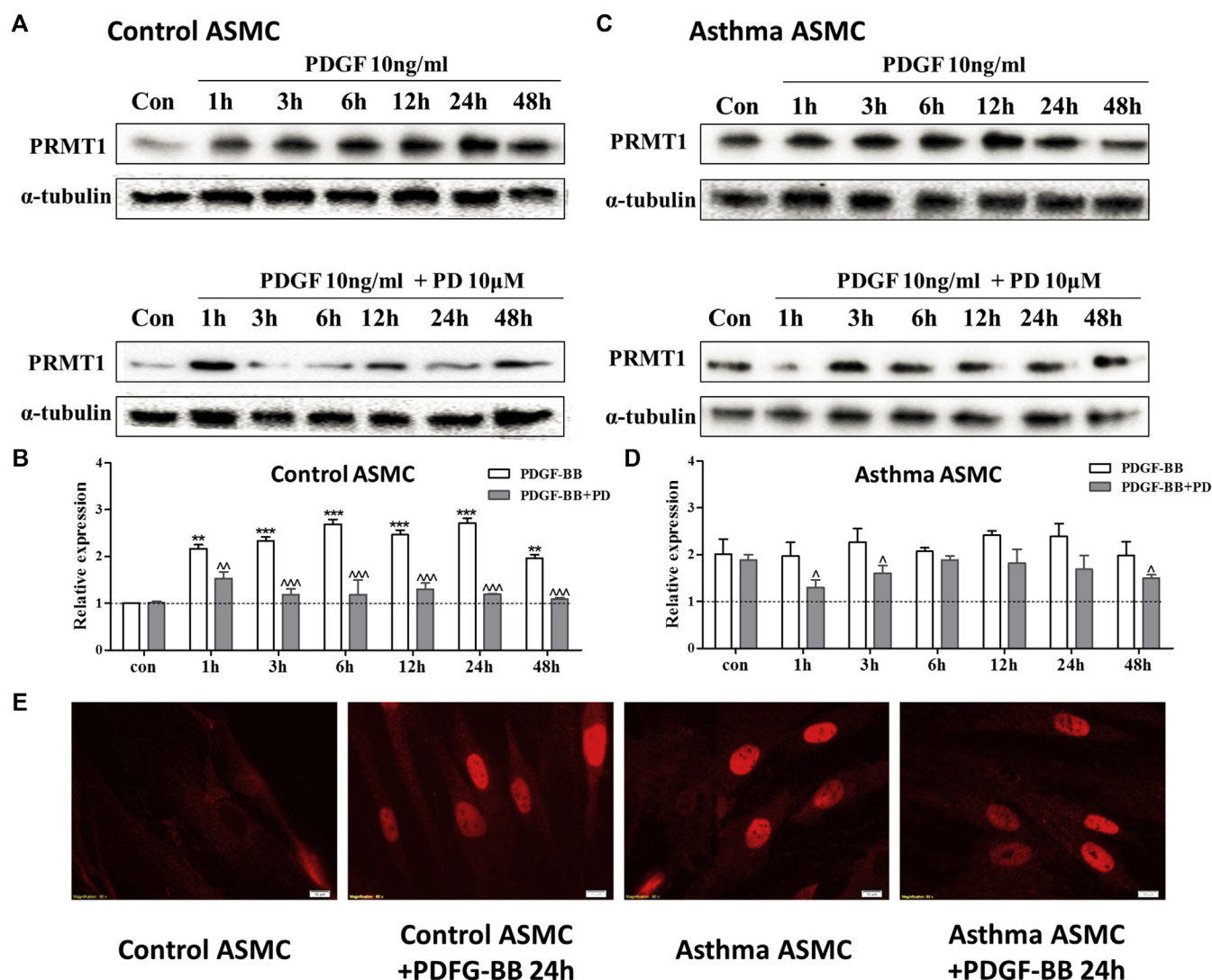


FIG 2. Asthma-specific PRMT1 expression in ASMCs. **A** and **C**, Representative Western blots of PDGF-BB induced PRMT1 expression and its regulation by ERK1/2 MAPK in ASMCs: control subjects (Fig 2, A) and asthmatic patients (Fig 2, C). PD, PD98059 (10 μ mol/L). **B** and **D**, Densitometry of Western blots (6 per group). **E**, Bars = means $\pm$ SEMs. Mann-Whitney *U* test for PDGF-BB-treated versus nontreated cells: ***P* < .01 and ****P* < .001. PDGF-BB versus PDGF-BB+PD: ^*P* < .05, ^^*P* < .01, and ^^*P* < .001.

MAPK1 mRNA by using miR computer-assisted target analysis programs (miRanda and TargetScan), which suggested that the 3' UTR of the *MAPK1* gene contains complementary binding sites for miR-101, miR-19a, miR-130a, miR-200a, miR-1, miR-21, and miR-181a. Because all miRs are highly conserved in different mammalian species, their critical role for miR-mRNA interaction is suggested (see Fig E1 in this article's Online Repository at www.jacionline.org). All candidate miRs were determined in ASMCs by using RT-qPCR and compared between 6 control subjects versus 6 asthmatic patients and 8 patients with COPD. As shown in Fig 5, expression of miR-101 and miR-19a showed a significant disease-specific decrease in numbers of asthmatic ASMCs; additionally, miR-101 and miR-200a decreased significantly in ASMCs from patients with COPD.

miR-19a deficiency increases PRMT1 expression in ASMCs from asthmatic patients through targeting ERK1/2 MAPK

We next examined whether miR-101 or miR-19a regulates expression of *MAPK1* and *PRMT1*. ASMCs from asthmatic patients were transfected with either miR-101 or miR-19a mimics and inhibitors, and protein levels of ERK1/2 MAPK and PRMT1 expression were measured by using Western blotting. As shown in Fig 6, A and B, in both control ASMCs and ASMCs from asthmatic patients, miR-19a double-stranded RNA mimics decreased the level of total ERK1/2 MAPK and thus the phosphorylation of ERK1/2 MAPK and STAT1. Subsequently, miR-19a mimics also reduced the expression of PRMT1 in both control ASMCs (Fig 6, A) and ASMCs from asthmatic patients (Fig 6, B). The transfection of control ASMCs and ASMCs

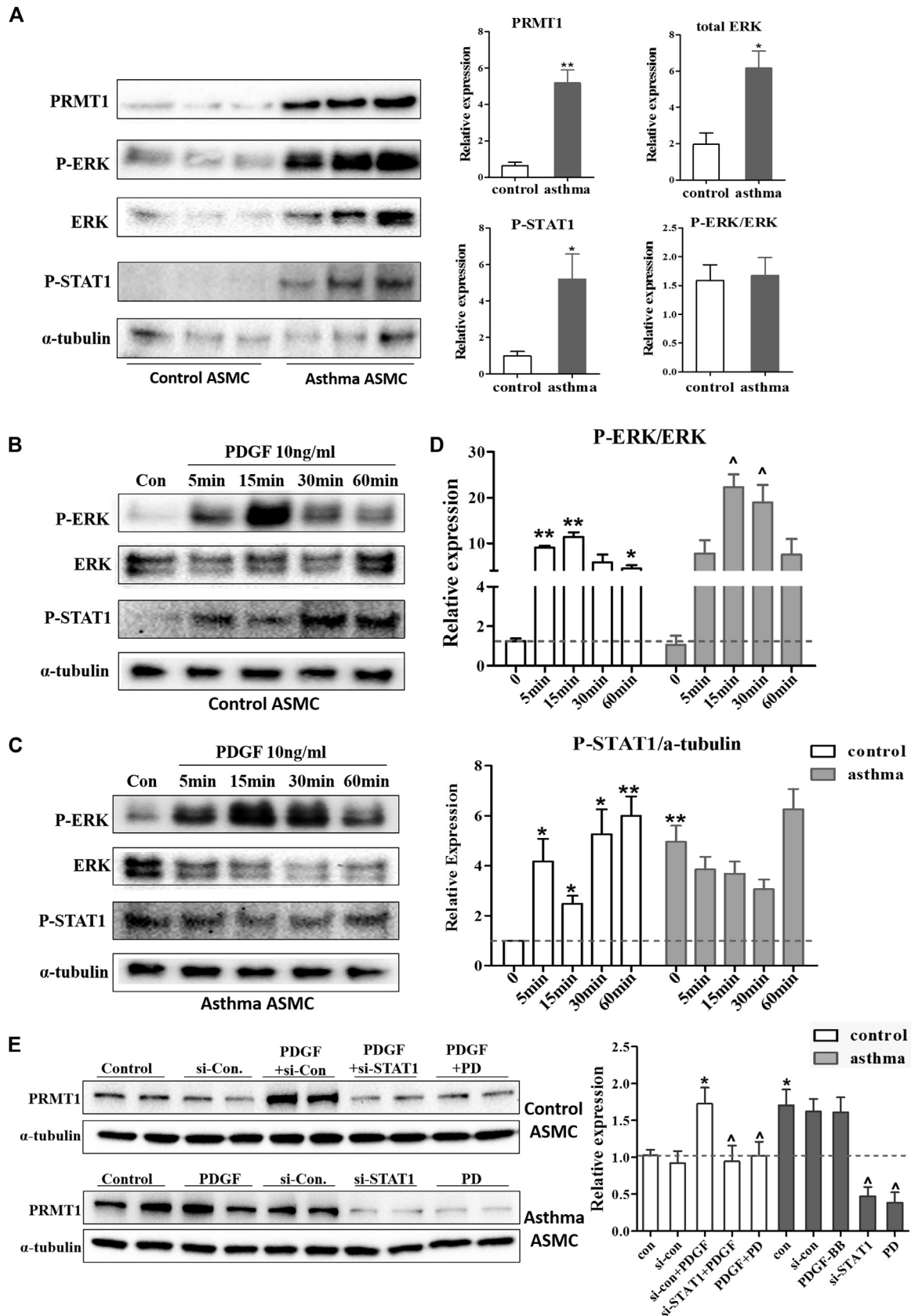


FIG 3. PDGF-BB upregulates PRMT1 through STAT1. **A**, Protein expression in unstimulated ASMCs was disease specific (3 asthmatic patients and 3 control subjects). **B** and **C**, PDGF-BB disease specifically modified protein expression and phosphorylation. **D** and **E**, Downregulation of PRMT1 by small interfering STAT1 or PD98059 (10 μ mol/L). Bars = means $\pm$ SEMs. Untreated versus PDGF-BB-treated cells: * P < .05 and ** P < .01. PDGF-BB versus inhibitor: $\wedge$ P < .05.

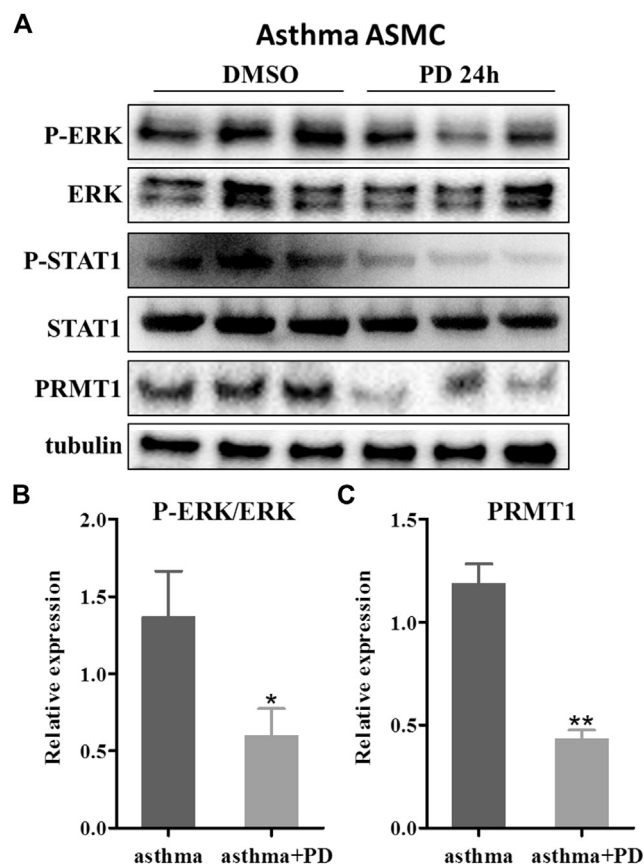


FIG 4. PRMT1 regulation by ERK1/2 in ASMCs from asthmatic patients. Three ASMC lines from asthmatic patients were incubated with or without PD98059 (PD; 10 μ mol/L), and expression of phospho-ERK, total ERK, phospho-STAT1, total STAT1, and PRMT1 was detected (A) and analyzed by using densitometry (B and C). Bars = means $\pm$ SEMs ($n = 3$). Mann-Whitney U test for asthma versus asthma + PD: * $P < .05$ and ** $P < .01$.

from asthmatic patients with the miR-19a inhibitor enhanced the production of ERK1/2 MAPK and the phosphorylation of ERK1/2 MAPK and phospho-STAT1, as well as increasing PRMT1 expression, compared with treatment with control miR or control inhibitor. The densitometric analysis of all Western blotting and the comparison between control ASMCs and ASMCs from asthmatic patients are displayed on a bar chart in Fig 6, C and D.

In contrast, the transfection of ASMCs with miR-101 did not show any significant effect on ERK1/2 MAPK, STAT1, or PRMT1 expression (see Fig E2 in this article's Online Repository at www.jacionline.org).

Inhibition of PRMT1 activity decreases ASMC cell activity, proliferation, ECM deposition, and cell migration

Increased overall activity in nonstimulated ASMCs from asthmatic patients was detected based on higher MTT uptake over 24 hours. In control cells MTT uptake was increased by PDGF-BB. Incubation with the PRMT1 inhibitor AMI-1 significantly reduced MTT uptake by ASMCs from asthmatic patients (Fig 7, A, left panel).

Proliferation was determined based on direct cell counts and was faster in ASMCs from asthmatic patients compared with control ASMCs (Fig 7, A, right panel). Inhibition of PRMT activity by AMI-1 significantly reduced proliferation of ASMCs from asthmatic patients (Fig 7, A, right panel).

In ASMCs from asthmatic patients, expression of α -SMA, collagen type I, and fibronectin was significantly increased compared with that in control ASMCs, and pretreatment with the pan-PRMT inhibitor AMI-1 significantly reduced the expression of all 3 remodeling parameters, as monitored by using Western blotting (Fig 7, B). Densitometric analysis of all Western blots is depicted as a bar chart in Fig 7, C. Increased expression of collagen type I and fibronectin was also detected by using ELISA in ASMCs from asthmatic patients compared with control ASMCs (Fig 7, D). The asthma-specific increased expression of collagen type I and fibronectin was significantly reduced by the PRMT1 inhibitor AMI-1 in ASMCs from asthmatic patients, as shown in Fig 7, D. We confirmed the inhibitory effect of AMI-1 on α -SMA, collagen type I, and fibronectin expression using immunofluorescence staining (Fig 7, E). Importantly, PDGF-BB had no further stimulatory effect on any of the 3 remodeling indicators in cells from asthmatic patients.

Finally, we assessed whether PRMT1 is involved in ASMC migration because it was suggested to occur in the airways of asthmatic patients after repetitive injury. Confluent ASMC layers were "wounded" by scratching, and the repopulation of this area with ASMC was monitored at different time points over 24 hours. As shown in Fig 8, PDGF-BB stimulated the migration of ASMCs into the wounded area (second row panels) in control ASMCs compared with untreated control ASMCs (first row panels). Sequential observation by using microscopy in regular time intervals indicated that the repopulation of the wounded area occurred through migration rather than proliferation. Interestingly, the migratory effect in nonstimulated ASMCs from asthmatic patients (third row panels) was similarly as strong as that achieved by PDGF-BB in control ASMCs. Importantly, the inhibition of PRMT1 by AMI-1 significantly reduced migration of ASMCs from asthmatic patients (lower row panels).

DISCUSSION

For the first time, this study shows in human subjects that ERK1/2 MAPK, STAT1, and PRMT1 are constitutively overexpressed in ASMCs from asthmatic patients because of the reduced expression of miR-19a. Unlike in ASMCs of control subjects, PDGF-BB does not further increase PRMT1 expression in ASMCs from asthmatic patients. PRMT1 expression is controlled by the same signaling cascade in ASMCs from healthy subjects and asthmatic patients; however, this signaling cascade is constitutively activated in ASMCs from asthmatic patients.

Our previous studies in animal models suggested that PRMT1 participates in both the inflammation and remodeling process in asthmatic patients, which was induced by either TGF- β or IL-4. The stimulatory effect on PRMT1 was cell type and stimulus specific, with IL-4 being active on epithelial cells but not on fibroblasts and TGF- β being active on both cell types.¹²⁻¹⁴ At early stages of acute lung inflammation, PRMT1 increased mainly in epithelial cells, exacerbating inflammation in the rat lung, whereas in the setting of chronic inflammation, PRMT1 expression was located in subepithelial cells, including fibroblasts and ASMCs.¹² Here we show that in human airway tissue from

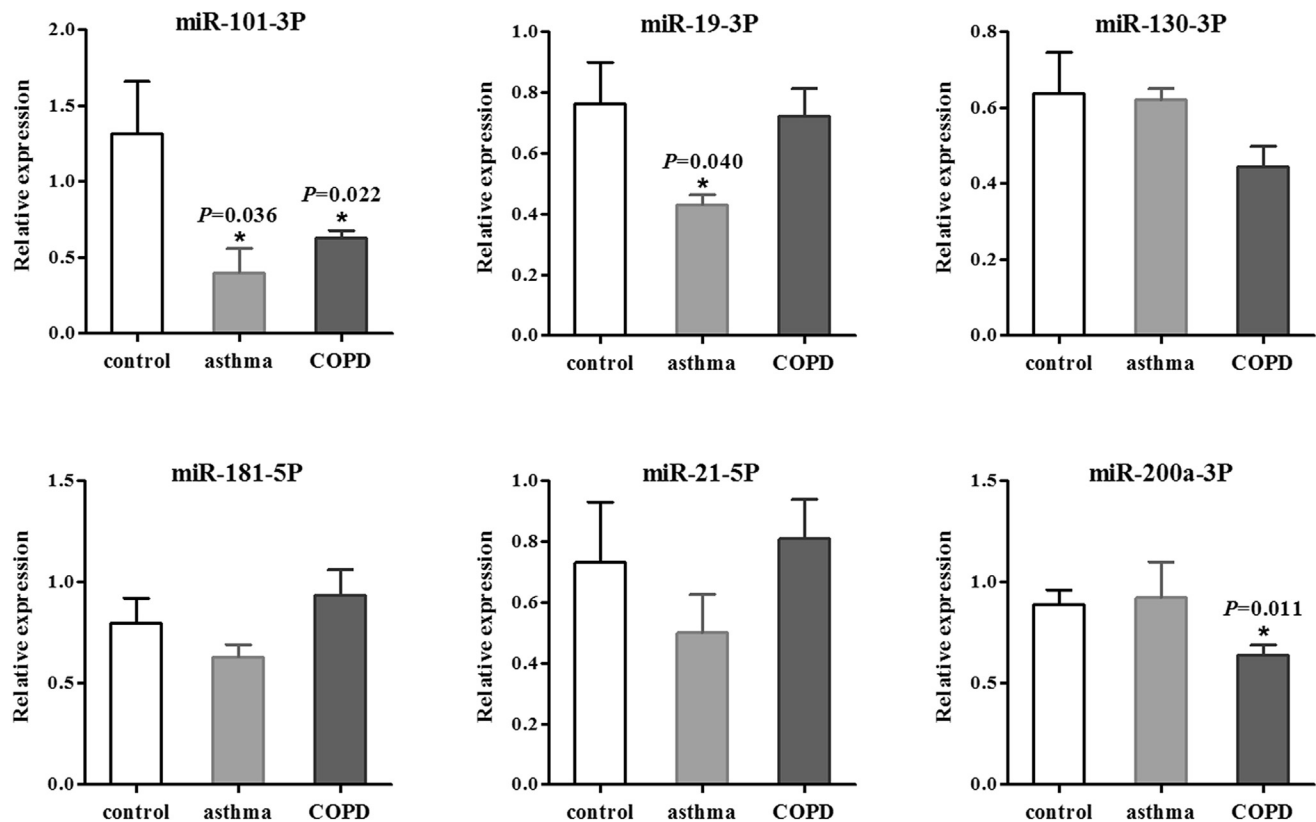


FIG 5. Expression of miRNAs binding to MAPK1 3' UTR. miR target prediction programs (miRanda, TargetScan) indicated miR-101-3p, miR-130-3p, miR-181-5p, miR-21-5p, and miR-200a-3p to bind MAPK1 3' UTR. Expression of miRNAs was detected by using quantitative RT-PCR in ASMCs from control subjects ($n = 6$), asthmatic patients ($n = 6$), and patients with COPD ($n = 9$). Bars = means $\pm$ SEMs. Mann-Whitney U test for control subjects versus asthmatic patients or patients with COPD: * $P < .05$.

asthmatic patients, PRMT1 is expressed at a higher level in epithelial and subepithelial cells. The increase of PRMT1 expression in ASMCs from asthmatic patients was detected on both the level of transcription and the protein level. The fact that this pathology was maintained in isolated ASMCs enabled us to further characterize the detailed regulatory mechanism of PRMT1 expression on asthma.

In asthmatic patients characteristic structure abnormalities of the airway wall include the accumulation of ASMCs, which express increased levels of connective tissue characteristics, and thus the cells are in a different stage of differentiation compared with ASMCs from subjects without asthma. Several studies have shown that this asthma-specific ASMC pathology correlates with the severity of asthma and is resistant to conventional therapies. The observation that asthma symptoms improve from reduced ASMC mass is supported by clinical data after thermoplasty therapy, which further supports the eminent role of this cell type in the pathogenesis of asthma.^{21,22} These data imply that removing or silencing of ASMCs might resolve asthma-associated airway wall remodeling and improve lung function and quality of life. Therefore other less invasive methods to reduce airway wall remodeling in asthmatic patients should be established.

In addition to other growth factors, PDGF-BB induced ASMC proliferation, as well as activating the secretion of proinflammatory ECM components.^{23,24} Interestingly, airway wall remodeling

by ASMCs can be induced by mechanical stress imposed during bronchoconstriction, ECM modification, and/or hypoxia,²⁵ which all might be related to increased PDGF-BB activity.²¹ Furthermore, the innate and adaptive immune response can trigger the proliferation and activation of subepithelial airway wall ASMCs.²⁵ The mechanism by which this pathology leads to increased thickness of the subepithelial basal membrane remains unclear, but its contribution to the pathogenesis of asthma, especially of reduced airway flexibility and increased constriction, has been demonstrated by others.²⁶

In asthmatic patients damaged bronchial epithelium secretes increased levels of proinflammatory growth factors and cytokines, including PDGF-BB, which activates ASMC and airway remodeling.²⁷ In asthmatic patients most PDGF-BB seems to originate from lung-infiltrating inflammatory cells, such as monocytes and macrophages, during an asthma attack and is accompanied by increased expression of the corresponding PDGF receptors on ASMCs.²⁸ ERK1/2 MAPK phosphorylation is a major downstream signal activated by the PDGF β receptor and mediates ASMC proliferation.^{29,30} In a previous study we reported that PDGF-BB induced PRMT1 through activation of ERK1/2 MAPK in primary human lung fibroblasts,¹⁴ and this mechanism is also functional in ASMCs, both from healthy subject and asthmatic patients, as we report here. Thus, once activated, PDGF-BB signaling can lead to chronic activation of ASMCs in asthmatic patients and present as

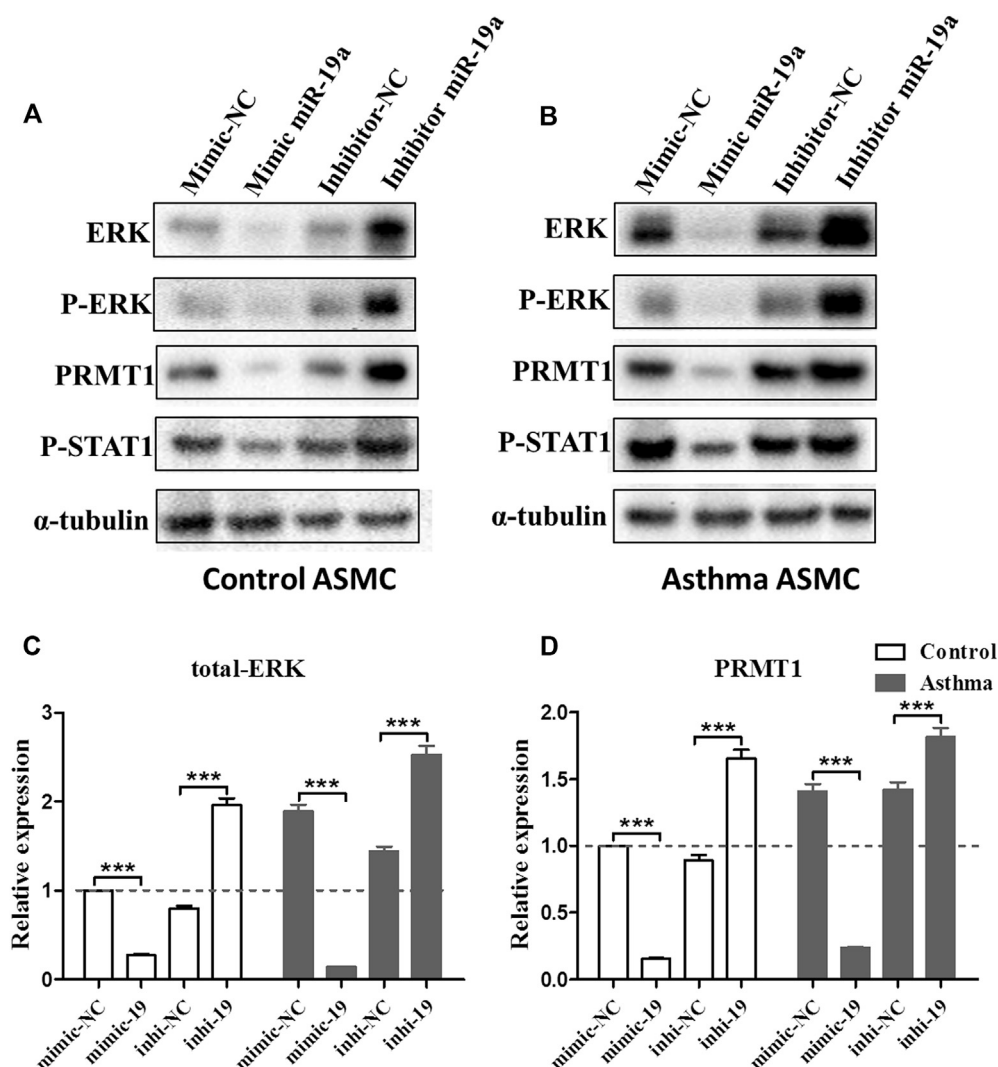


FIG 6. ERK1/2 as a putative target of miR-19a-3p. **A** and **B**, ASM cells from 3 control subjects (Fig 6, **A**) and 3 asthmatic patients (Fig 6, **B**) were transfected with either control double-stranded RNA, mimic miR-19a-3p (10 nmol/L), or miR-19a-3p control inhibitor (50 nmol/L). **C** and **D**, Expression of ERK, phospho-ERK, PRMT1, and phospho-STAT1 was detected after transfection by means of Western blotting, and analyzed by using densitometry. Results are expressed as means $\pm$ SEMs. *** $P < .001$.

constitutively reduced miR-19a, leading to constitutively activated phospho-ERK1/2 MAPK–STAT1–PRMT1, as suggested by our data.

In patients with other inflammatory diseases, there is evidence of cross-talk between ERK1/2 MAPK with STAT1 signaling, which results in radical oxygen generation and histone modification.^{31–33} In addition, ERK1/2 MAPK signaling plays an important role in the regulation of cell proliferation, migration, differentiation, and angiogenesis, suggesting a role for PRMT1 in these events.^{34,35} In this study we observed that inhibition of ERK1/2 MAPK suppressed STAT1 phosphorylation, which is consistent with the findings of others.³⁶ Furthermore, STAT1 activation was necessary to increase PRMT1 expression.

Enhanced phosphorylation of ERK1/2 MAPK was observed in airways obtained from asthmatic patients³⁷ and was greater in resting or suboptimal activated ASM cells from asthmatic patients.³⁸ Here we show that the constitutive activation of ERK1/2 MAPK is maintained in isolated ASM cells from asthmatic

patients over several cell division cycles. This indicates a correlation between the extent of phosphorylated ERK1/2 MAPK and increased proliferation of asthmatic ASM cells.^{19,38} In our experiments the inhibition of ERK1/2 MAPK activation reduced PRMT1 expression in cells from asthmatic patients and those from healthy subjects. Therefore it is suggested that PRMT1 inhibition might present a novel target for the therapy of asthma-associated airway wall remodeling.

Computer-assisted mRNA structure analysis suggested that ERK1/2 MAPK is a target for several miRs (see Fig E1). Studies on miR expression and function in ASM cells suggested their control function in cell proliferation, hypertrophy, and differentiation,³⁹ and aberrant expression of miRs was associated with various lung diseases, including asthma and COPD.⁴⁰ However, regulation of ERK1/2 MAPK by specific miRs in human ASM cells lacked experimental evidence. Our data indicated that the 3' UTR of the human *MAPK1* gene contains complementary sites for the seed region of miR-101, miR-19a,

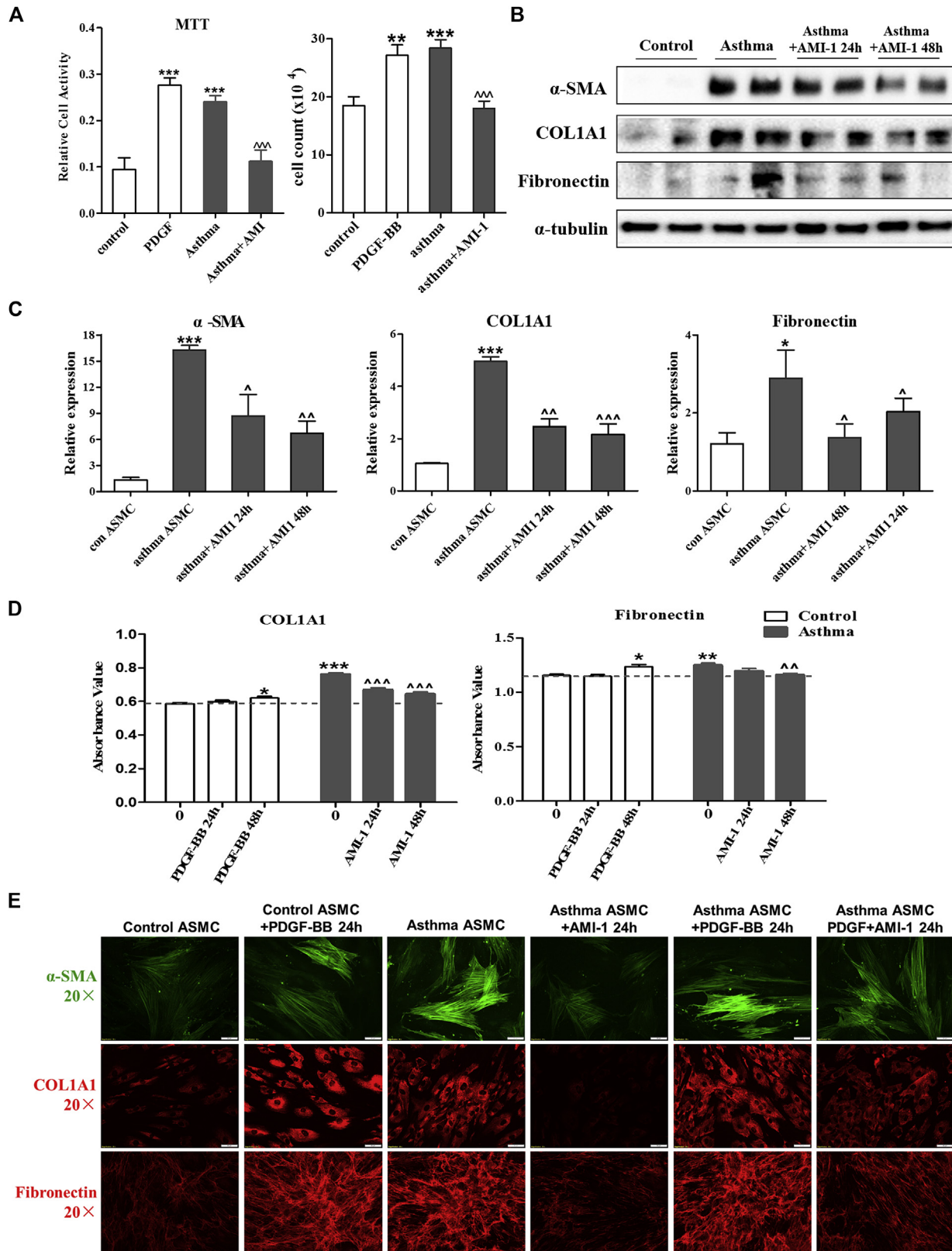


FIG 7. Inhibiting PRMT1 decreases ASMC proliferation and ECM production. PRMT1 inhibition reduced cell activity (MTT) and cell numbers (A), differentiation (α-SMA) (B), and collagen I and fibronectin deposition by using Western blotting (C) and ELISA (D) and by using immunofluorescence (E) in 4 ASMC lines. * $P < .05$, ** $P < .01$, and *** $P < .001$ between control ASMCs and other groups. Δ $P < .05$, ΔΔ $P < .01$, and ΔΔΔ $P < .001$ between ASMCs from asthmatic patients and asthmatic patients + AMI-1.

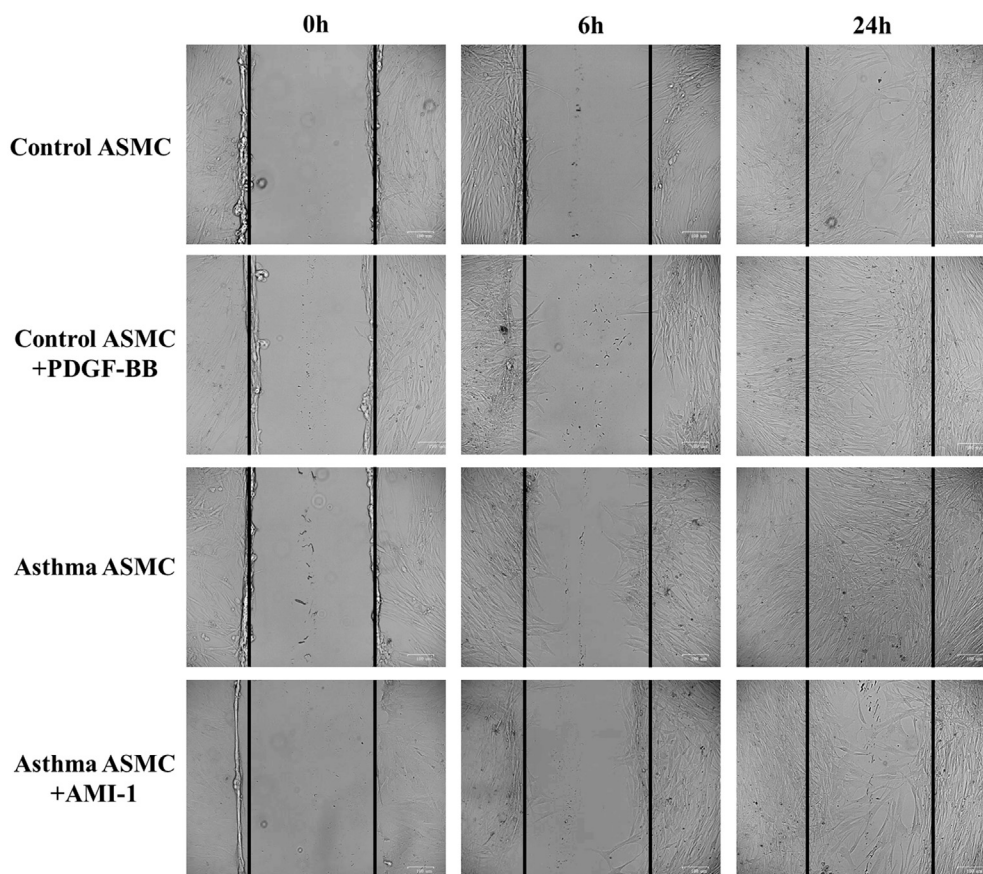


FIG 8. Inhibiting PRMT1 activity decreases ASMC migration. ASMCs (from control subjects and asthmatic patients) were injured and stimulated by PDGF-BB in the absence/presence of the PRMT1 inhibitor AMI-1. ASMC migration was monitored by cells within the wounded area at 6 and 24 hours. Representative pictures for one of 3 ASMC lines from asthmatic patients and one of 3 ASMC lines from control subjects are depicted.

miR-130a, miR-200a, miR-1, miR-21, and miR-181a. To prove the disease-specific role of certain miRs in asthmatic patients, we included ASMCs obtained from patients with COPD because both diseases show similar airway remodeling pathologies and have been discussed to represent the extreme opposing phenotypes of one basic disease. The fact that we detected a disease-specific pattern for specific miRs indicates a different pathogenesis at this level.

In ASMCs from asthmatic patients, miR-101 and miR-19a levels were significantly decreased compared with those in cells of control subjects and patients with COPD, which suggested that ERK1 MAPK might be deregulated in a disease-specific manner. Expression of miRs in ASMCs from patients with COPD was added to our study because COPD shares several pathologies with asthma and parameters for the distinction of both diseases are important. Downregulation of miRs in cells from asthmatic patients is not completely new because miR-19a, miR-27a, miR-106, miR-128, and miR-155 were downregulated in bronchial epithelial cells from asthmatic patients.⁴¹ However, this might be a cell type-specific effect because profiling of miR expression in human asthma airway-infiltrating T cells showed increased expression of miR-19a.⁴² Here we show that diminished miR-19a expression in ASMCs from asthmatic patients caused constitutive activation of ERK1/2 MAPK signaling, leading to consistently high expression of PRMT1,

and led to increased ASMC remodeling. Our data confirmed that miR-19a has cell type- and disease-specific functions in asthmatic patients and thus supports the hypothesis that it plays a directive role for the fine tuning of cell differentiation and pathogenesis.

Most chronic inflammatory pulmonary diseases are characterized by excessive deposition of specific ECM components in and around inflamed or damaged tissue, leading to thick and stiff airway walls.²⁵ PDGF-BB is a profibrotic cytokine that stimulates fibroblasts and ASMCs to synthesize ECM proteins.⁴³ In our study ASMCs from asthmatic patients versus control ASMCs produced more ECM components because of collagen type-I and fibronectin. Inhibition of PRMT1 activity by AMI-1 significantly reduced all profibrotic activities of ASMCs, including collagen type I and fibronectin deposition. None of the existing drugs for asthma therapy have such an effect, and thus PRMT1 inhibition might present a novel therapeutic target for reducing ECM accumulation during airway wall remodeling in asthmatic patients.

In addition, the higher level of fibrillar α -SMA arrangement in airways of asthmatic patients has been suggested to increase the contractile force of ASMCs during asthma attacks.⁴⁴ Our data show that inhibition of PRMT1 reduces the overall expression and fibrillar arrangement of α -SMA in ASMCs from asthmatic patients, which might reduce their contractility and also their ability to migrate.

PDGF-BB promoted ASMC motility through an incompletely understood mechanism.⁴⁵ Here we show data that ASMCs from asthmatic patients migrated faster than control cells and that the inhibition of PRMT reduced their motility. In addition, confirming the inductive role of PDGF-BB on ASMC migration, we show that *in vitro* PRMT1 inhibition markedly reduced ASMC migration into wounded areas. This observation strongly indicates that the molecular mechanism causing the asthmatic ASMC phenotype can be delineated from the nonasthmatic ASMC phenotype and thus has potential as a novel therapeutic target for the mitigation of asthma.

In conclusion, for the first time, we report constitutive reduced miR-19a expression, which leads to the consistent upregulation of ERK1/2 MAPK–STAT1–PRMT1 and causes overall activation of ASMCs from asthmatic patients (see the graphic abstract). Our data suggest that miR-19a and PRMT1 might be novel indicators of asthma and present new disease-specific therapeutic targets to reduce airway wall remodeling and inflammation in asthmatic patients.

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Key messages

- The lack of miR-19a in ASMCs from asthmatic patients leads to constitutive enhanced production and activity of ERK1/2 MAPK.
- Constitutive expression and activation of ERK1/2 MAPK increases PRMT1 expression in asthmatic smooth muscle cells.
- Enhanced PRMT1 aggravates airway remodeling in lungs from asthmatic patients by inducing ASMC proliferation, migration, and ECM deposition.

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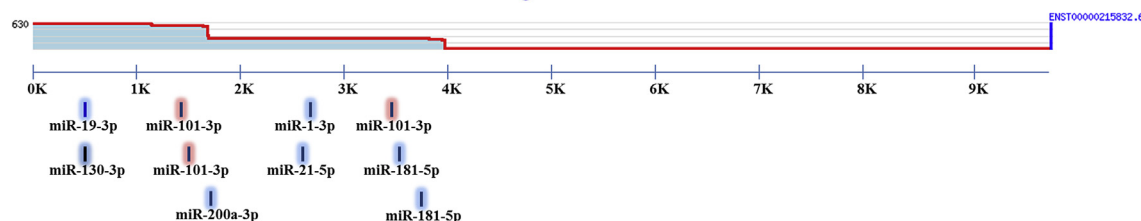
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METHODS

Based on computer-assisted target analysis, the depicted miRs were suggested as regulators for MAPK1 mRNA. In Fig E1 we depict the base pair match of the miRs with MAPK1 mRNA sequences. Data were generated by using the MiRanda (<http://www.microrna.org/microrna/home.do>) and TargetScan (http://www.targetscan.org/vert_71/) databases, which were used to predict the candidate miRs binding to MAPK1 3' UTR according to online instructions.

In addition to miR-19a, the computer-assisted analysis suggested that miR-101a is another regulator of Erk1/2 MAPK translation, and therefore the effects of inhibitors and mimics for miR-101a were assessed on the expression of total Erk1/2 MAPK, phospho-Erk1/2 MAPK, PRMT1, and phospho-STAT1. Expression of ERK, phospho-ERK, PRMT1, and phospho-STAT1 after transfection was detected and shown as representative immunoblotting. As shown in Fig E2, we did not observe any significant effect on the named signal-transducing proteins in healthy control cells or cells from asthmatic patients.

Human MAPK1 ENST00000215832.6 3' UTR length: 9750

Conserved Position 1363-1369 of MAPK1 3' UTR 5' ...GCCUUGUCAAUAAUACUGUAC...
hsa-miR-101-3p.1 3' AGUCAAUAGUGUCAUGACAUG

Conserved Position 3583-3589 of MAPK1 3' UTR 5' ...CUUUGUAUCUGCUUUGUACUGUU...
hsa-miR-101-3p.1 3' AAGUCAAUAGUGU--CAUGACAUG

Poorly Conserved Position 1266-1272 of MAPK1 3' UTR 5' ...UGUUCCUGCCACUGUGUACUGUG...
hsa-miR-101-3p.1 3' AAGUCAAUAGUGUCAUGACAUG

Conserved Position 472-478 of MAPK1 3' UTR 5' ...AGUGACAGCCCCUAAUUGCACU...
hsa-miR-19a-3p 3' AGUCAAACGUAUCUAAACGUGU

Conserved Position 473-479 of MAPK1 3' UTR 5' ...GUGACAGCCCCUAAUUGCACUU...
hsa-miR-130a-3p 3' UACGGGAAAUAUGUAAACGUGAC

Conserved Position 1678-1684 of MAPK1 3' UTR 5' ...AUUCAUGUAAAGAUUCAGUGUUG...
hsa-miR-200a-3p 3' UGUAGCAAUGGUCUGUCACAAU

Conserved Position 2746-2753 of MAPK1 3' UTR 5' ...AAAAUCCUGCUGAAACAUAUCCA...
hsa-miR-1-3p 3' UAUGUAUGAAGAAUUGUAAGGU

Conserved Position 2684-2690 of MAPK1 3' UTR 5' ...UAUUUGUAUGUCAAUAAGCUU...
hsa-miR-21-5p 3' AGUUGUAGUCAGACUAUUCGAU

Conserved Position 3685-3691 of MAPK1 3' UTR 5' ...AUUUUAUUAAGAAUCUGAAUGUU...
hsa-miR-181a-5p 3' UGAGUGGCUGUCGCAACUACAA

Conserved Position 3842-3848 of MAPK1 3' UTR 5' ...AAAAGAUGGAUUAUUUGAAUGUU...
hsa-miR-181a-5p 3' UGAGUGGCUGUCGCAACUACAA

FIG E1. miRs binding to MAPK-1 3' UTR. Pairing of the *MAPK1* 3' UTR with its targeting miRs is shown. Data collection and analysis revealed that the *MAPK1* 3' UTR contains complementary binding sites for seed regions of miR-101, miR-19a, miR-130a, miR-200a, miR-1, miR-21, and miR-181a. All miRs are highly conserved in different mammalian species.

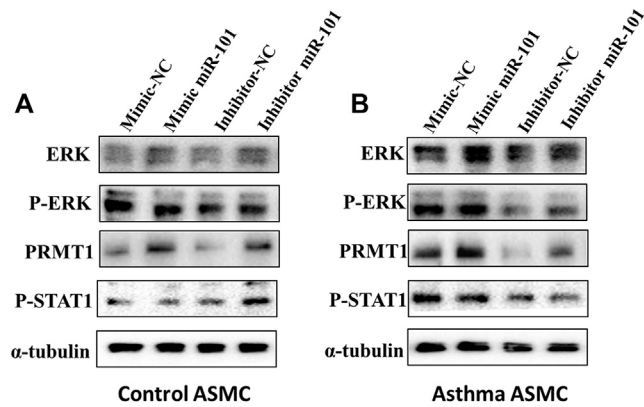


FIG E2. ERK1/2 might not be a putative target of miR-101. Representative immunoblots of the effect of miR-101a mimics and inhibitors in ASMCs are shown. ASMCs from nonasthmatic control subjects (**A**) and asthmatic patients (**B**) were used. Cells were transfected with either 10 nmol/L control mimic RNA or mimic miR-101, 50 nmol/L miR-101, or control inhibitor. Similar results were obtained in ASMCs of 2 additional control subjects and 2 additional asthmatic patients.