

5-Aza-2'-deoxycytidine inhibited PDGF-induced rat airway smooth muscle cell phenotypic switching

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Abstract Airway smooth muscle (ASM) cell phenotypic switching played an important role in airway remodeling in asthma. In vitro platelet-derived growth factor (PDGF) induced ASM cell phenotypic switching from a mature to proremodeling phenotype, but the mechanism remained incompletely understood. This study was to explore the effect of DNA methyltransferase inhibitor 5-Aza-2'-deoxycytidine (Aza-CdR) on PDGF-induced rat ASM cell phenotypic switching and biological behaviors. Rat airway smooth muscle (RASM) cells were obtained by primary explant techniques. Western blot, 3-dimensional gel contraction, transwell and wound healing assay, and MTT were applied to detect cell phenotypic switching, contractility, migration and proliferation, respectively. Cytoskeleton rearrangement was observed by immunofluorescence. Results showed Aza-CdR inhibited PDGF-induced down-regulation of contractile markers in RASM cells and increased cell contractility. Aza-CdR inhibited PDGF-induced RASM cell migration by abrogating cell morphology change and cytoskeletal reorganization and attenuated the effect of PDGF on proliferating cell nuclear antigen expression and cell cycle progression, ultimately cell proliferation. PDGF-induced DNA methyltransferase 1 (DNMT1) expression was mediated by activation of PI3K/Akt and ERK signaling in RASM cells. Selective depletion of DNMT1 protein by Aza-CdR inhibited PDGF-induced RASM cell phenotypic switching, revealing DNMT1-mediated DNA methylation was implicated in asthmatic ASM

remodeling. We proposed for the first time that DNMT1 played a key role in PDGF-induced RASM cell phenotypic switching and Aza-CdR is promising in intervening ASM remodeling in asthma. Although study of abnormal DNA methylation in PDGF-stimulated ASM cells is in its infancy, this work contributes to providing new insights into the mechanism of ASM remodeling and may be helpful for developing effective treatments for airway remodeling in asthma.

Keywords 5-Aza-2'-deoxycytidine · Airway smooth muscle cells · Phenotypic switching · DNA methylation · DNA methyltransferase 1

Abbreviations

RASMC	Rat airway smooth muscle cell
PDGF	Platelet-derived growth factor
Aza-CdR	5-Aza-2'-deoxycytidine
DNMT1	DNA methyltransferase 1
PCNA	Proliferating cell nuclear antigen
OD	Optical density
MRLC	Myosin regulatory light chain
MLCK	Myosin light-chain kinase
2-D	Two-dimensional
3-D	Three-dimensional
TBST	Tris-buffered saline with 0.1 % Tween-20
PI3K	Phosphatidylinositol 3-kinase

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Introduction

The incidence and severity of asthma is increasing worldwide. Airway structural remodeling, particularly airway smooth muscle (ASM) remodeling, is one of the characteristics of chronic severe asthma (James and Wenzel

2007). ASM cell proliferation exists before the clinical onset of asthma, and increased ASM mass or migration of ASM cells is correlated to severity of asthma (Zuyderduyn et al. 2008). ASM cells retain remarkable plasticity and undergo profound and reversible changes in phenotype from a “contractile” one (mature phenotype) to a “proliferative, migratory and synthetic” one (pro-remodeling phenotype) in response to different stimuli (Halayko et al. 2008). The switching from the mature phenotype to pro-remodeling one is defined as modulation (Chamley-Campbell et al. 1979). The phenotypic modulation of ASM cells contributed to the pathology of asthma by proliferation, secretion of inflammatory mediators and matrix deposition (Hirst et al. 2000; Moir et al. 2003; Halayko et al. 2006), and it is expected to become a potential target for the prevention and treatment of airway remodeling because of its reversibility. In vitro ASMC phenotypic modulation occurred in the presence of PDGF, but the mechanism remained incompletely understood.

Epigenetic changes such as DNA methylation, histone deacetylation and chromatin remodeling played important roles in both physiological and pathological conditions. DNA methylation in asthma has been concerned in recent years (Breton et al. 2011), and growing evidence showed it played an important role in increased susceptibility of mice to allergic airway disease (Prescott and Clifton 2009; Hollingsworth et al. 2008). The reversible nature of DNA methylation makes it a promising therapeutic target. However, little is known about its role in PDGF-induced phenotypic modulation in ASM cells. Aza-CdR, a potent DNA methyltransferase inhibitor, has been extensively used for epigenetic research (Zhu et al. 2004). Meanwhile, referred to decitabine clinically, Aza-CdR has been widely used in attempts to reverse abnormal DNA hypermethylation in pathological conditions (Steensma et al. 2009; Yang et al. 2006; Leone et al. 2003). We undertook the present studies to determine the effect of Aza-CdR on PDGF-induced RASM cell phenotypic modulation including migration, contraction and proliferation.

Materials and methods

Cell culture and reagents

Primary cultures of rat airway smooth muscle (RASM) cells from 8-week-old SD rats were isolated and identified as previously described (Ning et al. 2011). Animal handling and experimental procedures were approved by the Animal Experiments Ethics Committee of the Second Military Medical University and performed according to the Helsinki convention for the use and care of animals. The inhibitors Ly294002 and U0126 were obtained from

CST (Beverly, MA, USA). Primary antibodies against $\text{sm-}\alpha\text{-actin}$ and PI3K δ (Epitomic, USA), SM22 α and MRLC (Santa Cruz, CA, USA), p-Akt and p-mTOR (Signalway Antibody, Pearland, USA), MLCK (Bioworld, USA), proliferating cell nuclear antigen (PCNA) (Proteintech, Wuhan, Hubei, China), p-ERK and DNMT1 (CST, Beverly, MA, USA) and horseradish peroxidase (HRP)-conjugated GAPDH (Kangcheng Biotech, China) were applied. HRP-conjugated secondary antibodies (goat anti-rabbit and rabbit anti-mouse) were obtained from Abcam (Cambridge, UK). Rhodamine-conjugated phalloidin was obtained from Cytoskeleton (USA). Fetal bovine serum (Australian-sourced product) and Dulbecco's modified Eagle's medium/F-12 were purchased from Gibco (USA). Recombinant human PDGF-BB was purchased from Pepro Tech (Rocky Hill, NJ) and dissolved in PBS with 0.1 % bovine serum albumin. Aza-CdR was purchased from Sigma-Aldrich (USA) and dissolved in DMSO. RASM cells were cultured in DMEM/F-12 supplemented with 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin and 10 % FBS at 37 °C with 5 % CO₂ in a humidified incubator. Experiments were performed with cells at passages 2–4. For pretreatment, cells were cultured in DMEM/F-12 containing 0.1 % FBS and 10 $\mu\text{mol/l}$ Aza-CdR (unless otherwise stated) for 24 h. When treated, cells were cultured in DMEM/F-12 containing 2 % FBS with 20 ng/ml PDGF and/or 10 $\mu\text{mol/l}$ Aza-CdR. The control groups were treated in parallel with 0.1 % vehicle DMSO. The Aza-CdR-containing medium was freshly replaced every 24 h.

Western blot analysis

RASM cells from different treatments were lysed in RIPA buffer (Pierce) supplemented with 1 mmol/l PMSF at 4 °C for 20 min. The extracts were centrifuged at 12,000 rpm for 20 min at 4 °C, and the supernatants were used as total cell lysates. Protein concentrations were measured by BCA Protein Assay kit (Pierce, USA). Twenty microgram total protein per well was separated by 10 % SDS-PAGE and transferred onto PVDF membrane. After blocked with 5 % BSA in TBST at room temperature for 1 h, membranes were incubated with appropriate dilutions of primary antibodies at 4 °C overnight. After washed extensively with TBST, membranes were incubated with corresponding HRP-conjugated secondary antibodies and then detected by ECL kit (Invitrogen). The levels of interest proteins were normalized to the corresponding abundance of GAPDH.

Contraction of RASMCs-embedded collagen gels

For each group, collagen solution was prepared by mixing 450 μL ice-cold rat tail tendon collagen type I (5 mg/ml, Shengyou biotechnology, Hangzhou, China) with 53 μL

10 × PBS. The pH of each solution was adjusted to 7.4 with 0.1 mol/l NaOH. RASM cells in serum-free DMEM/F-12 medium were added to the neutralized collagen at a final concentration of 1 × 10⁶ cells/ml. The whole process of mixture is performed on ice. The collagen cell suspensions were quickly pipetted into a 35-mm cell culture dish (NEST, Wuxi, China), kept still for 20 min at room temperature and then incubated in CO₂ incubator for 2 h for the collagen gels to polymerize. After RASMCs-embedded collagen gels were incubated in DMEM/F-12 medium containing 2 % FBS for 12 h, the cells were synchronized and pretreated for 24 h. Then the gels were treated with PDGF in the presence or absence of Aza-CdR and released from the culture dish to initiate contraction. The gels were captured by camera at the indicated time points, and the reduced gel area was measured to evaluate the RASMCs-mediated gel contractility.

Transwell assay

Migration experiments were performed in 24-well plates using Millicell hanging cell culture inserts with 8-μm-pore PET membrane separating the inner and the outer chambers. After synchronized and pretreated, RASM cells were digested and resuspended gently in DMEM/F-12 medium containing 2 % FBS with or without Aza-CdR and inoculated into the inner chambers at a concentration of 5 × 10⁴ cells/well. Six hundred microliter of DMEM/F-12 medium containing 10 % FBS with or without PDGF was pipetted into each outer chamber. After incubated at 37 °C for 5 h, the PET membrane with cells was fixed. The nonmigrated cells were removed gently with a cotton swab, and the cells migrated onto the lower surface of the membrane were stained with Wright's stain. Six random microscopic fields at a magnification of ×200 were observed, and the cells were counted. Each treatment was performed in triplicate.

Wound healing assay

RASM cells in 24-well plates were cultured in DMEM/F-12 medium containing 10 % FBS until 100 % confluence. Then the cells were synchronized and pretreated for 24 h. After scraped by a sterile 1-ml pipette tip, the cells were washed with PBS and then treated with PDGF in the presence or absence of Aza-CdR for 36 h. The wounds were photographed at baseline and 36 h.

Cell proliferation assay

RASM cells at 60–70 % confluence in a 96-well plate were synchronized and pretreated. The growth-arrested cells were treated with or without PDGF in the presence or

absence of Aza-CdR for another 48 h. The rate of cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described earlier (Ning et al. 2011).

Flow cytometry

Subconfluent cells were treated with PDGF in the presence or absence of Aza-CdR for 48 h. Cells were then harvested and fixed with ice-cold 70 % (v/v) ethanol for 24 h. After centrifugation at 2,000g for 5 min, the cell pellet was washed with PBS and resuspended in PBS containing propidium iodide (50 μg/ml), Triton X-100 (0.1 %, v/v) and DNase-free RNase (1 μg/ml). Cells were then incubated for 1 h in the dark at room temperature. DNA content was determined by flow cytometry using a FACScan flow cytometer (Becton–Dickinson, San Jose, CA).

Immunofluorescence

RASM cells that migrated into the scratch area in the wound healing assay were fixed in 4 % paraformaldehyde for 30 min, washed with PBS, permeabilized in 0.3 % Triton X-100, blocked with 5 % BSA routinely in turn and then incubated with rhodamine-conjugated phalloidin in PBS for 30 min. Cell nuclei were identified with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Immunofluorescence images were captured using a Nikon Fluorescence Microscope.

Real-time RT-PCR

Total RNA was extracted from cells of each group using Trizol (Invitrogen) according to the manufacturer's protocol, followed by reverse transcription PCR using a PrimeScript RT Reagent kit (Takara). The transcriptional change of *DNMT1* was quantified by real-time RT-PCR using SYBR Premix Ex Taq (Takara) in Rotor-Gene 6000 thermal cycler (Corbett). The relative transcriptional level of *DNMT1* was normalized to that of *β-actin*. The primer sequences for *DNMT1* were as follows: forward: 5'-tcgtcttggttgagacctatg-3', reverse: 5'-ccgcgactgcaataca-aca-3'. The primer sequences for *β-actin* were as follows: forward: 5'-tccgtaaagacctctatgcc-3', reverse: 5'-ggactcatcgctactctgctt-3'.

Statistical analysis

Statistical analyses were performed using SPSS13.0 (Statistical Package for Social Sciences, Chicago, USA). All data are expressed as mean ± SD. *p* < 0.05 was considered statistically significant.

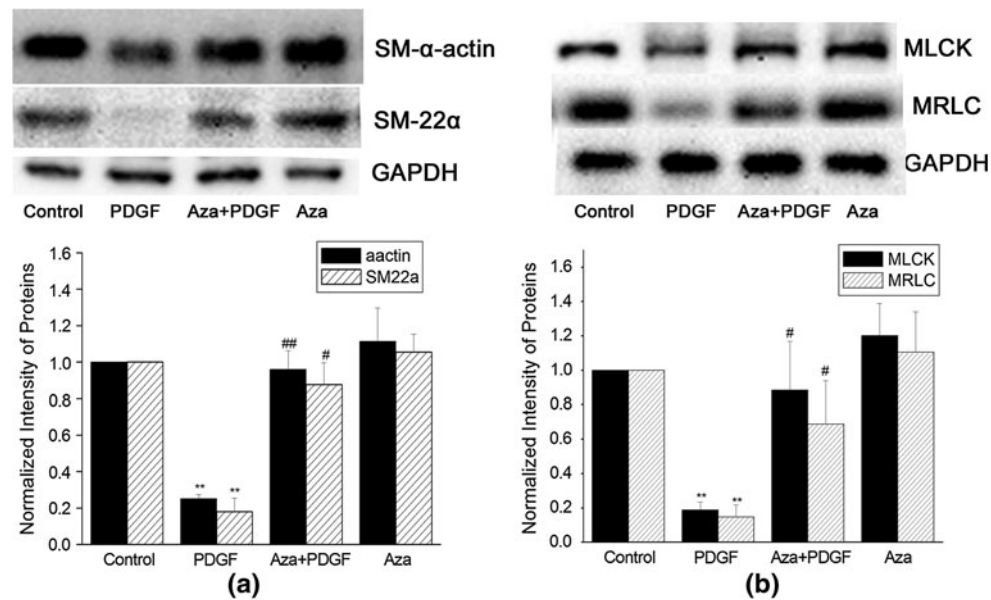


Fig. 1 Effect of Aza-CdR on the expressions of RASMC contractile markers and key regulatory proteins of contraction. **a** Representative Western blot analyses for contractile phenotypic markers SM22 α and sm- α -actin in RASM cells treated as indicated. **b** Representative Western blot analyses for MLCK and MRLC proteins. The levels of

interest proteins were normalized to the corresponding abundance of GAPDH. Data are presented as mean \pm SD from three independent experiments. * $p < 0.05$; ** $p < 0.01$ versus the control; # $p < 0.05$; ## $p < 0.01$ versus PDGF group. *MLCK* myosin light-chain kinase, *MRLC* myosin regulatory light chain

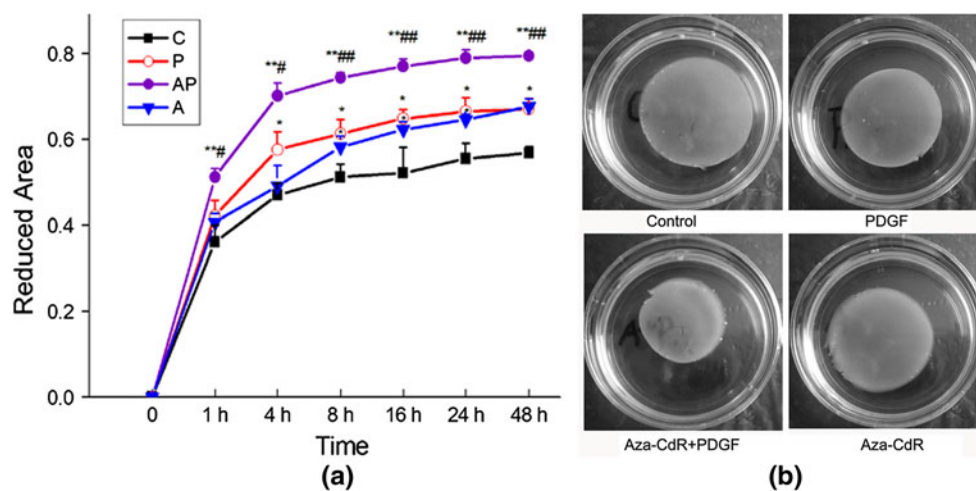


Fig. 2 Effect of Aza-CdR on RASMCs-mediated 3-dimensional collagen gel contraction. RASM cells were growth-arrested and pretreated for 24 h, followed by treatment with PDGF and/or Aza-CdR as indicated. The control group contained 0.1 % vehicle DMSO. **a** The reduced area of collagen gels mediated by RSAM cells in different treatments as indicated for the indicated times. The gel

contractility was expressed as the reduced area/initiative area. Data are presented as mean \pm SD of three independent experiments. * $p < 0.05$; ** $p < 0.01$ versus control; # $p < 0.05$; ## $p < 0.01$ versus PDGF group. **b** Representative pictures of 3-D collagen gel captured at 4 h after treatment

Results

Aza-CdR inhibited PDGF-induced down-regulation of contractile phenotype markers in RASM cells and increased cell contractility.

As a starting point for evaluating the effect of Aza-CdR on PDGF-induced RASM cell phenotypic modulation, we detected the expression changes of contractile phenotype

markers: SM22 α and sm- α -actin. As shown in Fig. 1a, PDGF significantly reduced the expressions of SM22 α and sm- α -actin proteins as compared to the control ($p < 0.01$), while pretreatment with Aza-CdR abrogated PDGF-induced down-regulation of both proteins. Meanwhile, we detected the expressions of myosin regulatory light-chain (MRLC) protein and myosin light-chain kinase (MLCK), the key activator of smooth muscle contraction (Somlyo and Somlyo

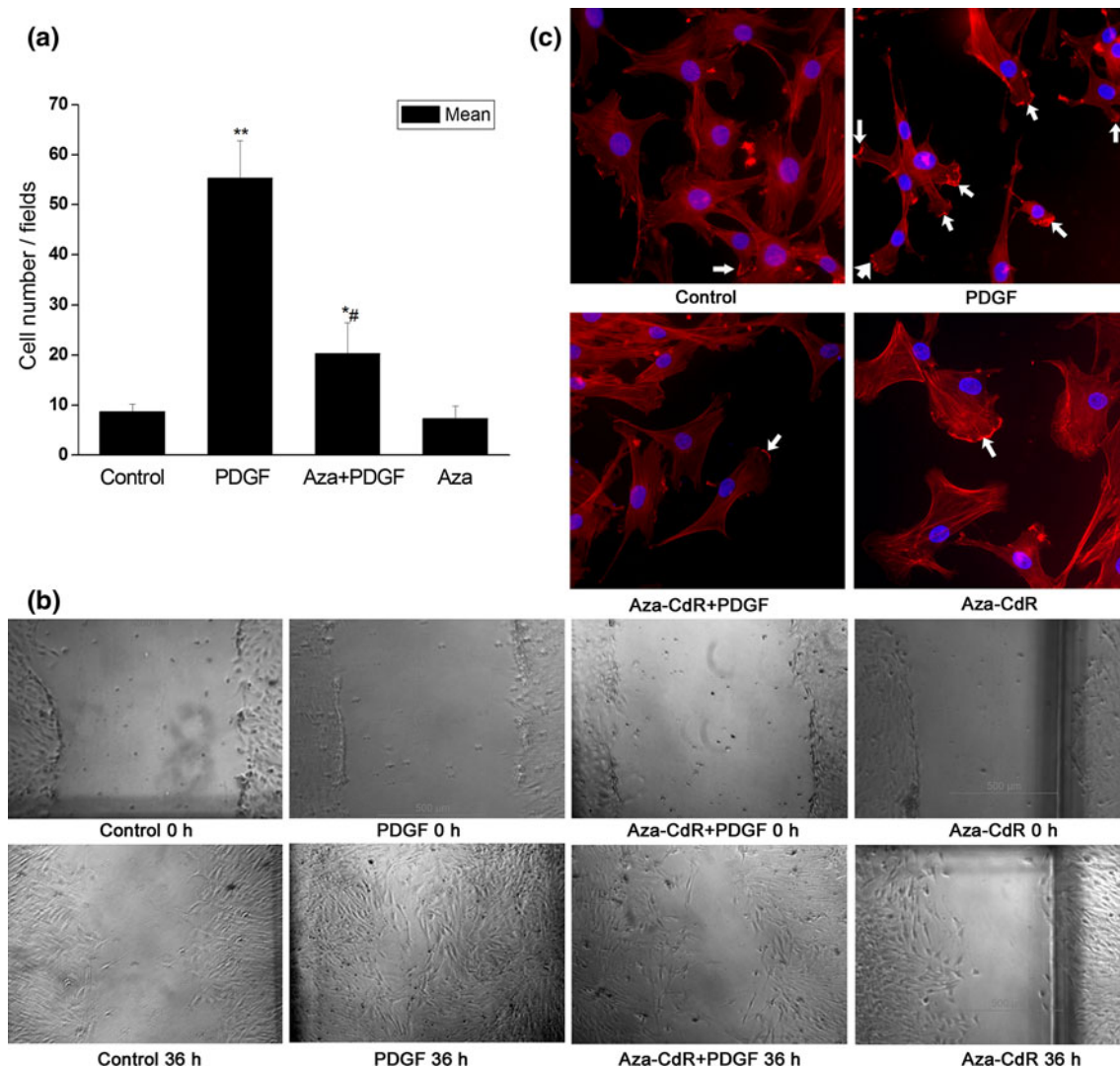


Fig. 3 **a** Effect of Aza-CdR on PDGF-induced RASM cell migration determined by transwell assay. RASM cells pretreated were inoculated into the inner chambers of the Millicell hanging inserts. The DMEM/F-12 medium out of the chambers contained 10 % FBS with or without PDGF. The cells that transmigrated were counted from six microscopic fields of each membrane after 5 h (magnification $\times 200$). Data from three replicates were presented as mean \pm SD of each microscopic field (* $p < 0.05$; ** $p < 0.01$ vs. control; # $p < 0.05$ vs. the PDGF group). **b** Aza-CdR inhibited PDGF-induced RASM cell migration determined by wound healing assay. When RASM cells reached 100 % confluence, the cells were growth-arrested and pretreated for 24 h. The monolayers were scratched to

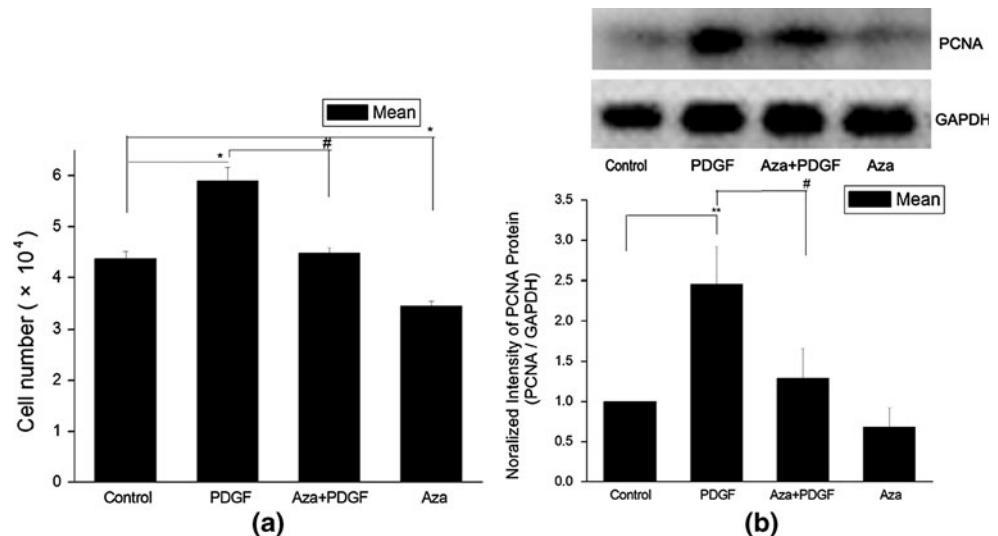
form a wound by a sterile 1-ml pipette tip. After washed with PBS, the cells were treated as indicated in DMEM/F-12 containing 2 % FBS for 36 h. The control group contained 0.1 % vehicle DMSO. The wound was photographed at 0 and 36 h (original magnification $\times 50$). **c** Effect of Aza-CdR on PDGF-induced F-actin rearrangement, lamellipodia formation and cell morphology change. Representative photomicrographs of RASM cells stained by rhodamine-conjugated phalloidin were captured by a Nikon Fluorescence Microscope. The cells were those migrated into the scratch area in the wound healing assay. The white arrows showed lamellipodia (original magnification $\times 200$)

1994). As shown in Fig. 1b, PDGF significantly down-regulated the expressions of MRLC and MLCK proteins as compared to the control ($p < 0.01$), while Aza-CdR pretreatment significantly inhibited the effect of PDGF on both proteins ($p < 0.05$ vs. the PDGF group). Aza-CdR alone slightly increased the expressions of all the four proteins.

The 3-dimensional (3-D) collagen matrix cell culture system (Grinnell 2000) was applied to detect the

contractility of RASM cells in vitro. The collagen gels of all groups contracted in a time-dependent manner after they were released (Fig. 2a). The basal reduced area of the control group reached 36.1 % 1 h after the gel was released. The cells treated with Aza-CdR or PDGF alone contracted the collagen lattices by 40.7 or 42.2 %, respectively, while Aza-CdR significantly reduced the gel area by 51.2 % in the presence of PDGF as compared to the

Fig. 4 Aza-CdR inhibited PDGF-induced RASM cell proliferation. **a** RASM cell proliferation was determined by MTT test after the cells were treated as indicated for 48 h. **b** Representative Western blot analysis for PCNA protein. Semi-quantitation of PCNA protein was normalized to the corresponding abundance of GAPDH. Data are presented as mean \pm SD from three independent experiments. * $p < 0.05$; ** $p < 0.01$ versus control; # $p < 0.05$ versus the PDGF group



PDGF-alone group ($p < 0.05$) or the control ($p < 0.01$). At 4 h the gel area of the PDGF plus Aza-CdR group dramatically reduced by 70.1 % versus that of the control by 47.0 % ($p < 0.01$) or that of the PDGF group by 57.6 % ($p < 0.05$). The gel area of the Aza-CdR-alone group reduced significantly as compared to that of the control after the gel was released to contract for 8 h ($p < 0.05$). All the gels shrank sharply on their release from the culture disks and tended to shrink steadily 4 h later.

Aza-CdR inhibited PDGF-induced RASM cell migration

The effect of Aza-CdR on PDGF-induced RASM cell migration was tested by transwell and wound healing assay. As what was expected, by transwell assay, PDGF significantly increased the number of cells transmigrated as compared to the control ($p < 0.01$), while Aza-CdR pretreatment significantly inhibited PDGF-induced RASM cell migration ($p < 0.05$ vs. PDGF group). Aza-CdR alone had little effect on the transmembrane migration of RASM cells (Fig. 3a).

As an alternative measure of cell motility, we also examined the effect of Aza-CdR on RASM cell migration toward PDGF in nondirectional wound healing assay (Liang et al. 2007). As shown in Fig. 3b, PDGF-stimulated RASM cells migrated into the wounded area and almost closed the wound at 36 h, while pretreatment with Aza-CdR slowed down the wound healing process induced by PDGF.

To further interpret the effect of Aza-CdR on PDGF-induced RASM cell migration, we observed the rearrangement of F-actin stained by rhodamine-conjugated phalloidin. As shown in Fig. 3c, PDGF induced actin rearrangement from regular F-actin fiber distributing along

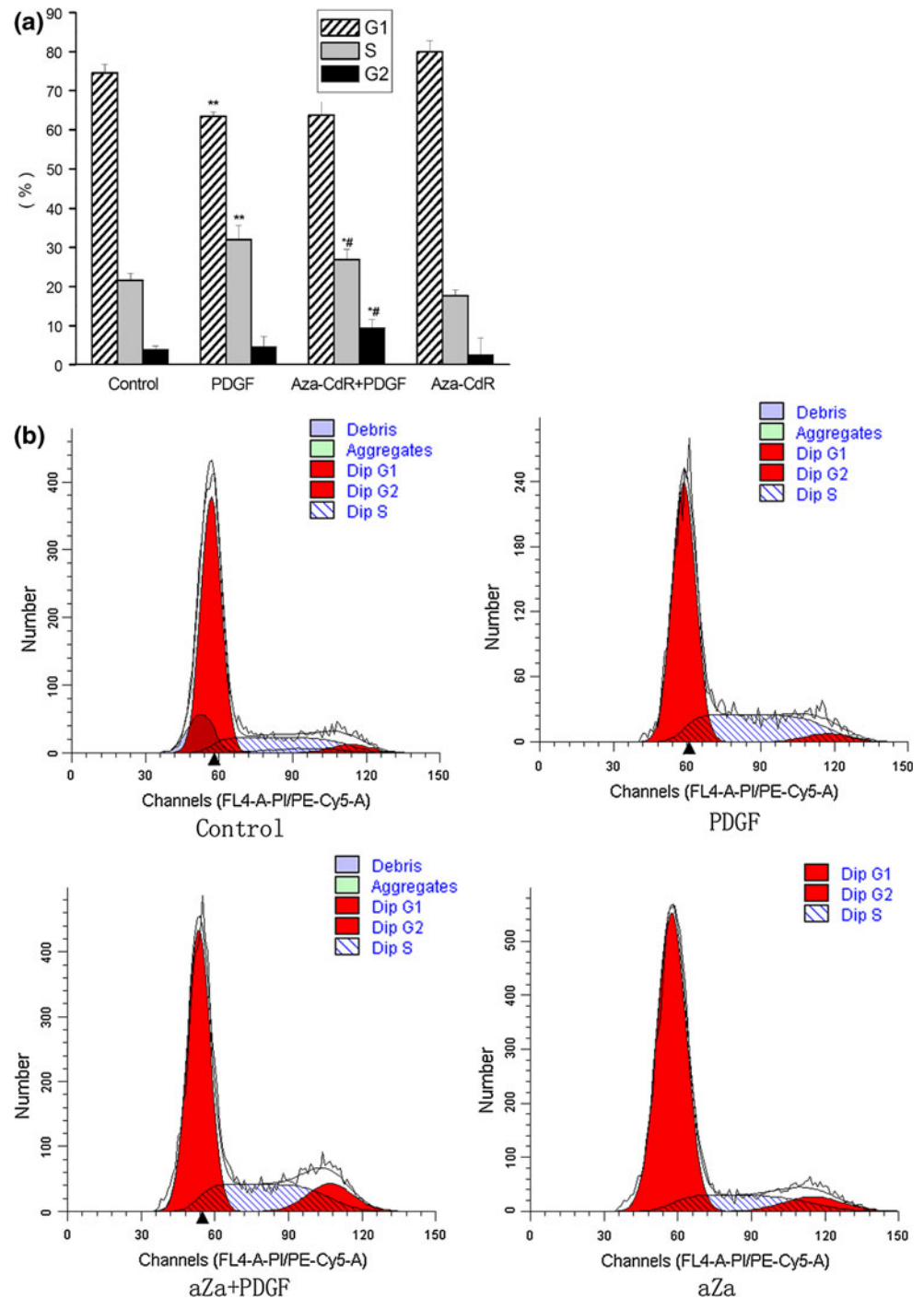
the longitudinal axis of cells into lamellipodia at the leading edges of migrating cells, while pretreatment with Aza-CdR attenuated PDGF-induced cytoskeleton rearrangement. The cells of the PDGF group tended to be elongated as compared to that of the control, while pretreatment with Aza-CdR inhibited PDGF-induced morphology change of RASM cells (Fig. 3c).

Aza-CdR inhibited PDGF-induced RASM cell proliferation

MTT assay was applied to determine RASM cell proliferation in response to different treatments. As shown in Fig. 4a, PDGF significantly stimulated RASM cell proliferation as compared to the control group ($p < 0.05$), while pretreatment with Aza-CdR significantly inhibited PDGF-induced RASM cell proliferation ($p < 0.05$ vs. PDGF group). The expression of PCNA in PDGF group was significantly up-regulated as compared to that in the control. Aza-CdR pretreatment significantly attenuated PDGF-induced up-regulation of PCNA protein, while Aza-CdR alone had little effect on the expression of PCNA protein (Fig. 4b). These results demonstrated that Aza-CdR pretreatment inhibited PDGF-induced RASM cell proliferation.

To further interpret the effect of Aza-CdR on PDGF-induced RASM cell proliferation, we investigated the cell cycle events by flow cytometry. As shown in Fig. 5a, PDGF induced the entry of RASM cells to synthesis phase (S-phase) to undergo proliferation, with 63.5 ± 1.0 % cells in G0/G1 phase ($p < 0.01$ vs. 74.54 ± 2.1 % in the control) and 32.0 ± 3.7 % in S-phase ($p < 0.01$ vs. 21.5 ± 1.9 % in the control), while pretreatment with Aza-CdR significantly arrested the cell cycle at G2/M phase (9.3 ± 2.4 % vs. 4.6 ± 2.7 % in the PDGF group, $p < 0.05$).

Fig. 5 Effect of Aza-CdR on PDGF-induced RASM cell cycle progression. **a** Cell cycle analysis was performed with 20,000 events per analysis using FACScan flow cytometer. Cell cycle distributions were expressed as the percentage of total cells. Data are mean \pm SD of three independent experiments; * $p < 0.05$; ** $p < 0.01$ versus control; # $p < 0.05$ versus the PDGF group. **b** Representative flow cytometric analysis of DNA content in RASM cells treated as indicated



Aza-CdR inhibited PDGF-induced up-regulation expression of DNMT1 in RASM cells

To explore the mechanism by which Aza-CdR affected PDGF-induced RASM cell phenotypic modulation, we detected the expression of DNMT1 stimulated by PDGF and the effect of Aza-CdR on it. PDGF stimulation resulted in time-dependent increases in DNMT1 mRNA (Fig. 6a) and

proteins (Fig. 6b). Different doses of Aza-CdR (0.1–100 $\mu\text{mol/l}$) dose-dependently depleted DNMT1 protein, and 1 $\mu\text{mol/l}$ Aza-CdR was able to deplete it effectively when RASM cells were treated for 48 h (Fig. 6c). Aza-CdR inhibited PDGF-induced up-regulation expression of DNMT1 both at transcriptional and at translational levels (Fig. 6d).

Depletion of DNMT1 protein by Aza-CdR did not affect PDGF-induced up-regulation of PI3K δ or phosphorylation

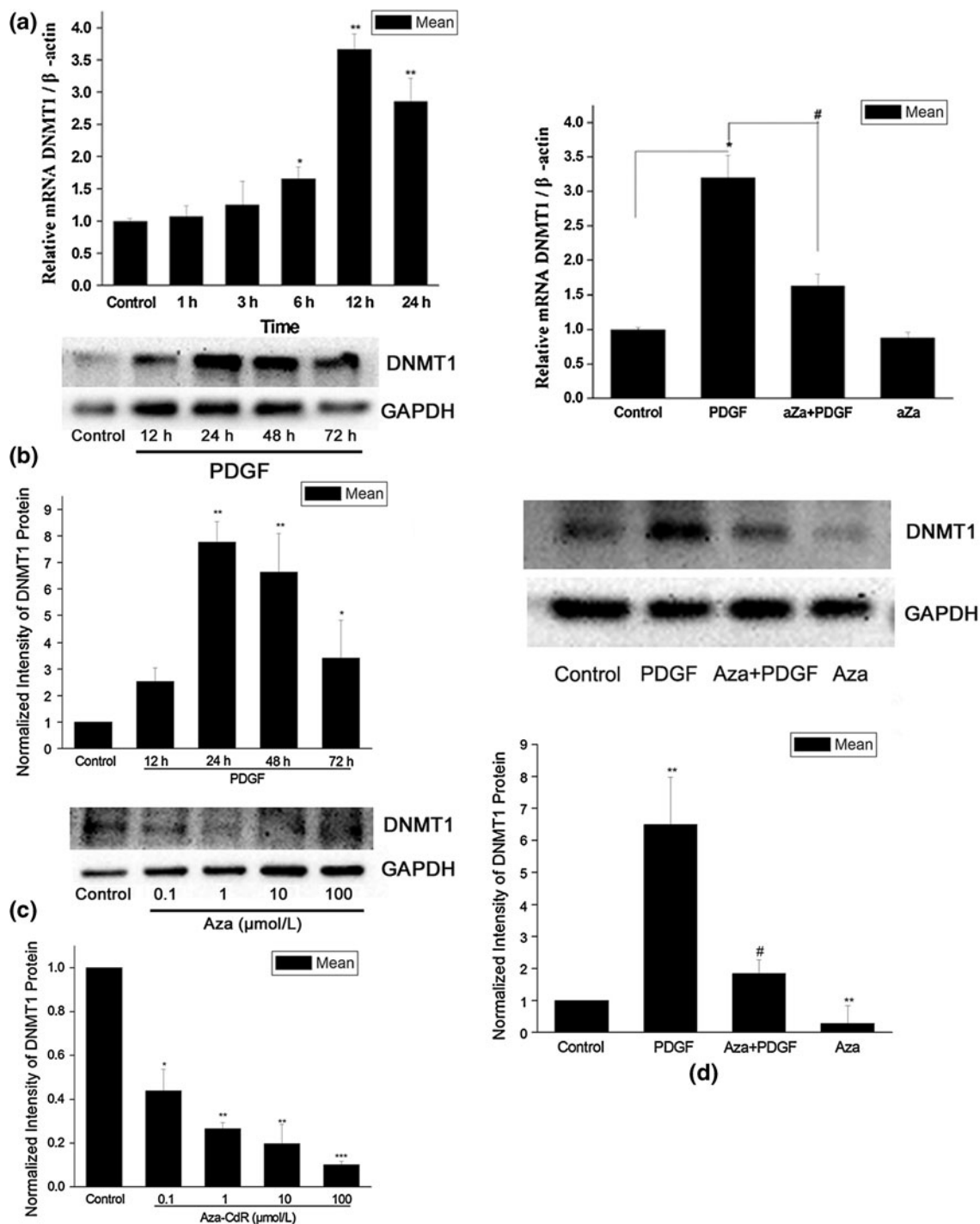


Fig. 6 Effect of Aza-CdR on DNMT1 gene expression in RASM cells. **a** Levels of DNMT1 mRNA in RASM cells stimulated by PDGF for the indicated times determined by real-time RT-PCR; **b** representative Western blot analysis for DNMT1 protein in RASM cells stimulated by PDGF for the indicated times. **c** DNMT1 protein was depleted in a dose-dependent manner by Aza-CdR for 48 h as determined by Western blot. **d** Aza-CdR pretreatment inhibited

PDGF-induced DNMT1 expression at both transcriptional and translational levels in RASM cells. The levels of DNMT1 mRNA or protein were normalized to the corresponding abundance of GAPDH. Data are mean \pm SD from three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus control; # $p < 0.05$ versus PDGF group

of Akt, mTOR and ERK, but when inhibitor Ly294002 or U0126 was applied to block the activation of Akt or ERK, PDGF-induced up-regulation of DNMT1 protein was

attenuated (Fig. 7). These results suggested that DNMT1 functioned downstream of PI3K-Akt-mTOR and ERK signaling pathway.

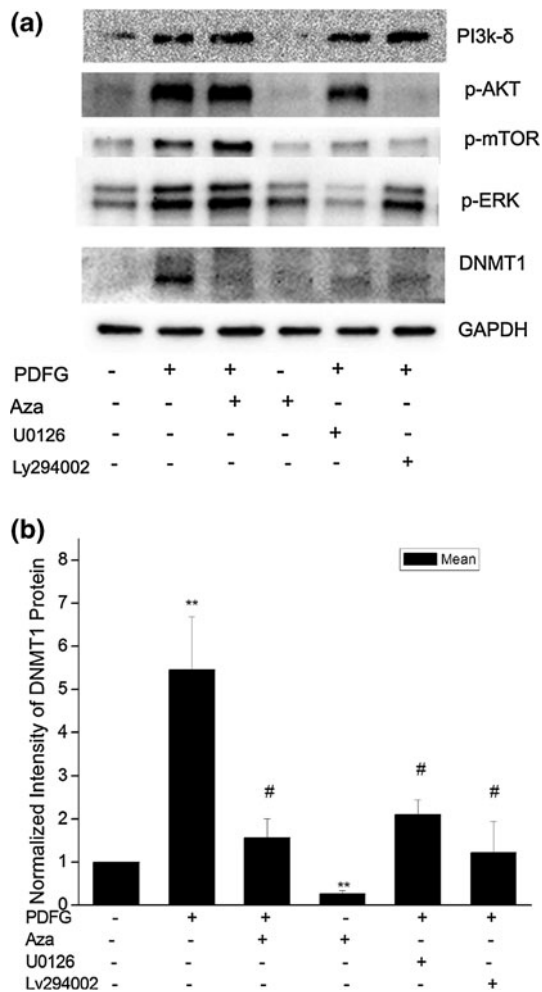


Fig. 7 PDGF-induced up-regulation of DNMT1 protein was mediated by the activation of PI3K/Akt and ERK signaling. RASM cells were growth-arrested and pretreated with Aza-CdR for 24 h or pretreated with 10 $\mu\text{mol/l}$ U0126 and 20 $\mu\text{mol/l}$ Ly294002, respectively, for 30 min, followed by PDGF stimulation for 24 h as indicated. The levels of DNMT1 protein were normalized to the corresponding abundance of GAPDH. Data are expressed as mean \pm SD from three independent experiments. ** $p < 0.01$ versus control; # $p < 0.05$ versus PDGF group

Discussion

Elevated levels of PDGF have been observed in asthma, and PDGF can induce ASM cell phenotypic modulation resulting in increased proliferation, migration and reduced contractility in vitro (Hirst et al. 2000; Carlin et al. 2003; Roscioni et al. 2010). The data presented here demonstrated for the first time that DNA methyltransferase inhibitor Aza-CdR inhibited PDGF-induced RASM cell switching from the contractile phenotype to a proliferative, migratory one. Aza-CdR promoted RASMCs-embedded 3-D collagen gel contraction, inhibited the effect of PDGF on PCNA expression and cell cycle progression, ultimately

cell proliferation, and inhibited PDGF-induced RASM cell migration by abrogating cell morphology change and cytoskeletal reorganization.

Airway remodeling is partially reversible in mild asthma but mostly irreversible in chronic severe asthma (Halwani et al. 2010). Airway smooth muscle remodeling is a dynamic process in long-standing asthma (Hassan et al. 2010). We focused our studies on airway smooth muscle cell phenotypic switching because it happened prior to asthma progression (Labonte et al. 2009) and played an important role in airway remodeling, and most of all, it was reversible under different circumstances (Ma et al. 1998).

DNA methylation contributes to expression regulation of many genes. Aberrant DNA promoter hypermethylation modification was reported to be implicated in the pathophysiology of asthma (Yang and Schwartz 2011; Tang et al. 2011). The results that Aza-CdR inhibited PDGF-induced decrease in contractile phenotype markers and increase in cell proliferation and migration in RASM cells revealed that DNA methylation was involved in PDGF-induced RASM cell phenotypic switching and Aza-CdR was promising in prevention and early intervention of ASM remodeling in asthma.

Mature airway smooth muscle cells in vivo are characterized by expressions of contractile marker proteins such as sm- α -actin, SM22 α and a low proliferative index. Freshly isolated ASM cells from adults exhibited a differentiated, contractile phenotype. When cultured in serum-rich conditions, ASM cells switched from a “contractile/differentiated” phenotype to a “proliferative/synthetic” one with reduced expressions of contractile proteins as passage numbers increased (Zuyderduyn et al. 2008). The RASM cells in this study were isolated from mature rats and applied at passages 2–4, so the RASMCs-embedded 3-D collagen gel contracted even in the control group. The results that Aza-CdR decreased RASMCs-embedded gel area significantly no matter in the presence or absence of PDGF as compared to the control demonstrated that Aza-CdR increased RASM cell contractility. It has been established that phosphorylation of MRLC at serine 19 by MLCK can activate myosin II to increase ASM cell contractility (Kamm and Stull 1985) and allow the cross-bridge to bind to the thin filaments and cycle to promote smooth muscle contraction (Wingard et al. 2001). The abundance expressions of MLCK and MRLC proteins in Aza-CdR-intervened or Aza-CdR-alone group were consistent with the contractile effect of Aza-CdR on RASMCs-embedded gels in the 3-D culture.

Hirota group (Hirota et al. 2011) reported that overexpression of PDGF-BB in vivo resulted in increased airway responsiveness and a decrease in lung compliance. As was reported elsewhere (Schaafsma et al. 2005), we also found that RASMCs-embedded matrix gel contracted greatly in

the PDGF group even though the cells dedifferentiated into the proliferative phenotype and the expressions of MLCK, MRLC and the contractile markers decreased significantly in the 2-D culture as compared to the control. Considering that the collagen gels were released from the culture disks and floated in the medium, the mechanism that led to the 3-D gel contraction in the PDGF group needs further study.

DNA methylation patterns are established and maintained by three major DNMTs: DNMT1, DNMT3A and DNMT3B. DNMT1 is the most abundant and considered to be the key maintenance of methyltransferase in mammalian cells. Ghoshal et al. (2004) reported that the gene expression profile of Aza-CdR-treated cells was very similar to that of DNMT1^{-/-} cells in their studies. Our results presented here suggested that Aza-CdR was able to selectively deplete DNMT1 in a dose-dependent manner in RASM cells. Although this study did not exclude the possibility that DNMT3A and DNMT3B might be implicated in, the result that selective depletion of DNMT1 protein by Aza-CdR inhibited PDGF-induced RASM cell phenotypic modulation demonstrated that DNMT1 played an important role in this process and revealed DNMT1-mediated DNA methylation was implicated in ASM remodeling in asthma.

It was reported that PDGF could activate PI3K-AKT-mTOR and ERK pathway (Chiou et al. 2006). Our results demonstrated that Aza-CdR pretreatment did not affect the activation of both signaling pathways, but DNMT1 functioned at downstream of PI3K-Akt-mTOR and ERK. As PI3K/Akt and ERK signaling pathways regulated many aspects of cellular function in a vast array of physiological and pathological processes such as migration, glucose metabolism and protein synthesis (Crowell et al. 2007; Torii et al. 2004), our results raised the possibility that inhibition of abnormal expression of DNMT1 could intervene ASM cell phenotypic modulation in asthma without affecting the normal physiological process mediated by PI3K-Akt-mTOR or ERK pathway. Considering that targeting DNMTs in cancer therapy was attractive and promising (Foulks et al. 2012), further studies on the role of DNMT1 in ASM remodeling are potentially important in developing therapeutic strategies to ameliorate or prevent asthma development.

In conclusion, Aza-CdR inhibited PDGF-induced RASM cells phenotypic switching from the mature phenotype to a proliferative, migratory one, increased cell contractility and attenuated PDGF-induced RASM cell proliferation and migration. We proposed for the first time that DNMT1 played a key role in PDGF-induced RASM cell phenotypic switching. Although the study of abnormal DNA methylation in PDGF-stimulated ASM cells is in its infancy, this work contributes to providing new insights into the mechanism of ASM remodeling and may be

helpful for developing effective treatments for airway remodeling in asthma.

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Conflict of interest None.

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