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Elevated protein arginine methyltransferase 1 expression regulates fibroblast motility in pulmonary fibrosis

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ABSTRACT

Objective: Idiopathic pulmonary fibrosis (IPF) is a devastating disease characterized by epithelial cell injury, fibroblast activation and excessive extracellular matrix deposition. Although protein arginine methyltransferase 1 (PRMT1) was found to regulate cell proliferation, differentiation and migration, its role in the development/progression of IPF has not yet been described.

Results: Expression of PRMT1 was elevated in lung homogenates from IPF patients. Significant upregulation of PRMT1 expression was also observed in the lungs of bleomycin-treated mice. Immunohistochemical analysis revealed PRMT1-positive staining in fibroblasts/myofibroblasts and alveolar type II cells of IPF lungs and in fibrotic lesions of bleomycin-injured lungs. Fibroblasts isolated from IPF lungs demonstrated increased PRMT1 expression. Interleukin-4 (IL-4), a profibrotic cytokine, enhanced the expression of PRMT1 and the migration of donor and IPF fibroblasts. Interference with the expression or the activity of PRMT1 diminished the migration of the cells in response to IL-4. Strikingly, even though the incubation of donor and IPF fibroblasts with IL-4 did not affect their proliferation, depletion, but not blockage of PRMT1 activity suppressed cell growth.

Conclusions: PRMT1 can contribute to the development of pulmonary fibrosis by regulating fibroblast activities. Thus, interference with its expression and/or activity may provide a novel therapeutic option for patients with IPF.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive interstitial lung disease with a median survival time of less than three years following diagnosis [1]. It is characterized by a histological pattern of usual interstitial pneumonia (UIP) with fibroblast foci consisting of activated, collagen producing myofibroblasts [2]. Although the fundamental mechanisms that initiate and propagate IPF have not yet been fully defined, it is believed that abnormal alveolar epithelial cell activation leads to the impairment of epithelial–mesenchymal crosstalk resulting in the accumulation of fibroblasts and extracellular matrix (ECM) proteins in the lung [2,3].

A growing body of evidence suggests that inflammatory cells recruited to the site of an injury might contribute to the fibrotic process through the production of profibrotic cytokines such as interleukin (IL)-4, IL-13 and transforming growth factor (TGF)-β [4]. In line with this notion, elevated levels of IL-4 were measured in IPF bronchial alveolar lavage fluid (BALF) and an increased expression of the IL-4 and IL-13 receptors was detected in IPF lung fibroblasts [5,6,7]. In addition, IL-4 deficient mice or mice with targeted disruption of the key components of the IL-4 signaling pathway were found to be protected against pulmonary fibrosis [8,9]. However, the anti-inflammatory therapies based on broad immunosuppression have been unsuccessful, and newly discovered pharmacotherapeutic options for IPF still remain limited [10,11].

Protein arginine methylation is a posttranslational modification which is catalyzed by a family of intracellular enzymes termed protein arginine methyltransferases (PRMT) [12]. In humans PRMTs have been classified into type I (PRMT1, 2, 3, 4/CARM1, 6 and 8) and type II (PRMT5, 7 and FBXO11) enzymes depending on their specific catalytic

Abbreviations: ADMA, asymmetric dimethylarginine; BALF, bronchoalveolar lavage fluid; Col, collagen; DAPI, 4′, 6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle's Medium; ECM, extracellular matrix; HIF, hypoxia-inducible factor; HLF, human lung fibroblast; IPF, idiopathic pulmonary fibrosis; IL, interleukin; LDH, lactate dehydrogenase; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; PBGD, porphobilinogen deaminase; PRMT, protein arginine methyltransferase; SDMA, symmetric dimethylarginine; proSP-C, prosurfactant protein C; SD, standard deviation; SEM, standard error of the mean; TBS, tris-buffered saline; UIP, usual interstitial pneumonia.

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activity [12]. Type I PRMTs catalyze the formation of asymmetric dimethylarginine (ADMA), whereas type II PRMTs form symmetric dimethylarginine (SDMA) [12,13]. PRMT1 is a predominant type I PRMT in mammalian cells that is responsible for approximately 85% of total protein arginine methylation [14]. Thus, PRMT1 is engaged in various cellular processes including signal transduction, gene transcription, DNA repair, and mRNA splicing [12]. Hence, dysregulated PRMT1 expression was found to contribute to the pathogenesis of a variety of diseases including cardiovascular and pulmonary disorders [13,15]. In our study, we sought to investigate whether dysregulated PRMT1

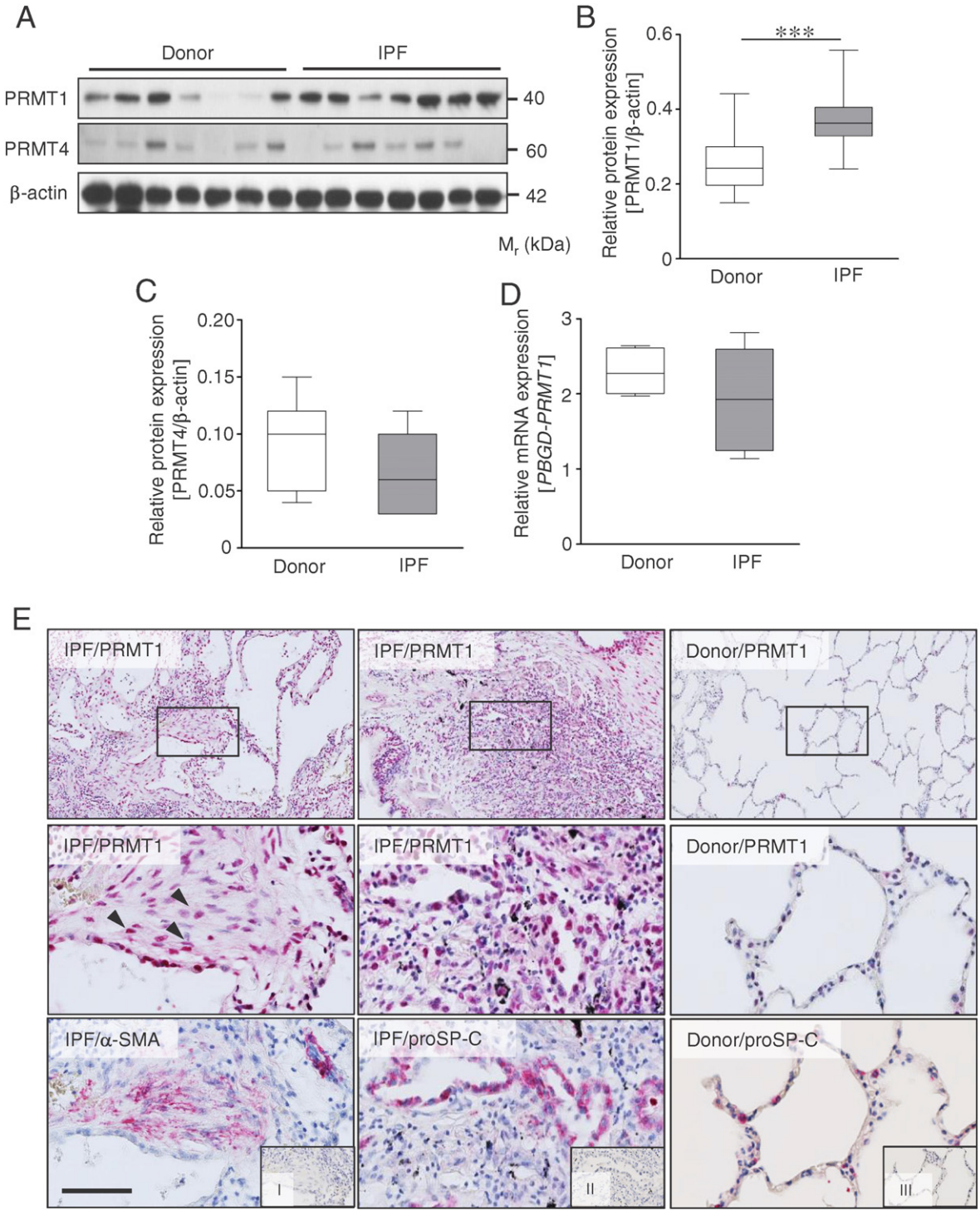


Fig. 1. Expression of PRMT1 is upregulated in the lungs of IPF patients. (A) Lung homogenates obtained from IPF patients and donors were subjected to Western blotting and the protein expression of PRMT1 and PRMT4 was analyzed. Representative donors (7/15) and patients (7/25) are shown. β-actin served as a loading control. (B, C) Densitometry analysis of (A); n = 15 (donors), n = 25 (IPF patients); ***, p ≤ 0.001. (D) PRMT1 mRNA expression in lung tissue of donors (n = 5) and IPF patients (n = 6) was assessed by RT-qPCR. RT-qPCR results are expressed as ΔCt using *PBGD* as the reference gene. (E) IPF and donor lung tissue sections were stained for PRMT1, α-SMA and proSP-C. Staining was performed on at least three independent sections obtained from at least three different patients or donors. Selected areas were magnified (boxed areas). Arrowheads indicate PRMT1-positive fibroblasts. Negative control was performed by omitting a primary antibody (I, II, III). Bar size 50 μm.

expression may regulate fibroblast activities and thus contribute to the development/progression of pulmonary fibrosis.

2. Materials and methods

2.1. Study population

The investigations have been conducted in accordance with the Declaration of Helsinki principles and were approved by the local ethics committee. Informed consent was obtained from either the patients or their next-of-kin. Lung tissue was obtained from 25 IPF patients who underwent lung transplantation at the Department of Cardiothoracic Surgery, Medical University of Vienna, Austria. IPF diagnosis was based on both clinical criteria as well as proof of a usual interstitial pneumonia (UIP) pattern in histopathological specimens from the explanted lungs [16]. Non-utilized donor lungs served as a control (n = 15). Inflammatory processes were not observed in the donor lungs following histopathological evaluation. For demographic and clinical characteristics of the patient cohort, please refer to [17].

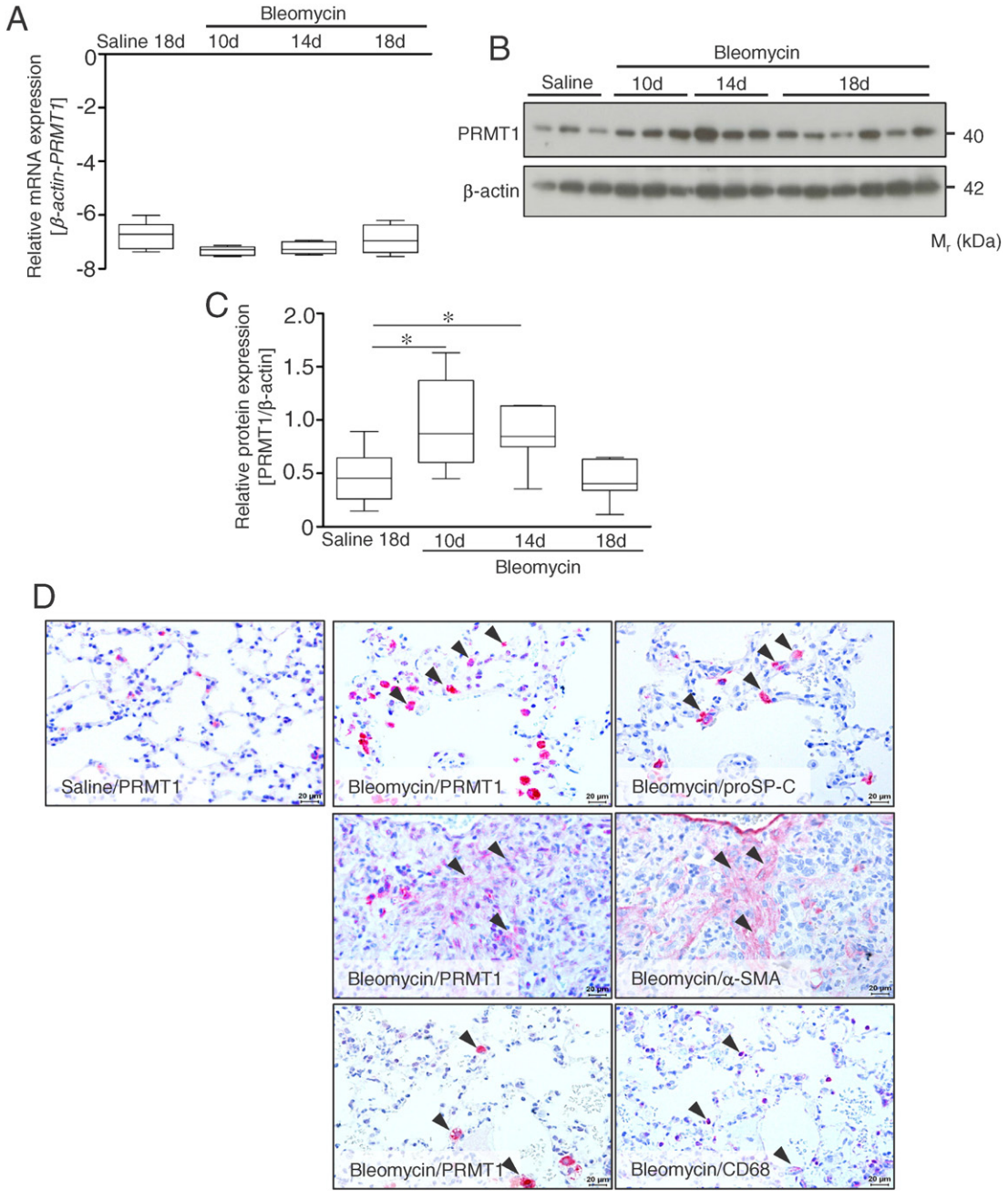


Fig. 2. Pulmonary PRMT1 expression is enhanced in a mouse model of bleomycin-induced lung fibrosis. (A) PRMT1 mRNA expression in lung tissue of control (saline 18d) and bleomycin-treated mice at day 10, 14 and 18 post-application was assessed by RT-qPCR. RT-qPCR results are expressed as ΔCt using β-*actin* as the reference gene; n = 4–5 mice/group. (B) PRMT1 protein expression in lung homogenates of control (saline 18d) and bleomycin-treated mice at day 10, 14 and 18 post-application. Representative control mice (3/7) and bleomycin-challenged animals (3–5/7–9) are shown. (C) Densitometry analysis of (B); n = 7–9 mice/group; *, p ≤ 0.05. (D) Representative lung tissue sections from control (saline) and bleomycin-treated mice at day 18 post-application stained for PRMT1, proSP-C, α-SMA and CD68. Arrowheads indicate cells that demonstrate a colocalization of PRMT1 with α-SMA, proSP-C and CD68. Bar size 20 μm.

2.2. Bleomycin administration

Six-ten week old mice (C57BL/6 N) were used in all experiments. Bleomycin (Almirall Prodesfarma, Barcelona, Spain) was applied by microsprayer (Penn-Century Inc., Philadelphia, PA) as a single dose of 5 U/kg bw. Age and sex-matched controls received saline. At different time points after bleomycin application, mice were euthanized with a lethal dose of pentobarbital. The animals were kept in accordance with NIH guidelines and the experiments were undertaken with the permission of the local authorities.

2.3. Cell isolation

Primary human lung fibroblasts (HLF) were isolated from donor (n = 8) and IPF (n = 8) lungs as previously described [17].

2.4. Cell culture and cell stimulation

HLF were cultured in a 5% CO_2 incubator at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Cramlington, UK) and 1% Penicillin/Streptomycin (Invitrogen Life Technologies). Before stimulation with different concentrations of IL-4 (R&D Systems GmbH, Wiesbaden, Germany), HLF were incubated overnight in serum-free DMEM/F12. Treatment of HLF with 10 μM AMI-1 (Sigma-Aldrich, Taufkirchen, Germany) was performed in DMEM/F12 supplemented with 10% FBS.

2.5. Protein isolation and Western blotting

For details, please refer to the online supplement.

2.6. Immunocytochemistry/immunohistochemistry

Immunocytochemical and immunohistochemical analyses were performed as previously described [17]. For details, please refer to the online supplement.

2.7. Antisense oligonucleotides

Commercially available siRNA sequence directed against human PRMT1 and a universal negative-control siRNA (both from Santa Cruz Biotechnology, Santa Cruz, CA) were employed. Overnight serum-starved cells were transfected with siRNA (100 nM) using the siLentFect™ Lipid transfection reagent (Bio-Rad Laboratories GmbH, Munich, Germany) in accordance with the manufacturer's instruction. The siRNA-mediated downregulation of PRMT1 expression was assessed 12 h and 24 h post-transfection by real-time PCR (qPCR) and Western blotting, respectively.

2.8. RNA isolation and RT-qPCR

RNA isolation and RT-qPCR were performed as previously described [17]. Details are outlined in the online supplement.

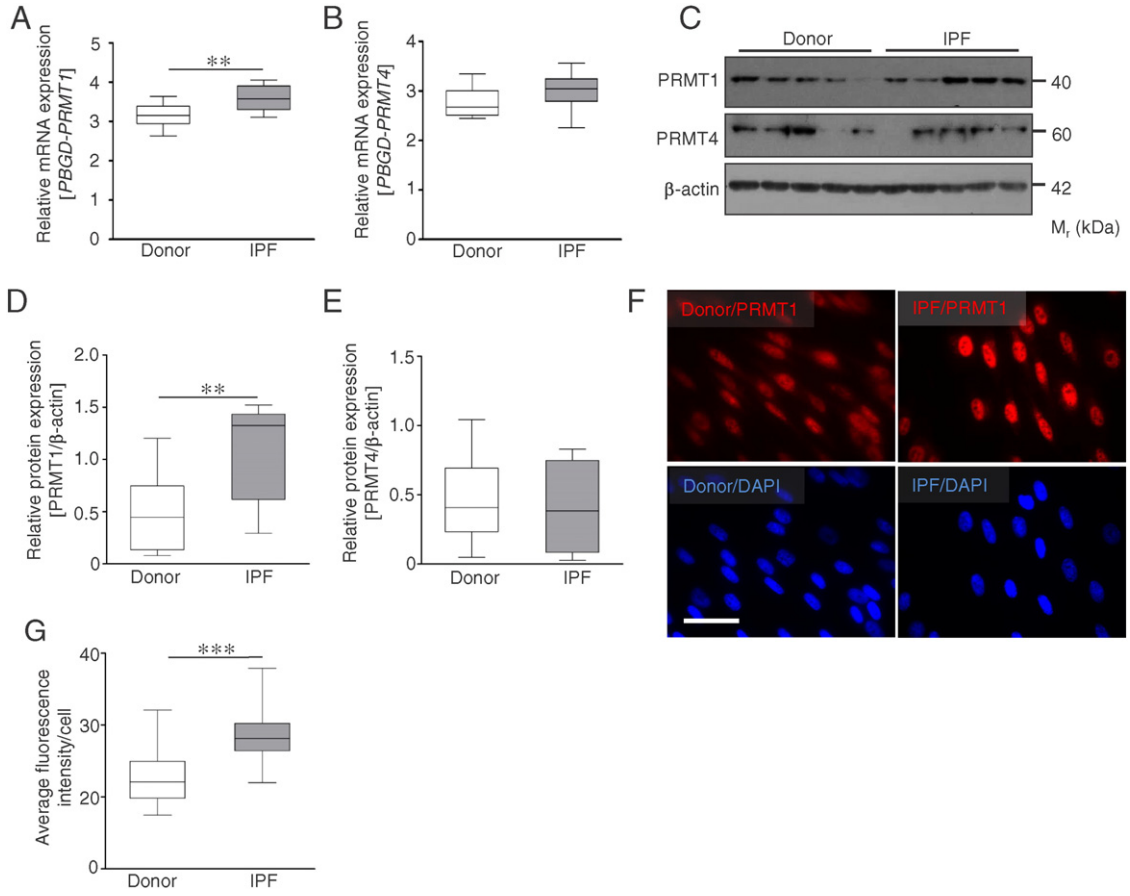


Fig. 3. Expression of PRMT1 is increased in IPF lung fibroblasts. (A, B) PRMT1 (A) and PRMT4 (B) mRNA expression in fibroblasts isolated from donor (n = 8) and IPF (n = 10) lungs as assessed by RT-qPCR. RT-qPCR results are expressed as ΔCt using *PBGD* as the reference gene; **, p ≤ 0.01. (C) PRMT1 and PRMT4 protein expression in human lung fibroblasts isolated from donor and IPF lungs. β-actin served as a loading control. (D, E) Densitometry analysis of (C); n = 8; **, p ≤ 0.01. (F) Human lung fibroblasts isolated from IPF and donor lungs stained for PRMT1. DAPI was used to demonstrate the nuclei. Bar size 20 μm. (G) Semiquantitative analysis of (F); n ≥ 20; ***, p ≤ 0.001.

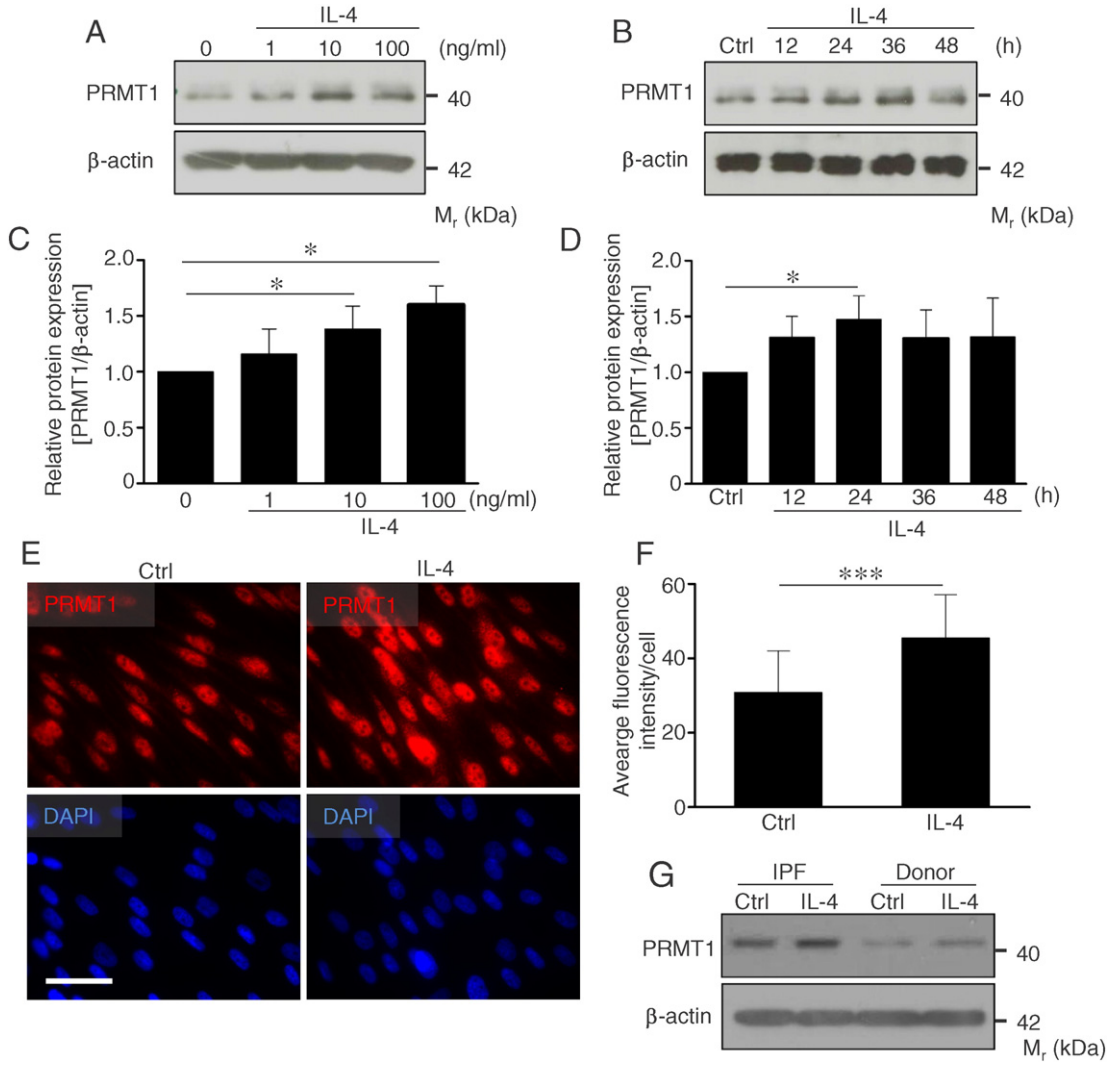


Fig. 4. IL-4 stimulates PRMT1 expression in a dose- and time-dependent manner in HLF. (A) Fibroblasts were isolated from human donor lungs and stimulated with different concentrations of IL-4. After 24 h cell crude extracts were subjected to Western blotting. Representative Western blots are shown (n = 6). β-actin served as a loading control. (B) Human lung fibroblasts stimulated with 10 ng/ml IL-4 were harvested at indicated time points and subjected to Western blotting using an anti-PRMT1 antibody. Representative Western blots are shown (n = 5). β-actin served as a loading control. (C, D) Densitometry analysis of (A) and (B), respectively; *, $p \le 0.05$. (E) Human lung fibroblasts isolated from donor lungs, stimulated with 10 ng/ml IL-4 for 36 h and stained for PRMT1. DAPI was used to demonstrate the nuclei. Bar size 20 μm. (F) Semiquantitative analysis of (E); n ≥ 20; ***, $p \le 0.001$. (G) Western blot analysis of PRMT1 expression in donor and IPF lung fibroblasts stimulated with IL-4 (10 ng/ml) for 36 h. β-actin served as a loading control. Ctrl, control.

2.9. Lactate dehydrogenase (LDH) release

HLF were transfected with PRMT1 or control siRNAs, or treated with 10 μM AMI-1 or 0.01% DMSO (vehicle control). After 24 h LDH release into cell culture media was measured using a Cytotoxicity Detection Kit (Roche Diagnostic Roche Applied Science, Mannheim, Germany) in accordance with the manufacturer's instruction. HLF treated with 1% Triton X-100 for 3 min served as a positive control.

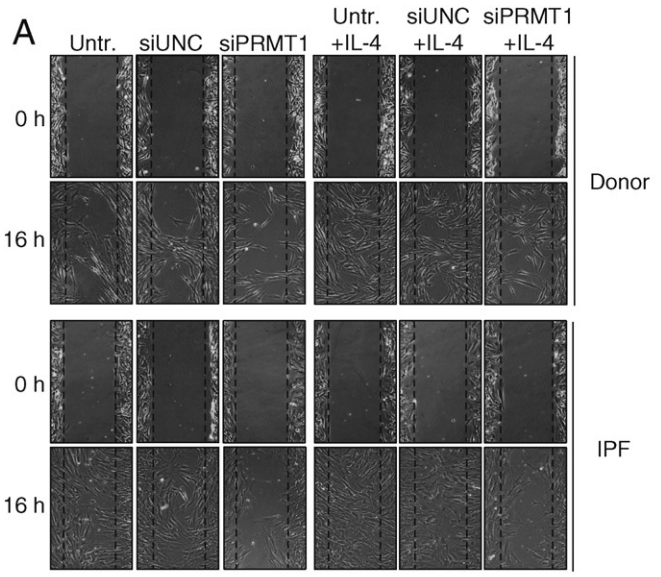
2.10. Scratch wound-healing assay

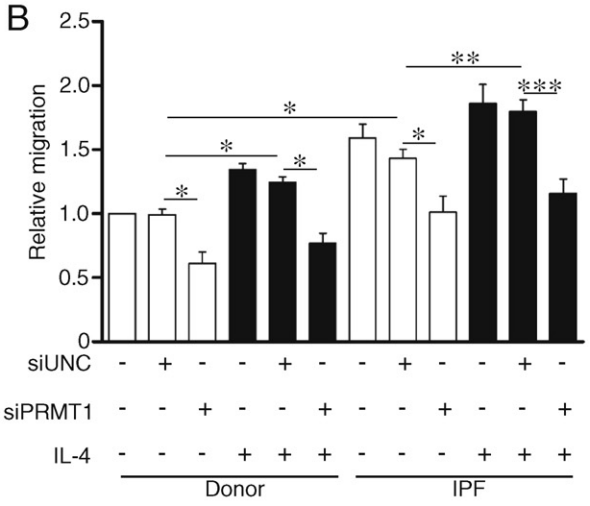
Cells were seeded into 6-well tissue culture plates at a density of 1×10^6 cells/well and starved overnight. siRNA-transfected HLF, AMI-treated cells and respective controls were stimulated with 10 ng/ml IL-4. Twenty four hours after stimulation, HLF were seeded into silicone inserts with a defined cell-free gap (Ibidi, Planegg–Martinsried, Germany). Images of the gap area were taken after removing the inserts at 0 and 16 h. The rate of wound closure was assessed by counting the cells that had migrated into the same-sized square fields.

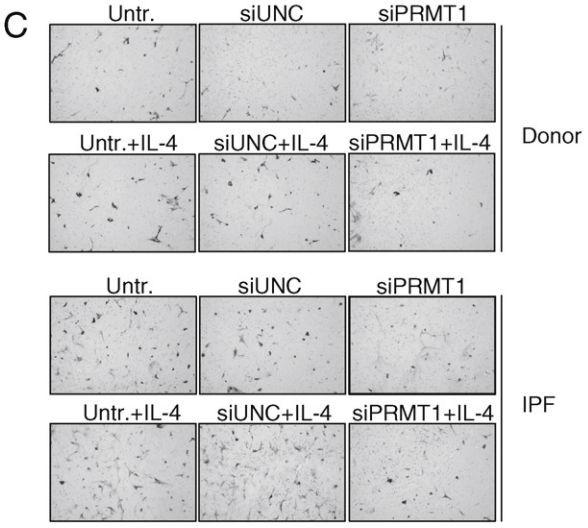
2.11. Transwell-filter migration assay

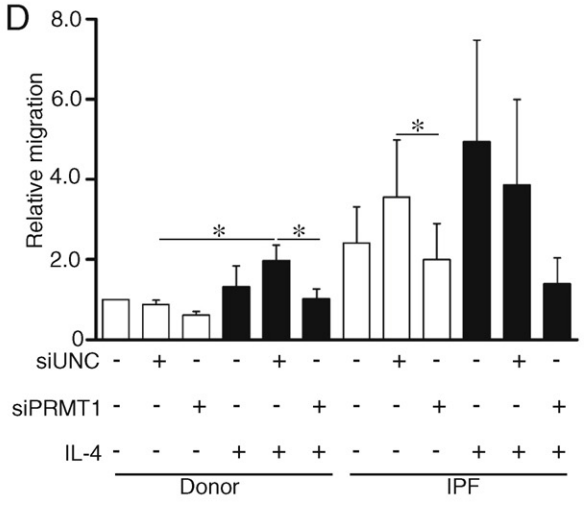
A transwell-filter migration assay was performed as previously described [18]. Further details are provided in the online supplement.

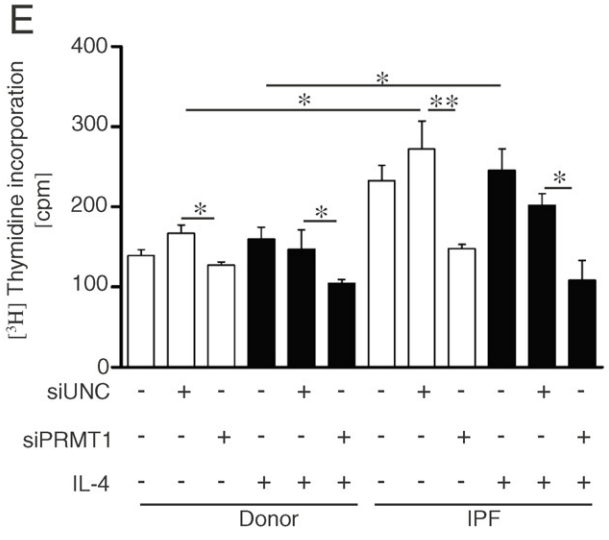
Fig. 5. PRMT1 knockdown inhibits IL-4-driven lung fibroblast migration. (A) Donor and IPF human lung fibroblasts were transfected with universal control siRNA (siUNC) or RNA directed against PRMT1 (siPRMT1). After 24 h cells were stimulated with IL-4 (10 ng/ml) for 16 h and subjected to a scratch wound healing assay in serum-free DMEM/F12. Representative pictures from the scratch wound healing assay at time 0 h and 16 h are shown. (B) The rate of wound closure was assessed by counting the cells that had migrated into the same-sized square fields. Data represent mean values ± SEM, n = 4; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (C) Fibroblasts were transfected with siUNC or siPRMT1. After 24 h cells were plated onto the upper side of the transwell-filter membrane and allowed to invade the bottom side overnight. Representative images of the cells on the underside of the transwell-filter membrane are demonstrated. (D) The invading cells were counted. Data represent mean values ± SEM, n = 3; *, $p < 0.05$. (E) Proliferation of siUNC- and siPRMT1-transfected donor and IPF HLF as assessed by [^{3}H] thymidine incorporation. Data represent mean values ± SEM; n = 3; *, $p \le 0.05$; **, $p \le 0.01$. (F) Efficiency of PRMT1 knockdown as assessed by Western blotting using anti-PRMT1 and anti-ASYM24 antibodies. β-actin served as a loading control. Asterisks indicate reduction in protein methylation. Untr., untreated.

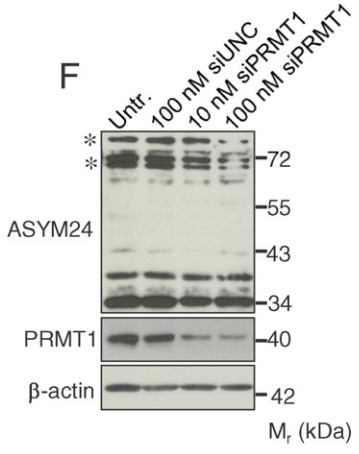

A
Untr.
siUNC
siPRMT1
Untr. +IL-4
siUNC +IL-4
siPRMT1 +IL-4
0 h
16 h
Donor
IPF

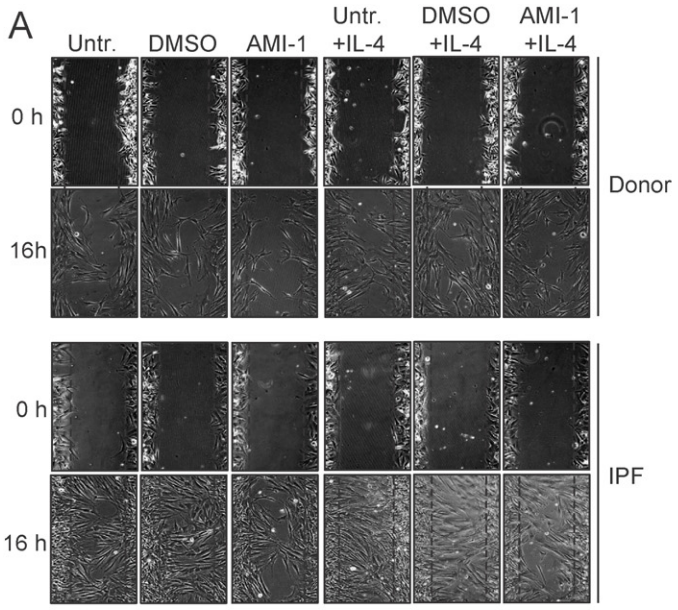

B
Relative migration
siUNC
siPRMT1
IL-4
Donor
IPF

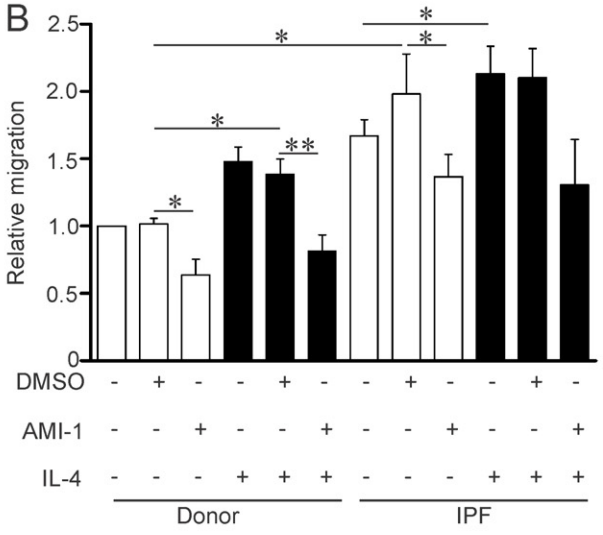

C
Untr.
siUNC
siPRMT1
Untr.+IL-4
siUNC+IL-4
siPRMT1+IL-4
Donor
IPF

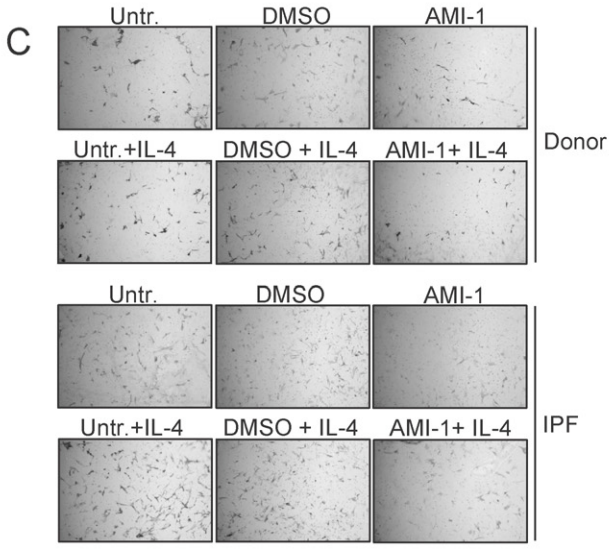

D
Relative migration
siUNC
siPRMT1
IL-4
Donor
IPF

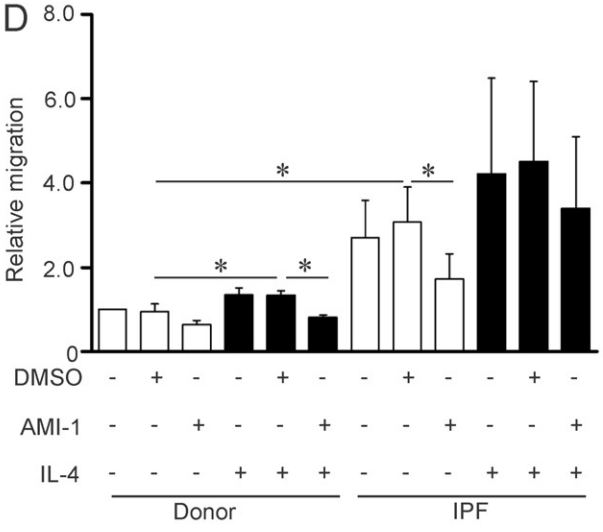

E
[3H] Thymidine incorporation [cpm]
siUNC
siPRMT1
IL-4
Donor
IPF

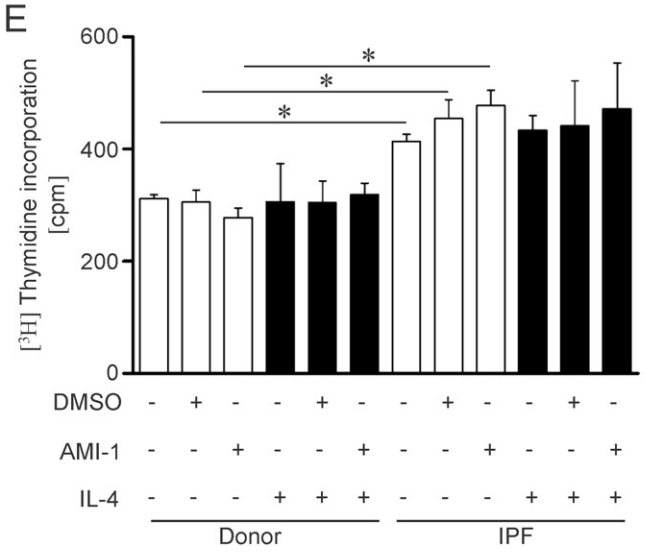

F
Untr.
100 nM siUNC
10 nM siPRMT1
100 nM siPRMT1
ASYM24
PRMT1
β-actin
Mr (kDa)

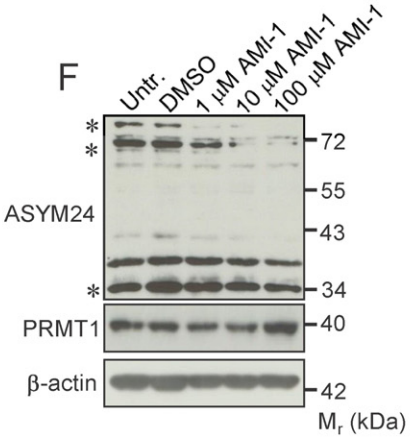

A
Untr.
DMSO
AMI-1
Untr. +IL-4
DMSO +IL-4
AMI-1 +IL-4
0 h
16h
Donor
0 h
16 h
IPF


B
Relative migration
2.5
2.0
1.5
1.0
0.5
0
DMSO
AMI-1
IL-4
Donor
IPF


C
Untr.
DMSO
AMI-1
Untr.+IL-4
DMSO + IL-4
AMI-1+ IL-4
Donor
IPF


D
Relative migration
8.0
6.0
4.0
2.0
0
DMSO
AMI-1
IL-4
Donor
IPF


E
[3H] Thymidine incorporation [cpm]
600
400
200
0
DMSO
AMI-1
IL-4
Donor
IPF


F
Untr.
DMSO
1 μM AMI-1
10 μM AMI-1
100 μM AMI-1
ASYM24
PRMT1
β-actin
72
55
43
34
40
42
Mr (kDa)

2.12. Proliferation assay

Proliferation of HLF was determined by a DNA synthesis assay based on the uptake of [^{3}H]thymidine (Amersham Biosciences, Freiburg, Germany). Twenty four hours after transfection of HLF with siRNAs or after overnight exposure of cells to AMI-1 and/or IL-4, the cells were seeded onto a 48-well tissue culture plate. Subsequently, the cells were pulsed with 1.2 μCi/ml [^{3}H]thymidine. After 16 h, the cells were solubilized in 0.5 M NaOH and [^{3}H]thymidine incorporation was determined by liquid scintillation spectrometry.

2.13. Statistics

The statistical analyze was performed using GraphPad Prism, version 5.02 for Windows (GraphPad Software, La Jolla, CA). Patient data are reported as medians and interquartile ranges, and are shown in box plots according to Tukey's definition. Differences between groups were assessed using the Student's *t*-test or the Mann–Whitney *U*-test. When three or more groups were compared, analysis of variance (ANOVA) followed by Tukey's *post hoc* test was used. Experimental data are presented as mean (± SEM). A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. PRMT1 expression is upregulated in IPF lungs

Initially, we investigated the expression of PRMT1 in the lung tissue of IPF patients. While elevated PRMT1 protein expression was noted in IPF lungs as compared to donor lungs (Fig. 1A, B), PRMT1 mRNA levels remained unchanged (Fig. 1D). Since PRMT4 shares structural and functional similarities with PRMT1 [19], we also analyzed the expression of this enzyme in IPF lungs. However, no changes in PRMT4 protein levels were observed (Fig. 1C). Immunohistochemical analysis of donor lung sections demonstrated a PRMT1-positive staining in alveolar (Fig. 1E) and bronchial (data not shown) epithelial cells. In IPF lungs, PRMT1 was associated with hyperplastic alveolar epithelial type II cells (ATII), bronchial epithelial cells and fibroblasts/myofibroblasts in fibrotic foci (Fig. 1E).

3.2. PRMT1 expression is increased in the lungs of bleomycin-treated mice

Next, we investigated the expression of PRMT1 in the lungs of mice exposed to bleomycin. Although the mRNA level of PRMT1 was not changed in the lungs of affected animals (Fig. 2A), PRMT1 protein expression was upregulated at day 10 and 14 post-application (Fig. 2B, C). Immunohistochemical inspection of bleomycin-challenged mouse lungs revealed PRMT1- positive staining in monocyte/macrophages (identified by CD68 staining), ATII (identified by proSP-C staining) and myofibroblasts (identified by α-SMA staining) suggesting that PRMT1 is ubiquitously expressed in the injured lung (Fig. 2D).

3.3. IPF fibroblasts are characterized by increased PRMT1 expression

As fibroblasts are key effector cells in the development of fibrosis [2], we focused further studies on the role of PRMT1 in the regulation of various processes in this cell population. First, we sought to characterize PRMT1 and PRMT4 expression in fibroblasts isolated from IPF and donor lungs. IPF HLF demonstrated enhanced PRMT1 mRNA (Fig. 3A) and protein expression (Fig. 3C, D) as compared to donor HLF. In contrast, no changes in PRMT4 mRNA (Fig. 3B) and protein (Fig. 3C, E) levels were observed. Immunocytochemical analysis verified augmented PRMT1 expression in IPF HLF and revealed a predominant localization of PRMT1 in the nucleus and a moderate PRMT1 abundance in the cytoplasm (Fig. 3F). Analysis of the immunostaining intensity confirmed increased PRMT1 expression in IPF HLF when compared to donor cells (Fig. 3G).

3.4. IL-4 stimulates PRMT1 expression in human lung fibroblasts

As previous studies demonstrated the induction of PRMT1 expression following stimulation of lung adenocarcinoma cells with IL-4 [20], we next investigated whether exposure of HLF to this profibrotic cytokine regulates PRMT1 synthesis. Treatment of donor HLF with IL-4 stimulated PRMT1 protein expression in a dose- and time-dependent manner (Fig. 4A–D). The maximal PRMT1 expression was achieved within 24 h of stimulation (Fig. 4D). Immunocytochemical analysis also revealed enhanced PRMT1 staining in HLF exposed for 24 h to IL-4 when compared to unstimulated cells (Fig. 4E). Analysis of the immunostaining intensity validated these results (Fig. 4F). An increase in PRMT1 protein expression was also observed when IPF fibroblasts were exposed to IL-4 (Fig. 4G).

3.5. IL-4-induced PRMT1 expression regulates donor and IPF fibroblast motility

IPF is a devastating disease that is characterized by an excessive proliferation and migration of parenchymal fibroblasts [2]. Thus, in further studies we evaluated the importance of altered PRMT1 expression for motility of HLF. A scratch wound healing assay revealed the increased migration of IPF HLF as compared to donor HLF (Fig. 5A, B). The addition of IL-4 enhanced the migration of both cell types (Fig. 5A, B). The depletion of PRMT1 reduced the motility of unstimulated and IL-4-stimulated donor and IPF HLF; the latter to the level observed in the cells isolated from donor lungs (Fig. 5A, B). Similar effects were observed when the transwell filter migration assay was applied (Fig. 5C, D). Here, a clear tendency towards a reduction of PRMT1-depleted HLF migration under basal conditions and upon IL-4 stimulation was observed (Fig. 5C, D). Furthermore, although PRMT1 knockdown of donor HLF resulted in downregulation of the expression of profibrotic genes such as *Col1a1* and *MMP-2*, (Supplementary Fig. 1A), it did not have any effect on the MMP-2 and MMP-9 activities (Supplementary Fig. 1B). The expression of *Col3a1* was not influenced by PRMT1 knockdown (Supplementary Fig. 1A). Strikingly, even though the incubation of HLF with IL-4 did not affect donor and IPF HLF proliferation, the depletion of PRMT1 significantly reduced cell growth (Fig. 5E). PRMT1 knockdown neither changed cell morphology nor altered cell survival (Supplementary Fig. 2A, B, respectively). The efficiency of PRMT1 knockdown was assessed by RT-qPCR (Supplementary Fig. 1A) and Western blotting using an anti-ASYM24 antibody which recognizes asymmetrically methylated proteins (Fig. 5F). Altogether, these results demonstrate the pivotal

Fig. 6. Inhibition of PRMT activity blocks IL-4-induced lung fibroblast migration. (A) Primary human lung fibroblasts were pretreated with DMSO or 10 μM AMI-1. Cells were stimulated with IL-4 (10 ng/ml) for 16 h and subjected to a scratch wound healing assay in serum-free DMEM/F12. Representative pictures from the wound healing assay at time 0 h and 16 h are shown. (B) The rate of wound closure was assessed by counting the cells that had migrated into the same-sized square fields. Data represent mean values ± SEM, n = 4; *, p ≤ 0.05; **, p ≤ 0.01. (C) Fibroblasts were pretreated overnight with DMSO or 10 μM AMI-1. Afterwards, the cells were plated onto the upper side of the transwell-filter membrane and allowed to invade the bottom side overnight. Representative images of cells on the underside of the transwell-filter membrane are demonstrated. (D) The invading cells were counted. Data represent mean values ± SEM, n = 3; *, p ≤ 0.05. (E) Proliferation of untreated and AMI-1 (10 μM)-treated HLF as assessed by [^{3}H]thymidine incorporation. Data represent mean values ± SEM, n = 3. Data represent mean values ± SEM; n = 3; *, p ≤ 0.05. (F) Efficiency of PRMT1 inhibition as assessed by Western blotting using anti-ASYM24 antibodies. β-actin served as a loading control. Asterisks indicate reduction in protein methylation. Untr., untreated.

role of PRMT1 in donor and IPF HLF migration under basal conditions and upon exposure to IL-4.

3.6. Catalytic activity of PRMT controls IL-4-triggered donor and IPF fibroblast migration

To evaluate whether PRMT activity is involved in the regulation of IL-4-induced lung fibroblast migration, an inhibitor of PRMT, AMI-1, was utilized [20,21]. Although, AMI-1 interacts with the substrate-binding pocket of the enzymes, and thus inhibits total activity of PRMTs [22], the IC_{50} value of AMI-1 towards PRMT1 is 20 times lower than towards PRMT4 (8.8 μM *versus* 169.8 μM, respectively) [22]. A scratch wound healing assay revealed an increased migration of IPF HLF as compared to donor HLF (Fig. 6A, B). Treatment of donor and IPF HLF with AMI-1 attenuated the migration of cells under basal conditions and upon exposure to IL-4. (Fig. 6A, B). Moreover, as depicted in Fig. 6C and D, AMI-1 treatment reduced motility of unstimulated donor and IPF HLF and IL-4-stimulated donor HLF in a transwell migration assay. The inhibition of PRMT activity neither altered the expression of *PRMT1*, *Col1a1*, *Col3a1* and *MMP-2* (Supplementary Fig. 3A) nor influenced MMP-2 and MMP-9 activities (Supplementary Fig. 3B). Furthermore, AMI-1 treatment did not affect HLF proliferation, supporting the notion that PRMT activity is required for cell migration but dispensable for cell growth (Fig. 6E). Importantly, the incubation of HLF with AMI-1 did not change their morphology (Supplementary Fig. 4A) and did not induce LDH release (Supplementary Fig. 4B). The efficacy of AMI-1 was demonstrated by Western blot analysis using the anti-ASYM24 antibody (Fig. 6F). Collectively, our results imply that interference with PRMT1 activity reduces the motility of HLF, but does influence their growth.

4. Discussion

In our present study we sought to investigate the role of PRMT1 in the pathogenesis of IPF. PRMT1 expression was found to be significantly enhanced in the lungs of IPF patients when compared to controls. PRMT1 was localized in IPF lungs to bronchial and alveolar epithelial cells, as well as to fibroblasts/myofibroblasts present in fibrotic foci. Using the mouse model of bleomycin-induced pulmonary fibrosis, we demonstrated a time-dependent increase of PRMT1 protein expression in the lungs of affected animals. Furthermore, we identified IL-4 as a trigger of enhanced PRMT1 expression in HLF. Finally, we showed, for the first time, that the interference with PRMT1 expression and/or activity may decrease the IL-4-driven migratory properties of donor and IPF HLF. Altogether, our results imply that PRMT1 may play an important role in the development/progression of IPF.

The fibrotic processes that occur in the lungs of IPF patients are the result of a recurrent injury to the alveolar epithelium followed by an uncontrolled proliferation of fibroblasts [2]. However, a large body of evidence suggests that IPF might be a neoproliferative disorder since it exhibits several pathogenic features similar to cancer including epigenetic and genetic abnormalities, abnormal activation of specific signal transduction pathways, and dysregulated proliferation of the cells [23]. A number of studies point out a fundamental role of protein arginine methylation and PRMT1 in the aforementioned processes and thus in the pathobiology of various types of human cancer including lung carcinoma [15]. While the causative role of PRMTs has already been extensively studied in lung cancer pathogenesis, little is known about the involvement of PRMTs in the development of chronic fibroproliferative lung disorders, in particular IPF. PRMT1 is highly expressed in the lung tissue as compared to other organs [24], and it accounts for the bulk of total protein arginine methylation in mammalian cells [14]. Hence, any dysregulation of PRMT1 expression and/or activity may have a great impact on lung homeostasis [13]. Abnormal PRMT1 expression has recently been implicated in the pathogenesis of asthma and pulmonary hypertension by influencing the eosinophil infiltration of the airways [20] and hypoxia-inducible factor (HIF)-dependent responses, respectively [25]. Our study demonstrates, for the first time, that PRMT1 protein expression is enhanced in IPF lungs and that this may have potential consequences for the migratory properties of the cells. Although no change in the PRMT1 mRNA level in IPF lung homogenates was observed, a clear alteration of PRMT1 mRNA expression was visible in the fibroblasts isolated from diseased lungs. This suggests that PRMT1 mRNA levels may vary among different types of lung cells. Furthermore, the increased PRMT1 mRNA level detected in isolated IPF HLF and upon stimulation of the cells with IL-4 can be a result of either enhanced PRMT1 promotor activity or altered PRMT1 mRNA stability. In this regard, it was demonstrated that IL-4, on the one hand, stabilizes 15-lipoxygenase-1 mRNA in human orbital fibroblasts [26], and, on the other, regulates promotor activity of the IL-13 receptor α2 in the HaCaT human keratinocyte cell line [27]. Moreover, studies by Seegmuller et al. revealed that IL-4 may simultaneously modulate promotor activity and mRNA stability, thereby influencing overall mRNA levels in the cell [28]. Yet, further studies focusing on the molecular mechanism of the IL-4-triggered upregulation of PRMT1 mRNA level in HLF are pending.

Although IL-4 levels were found to be increased in BALF of IPF patients, its profibrotic properties have not yet been entirely defined [4]. Here, we demonstrate that IL-4 by enhancing the expression of PRMT1 can modulate the migratory properties of human lung fibroblasts and thereby contribute to the pathogenesis of pulmonary fibrosis. Albeit, IPF is considered a disease of disturbed epithelial–mesenchymal crosstalk, recent studies have drawn attention to the role of inflammatory cells in the pathobiology of this disorder [4]. It has been postulated, for example, that T-lymphocyte and mast cells may contribute to the development of pulmonary fibrosis by producing a number of pro-fibrotic mediators such as tryptase, TGF-β and IL-4 [29,30]. All the aforementioned molecules have pleiotropic properties with biological effects on a variety of target cells including B and T lymphocytes, macrophages, hematopoietic cells, mast cells, and fibroblasts [4,29]. In this regard, it has been demonstrated that IL-4 is a powerful inducer of TGF-β1 expression leading thereby to increased extracellular matrix synthesis [31] and thus to enhanced movement of pulmonary fibroblasts [32]. In line with these findings we observed the augmented migration of HLF in response to IL-4. Interestingly, the pro-migratory properties of HLF were abolished by either depletion of PRMT1 or treatment of the cells with the inhibitor of PRMT activity, AMI-1. As PRMT1 knockdown decreased HLF proliferation possibly by the mechanism involving its ability to regulate telomere length and/or stability and thus induce growth arrest [33], we cannot exclude that this effect could, in part, account for the reduction in IL-4-driven cell migration [34]. Furthermore, PRMT1-controlled changes in ECM and ECM-associated proteins might also have an influence on the observed alteration in cell migration. Our results point towards the involvement of PRMT1 in the modulation of *Col1a1* and *MMP-2* mRNA expression. In this respect, the direct effect of PRMT1 on the regulation of MMP-2 transcription and its capability to serve as a co-activator of the nuclear factor-κB (NF-κB) and thus as a regulator of MMP-2 expression have already been described [35,36]. Moreover, the ability of PRMT1 to methylate histones provides the possibility for its direct participation in the epigenetic regulation of gene transcription [12]. Methylation of the arginine 3 at histone H4 by PRMT1 [12,37] is largely considered as a mark of transcriptionally activated genes [37,38].

The inhibitory effect of AMI-1 on IL-4-induced HLF migration differed substantially from that observed when PRMT1 was depleted. AMI-1 treatment did not alter cell proliferation and mRNA levels of *Col1a1*, *Col3a1* and *MMP-2*, but clearly influenced intracellular protein arginine methylation thereby inhibiting IL-4-induced fibroblasts migration. In line with this supposition, the blockage of PRMT1-dependent methylation of the transcription factor Twist1 attenuated cell migration of A549 lung epithelial cell line [39]. Furthermore, PRMT1-mediated

methylation of nuclear factor of activated T cells cofactor protein NIP45 was found to be essential for the synthesis and release of IL-4 from T-lymphocytes [40], suggesting the involvement of PRMT1 in the IL-4 production and in the IL-4-driven cellular effects. Thus, it is tempting to speculate that the imbalance in cellular protein arginine methylation ("protein methylome") manifested by disturbed chromatin remodeling and dysregulated function of a number of nuclear and cytoplasmic non-histone proteins culminated in uncontrolled fibroblast behavior.

In conclusions, our study provides compelling evidence that PRMT1 drives proliferation and migration of human lung fibroblasts and thereby links intracellular protein arginine methylation to fibrogenic processes. Thus, we present novel rational for strategies aimed at blocking PRMT1 in IPF.

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Conflict of interest

The authors declare no conflict of interest.

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References

[1] W.D. Travis, U. Costabel, D.M. Hansell, T.E. King Jr., D.A. Lynch, A.G. Nicholson, C.J. Ryerson, J.H. Ryu, M. Selman, A.U. Wells, J. Behr, D. Bouros, K.K. Brown, T.V. Colby, H.R. Collard, C.R. Cordeiro, V. Cottin, B. Crestani, M. Drent, R.F. Dudden, J. Egan, K. Flaherty, C. Hogaboam, Y. Inoue, T. Johkoh, D.S. Kim, M. Kitaichi, J. Loyd, F.J. Martinez, J. Myers, S. Protzko, G. Raghu, L. Richeldi, N. Sverzellati, J. Swigris, D. Valeyre, An official American Thoracic Society/European Respiratory Society statement: update of the international multidisciplinary classification of the idiopathic interstitial pneumonias, Am. J. Respir. Crit. Care Med. 188 (2013) 733–748.
[2] T.E. King Jr., A. Pardo, M. Selman, Idiopathic pulmonary fibrosis, Lancet 378 (2011) 1949–1961.
[3] I.E. Fernandez, O. Eickelberg, New cellular and molecular mechanisms of lung injury and fibrosis in idiopathic pulmonary fibrosis, Lancet 380 (2012) 680–688.
[4] T.A. Wynn, Integrating mechanisms of pulmonary fibrosis, J. Exp. Med. 208 (2011) 1339–1350.
[5] C. Jakubzick, E.S. Choi, K.J. Carpenter, S.L. Kunkel, H. Evanoff, F.J. Martinez, K.R. Flaherty, G.B. Toews, T.V. Colby, W.D. Travis, B.H. Joshi, R.K. Puri, C.M. Hogaboam, Human pulmonary fibroblasts exhibit altered interleukin-4 and interleukin-13 receptor subunit expression in idiopathic interstitial pneumonia, Am. J. Pathol. 164 (2004) 1989–2001.
[6] S. Chandriani, D.J. DePianto, E.N. N'Diaye, A.R. Abbas, J. Jackman, J. Bevers 3rd, V. Ramirez-Carrozzi, R. Pappu, S.E. Kauder, K. Toy, C. Ha, Z. Modrusan, L.C. Wu, H.R. Collard, P.J. Wolters, J.G. Egen, J.R. Arron, Endogenously expressed IL-13Ralpha2 attenuates IL-13-mediated responses but does not activate signaling in human lung fibroblasts, J. Immunol. 193 (2014) 111–119.
[7] C. Jakubzick, E.S. Choi, S.L. Kunkel, H. Evanoff, F.J. Martinez, R.K. Puri, K.R. Flaherty, G.B. Toews, T.V. Colby, E.A. Kazerooni, B.H. Gross, W.D. Travis, C.M. Hogaboam, Augmented pulmonary IL-4 and IL-13 receptor subunit expression in idiopathic interstitial pneumonia, J. Clin. Pathol. 57 (2004) 477–486.
[8] F. Huaux, T. Liu, B. McGarry, M. Ullenbruch, S.H. Phan, Dual roles of IL-4 in lung injury and fibrosis, J. Immunol. 170 (2003) 2083–2092.
[9] C.T. Migliaccio, M.C. Buford, F. Jessop, A. Holian, The IL-4Ralpha pathway in macrophages and its potential role in silica-induced pulmonary fibrosis, J. Leukoc. Biol. 83 (2008) 630–639.
[10] T.E. King Jr., W.Z. Bradford, S. Castro-Bernardini, E.A. Fagan, I. Glaspole, M.K. Glassberg, E. Gorina, P.M. Hopkins, D. Kardatzke, L. Lancaster, D.J. Lederer, S.D. Nathan, C.A. Pereira, S.A. Sahn, R. Sussman, J.J. Swigris, P.W. Noble, A phase 3 trial of pirfenidone in patients with idiopathic pulmonary fibrosis, N. Engl. J. Med. 370 (2014) 2083–2092.
[11] L. Richeldi, R.M. du Bois, G. Raghu, A. Azuma, K.K. Brown, U. Costabel, V. Cottin, K.R. Flaherty, D.M. Hansell, Y. Inoue, D.S. Kim, M. Kolb, A.G. Nicholson, P.W. Noble, M. Selman, H. Taniguchi, M. Brun, F. Le Maulf, M. Girard, S. Stowasser, R. Schlenker-Herceg, B. Disse, H.R. Collard, Efficacy and safety of nintedanib in idiopathic pulmonary fibrosis, N. Engl. J. Med. 370 (2014) 2071–2082.
[12] M.T. Bedford, S.G. Clarke, Protein arginine methylation in mammals: who, what, and why, Mol. Cell 33 (2009) 1–13.
[13] D. Zakrzewicz, O. Eickelberg, From arginine methylation to ADMA: a novel mechanism with therapeutic potential in chronic lung diseases, BMC Pulm. Med. 9 (2009) 5.
[14] J. Tang, A. Frankel, R.J. Cook, S. Kim, W.K. Paik, K.R. Williams, S. Clarke, H.R. Herschman, PRMT1 is the predominant type I protein arginine methyltransferase in mammalian cells, J. Biol. Chem. 275 (2000) 7723–7730.
[15] D. Zakrzewicz, A. Zakrzewicz, K.T. Preissner, P. Markart, M. Wygrecka, Protein arginine methyltransferases (PRMTs): promising targets for the treatment of pulmonary disorders, Int. J. Mol. Sci. 13 (2012) 12383–12400.
[16] G. Raghu, H.R. Collard, J.J. Egan, F.J. Martinez, J. Behr, K.K. Brown, T.V. Colby, J.F. Cordier, K.R. Flaherty, J.A. Lasky, D.A. Lynch, J.H. Ryu, J.J. Swigris, A.U. Wells, J. Ancochea, D. Bouros, C. Carvalho, U. Costabel, M. Ebina, D.M. Hansell, T. Johkoh, D.S. Kim, T.E. King Jr., Y. Kondoh, J. Myers, N.L. Muller, A.G. Nicholson, L. Richeldi, M. Selman, R.F. Dudden, B.S. Griss, S.L. Protzko, H.J. Schunemann, An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management, Am. J. Respir. Crit. Care Med. 183 (2011) 788–824.
[17] M. Wygrecka, G. Kwapiszewska, E. Jablonska, S. von Gerlach, I. Henneke, D. Zakrzewicz, A. Guenther, K.T. Preissner, P. Markart, Role of protease-activated receptor-2 in idiopathic pulmonary fibrosis, Am. J. Respir. Crit. Care Med. 183 (2011) 1703–1714.
[18] D. Zakrzewicz, M. Didiasova, A. Zakrzewicz, A.C. Hocke, F. Uhle, P. Markart, K.T. Preissner, M. Wygrecka, The interaction of enolase-1 with caveolae-associated proteins regulates its subcellular localization, Biochem. J. 460 (2014) 295–307.
[19] M.T. Bedford, S. Richard, Arginine methylation an emerging regulator of protein function, Mol. Cell 18 (2005) 263–272.
[20] Q. Sun, X. Yang, B. Zhong, F. Jiao, C. Li, D. Li, X. Lan, J. Sun, S. Lu, Upregulated protein arginine methyltransferase 1 by IL-4 increases eotaxin-1 expression in airway epithelial cells and participates in antigen-induced pulmonary inflammation in rats, J. Immunol. 188 (2012) 3506–3512.
[21] D. Cheng, N. Yadav, R.W. King, M.S. Swanson, E.J. Weinstein, M.T. Bedford, Small molecule regulators of protein arginine methyltransferases, J. Biol. Chem. 279 (2004) 23892–23899.
[22] K. Bonham, S. Hemmers, Y.H. Lim, D.M. Hill, M.G. Finn, K.A. Mowen, Effects of a novel arginine methyltransferase inhibitor on T-helper cell cytokine production, FEBS J. 277 (2010) 2096–2108.
[23] C. Vancheri, M. Failla, N. Crimi, G. Raghu, Idiopathic pulmonary fibrosis: a disease with similarities and links to cancer biology, Eur. Respir. J. 35 (2010) 496–504.
[24] P. Bulau, D. Zakrzewicz, K. Kitowska, J. Leiper, A. Gunther, F. Grimminger, O. Eickelberg, Analysis of methylarginine metabolism in the cardiovascular system identifies the lung as a major source of ADMA, Am. J. Physiol. Lung Cell. Mol. Physiol. 292 (2007) L18–L24.
[25] S.K. Lim, Y.W. Jeong, D.I. Kim, M.J. Park, J.H. Choi, S.U. Kim, S.S. Kang, H.J. Han, S.H. Park, Activation of PRMT1 and PRMT5 mediates hypoxia- and ischemia-induced apoptosis in human lung epithelial cells and the lung of miniature pigs: the role of p38 and JNK mitogen-activated protein kinases, Biochem. Biophys. Res. Commun. 440 (2013) 707–713.
[26] B. Chen, S. Tsui, W.E. Boeglin, R.S. Douglas, A.R. Brash, T.J. Smith, Interleukin-4 induces 15-lipoxygenase-1 expression in human orbital fibroblasts from patients with Graves disease. Evidence for anatomic site-selective actions of Th2 cytokines, J. Biol. Chem. 281 (2006) 18296–18306.
[27] M.D. David, J. Bertoglio, J. Pierre, Functional characterization of IL-13 receptor alpha2 gene promoter: a critical role of the transcription factor STAT6 for regulated expression, Oncogene 22 (2003) 3386–3394.
[28] I. Seegmuller, H. Hacker, H. Wagner, IL-4 regulates IL-12 p40 expression post-transcriptionally as well as via a promoter-based mechanism, Eur. J. Immunol. 33 (2003) 428–433.
[29] M. Wygrecka, B.K. Dahal, D. Kosanovic, F. Petersen, B. Taborski, S. von Gerlach, M. Didiasova, D. Zakrzewicz, K.T. Preissner, R.T. Schermuly, P. Markart, Mast cells and fibroblasts work in concert to aggravate pulmonary fibrosis: role of transmembrane SCF and the PAR-2/PKC-alpha/Raf-1/p44/42 signaling pathway, Am. J. Pathol. 182 (2013) 2094–2108.
[30] C. Overed-Sayer, L. Rapley, T. Mustelin, D.L. Clarke, Are mast cells instrumental for fibrotic diseases? Front. Pharmacol. 4 (2013) 174.
[31] C.M. Hogaboam, C.L. Bone-Larson, S. Lipinski, N.W. Lukacs, S.W. Chensue, R.M. Strieter, S.L. Kunkel, Differential monocyte chemoattractant protein-1 and chemokine receptor 2 expression by murine lung fibroblasts derived from Th1- and Th2-type pulmonary granuloma models, J. Immunol. 163 (1999) 2193–2201.
[32] A.E. Postlethwaite, J.M. Seyer, Fibroblast chemotaxis induction by human recombinant interleukin-4. Identification by synthetic peptide analysis of two chemotactic domains residing in amino acid sequences 70–88 and 89–122, J. Clin. Invest. 87 (1991) 2147–2152.

[33] T.R. Mitchell, K. Glenfield, K. Jeyanthan, X.D. Zhu, Arginine methylation regulates telomere length and stability, Mol. Cell. Biol. 29 (2009) 4918–4934.
[34] Z. Yu, T. Chen, J. Hebert, E. Li, S. Richard, A mouse PRMT1 null allele defines an essential role for arginine methylation in genome maintenance and cell proliferation, Mol. Cell. Biol. 29 (2009) 2982–2996.
[35] T. Zhang, G. Cui, Y.L. Yao, Y. Guo, Q.C. Wang, X.N. Li, W.M. Feng, Inhibition of nonsmall cell lung cancer cell migration by protein arginine methyltransferase 1-small hairpin RNA through inhibiting epithelial–mesenchymal transition, extracellular matrix degradation, and Src phosphorylation in vitro, Chin. Med. J. 128 (2015) 1202–1208.
[36] P.O. Hassa, M. Covic, M.T. Bedford, M.O. Hottiger, Protein arginine methyltransferase 1 coactivates NF-kappaB-dependent gene expression synergistically with CARM1 and PARP1, J. Mol. Biol. 377 (2008) 668–678.
[37] H. Wang, Z.Q. Huang, L. Xia, Q. Feng, H. Erdjument-Bromage, B.D. Strahl, S.D. Briggs, C.D. Allis, J. Wong, P. Tempst, Y. Zhang, Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor, Science 293 (2001) 853–857.
[38] M.A. Kleinschmidt, G. Streubel, B. Samans, M. Krause, U.M. Bauer, The protein arginine methyltransferases CARM1 and PRMT1 cooperate in gene regulation, Nucleic Acids Res. 36 (2008) 3202–3213.
[39] S. Avasarala, M. Van Scoyk, M.K. Karuppusamy Rathinam, S. Zerayesus, X. Zhao, W. Zhang, M.R. Pergande, J.A. Borgia, J. DeGregori, J.D. Port, R.A. Winn, R.K. Bikkavilli, PRMT1 is a novel regulator of epithelial–mesenchymal-transition in non-small cell lung cancer, J. Biol. Chem. 290 (2015) 13479–13489.
[40] K.A. Mowen, B.T. Schurter, J.W. Fathman, M. David, L.H. Glimcher, Arginine methylation of NIP45 modulates cytokine gene expression in effector T lymphocytes, Mol. Cell 15 (2004) 559–571.