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MeCP2 epigenetically regulates alpha-smooth muscle actin in human lung fibroblasts

Zheyi Xiang^{1,2}, Qingxian Zhou², Min Hu¹, Yan Y. Sanders²

¹Laboratory of Clinical Medicine, The Second Xiangya Hospital of Central South University, Changsha, Hunan, China

²Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama

Abstract

Background—A critical feature for fibroblasts differentiation into myofibroblasts is the expression of alpha-smooth muscle actin (α -SMA) during the tissue injury and repair process. The epigenetic mechanism, DNA methylation, is involved in regulating α -SMA expression. It is not clear how methyl-CpG-binding protein 2 (MeCP2) interacts with CpG-rich region in α -SMA, and if the CpG methylation status would affect MeCP2 binding and regulation of α -SMA expression.

Methods—The association of MeCP2 with α -SMA CpG rich region were examined by chromatin immunoprecipitation (ChIP) assays in primary fibroblasts from idiopathic pulmonary fibrosis (IPF) and non-IPF control individuals, and in the lung fibroblasts treated with profibrotic cytokine transforming growth factor β 1 (TGF- β 1). The regulation of α -SMA by MeCP2 was examined by knocking down MeCP2 with small interfering RNA (siRNA). To explore the effects of the DNA methylation status of the CpG rich region on α -SMA expression, the cells were treated with DNA methyltransferase inhibitor, 5'-azacytidine (5'-aza). The expression of α -SMA was examined by Western blot and quantitative polymerase chain reaction, the association with MeCP2 was assessed by ChIP assays, and the methylation status was checked by bisulfate sequencing.

Results—The human lung fibroblasts with increased α -SMA showed an enriched association of MeCP2, while knockdown MeCP2 by siRNA reduced α -SMA upregulation by TGF- β 1. The 5'-Aza-treated cells have decreased α -SMA expression with reduced MeCP2 association. However,

Correspondence Min Hu, Laboratory of Clinical Medicine, The Second Xiangya Hospital of Central South University, Changsha, 410011 Hunan, China., huminjyk@csu.edu.cn, Yan Y. Sanders, Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, University of Alabama at Birmingham, 901 19th St S, BMR II Room 408, Birmingham, AL 35294. yans@uab.edu.

AUTHOR CONTRIBUTIONS

XZ carried out the experiments and analyzed the data; QZ carried out the experiments; MH coordinated the study and analyzed the data; YYS designed, coordinated the study, analyzed the data, and wrote the manuscript.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

bisulfite sequencing revealed that most CpG sites are unmethylated despite the different expression levels of α -SMA after being treated by TGF- β 1 or 5'-aza.

Conclusion—Our data indicate that the methyl-binding protein MeCP2 is critical for α -SMA expression in human lung myofibroblast, and the DNA methylation status at the CpG rich region of α -SMA is not a determinative factor for its inducible expression.

Keywords

DNA methylation; epigenetic regulation; fibrosis; human lung fibroblast; IPF; MeCP2; α -SMA

1 | INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive interstitial lung disease with high mortality and unknown etiology.¹ IPF is characterized by scar formation and lung tissue remodeling, and the differentiation of fibroblast into myofibroblast is a critical feature in this process.² During the tissue repair process, transforming growth factor β 1 (TGF- β 1) is extensively involved as a profibrotic cytokine³; it induces fibroblast differentiation into myofibroblast.³ Myofibroblasts are the main effector cells for tissue remodeling and repair,⁴ characterized by the expression of alpha-smooth muscle actin (α -SMA).² Regulation of α -SMA, especially the epigenetic regulation of the gene in human lung fibroblasts, is not completely understood.

Epigenetic regulation is defined as the alterations of gene expression without a change of genomic sequence.⁵ It includes DNA methylation, histone modification, and noncoding RNA, which are all reported in IPF.^{6–8} DNA methylation is the addition of a methyl group from S-adenosyl methionine to a nucleotide of DNA, which usually occurs on the cytosine of the CpG dinucleotide.⁹ DNA methylation at the promoter region of the genes usually represses gene expression.¹⁰ Methyl-CpG-binding domain family proteins are the readers of DNA methylation; these proteins are often associated with gene repression, which would recruit other remodelers, enzymes to the methylated DNA.¹¹ As a member of this family, methyl-CpG-binding protein 2 (MeCP2) selectively binds with methyl-CpG residues and was thought to be a transcriptional repressor.¹² However, recent studies found that MeCP2 could function as a transcriptional activator by associating with cAMP response element-binding protein 1 is a major transcriptional activator.¹³ Thus, the role of MeCP2 in regulating target gene expression is complicated. MeCP2 was reported to be involved in various fibrotic diseases, including lung fibrosis,¹⁴ liver fibrosis,¹⁵ and scleroderma.¹⁶

In the cellular model of scleroderma,¹⁶ MeCP2 is reported to mediate antifibrotic effects. However, in the animal model of lung fibrosis, MeCP2 was reported to be profibrotic and MeCP2-deficient mice showed reduced fibrotic response to bleomycin-induced lung fibrosis.¹⁴ Together, these indicate that MeCP2 may regulate target genes through significantly different pathways in different organs or species. In this study, we investigated if MeCP2 is associated with α -SMA expression in human primary lung fibroblasts derived from IPF and non-IPF subjects, as well as normal lung fibroblasts in response to TGF- β 1 treatment. We further examined the DNA methylation status at the CpG rich region of α -SMA in TGF- β 1 treated cells.

2 | MATERIALS AND METHODS

2.1 | Cell culture and treatments

The primary human IPF and non-IPF fibroblasts were obtained from deidentified tissues from the University of Alabama at Birmingham (UAB) Institutional Review Board approved Tissue Procurement Facility. The American Thoracic Society/European Respiratory Society guidelines¹⁷ were used for a multidisciplinary approach for the diagnosis of IPF. Early passages (<5) were used for all primary cell lines. Human lung fibroblasts cell line IMR-90 were purchased from Coriell Institute for Medical Research (Camden, NJ), and were used at low passages. The cells were kept in 5% CO₂ at 37°C incubator with Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY), with 10% fetal bovine serum (FBS; Life Technologies), 100 units/mL penicillin, 100 µg/mL streptomycin, 1.25 µg/mL amphotericin B, and 2 mM L-glutamine (G1251; Sigma, St Louis, MO). The cells were collected at 80% confluence for various experiments.

For cells treated with TGF-β1, 1% FBS culture medium was used overnight before adding 2 ng/mL of TGF-β1 (R&D Systems, Minneapolis, MN) for 24 hours. The treatment of 5'-aza-2'-deoxycytidine (5'-aza; Sigma) was added in the culture medium after the cells were seeded for 24 hours. The medium was changed daily with freshly added 5'-aza at 5 µM as previously described.¹⁸ After 3 days, the cells were either collected for assays or were subjected to 1% FBS medium overnight, then the TGF-β1 was added at 2 ng/mL for 24 hours and the cells were collected for assays.

2.2 | Extraction of DNA/RNA and quantitative real-time polymerase chain reaction

Genomic DNA was extracted with DNeasy Tissue Kit, while RNA was with RNeasy Mini Kit, both were from Qiagen (Valencia, CA). A cDNA Synthesis Kit (Promega, Madison, WI) was used to transcribe RNA into complementary DNA. The quantitative real-time polymerase chain reaction (RT-PCR) was done in triplicate, and the results were normalized to β-actin and calculated with the C_t method.¹⁹ All the primers used are in Table 1.

2.3 | Transfection of small interfering RNA

The transfection of small interfering RNA (siRNA) was carried out as per the instructions of the manufacturer of Opti-MEM (Thermo Fisher Scientific) with Lipofectamine RNAi/MAX (Invitrogen) as described before.²⁰ The sequences for MeCP2 siRNA were: sense, 5'-GGAAAGG ACUGACCUGUUU-3', antisense, 5'-AACAGGUCUUC AGUCCUUUCCUU-3'; the sequences for nontargeting are as in previous studies.²¹ After transfection for 48 hours, the cells were subjected to experiments.

2.4 | Protein lysate and immunoblotting

The whole-cell lysate was collected and the concentration was measured by a Micro BCA Protein Assay Kit (Thermo Fisher Scientific). Western blots were carried out as previously described.⁷ Amersham Biosciences 600 Imager (GE Healthcare) was used for immunoblot images, and ImageQuant TL software was used to quantitate the signals. The anti-α-SMA (#03-61001; ARP, MA), and anti-β-actin (#2128) antibodies from Cell Signaling (Beverly, MA) were used to detect the corresponding proteins.

2.5 | Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed according to the manufacturer's protocol (cat# ab500; Abcam, Cambridge, MA) with modifications.²¹ ChIP grade antibody against MeCP2 was from Active Motif (cat# 61285; Carlsbad, CA). ChIP-DNA was subjected to quantitative PCR using SYBR Green PCR Master Mix (Life Technologies), the primers used in PCR are listed in Table 1. The results were calculated using C_t method and normalized to input DNA.

2.6 | Bisulfite conversion and sequencing

An EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA) was used to bisulfite-convert the DNA samples as per the manufacturer's instructions. The unmethylated cytosine would convert to uracil, and the methylated cytosine would remain unchanged. The methylation status of α -SMA in IMR-90 lung fibroblasts treated with and without TGF- β 1, as well as with or without 5'-aza was examined by direct bisulfite sequencing PCR (BSP). The α -SMA sequence was retrieved from Ensembl (www.ensembl.org) with human ACTA2 (α -SMA) ID ENSG00000107796. CpG rich regions were identified by Methprimer software,²² specific sets of BSP primers were designed by Methprimer as shown in Table 1. The conditions used for PCRs were: after 5 minutes of 95°C, 38 cycles of 30 seconds of 95°C, 30 seconds of 60°C, and 30 seconds of 72°C, then 5 minutes of 72°C extension. The PCR products were checked on an agarose gel and cleaned before being sent for sequencing at the sequencing core facility at UAB.

2.7 | Statistical analysis

For a comparison between multiple groups, one-way analysis of variance was applied; for comparison between two groups, the Student paired *t* test was used. The data were analyzed with GraphPad Prism 5.0 (La Jolla, CA), and presented as the mean $\pm$ standard deviation. Statistical significance was considered at a *P* value less than .05.

3 | RESULTS

3.1 | MeCP2 association is enriched with increased α -SMA expression in IPF (myo)fibroblasts

IPF lung fibroblasts are characterized by significantly increased α -SMA expression as myofibroblasts.² We confirmed the upregulated α -SMA expression in our primary IPF fibroblasts (Figures 1A and S1). We then examined the presence of any association of MeCP2 with the α -SMA gene in human lung fibroblasts. CpG rich regions in the human α -SMA gene sequence (ENSG00000107796) were identified by MethPrimer.²² We designed two sets of PCR primers to check the binding of MeCP2 at this region; we also designed a set of primers at the region near the CArG elements of α -SMA gene²³ (Figure 1B). By ChIP assays, a significantly enriched association of MeCP2 at α -SMA gene CpG rich regions as well as near CArG elements are present in human lung fibroblasts derived from IPF patients compared with non-IPF controls (Figure 1C). These data indicate that MeCP2 is associated with the α -SMA gene in human lung (myo)fibroblasts and the enrichment correlates with α -SMA upregulation.

3.2 | MeCP2 is critical for upregulation of α -SMA by TGF- β 1 in lung fibroblasts

As the MeCP2 is associated with intrinsic α -SMA expression, we further examined if the inducible upregulation of α -SMA by TGF- β 1 is regulated by MeCP2 in a similar pattern. In human primary non-IPF fibroblasts, in response to TGF- β 1 treatment, α -SMA is significantly upregulated (Figures 2A and S2). The association of MeCP2 with α -SMA was examined by ChIP assays. Enriched MeCP2 association with α -SMA was noticed in the cells treated with TGF- β 1 (Figure 2B). We then knocked down MeCP2 by transfecting siRNA MeCP2 (Figure 3A), and treated the transfected cells with TGF- β 1. The upregulation of α -SMA by TGF- β 1 is significantly reduced in the cells with MeCP2 knockdown (Figure 3B,C). Our data demonstrated that MeCP2 is critical for α -SMA upregulation by TGF- β 1.

3.3 | Reduced MeCP2 association with α -SMA contribute to the decreased α -SMA expression in primary IPF fibroblasts treated with 5'-aza

To explore if DNA methylation status at the CpG rich region of α -SMA may affect MeCP2 binding and/or regulate α -SMA expression, we treated the IPF fibroblasts with DNA methyltransferase inhibitor 5'-aza for 3 days as previously described¹⁸ before examining the α -SMA expression. These IPF primary fibroblasts showed decreased α -SMA expression at the messenger RNA (Figure 4A) and protein (Figure 4B) levels. By ChIP assays with MeCP2 pulldown, we noticed a significantly depleted association of MeCP2 with the α -SMA gene (Figure 4C). This suggests that 5'-aza treatment downregulates α -SMA expression, and depletes the MeCP2 association with α -SMA.

3.4 | 5'-Aza treatment partially blocked the upregulation of α -SMA by TGF- β 1 with reduced α -SMA association with MeCP2

We further studied if the DNA demethylation agent 5'-aza can block the inducible α -SMA expression by TGF- β 1 in lung fibroblasts. Unlike the heterogeneous primary fibroblasts that have different sensitivity to TGF- β 1 (Figure S2), lung fibroblast cell line IMR-90 is highly responsive to TGF- β 1 treatment for α -SMA expression.^{24,25} We used this cell line to examine if 5'-aza interferes with TGF- β 1 upregulation of α -SMA. After being treated with 5'-aza for 3 days, the cells were then treated with TGF- β 1 at 2 ng/mL for 24 hours. The pretreatment with 5'-aza reduced the upregulation of α -SMA compared with the cells treated with TGF- β 1 only (Figure 5A,B). In the IMR-90 cells, the association of MeCP2 is significantly enriched with α -SMA with TGF- β 1 (Figure 5C), corresponding to the robust upregulation of α -SMA by TGF- β 1 in these cells (Figure 5B); similar to what we showed in the primary non-IPF fibroblasts (Figures 2 and S2). The 5'-aza alone downregulates the α -SMA expression and diminished its association with MeCP2 (Figure 5C). Compared with TGF- β 1 upregulated α -SMA, the 5'-aza pretreatment significantly reduced the upregulation, which corresponded to the reduced association of MeCP2 with α -SMA (Figure 5C). These data revealed that DNA demethylation agent 5'-aza downregulates α -SMA expression and reduces its association with MeCP2.

3.5 | Methylation status of CpG rich region of α -SMA gene in lung fibroblasts that are treated with TGF- β 1, 5'-aza, or TGF- β 1 and 5'-aza

Our data from the 5'-aza-treated cells suggests that DNA methylation may play a role in α -SMA expression (Figure 5), from which we further analyzed the DNA methylation status of the CpG rich region of α -SMA. By bisulfite sequencing, we screened approximately 36 CpG sites as mapped out in Figure 6A-C. To avoid heterogeneity of primary fibroblasts, IMR-90 was used for the study, and the cells were prepared in the same way as shown in Figure 5. The DNA was collected and subjected to bisulfite modification for bisulfite sequencing with three specifically designed primer sets by MethPrimer²² (Table 1). For each condition, at least 10 samples were sent for sequencing (Figure S3). Most of the CpG sites are unmethylated at the baseline condition, while with the demethylation agent 5'-aza, almost all of the sequenced samples showed unmethylated CpGs (Figure 6D,E). A few methylated CpG sites are observed under baseline and with TGF- β 1, no matter pretreated with or without adding 5'-aza (Figure 6D,E). This data demonstrated that DNA methylation status is similar in the cells with different levels of α -SMA expression, indicating that DNA methylation status at these CpG sites may not be the determinative factor in α -SMA expression in response to TGF- β 1 and/or to 5'-aza.

4 | DISCUSSION

In this study, we explored if the methyl-binding protein MeCP2 is associated with α -SMA expression in human lung fibroblasts. We further examined the DNA methylation status at the CpG rich region and if it affects the α -SMA expression. Our data demonstrated that MeCP2 is important for α -SMA constitutive or inducible expression in lung fibroblasts. Knockdown of MeCP2 by siRNA significantly reduced the upregulation of α -SMA by TGF- β 1 in lung fibroblasts. We demonstrated that DNA methylation is involved in regulating α -SMA expression, however, likely through indirect effects; as in most screened α -SMA, CpG rich region DNA methylation status is similar in human fibroblasts at baseline, with TGF- β 1 or 5'-aza treatment, where in either case the α -SMA expression is altered.

Our findings that MeCP2 is an activator for α -SMA in human lung fibroblasts is consistent with studies using lung fibroblasts derived from animal tissues.¹⁴ In those studies, the authors used lung fibroblasts from rat lung tissues and used a bleomycin-induced lung fibrosis model in MeCP2 null mice, which showed significantly reduced fibrotic response.¹⁴ MeCP2 was also involved in liver fibrosis when knockdown of MeCP2 attenuated fibrogenesis.¹⁵ However, MeCP2 showed antifibrotic effects in a study with human dermal fibroblasts from patients with diffuse cutaneous scleroderma.¹⁶ They indicated that MeCP2 attenuated profibrotic responses through its overexpression. These studies suggested that the role of MeCP2 is complex. MeCP2 was initially reported to be a transcriptional repressor in the normal development of the nervous system; MeCP2 gene mutation is associated with Rett syndrome.²⁶ Emerging data suggested that MeCP2 is a multifunctional nuclear protein, important in regulating RNA splicing and active transcription.²⁶ Our data demonstrated that MeCP2 is involved in the upregulation of α -SMA. The cells transfected with siRNA MeCP2 significantly reduced the upregulation of α -SMA by TGF- β 1. Thus, MeCP2 is likely a transcriptional activator in our cellular model.

Besides MeCP2, DNA methylation was reported in control of α -SMA gene expression in rat lung tissues.²⁷ Different levels of CpG methylation in α -SMA genes in fibroblasts, myofibroblasts, and alveolar epithelial type II cells were reported, which corresponded to different levels of α -SMA gene expression in these cells.²⁷ We examined the inducible α -SMA in response to TGF- β 1 and to 5'-aza in lung fibroblasts. The reduced expression of α -SMA by 5'-aza is similar to a report that examined α -SMA expression in hypoxia-induced DNA methylation, which 5'-aza treatment reduced its expression.²⁸ Although the α -SMA expression is downregulated by 5'-aza and upregulated by TGF- β 1, we did not notice significant changes in DNA methylation at the CpG sites we examined. This suggests that the downregulation of α -SMA by 5'-aza maybe due to the indirect effects of the nonspecific demethylation agent 5'-aza.⁹ It is likely caused by other regulators affected by 5'-aza that control α -SMA expression.

The upregulation of α -SMA in response to TGF- β 1 may not primarily be controlled by DNA methylation either. Studies show that DNA methylation usually marks the silent genes and bears the long-term memory of epigenomic programming.²⁹ However, we did notice there are a few methylated CpG sites at baseline and in TGF- β 1-treated cells with enriched association with MeCP2 compared with 5'-aza treatment. We are not sure if these specific CpG methylation status would affect MeCP2 binding. Generally, most of the DNA methyl-binding proteins preferentially bind methylated rather than unmethylated DNA.³⁰ In vitro experiments showed that MeCP2 binds A/T-rich sequence following CpG methylation site in the target genes it represses,³¹ yet MeCP2 is likely an activator for α -SMA in our model. Although it is not clear if the MeCP2 binding mechanism would be different in diverse target genes, MeCP2 was reported to bind mostly actively expressed genes, only a small percentage of the genes it binds are methylated.³²

In this study, we used non-IPF and IPF patient-derived primary lung fibroblasts and a well-documented human lung fibroblast cell line IMR-90. Due to the heterogeneity of the human primary cells, the response of the primary cells to TGF- β 1 varies and some are not as robust as the IMR-90 cell line. Similarly, the response to the 5'-aza treatment in primary cells also demonstrates different levels of sensitivity. Despite the heterogeneity from the individual cells, MeCP2 demonstrates an important role in regulating the expression of intrinsic and inducible α -SMA in human primary lung fibroblasts. Our current studies indicate that MeCP2 is an activator in α -SMA expression in human lung myofibroblasts. MeCP2 may be an important target for blocking myofibroblast differentiation in lung injury and repair processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- Martinez FJ, Collard HR, Pardo A, et al. Idiopathic pulmonary fibrosis. *Nat Rev Dis Primers*. 2017;3:17074 10.1038/nrdp.2017.74 [PubMed: 29052582]
- Habiel DM, Hogaboam CM. Heterogeneity of fibroblasts and myofibroblasts in pulmonary fibrosis. *Curr Pathobiol Rep*. 2017; 5(2):101–110. 10.1007/s40139-017-0134-x [PubMed: 29082111]
- Fernandez IE, Eickelberg O. The impact of TGF-beta on lung fibrosis: from targeting to biomarkers. *Proc Am Thorac Soc*. 2012;9(3):111–116. 10.1513/pats.201203-023AW [PubMed: 22802283]
- Lyu X, Hu M, Peng J, Zhang X, Sanders YY. HDAC inhibitors as antifibrotic drugs in cardiac and pulmonary fibrosis. *Ther Adv Chronic Dis*. 2019;10:1–19. 10.1177/2040622319862697
- Brunet A, Berger SL. Epigenetics of aging and aging-related disease. *J Gerontol A Biol Sci Med Sci*. 2014;69(suppl 1):S17–S20. 10.1093/gerona/glu042 [PubMed: 24833581]
- Sanders YY, Hagood JS, Liu H, Zhang W, Ambalavanan N, Thannickal VJ. Histone deacetylase inhibition promotes fibroblast apoptosis and ameliorates pulmonary fibrosis in mice. *Eur Respir J*. 2014;43(5):1448–1458. 10.1183/09031936.00095113 [PubMed: 24603818]
- Sanders YY, Kumbha P, Hagood JS. Enhanced myofibroblastic differentiation and survival in Thy-1(–) lung fibroblasts. *Am J Respir Cell Mol Biol*. 2007;36(2):226–235. 10.1165/rcmb.2006-0178OC [PubMed: 16960126]
- Yang S, Banerjee S, de Freitas A, et al. Participation of miR-200 in pulmonary fibrosis. *Am J Pathol*. 2012;180(2): 484–493. 10.1016/j.ajpath.2011.10.005 [PubMed: 22189082]
- Zhang X, Hu M, Lyu X, Li C, Thannickal VJ, Sanders YY. DNA methylation regulated gene expression in organ fibrosis. *Biochim Biophys Acta Mol Basis Dis*. 2017;1863(9):2389–2397. 10.1016/j.bbadis.2017.05.010 [PubMed: 28501566]
- Schubeler D Function and information content of DNA methylation. *Nature*. 2015;517(7534):321–326. 10.1038/nature14192 [PubMed: 25592537]
- Della Ragione F, Vacca M, Fioriniello S, Pepe G, D'Esposito M. MECP2, a multi-talented modulator of chromatin architecture. *Brief Funct Genomics*. 2016;15(6):420–431. 10.1093/bfpg/elw023 [PubMed: 27296483]
- Jones PL, Veenstra GJ, Wade PA, et al. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet*. 1998;19(2):187–191. 10.1038/561 [PubMed: 9620779]
- Chahrour M, Jung SY, Shaw C, et al. MeCP2, a key contributor to neurological disease, activates and represses transcription. *Science*. 2008;320(5880):1224–1229. 10.1126/science.1153252 [PubMed: 18511691]
- Hu B, Gharraee-Kermani M, Wu Z, Phan SH. Essential role of MeCP2 in the regulation of myofibroblast differentiation during pulmonary fibrosis. *Am J Pathol*. 2011;178(4):1500–1508. 10.1016/j.ajpath.2011.01.002 [PubMed: 21435439]
- Mann J, Chu DC, Maxwell A, et al. MeCP2 controls an epigenetic pathway that promotes myofibroblast transdifferentiation and fibrosis. *Gastroenterology*. 2010;138(2):705.e4–714.e4. 10.1053/j.gastro.2009.10.002 [PubMed: 19843474]
- He Y, Tsou PS, Khanna D, Sawalha AH. Methyl-CpG-binding protein 2 mediates antifibrotic effects in scleroderma fibroblasts. *Ann Rheum Dis*. 2018;77(8):1208–1218. 10.1136/annrheumdis-2018-213022 [PubMed: 29760157]
- Raghu G, Remy-Jardin M, Myers JL, et al. Diagnosis of idiopathic pulmonary fibrosis. An official ATS/ERS/JRS/ALAT clinical practice guideline. *Am J Respir Crit Care Med*. 2018; 198(5):e44–e68. 10.1164/rccm.201807-1255ST [PubMed: 30168753]
- Sanders YY, Pardo A, Selman M, et al. Thy-1 promoter hypermethylation: a novel epigenetic pathogenic mechanism in pulmonary fibrosis. *Am J Respir Cell Mol Biol*. 2008;39(5):610–618. [PubMed: 18556592]
- Zhang X, Liu H, Hock T, Thannickal VJ, Sanders YY. Histone deacetylase inhibition downregulates collagen 3A1 in fibrotic lung fibroblasts. *Int J Mol Sci*. 2013;14(10):19605–19617. 10.3390/ijms141019605 [PubMed: 24084714]

20. Bai L, Bernard K, Tang X, et al. Glutaminolysis epigenetically regulates anti-apoptotic gene expression in ipf fibroblasts. *Am J Respir Cell Mol Biol*. 2018;60:49–57. 10.1165/rcmb.2018-0180OC
21. Sanders YY, Liu H, Liu G, Thannickal VJ. Epigenetic mechanisms regulate NADPH oxidase-4 expression in cellular senescence. *Free Radic Biol Med*. 2015;79:197–205. 10.1016/j.freeradbiomed.2014.12.008 [PubMed: 25526894]
22. Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. *Bioinformatics*. 2002;18(11):1427–1431. [PubMed: 12424112]
23. Hirschfeld J, Maurer J, Jung D, et al. Targeting myofibroblasts in model systems of fibrosis by an artificial alpha-smooth muscle-actin promoter hybrid. *Mol Biotechnol*. 2009;43(2): 121–129. 10.1007/s12033-009-9186-4 [PubMed: 19551523]
24. Sanders YY, Cui Z, Le Saux CJ, et al. SMAD-independent down-regulation of caveolin-1 by TGF-beta: effects on proliferation and survival of myofibroblasts. *PLoS One*. 2015;10(2): e0116995 10.1371/journal.pone.0116995 [PubMed: 25658089]
25. Thannickal VJ, Lee DY, White ES, et al. Myofibroblast differentiation by transforming growth factor-beta1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. *J Biol Chem*. 2003;278(14):12384–12389. 10.1074/jbc.M208544200 [PubMed: 12531888]
26. Hite KC, Adams VH, Hansen JC. Recent advances in MeCP2 structure and function. *Biochem Cell Biol*. 2009;87(1):219–227. 10.1139/O08-115 [PubMed: 19234536]
27. Hu B, Gharaee-Kermani M, Wu Z, Phan SH. Epigenetic regulation of myofibroblast differentiation by DNA methylation. *Am J Pathol*. 2010;177(1):21–28. 10.2353/ajpath.2010.090999 [PubMed: 20489138]
28. Rodriguez-Jimenez FJ, Moreno-Manzano V, Lucas-Dominguez R, Sanchez-Puelles JM. Hypoxia causes downregulation of mismatch repair system and genomic instability in stem cells. *Stem Cells*. 2008;26(8):2052–2062. [PubMed: 18511603]
29. Bogdanovic O, Lister R. DNA methylation and the preservation of cell identity. *Curr Opin Genet Dev*. 2017;46:9–14. 10.1016/j.gde.2017.06.007 [PubMed: 28651214]
30. Bogdanovic O, Veenstra GJ. DNA methylation and methyl-CpG binding proteins: developmental requirements and function. *Chromosoma*. 2009;118(5):549–565. 10.1007/s00412-009-0221-9 [PubMed: 19506892]
31. Klose RJ, Sarraf SA, Schmiedeberg L, McDermott SM, Stancheva I, Bird AP. DNA binding selectivity of MeCP2 due to a requirement for A/T sequences adjacent to methyl-CpG. *Mol Cell*. 2005;19(5):667–678. 10.1016/j.molcel.2005.07.021 [PubMed: 16137622]
32. Yasui DH, Peddada S, Bieda MC, et al. Integrated epigenomic analyses of neuronal MeCP2 reveal a role for long-range interaction with active genes. *Proc Natl Acad Sci U S A*. 2007; 104(49):19416–19421. 10.1073/pnas.0707442104 [PubMed: 18042715]

FIGURE 1.

Association of α -SMA with MeCP2 in primary human non-IPF and IPF fibroblasts. The cells were collected at 80% confluence for mRNA or ChIP assays with MeCP2 pulldown. A, Real-time quantitative RT-PCR for α -SMA mRNA expression. The results were averaged from a representative cell line from non-IPF or IPF lung fibroblasts with three independent experiments, expressed as mean $\pm$ SD. * P < .05, compared with the non-IPF cell control. B, Sketch of a human α -SMA gene, the CpG rich region identified by MethPrimer.²² The approximate location of PCR primer sets for ChIP (dark bars) or for RNA (RT-FR, green bar) are marked. C, MeCP2 association with α -SMA by ChIP assays. The association of MeCP2 and α -SMA gene was determined by ChIP assays. DNA was immunoprecipitated by the MeCP2 antibody. Bars are the relative values of the PCR product of α -SMA associated with MeCP2 by three different PCR primer sets (as indicated in B). The 2^{-C_t} method was used for quantitative PCR, normalized to input. The results are relative to primer set FaRa of non-IPF cells, expressed as fold changes. The bars are values of mean $\pm$ SD, obtained from three independent experiments of a representative cell line. * P < .05 compared with the non-IPF control cells. ChIP, chromatin immunoprecipitation; IPF, idiopathic pulmonary fibrosis; MeCP2, methyl-CpG-binding protein 2; mRNA, messenger RNA; RT-PCR, reverse transcription polymerase chain reaction; SD, standard deviation; α -SMA, alpha-smooth muscle actin

FIGURE 2.

The upregulation of α -SMA by TGF- β 1 in primary non-IPF fibroblasts is associated with enriched MeCP2. A, mRNA levels of α -SMA in non-IPF lung fibroblasts treated with TGF- β 1 by quantitative PCR (qPCR). The results were average of three independent experiments from one representative cell line, expressed as mean $\pm$ SD. * P < .05, compared with its baseline control (Ctrl). B, MeCP2 association with α -SMA by ChIP assays. The values of the bars are relative levels of qPCR of α -SMA associated with MeCP2 by three different PCR primer sets, all relative to FaRa of non-IPF at baseline control (Ctrl). The values of PCR are normalized to input DNA and expressed as fold changes. The bars are values of mean $\pm$ SD, obtained by three independent experiments from one representative cell line. * P < .05 vs non-IPF cells at baseline (Ctrl) of each primer set. ChIP, chromatin immunoprecipitation; IPF, idiopathic pulmonary fibrosis; MeCP2, methyl-CpG-binding protein 2; mRNA, messenger RNA; PCR, polymerase chain reaction; SD, standard deviation; TGF- β 1, transforming growth factor β 1; α -SMA, alpha-smooth muscle actin

FIGURE 3.

Non-IPF fibroblasts with MeCP2 knockdown by siRNA failed to upregulate α-SMA in response to TGF-β1 treatment. A, Transfected non-IPF fibroblasts with siRNA of nontargeting (NT) or MeCP2. The expression of MeCP2 by mRNA was determined by quantitative real-time RT-PCR to confirm the knockdown of MeCP2. * $P < .05$, vs siRNA NT. B, siRNA transfected non-IPF fibroblasts were subjected to TGF-β1 at 2 ng/mL for 24 hours, the mRNA levels of α-SMA were examined by real-time RT-PCR. * $P < .05$, compared with NT without TGF-β1 treatment. C, The same treatment as in B, while the cells were collected to check α-SMA expression at protein levels by Western blots. Loading control is β-actin. IPF, idiopathic pulmonary fibrosis; MeCP2, methyl-CpG-binding protein 2; mRNA, messenger RNA; RT-PCR, reverse transcription polymerase chain reaction; siRNA, small interfering RNA; TGF-β1, transforming growth factor β1; α-SMA, alpha-smooth muscle actin

FIGURE 4.

DNA methylation inhibitor 5'-aza treated IPF primary fibroblasts. A, B, Primary IPF fibroblasts from three different patients were treated with or without 5'-aza at 5 μ M in fresh medium daily for 3 days. The expression of α -SMA was examined at RNA levels by quantitative real-time RT-PCR (A), and at protein levels by Western blots (B). Control (Ctrl) is the cells with a vehicle only under the same condition as with 5'-aza (AZA) treatment. mRNA expression is expressed as the mean $\pm$ SD from three independent experiments. * $P < .05$, vs its own Ctrl. C, MeCP2 association with α -SMA by ChIP assays, with the same treatment as in A. Bars are the values of PCR product of α -SMA associated with MeCP2 by three different PCR primer sets, all relative to FaRa Ctrl. Input DNA was used to normalize the PCR results; the data were presented as fold changes. The bars are values of mean $\pm$ SD, obtained by three independent experiments of one representative cell line (IPF#3). * $P < .05$ vs cells at Ctrl under each primer set. 5'-aza, 5'-aza-2'-deoxycytidine; ChIP, chromatin immunoprecipitation; IPF, idiopathic pulmonary fibrosis; MeCP2, methyl-CpG-binding protein 2; mRNA, messenger RNA; RT-PCR, reverse transcription polymerase chain reaction; SD, standard deviation; α -SMA, alpha-smooth muscle actin

FIGURE 5.

α-SMA expression and association with MeCP2 with or without 5'-aza and TGF-β1 treatment. Human lung fibroblast cell line IMR-90 were pretreated with or without 5 μM 5'-aza for three days, then the medium was changed into 1% FBS for overnight before adding 2 ng/mL TGF-β1 for 24 hours; the cells were collected for whole-cell lysate, RNA, or ChIP assays. A, α-SMA protein-level expression was examined by Western blots, the loading control is β-actin. B, α-SMA mRNA expression by real-time quantitative RT-PCR. The results are expressed as mean ± SD from three independent experiments, all relative to baseline vehicle control (Ctrl). **P* < .05, compared with vehicle only (Veh1) or 5'-aza (AZA) both conditions without TGF-β1 (Ctrl). C, MeCP2 association with α-SMA by ChIP. Bars are the relative values of the PCR product of α-SMA associated with MeCP2 by three different PCR primer sets. Quantitative real-time PCR results were expressed as fold changes after being normalized to input DNA, all relative to FaRa of the vehicle only control (Ctrl). The bars are values from three independent experiments, expressed as mean ± SD. **P* < .05 compared with cells at vehicle only Ctrl (white bars) of each primer set. 5'-aza, 5'-aza-2'-deoxycytidine; ChIP, chromatin immunoprecipitation; FBS, fetal bovine serum; MeCP2, methyl-CpG-binding protein 2; mRNA, messenger RNA; RT-PCR, reverse transcription polymerase chain reaction; SD, standard deviation; TGF-β1, transforming growthfactor β1; α-SMA, alpha-smooth muscle actin

FIGURE 6.

α -SMA DNA methylation status of IMR-90 lung fibroblasts treated with or without 5'-aza and TGF- β 1. The cells were treated as described in Figure 5, and the DNA was collected and modified with bisulfite to examine the DNA methylation status. The modified DNA was subjected to bisulfite sequencing. The sequenced regions are labeled in A (light and dark gray bars at region A or region B, respectively, with three sets of BSP PCR primers). The CpG sites we examined are labeled from A1 to A9 (B) or B1 to B27 (C), corresponding to the highlights marked in region A or region B. D, E, Schematic illustrations of region A (D) or region B (E) of the α -SMA CpG rich region based on sequencing of at least 10 bisulfite sequencing PCR (BSP) samples with indicated treatments. Different shades of gray indicate methylated CpG from 10% to 30% of the examined copies, the darker the more methylated CpG; white circle indicates unmethylated CpG. 5'-aza, 5'-aza-2'-deoxycytidine; TGF- β 1, transforming growth factor β 1; α -SMA, alpha-smooth muscle actin

TABLE 1

Primers sequences for PCR

Name	Primers	Sequence
Human ACTA2(α-SMA) (ENSG00000107796)	RT-PCR (RT-FR)	F: 5'-TCCTCATCTCCCTTGAGAA-3' R: 5'-ATGAAGGATGGCTGGAACAG-3'
	ChIP-PCR	Fa: 5'-CAAGCCTCCAGAAAGCTCATT-3' Ra: 5'-TGCAGCCCTTCAGAACAGATATT-3' Fb: 5'-GCAATCCTCCGAAAGTGAAAGA-3' Rb: 5'-GAGTGAGGGAAGCGGTTTAC-3' Fc: 5'-GAGGTCCCTATATGGTTGTGTAG-3' Rc: 5'-AGCTGAAAGCTGAAGGTTAT-3'
MeCP2	Bisulfite sequencing PCR	BSP_Fa: 5'-AGGGTAGAAAAATAAAATGAAATT-3' BSP_Ra: 5'-AAAAAAACCTACACCTTCAAAAC-3' BSP_Fb: 5'-AGGTTTAAAGAAAAGTAAGTTTATTAG-3' BSP_Rb: 5'-TACCTCTAATAAACCTCTCTTACC-3' BSP_Fc: 5'-AGGAGAGGTTTATTAGAGGTAGGAG-3' BSP_Rc: 5'-ACACCCTAACCAACCTAACTAC-3'
		F: 5'-GCAGGCAAGCAGAGACATCAGAA-3' R: 5'-CCTTTGCTTAAAGCTTCCGTGTCCA-3'
		F: 5'-TGCTATCCAGGCTGTGCTAT-3' R: 5'-AGTCCATCAGATGCCAGT-3'
β-Actin	RT-PCR	

Abbreviations: MeCP2, methyl-CpG-binding protein 2; RT-PCR, reverse transcription polymerase chain reaction; α-SMA, alpha-smooth muscle actin.