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ORIGINAL ARTICLE

MeCP2 Regulates PTCH1 Expression Through DNA Methylation in Rheumatoid Arthritis

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Abstract—Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease, in which pathogenesis is not clear. Many research demonstrated that fibroblast-like synoviocytes (FLSs) play a key role in RA pathogenesis, join in the cartilage injury and hyperplasia of the synovium, and contribute to the release of inflammatory cytokines. We used adjuvant arthritis (AA) rats as RA animal models. The methyl-CpG-binding protein 2 (MeCP2) enables the suppressed chromatin structure to be selectively detected in AA FLSs. Overexpression of this protein leads to an increase of integral methylation levels. Some research has confirmed the hedgehog (Hh) signaling pathway plays an important role in RA pathogenesis; furthermore, patched 1 (PTCH1) is a negative fraction of Hh signaling pathway. We used 5-aza-2′-deoxycytidine (5-azadc) as DNA methylation inhibitor. In our research, we found MeCP2 reduced PTCH1 expression in AA FLSs; 5-azadc obstructed the loss of PTCH1 expression. 5-Azadc, treatment of AA FLSs, also blocks the release of inflammatory cytokines. In order to probe the potential molecular mechanism, we assumed the epigenetic participation in the regulation of PTCH1. Results demonstrated that PTCH1 hypermethylation is related to the persistent FLS activation and inflammation in AA rats. Knockdown of MeCP2 using small-interfering RNA technique added PTCH1 expression in AA FLSs. Our results indicate that DNA methylation may offer molecule mechanisms, and the reduced PTCH1 methylation level could regulate inflammation through knockdown of MeCP2.

KEY WORDS: DNA methylation; PTCH1; fibroblast-like synoviocytes; methyl-CpG-binding protein 2; hedgehog signaling pathway; rheumatoid arthritis; inflammation.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease [1]. Its characteristics are synovial hyperplasia and progressive joint inflammation, which finally contribute to cartilage and bone destruction [2]. Synovial tissue includes intimal lining and sublining. During RA pathogenesis, the synovium forms a relatively static non-cellular tissue to a thickened, aggressive tissue full of immunocytes [3]. Studies showed that fibroblast-like synoviocytes (FLSs) exhibit unique features in RA, including an outstanding synovial hyperplasia to form a pannus which eventually destroys articular bone and cartilage [4, 5]. In addition,

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inflammatory cytokine secretion was contributed to imbalance of cytokines [6]. These pathological changes were involved in the RA pathological process. However, cytokines were regarded as a key feature of RA [7].

Methyl-CpG-binding proteins (MeCPs) recruit histone deacetylases, chromatin remodelers, and methylases to methylated DNA, leading to transcriptional repression [8]. Methyl-CpG-binding protein 2 (MeCP2) belong to methyl-CpG-binding domain (MBD) protein family which can recognize methylated CpG islands specifically. It exerts the ability to convene inhibitor complexes at methylated CpG islands to methylate DNA, which results in gene transcriptional suppression [9]. DNA methylation is a kind of epigenetic mechanisms, recruiting proteins involved in transcriptional repression to controls gene expression [10]. In many cancers, cellular DNA undergoes profound changes in methylation. DNA methylation also causes other genes repression which important for cellular growth, regulation, differentiation, and the silencing of tumor suppressors [11–15]. Growing evidence indicated DNA methylation plays a crucial role in RA pathogenesis, providing a new perspective to study RA [16, 17].

Similarly, FLSs showed aberrant proliferation in RA, leading to joint destruction. Therefore, activated FLS was a key factor in RA [18]. These functions of activated FLSs are associated with a variety of signaling pathways. Patched 1 (PTCH1) is the suppressant protein in hedgehog (Hh) signaling pathway, which could regulate cell differentiation and proliferation [19]. When sonic hedgehog (Shh) protein is deficient, PTCH1 suppresses the smoothened (Smo) activity, inhibiting the Hh signal pathway. When Shh protein binds to PTCH1 and releases the suppressive Smo, downstream transcription factors was activated through glioma-associated oncogene homolog (Gli1) [19, 20]. The Hh signaling pathway activity is based on the levels of Hh genes (Gli1, PTCH1) transcription [21]. Evidence has revealed that Hh signaling pathway participates in RA pathogenesis [22, 23].

Here we used the adjuvant arthritis (AA) rats to explore the relationship between the DNA methylation and the molecular mechanism for MeCP2 regulated PTCH1 expression, and explore whether MeCP2 affects the Hh signaling pathway.

MATERIALS AND METHODS

Materials

Australian fetal bovine serum (FBS) comes from Gbico (Australia). High-glucose DMEM comes from Hyclone. Mouse anti-MeCP2 and mouse anti-Shh come from abcam (Cambridge, UK). Mouse anti-β-actin comes from Zhongshan Jinqiao (Beijing, China). Rabbit anti-PTCH1 and rabbit anti-Gli1 come from Beijing Bioiss (Beijing, China). Enzyme-linked immunosorbent assay kit of IL-6 and TNF-α comes from Elabscience (Wuhan, China). Secondary antibody for goat anti-rabbit immunoglobulin (Ig)G horse radish peroxidase (HRP) and goat anti-mouse IgG HRP come from Zhongshan Jinqiao (Beijing, China). 5-Aza-2′-deoxycytidine (5-azadc) comes from Sigma Inc. (St. Louis, MO, USA).

The Preparation of Rheumatoid Arthritis Rat Model

Adult Sprague-Dawley (SD) rats (180–210 g) come from the Animal Experiment Center of Anhui Medical University. AA rats were induced by complete Freund's adjuvant (CFA) (per 1 ml contain 1 mg *Mycobacterium butyricum*) while control rats were injected with PBS. Each rat was 0.1 ml/100 g body weight. Then, synovium was isolated from knee at 24 days. All the animal experiment processing was supervised and approved by the Animal Ethics Management Committee and Animal Experiments Guidelines.

The Extraction of Primary Cell

Animals were killed on day 24 after treatment with complete Freund's adjuvant. Fibroblast-like synoviocytes were extracted from isolated synovium and cultivated in culture flanks. Synovium was separated from the joint of AA rats and control rats, cultivated in the bottom of cell culture bottle, and incubated in DMEM including 20% (FBS) and 1% penicillin-streptomycin solution (Beyotime, China) at 5% CO_2 and 37 °C for 6 to 8 h. Three days later, scarce synovial cells around individual organizations were visible. Six days later, cell growth closely around some organizations, namely, can remove tissues. Then, synovial tissues were removed and attached cells were cultivated in medium. Covered with about 70 to 80% of a bottomed culture bottle, cells were trypsinized and cultured in the previous medium. Then, in the following experiment, we used the pure FLSs which undergo three trypsinizations.

Immunostaining

The knee joint from AA rats and control rats were fixed in 4% paraformaldehyde, embedded in paraffin, and used to immunostaining assay. The sections were handled; we used xylene to dewax and alcohol to dehydrate, and used microwave for antigen retrieval in citric saline for

15 min. We used 0.3% hydrogen peroxide to treat the sections for 15 min, so the activity of endogenous peroxidase was blocked. We used 1% bovine serum albumin to block the thin sections and used primary antibody against PTCH1 (1:500) and MeCP2 (1:300) to incubate sections. After repeatedly washing, we used biotinylated secondary antibody to incubate sections 25 min at room temperature. Then, we used 3,3′-diaminobenzidinetetrahydrochloride (DAB) staining to detect PTCH1 and MeCP2 expression. Before dehydration, the sections were counter stained with hematoxylin for 30 s, and then PTCH1 and MeCP2 positive areas were visible.

Knee Joint Histopathology

The rats were sacrificed after treatment of CFA 24 days. We separate the knee joint synovial tissues to do hematoxylin and eosin (H&E) staining. Knee joint synovial tissues from AA rats and control rats were fixed in 4% paraformaldehyde for 3 day and decalcification of knee joint was done in 10% Ethylene Diamine Tetraacetic Acid (EDTA) for 20 days. Then, the knee joint specimens were embedded in paraffin. The tissue sections (5 μm thick) were used for pathological and morphological evaluation. These sections were observed at 10 × 10 light microscopic fields.

Immunofluorescence

FLSs were seeded on cover glass and washed by PBS three times, fixed with acetone for 10 min, and blocked with 10% BSA for 10 min at room temperature. We used MeCP2 (1:200 dilution; abcam, Cambridge, UK) and PTCH1 (1:200 dilution; Bioss, China) to incubate cells at 4 °C overnight. Then, goat anti-mouse and goat anti-rabbit IgG, as well as FITC conjugate (1:100 dilution; Zhongshan Jinqiao, Beijing, China) were used to combine primary antibodies for 2 h at room temperature. Finally, 40,60-diamidino-2-phenylindole (DAPI) (Sigma, MO, USA) was used for counterstaining, cover glass was mounted onto slides glass with glycerine, and pictures were taken by Fluorescence Inversion Microscope System (Zeiss, Axiovertt200).

Western Blotting

We get the total protein from control FLSs and AA FLSs, which were lysed with RIPA (Beyotime, China) including 1% phenylmethanesulfonyl fluoride (PMSF). In order to gain the protein concentration, we used BCA protein kit (Boster, China) to detect the extraction according to the operating manual. Protein sample (20 or 60 mg) was separated by electrophoresis using SDS-PAGE and transferred to PVDF membranes (Millipore Corp., Billerica, MA, USA). Membranes were blocked by milk, blot strips were incubated for 12 h in antibody diluents (Beyotime, China) including primary antibodies. Anti-MeCP2, anti-Shh (abcam, Cambridge, UK), and anti-β-actin (Zhongshan Jinqiao, Beijing, China) were diluted 1:1000. Anti-PTCH1 and anti-Gli1 were diluted 1:200. After incubating, we used TBS/Tween 20 (0.075%) to rinse membranes three times and then used secondary antibodies (1:10,000) which were conjugated with horseradish-peroxidase to incubate these membranes for 1 h. Finally, proteins bands were showed by ECL-chemiluminescent kit (ECL-plus, Thermo Scientific).

Methylation-Specific PCR

We used methylation-specific PCR (MSP) to detect the PTCH1 promoter methylation level through bisulfite-modified DNA. High-molecular-weight genome DNA was extract by standard protocols from FLSs through DNA extraction kit (Axygen). The primers were designed for amplifying PTCH1 CpG-island: one specific sequence is to methylated DNA and the other specific sequence is to unmethylated DNA. The sequences used to amplify the methylated PTCH1 gene were 5′-GAGCGGAGTTTTTA GGTTTC-3′ (forward) and 5′-ATCTCAACGATACC GAACC-3′ (reverse), and sequences used for unmethylated PTCH1 gene were 5′-ATAG AGTGGAGTTTTTAGGTTTT-3′ (forward) and 5′-ATCT CAACAATACCAAACCAAA-3′ (reverse). We used the primers to detect the PTCH1 gene promoter region specifically. PCR reactions were proceeding at final 25 μl volume containing the following: 12.5 μl KAPA 2G Robust HS ReadyMix (2×), 2 μl primers, and 2 μl DNA sample. The amplification conditions were as follows: 95 °C 3 min, cycled at 95 °C 15 s,57 °C 15 s, and 72 °C 15 s (35 cycles) and extension at 72 °C 7 min. MSP detection was processed in duplicate.

Pyrosequencing

The site-specific methylation level of PTCH1 promoter region was detected quantificationally by pyrosequencing. We get the PCR products from bisulfite-treated genome DNA extraction. The specific primers were designed to recognize the GpG islands. Primer sequences: forward PCR Primer 5′-AGTTGGGAGTTTTTTAGATG ATAGG-3′, reverse PCR Primer 5′-CCAA AAAACCCCACCTTAATCTCTTTCA-3′, sequencing

Primer 5′-GGAGTTTTTTAGATGATAGGTTTA-3′. Pyrosequencing was performed according to the operating instructions (Qiagen). CpG islands were assessed by transforming the pyrograms to quantitative consequence for hump heights. We are presenting a pyrosequencing diagram in Fig. 4d.

Small RNAi Transfection

In order to perform the gene knockdown of MeCP2, FLS density is 0.6–1 × 10^5 cells/ml, which cultivated in DMEM including 20% (FBS). On the second day after culturing, cells were transfect with MeCP2-small-interfering RNA (siRNA) or a Scrambled-siRNA (Shanghai, China) through Lipofectamine 2000 (Invitrogen, USA) and opti-MEM on the basis of operating instructions. The MeCP2-siRNA sense strand is 5′-GGGA CCUAUGUAUGAUGACTT-3′ and antisense strand is 5′-GUCAUCAUACAUAGGUCCCTT-3′. The scrambled-siRNA sense strand is 5′-UUCUCCGA ACGUGUCACGUTT-3′, and antisense strand is 5′-ACGUGACACGUUCGGAGAATT-3′. Transfection was for 6 h, and then we change the opti-MEM by DMEM including 20% (FBS).

Statistical Analysis

Results were represented as mean ± SD. SPSS 16.0 was used to calculate data statistic difference. Paired *t* test was used to do difference comparison within intra-group; we used ANOVA to compare the difference among groups. $P < 0.05$ was identified as statistical differences.

RESULTS

MeCP2 Is Upregulated in AA Rat's Synovial Tissues

The establishment of AA rats was determined by H&E and the degree of foot swelling ($P < 0.01$) (Fig. 1a, b). Histopathological analysis (Fig. 1a) confirmed that AA rats have an obvious synovial hyperplasia, an inflammatory cytokine infiltration, and the formation of pannus compared with control rats. Immunohistochemical results (Fig. 1c) demonstrated that MeCP2 expression significantly increased in AA rat synovium. Furthermore, to confirm the changes in MeCP2 protein expression during the progression of RA, FLS was isolated from AA rat synovium. Western blotting and immunofluorescence results ($P < 0.01$) (Fig. 1d, e) revealed that MeCP2 protein level was indeed increased in AA FLSs. To explore the function assessment, the cell (5 × 10^4 cells/100 mm^2 dish) culture supernatant was collected and centrifuged (1000 g for 1 min) for detection of cytokines such as IL-6 and TNF-α. ELISA results ($P < 0.01$) (Fig. 1f) indicated that cytokines IL-6 and TNF-α were markedly increased in AA FLS medium.

Reduce MeCP2 Protein Level by the Treatment of 5-Azadc

Then, we explore the potential effect of MeCP2 during RA pathogenesis; the meaning of MeCP2 expression was studied in AA rats. It was assumed that MeCP2 was participated in DNA methylation. To prove the point, AA FLSs were cultured in serum-containing media and used 5-azadc (2 μm) to stimulate for 48 h. 5-Azadc is an efficient methylation inhibitor. Western blotting results ($P < 0.01$) (Fig. 2a) showed that 5-azadc inhibited MeCP2 protein expression. Interestingly, when the FLSs (5 × 10^4 cells/100 mm^2 dish) cover the plate, culture supernatant was collected and ELISA analysis ($P < 0.01$) (Fig. 2b) showed that 5-azadc inhibits inflammation cytokines release, such as IL-6 and TNF-α. These results suggest that increased MeCP2 protein in AA rats may be involved in reversible epigenetic mechanisms.

PTCH1 Was Downregulated in AA Rat Synovial Tissues

In our research, we identify the change of PTCH1 expression between AA rat's synovium and control rat's synovium. Three normal synovial tissue samples and three AA synovial tissue samples were used for immunohistochemistry. Immunohistochemical analysis (Fig. 3a) showed that PTCH1 expression was significantly decreased in AA rat synovium. Furthermore, Western blotting ($P < 0.01$) (Fig. 3b) and immunofluorescence (Fig. 3c) results revealed that PTCH1 was also markedly decreased in AA FLSs, which was compared with control group. Furthermore, Western blotting analysis ($P < 0.01$) (Fig. 3d) showed that Gli1 and Shh protein expression was increased in AA FLSs. According to these results, the expression of PTCH1 protein in AA rats was reduced, accompanied by Hh signal pathway activation.

Epigenetics Regulates PTCH1 Protein Expression That Contributes to Activate the Hh Signal Pathway

For confirming that DNA methylation resulted in the decreased of PTCH1 expression *in vitro*, AA FLSs were treated by 5-azadc which is methylation specific inhibitor. Western blotting analysis ($P < 0.05$ and $P < 0.01$) (Fig. 4a)

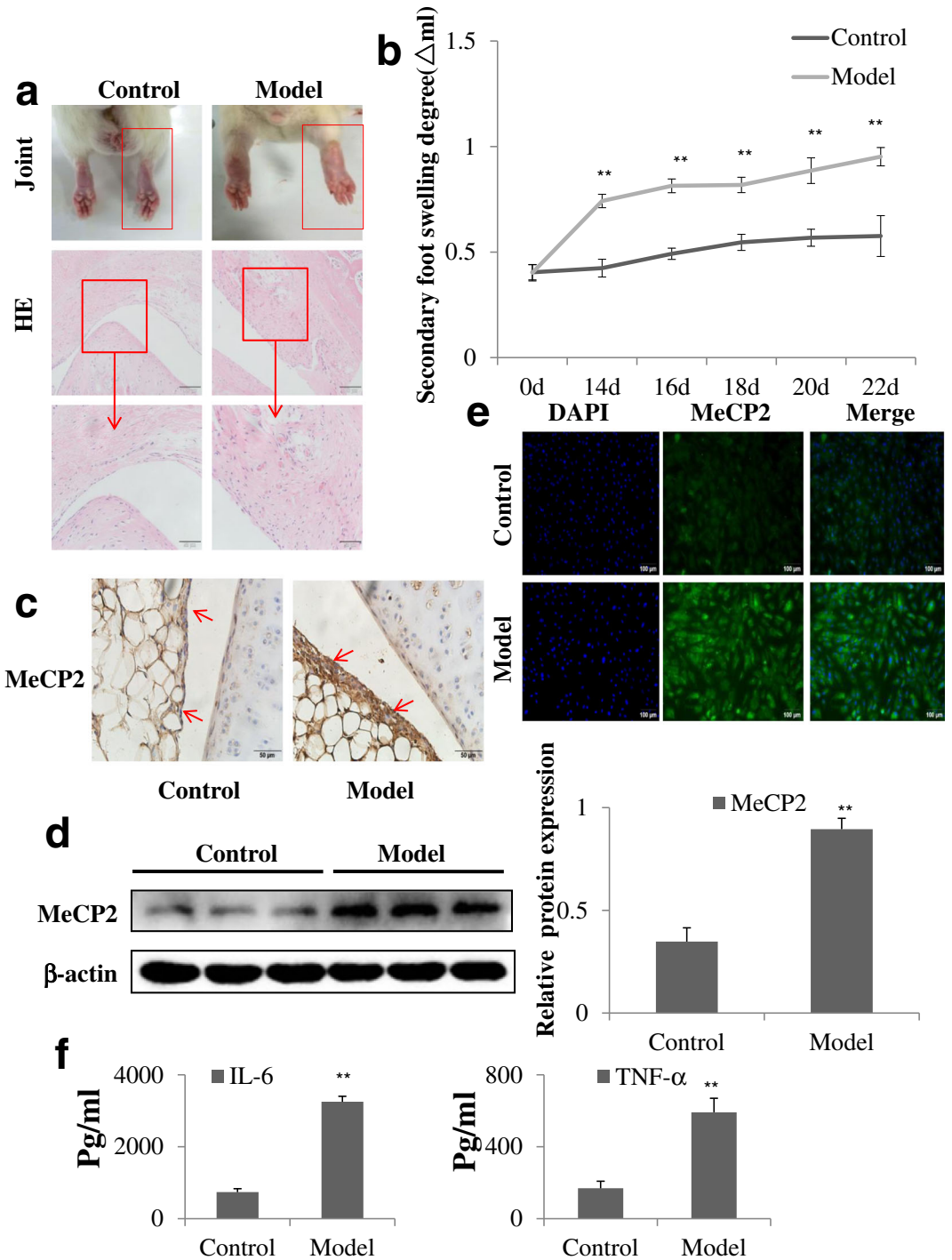

Fig. 1. MeCP2 is upregulated in AA rat synovial tissues. **a** AA rats' synovium and control synovium were measured by HE staining (×50). **b** Secondary parapodum swelling was detected by tester. Representative agent from each group ($n = 5$/group) is presented. **c** AA rats' synovial tissues and control synovial tissues were tested by immunohistochemistry (×50). **d** MeCP2 protein in FLSs was detected through Western blotting. **e** The levels of MeCP2 in FLSs were detected by immunofluorescence (×100). **f** After culturing for 48 h (5×10^4 cells/100 mm^2 dish), culture supernatant was collected; IL-6 and TNF-α were detected according to the description in the "MATERIALS AND METHODS" section. These results are expressed as the mean ± SD of three different experiments. $^{**}P < 0.01$ compared to the control group.

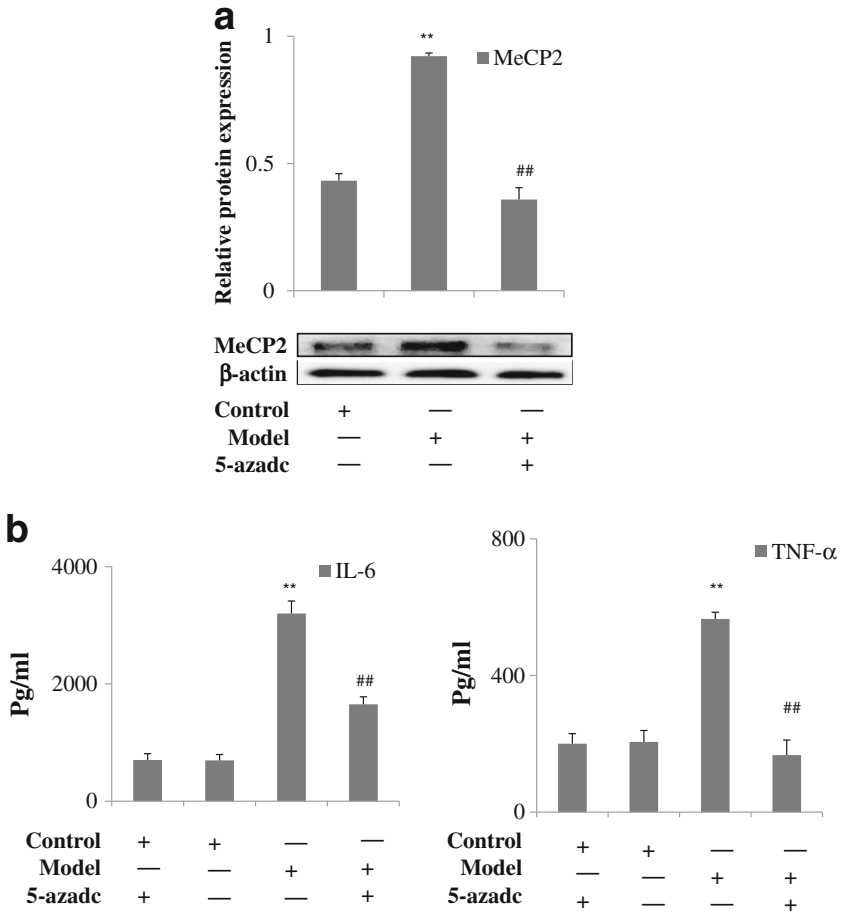

Fig. 2. Reduce MeCP2 protein level by the treatment of 5-azadc. **a** After treatment with 5-azadc for 48 h, MeCP2 protein in FLSs was detected through Western blotting. **b** After treatment with 5-azadc for 48 h (5×10^4 cells/100 mm^2 dish), culture supernatants IL-6 and TNF-α were detected according to the description in operating instruction. These results are expressed as the mean ± SD of three different experiments. $^{**}P < 0.01$ compared to the control group, $^{\#\#}P < 0.01$ compared to the model group.

revealed that PTCH1 protein level was upregulated after 5-azadc application, while Shh and Gli1 protein level were markedly decreased. ELISA (Fig. 2b) was performed to detect the cytokines in medium supernatant. The results indicated that the level of IL-6 and TNF-α was significantly reduced after treating 5-azadc. These results provide additional evidence that 5-azadc may restrain the Hh signaling pathway by inhibiting PTCH1 gene methylation. For further exploring why PTCH1 protein was reduced, prediction was done to determine the CpG island of PTCH1 gene. Projections were suggested near the transcript first exon and first exon upstream; the CG point rich could form a big CpG island (Fig. 4b). It becomes obvious that the decrease of PTCH1 gene expression may be related to CpG island methylation. MSP result (Fig. 4c) showed that PTCH1 gene promoter in AA FLSs were obviously methylation, but treatment with 5-azadc markedly reduced the PTCH1 promoter methylation level. These phenomenons indicated that DNA methylation regulates the PTCH1 protein expression. Likewise, pyrosequencing ($P < 0.01$) (Fig. 4d) demonstrated PTCH1 gene promoter methylation to be more serious in AA FLSs compared with control group; after treatment with 5-azadc, the methylation level of PTCH1 was declined. These data revealed that the reduced PTCH1 protein level results from DNA methylation.

Transfecting MeCP2-siRNA Contributed to the High Expression of PTCH1 in AA FLSs

To explore the potential relationship between MeCP2 and PTCH1, the effects of MeCP2 knockdown on the activation of Hh signaling pathway and the expression of

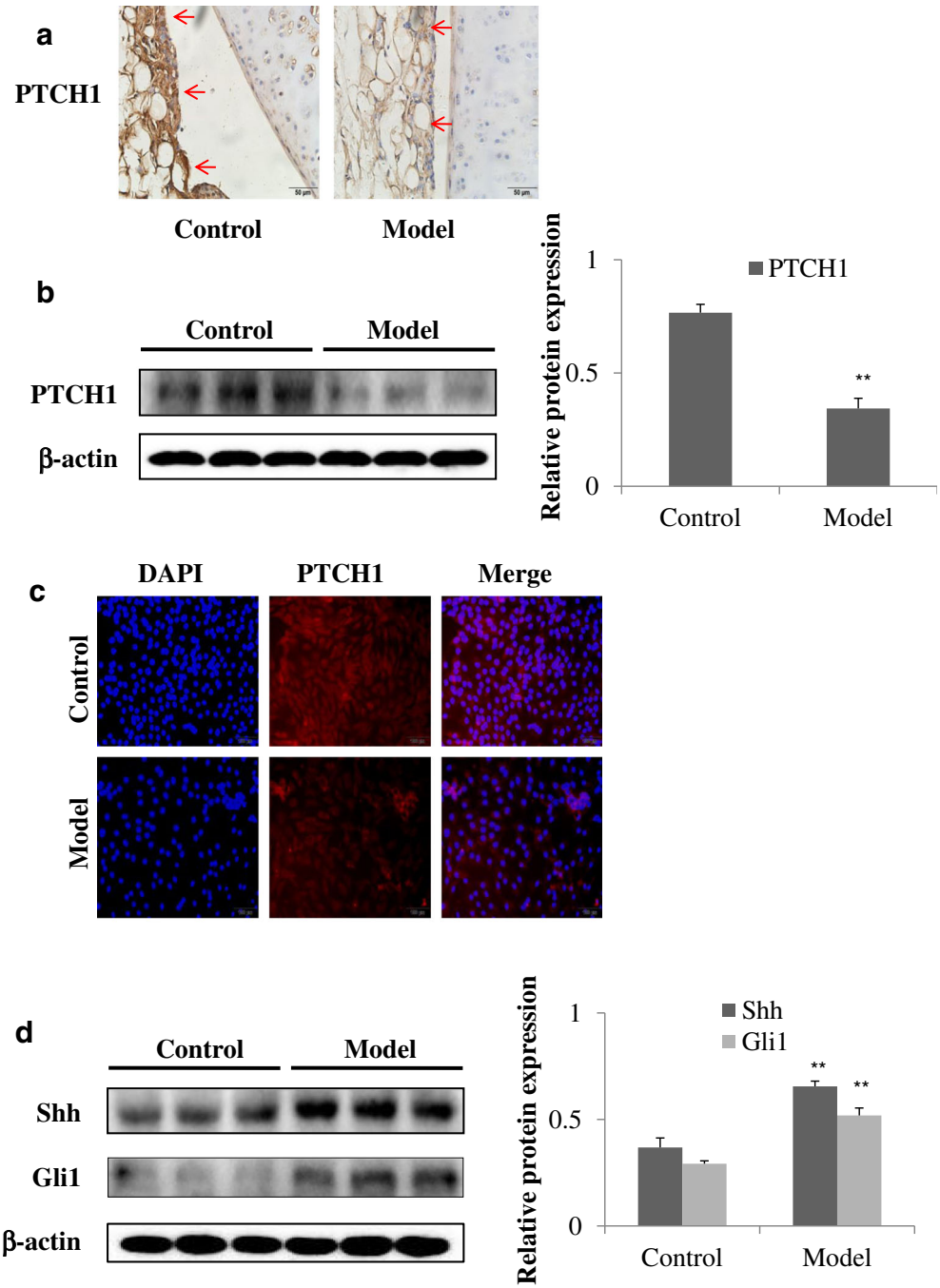

Fig. 3. PTCH1 was downregulated in AA rat synovial tissues. **a** AA rats' synovial tissues (three samples) and control synovial tissues (three samples) were tested by immunohistochemistry (×50). **b** PTCH1 protein in FLSs was measured through Western blotting. **c** PTCH1 protein in FLSs was detected by immunofluorescence (×100). **d** Gli1 and Shh protein in FLSs were detected through Western blotting. These results are expressed as the mean ± SD of three different experiments. $^{**}P < 0.01$ compared to the control group.

PTCH1 were examined in AA FLSs. FLSs were inoculated in six pore plates and transfect 0.5 mg MeCP2-siRNA and 0.5 mg scrambled-siRNA in Opti-MEM for 6 h, then dye transfer after 24 h; we used fluorescence microscope to photograph the FLSs at ×200 amplification (Fig. 5a). After 48 h of siRNA transfection, FLS (5×10^4 cells/100 mm^2

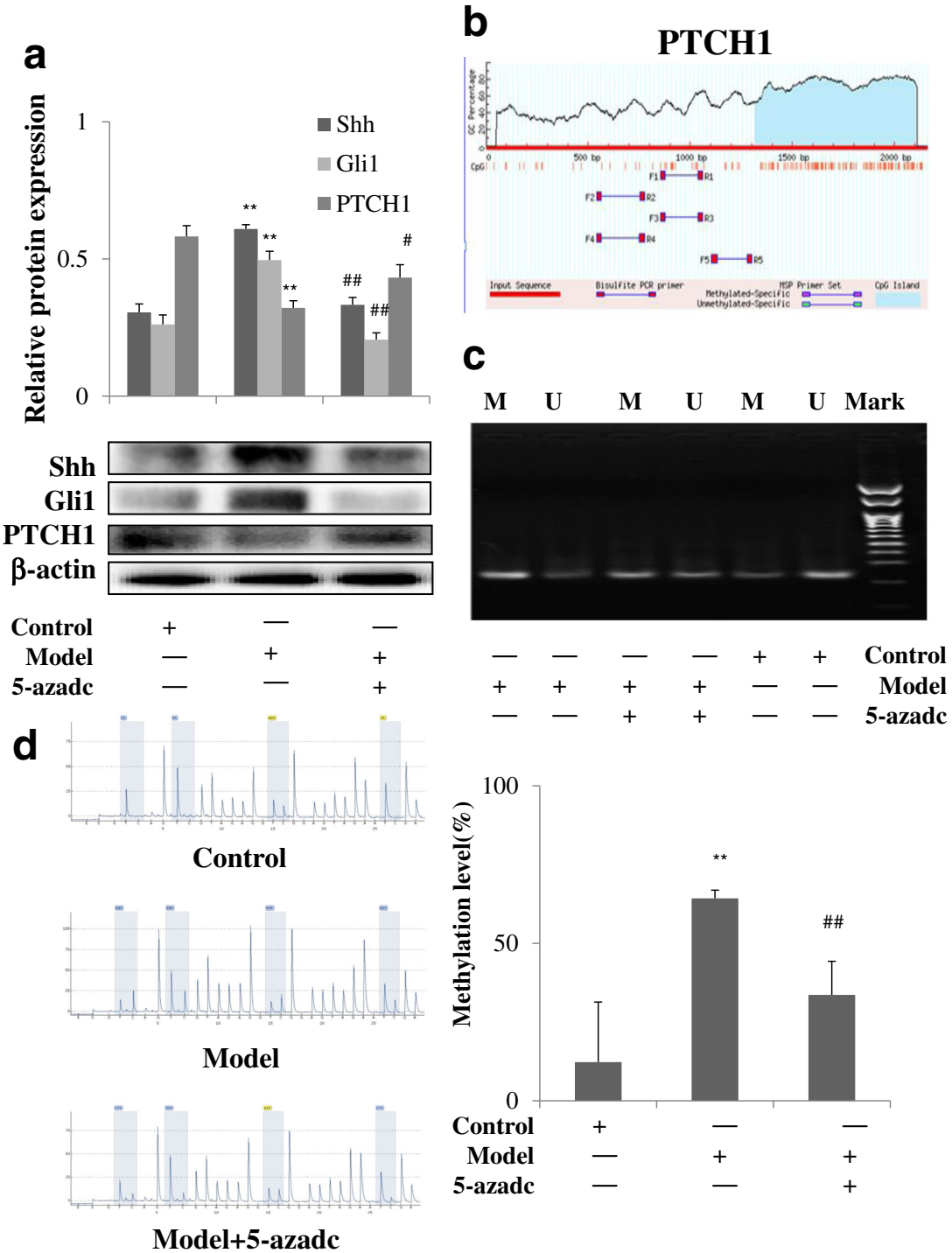

Fig. 4. Epigenetics regulate PTCH1 protein expression contributes to activate the Hh signal pathway. **a** Treatment with 5-azadc for 48 h, *Shh*, *Gli1*, and *PTCH1* protein were detected through Western blotting. **b** PTCH1 methylation CpG sites were predicted. **c** Treatment with 5-azadc for 48 h, MSP results of PTCH1 in control FLSs and AA FLSs (*M* and *U*, PCR products of methylated and unmethylated alleles, respectively). **d** After treatment with 5-azadc for 48 h, FLSs were collected and used to detect the degree of methylation of PTCH1 by the pyrosequencing. These results are expressed as the mean ± SD of three different experiments. $^{**}P < 0.01$ compared to the control group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to the model group.

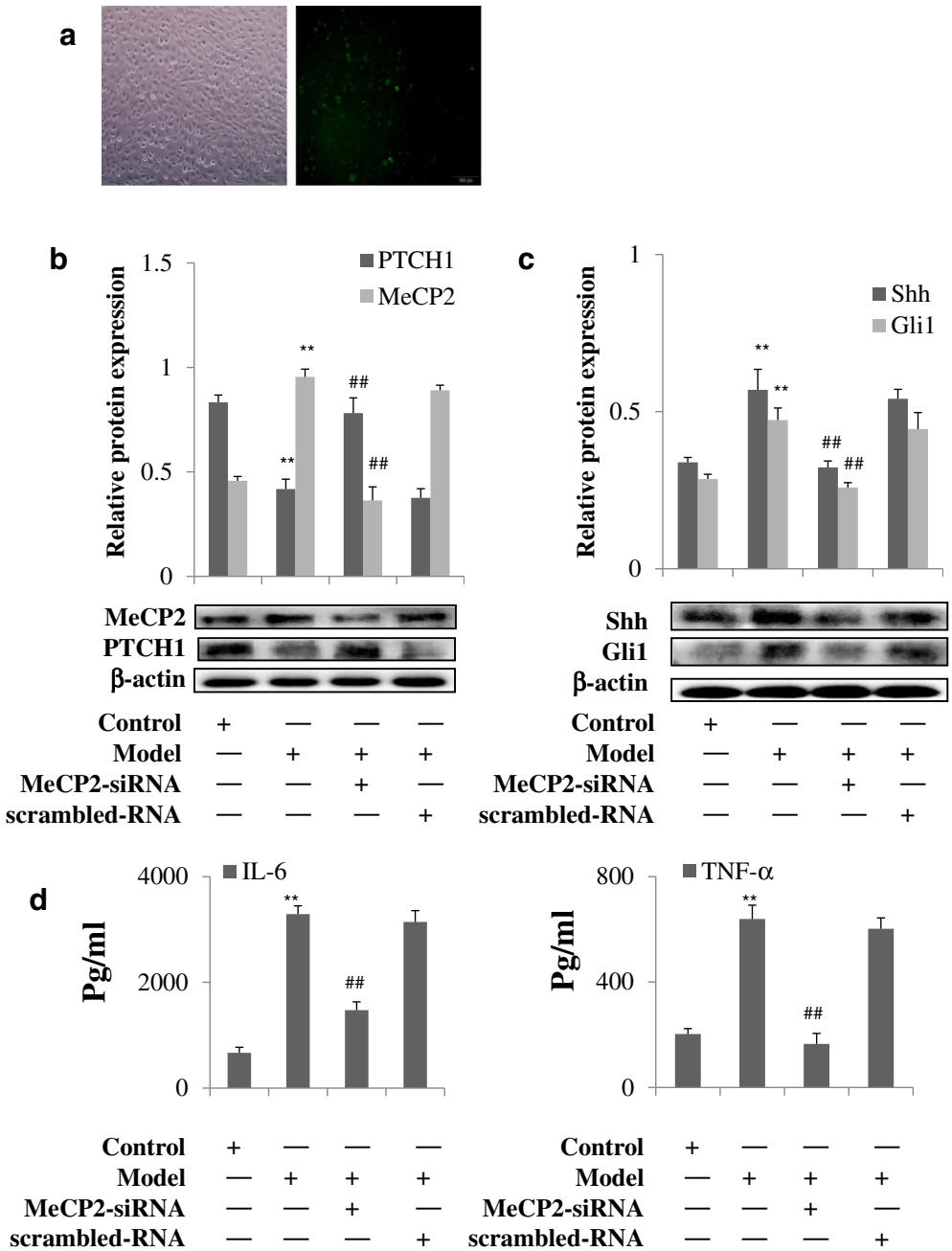

Fig. 5. Transfecting MeCP2-siRNA contributed to the high expression of PTCH1 in AA FLSs. **a** FLSs were seeded onto six well plates and were treated with 0.5 mg MeCP2-siRNA and 0.5 mg scrambled-siRNA. The MeCP2-siRNA and scrambled-siRNA both have GFP, which could be seen under fluorescent microscope (×200). After 48 h, protein extracts were collected. **b** MeCP2 and PTCH1 protein in FLSs were detected through Western blotting. **c** Gli1 and Shh protein in FLSs were detected through Western blotting. **d** Culture supernatants IL-6 and TNF-α were detected according to the description in the "MATERIALS AND METHODS" section. These results are expressed as the mean ± SD of three different experiments. $^{**}P < 0.01$ compared to the control group. $^{\#\#}P < 0.01$ compared to the model group.

dish) was used to extract total protein for Western blotting and the cell culture supernatant was collected and centrifuged (1000×*g* for 1 min) for detection of cytokines. The Western blotting result ($P < 0.01$) (Fig. 5b) showed that MeCP2 protein expression was reduced by the MeCP2-siRNA, but the AA FLS transfect

scrambled-siRNA was not obviously reduced. Both of the results were proved successful transfection. Interestingly, Western blotting ($P < 0.01$) (Fig. 5b) revealed that MeCP2 regulates the PTCH1 protein expression; PTCH1 protein expression was markedly increased after AA FLSs were treated with MeCP2-siRNA, but there is no significant difference between AA FLS and the AA FLS transfected with scrambled-siRNA. These phenomenons demonstrated that MeCP2 might the reason of changed PTCH1 protein level. Simultaneously, Western blotting results ($P < 0.01$) (Fig. 5c) showed that the Shh and Gli1 protein expressions were decreased after AA FLSs were treated with MeCP2-siRNA. This phenomenon indicated that Hh signal pathway was inhibited. Furthermore, the ELISA result ($P < 0.01$) (Fig. 5d) indicates that the level of IL-6 and TNF-α was significantly reduced after MeCP2-siRNA transfection. Finally, we draw the conclusion that MeCP2 through DNA methylation regulates PTCH1 in RA pathogenesis.

DISCUSSION

RA is a chronic autoimmune inflammatory disease with undefined pathogenesis. It causes destruction of joint and bone damage. Many evidences showed that FLS is a key cell in promoting RA occurrence. Once activated, various cytokines, chemokines, and matrix-degrading enzymes were produced by FLS to join in the damage of joint cartilage and bone [24]. We used AA rats as animal models which induced by complete Freund's adjuvant (CFA). This model was widely used for studying the RA pathogenesis.

MeCP2 is the member of the MBD protein family, which participates in recruiting transcriptional inhibitors and combine with methylated CpG islands specifically in genome DNA. It contains a transcription repression domain, a methyl-CpG-binding domain, a C-terminal segment, and two nuclear localization signals [25, 26]. These components cause chromatin condensation and deacetylation of histones, which finally prevent gene transcription. MeCP2 usually represents the global level of methylation. Immunohistochemical analysis showed that MeCP2 is upregulated in AA rat synovium. Likewise, Western blot and immunofluorescence analysis showed that MeCP2 protein level was increased in AA FLSs. The upregulation of MeCP2 activates the Hh signaling pathway in AA FLSs while suppressed after treatment 5-azadc for 48 h.

Our studies present that Hh signaling pathway can be the therapeutic target for RA, in accordance with previous researches. PTCH1 is a suppressive receptor in Hh signaling pathway [27]. When the Hh signaling pathway is activated, overexpressed Shh binds to PTCH1, and then Smo leads to Gli1 translocation from the cytoplasm to the nucleus. Therefore, Gli1 has usually been identified as a mark to judge whether the Hh signaling pathway is activated [28]. In the present study, immunohistochemical analysis indicated that PTCH1 expression was downregulated in AA synovium which compared with control group. Western blot and immunofluorescence analysis also showed that PTCH1 protein level was decreased in AA FLSs. But in other RA studies, the PTCH1 changes were different. PTCH1 expression changes are related with organ specific and cell type specific. Tissue and cell function is different; the role of the same protein in different tissues and cells is variant. Wang's research that used human synovial tissue and FLSs found that Shh, Smo, and Gli1 were of higher expression in the AA rats compared with control rats, but PTCH1 expression was not reduced [23]. Li's research, which used rats' cartilage tissues and chondrocytes, found that Shh, Smo, Gli1, and PTCH1 were of higher expression in the AA rats compared with control rats [22]. Our study, which used rats' synovial tissues and FLSs, found that Shh and Gli1 expression was increased in RA model rats, but the PTCH1 expression was reduced. The expression of Gli1 and Shh is similar in those studies, but PTCH1 may have different roles in the articular cartilage and synovium. Furthermore, species differences may be the reason for various PTCH1 expressions. It might be the diversity of PTCH1 expression that results in the complexity of RA. So, we explore the reason of reduced PTCH1 protein level. Studies have found many kinds of cancer subject to DNA methylation, such as breast cancer and gastric cancer [29, 30]. Moreover, there are a lot of researches that emphasize common point between the development of cancer and the RA progression. It might be that the common epigenetic modifications are participating in cancer and RA. Therefore, we hypothesize that hypermethylation of PTCH1 genes contribute to reduced protein level. To explore the underlying molecular mechanisms, AA FLSs used DNA methylation inhibitor 5-azadc to treatment for 48 h, adding 5-azadc that increased the PTCH1 expression. Furthermore, increased PTCH1 inhibited Hh signal pathway key gene Shh and Gli1 expression.

PTCH1 protein level was decreased in AA rats, and overexpression of MeCP2 activated the Hh signaling pathway through the PTCH1 inhibition, indicating a crucial role in the signal transduction process. Changes of MeCP2 expression and activity may offer an explanation for the reduced PTCH1 protein level. Concurrently, we found that

5-azadc decreased the cytokines release in AA FLSs. ELISA analysis indicated that 5-azadc treatment resulted in less IL-6 and TNF-α expressions. Thus, we assume that PTCH1 expression would be regulated by DNA methylation. The most compellent evidence for DNA methylation regulating PTCH1 protein expression was pyrosequencing and methylation-specific polymerase chain reaction (MSP), which verified that 5-azadc can inhibit the PTCH1 gene promoter hypermethylation.

It let us imagine whether this kind change of PTCH1 receives MeCP2 regulation. In order to continue to explore the mechanism, we transfect MeCP2-siRNA and scrambled-siRNA into AA FLSs. Knockdown of MeCP2 enhanced PTCH1 protein expression in AA FLSs. This phenomenon indicated that MeCP2 might be involved in differential expression of PTCH1. MeCP2 knockdown has prevented Hh signaling pathway in AA FLSs. Interestingly, MeCP2-siRNA treatment decreased elevation of IL-6 and TNF-α expression. Based on the experimental data, we demonstrated the increased MeCP2 protein involvement of the decreased PTCH1 expression in RA and that it can positively regulate proinflammatory cytokine secretion. Methylation plays an important role in disease processes, such as the liver fibrosis, renal fibrosis, and cancer. 5-AzadC, as a non-specific methylation inhibitor, can inhibit DNMT1, DNMT3a, DNMT3b, MeCP2, and other methylation-related proteins. Our study showed that PTCH1 gene was hypermethylated in RA; 5-azadc could increase the expression of PTCH1 protein. Further study found that knockdown of MeCP2 could promote PTCH1 protein expression. It is suggested that MeCP2 may regulate the methylation status of PTCH1 gene. As a non-specific methylation inhibitor, 5-azadc has a wide range of inhibitory effects on a variety of methylation-related proteins. The specific role of each methylation-related protein also needs other pharmacological methods for research.

In summary, our work shows that hypermethylation of specific genes occur in RA pathogenesis. Knockdown of MeCP2 enhanced PTCH1 expression. In addition, the Hh signaling pathway plays a crucial role in synovium proliferation and cytokines release. MeCP2 activated the Hh signaling pathway through downregulated PTCH1. Our research showed that MeCP2 increased and PTCH1 obviously reduced in RA. After the application of DNA methylation inhibitor such as 5-azadc, it let us believe that MeCP2 regulates PTCH1 through methylation of the gene promoter. It appears to have the potential of this kind of drugs that could be used for treating RA and chronic inflammatory. Our research also provides a new idea that we can target Hh signaling pathway to treat RA. It may be useful for RA patients targeting the epigenetic regulation of specific gene as a promising therapeutic approach.

CONCLUSION

- Epigenetic PTCH1 loss was beneficial to rheumatoid arthritis.
- MeCP2 regulates PTCH1 expression in the progression of rheumatoid arthritis.
- PTCH1 affects the release of inflammatory mediators by influencing hedgehog signaling pathway.

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COMPLIANCE WITH ETHICAL STANDARDS

All the animal experiment processing was supervised and approved by the Animal Ethics Management Committee and Animal Experiments Guidelines.

Conflict of Interest. The authors declare that they have no conflicts of interest.

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