Histone deacetylase 1 is increased in rheumatoid arthritis synovium and promotes synovial cell hyperplasia and synovial inflammation in the collagen-induced arthritis mouse model *via* the microRNA-124-dependent MARCKS-JAK/STAT axis

Q. Meng, B. Pan, P. Sheng

Department of Joint Surgery, The First Affiliated Hospital, Sun Yat sen University, Guangdong, China.

Abstract

Objective

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease featured by synovial joint inflammation. Increasing evidence has highlighted microRNAs (miRNAs) and histone deacetylase 1 (HDAC1) as active participants in RA progression. Hence, the present study aims to explore the functions of HDAC1 and miR-124 on synovial cell hyperplasia and synovial inflammation in RA.

Methods

The expression of HDAC1, miR-124 and MARCKS was determined in the synovial tissues collected from 25 RA patients by RT-qPCR and Western blot analysis. Next, a mouse model with collagen-induced arthritis (CIA) was established, from which fibroblast-like synovial cells (FLSs) were isolated. Then the effect of HDAC1, miR-124 and MARCKS on synovial cell hyperplasia and synovial inflammation in CIA mice was evaluated by HE staining, ELISA, and EdU assays. Afterwards, the interaction among HDAC1, miR-124, MARCKS and the JAK/STAT signalling pathway was assessed by ChIP and dual luciferase reporter assay. Finally, the effect of HDAC1 on RA was further verified by establishing a CIA mouse model.

Results

HDAC1 was highly expressed and miR-124 and MARCKS were poorly expressed in synovial tissues of CIA. Silencing HDAC1 inhibited synovial cell hyperplasia and synovial inflammation by elevating MARCKS and miR-124 both in vitro and in vivo. Deficiency of HDAC1 promoted H3 and H4 acetylation of miR-124 and MARCKS promoter region. miR-124 alleviated synovial cell hyperplasia and synovial inflammation by repressing the JAK/STAT signalling pathway in CIA.

Conclusion

To sum up, silencing HDAC1 mitigates synovial cell hyperplasia and synovial inflammation in mice with CIA by elevating miR-124 and MARCKS expression, thus highlighting a promising competitive new target for RA treatment.

Key words

rheumatoid arthritis, histone deacetylase 1, microRNA-124, MARCKS, JAK/STAT signalling pathway, synovial inflammation

Qing Meng, MD
Boqi Pan, MD
Puyi Sheng, MD

Please address correspondence to:
Puyi Sheng,
Department of Joint Surgery,
The First Affiliated Hospital,
Sun Yat sen University,
58 Zhongshan 2nd Road,
510080 Guangdong,
Guangzhou Province, China.
E-mail: shengpy@mail.sysu.edu.cn

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that is influenced by environmental factors, epigenetic markers and high-risk genetic background (1). The major characteristics of RA include development of pathogenic cellular and humoral autoimmunity to citrullinated proteins and the inflammation of synovial tissues in joints (2). RA can lead to progressive functional loss, articular injury, and other complications. The pathogenesis of RA is associated with fibroblast-like synovial cell (FLS), a cell type commonly occurring at the junction of pannus and cartilage, which can cause joint degeneration by migrating to or sometimes invading joint cartilage, and then producing abnormal chemokines, cytokines, and matrix-degrading molecules (3). Although the clinical outcomes of RA patients has been greatly improved by treatment with small-molecule kinase inhibitors and efficient biologics (4), the early diagnosis of RA is still problematic, resulting in failure to initiated early treatment (5).

Recently, histone deacetylase (HDAC) has attracted great research attention for its novel role in the regulation of FLS in RA (6). HDACs are a family of crucial transcriptional modulatory enzymes, which exert their functions through temporally and spatially controlling gene expression (7). HDAC1 functions as a primary regulator of tissue damage in RA, which presents it as a promising therapeutic target in diagnostics RA therapy (8). Elevated expression and activity of HDAC1 have been detected in synovial tissue of RA, to an extent correlating with the level of tumour necrosis factor-α (TNF-α) (9). Moreover, HDAC1 is also an indispensable part of the Drosha/DGCR8 complex, which affiliates DGCR8 to primary microRNA (miRNA) transcripts to promote the processing of miRNA (10). miRNAs are newly recognised to regulate the inflammatory and immune responses in RA and other autoimmune disorders (11). For example, miR-124 expression correlates with that of matrix metalloproteinase 3 in joint tissues to modulate the age of RA onset (12). miR-124 also plays an inhibitory role in the development of adjuvant-induced arthritis in rats (13).

Myristoylated alanine-rich C-kinase substrate (MARCKS), a dominant substrate of protein kinase C, is located in the plasma membrane and contributes to multiple cellular processes (14). Notably, MARCKS protein is associated with regulation of the migration of fibroblasts (15). Furthermore, the JAK/STAT signalling pathway is a major regulator of growth factor and cytokine activation, which plays roles in the development of several diseases (16). The JAK/STAT signalling pathway can also serve as a therapeutic target for autoimmune and inflammatory diseases including RA (17).

In the current study, we conduct a series of experiments to show that HDAC1 acts as a promoter of RA by regulating MARCKS and the miR-124-dependent JAK/STAT signalling pathway. These findings provide a theoretical foundation for developing rationalised new RA treatments.

Materials and methods

Ethical statement

Written informed consent was obtained from all patients prior to the study. Study protocols were approved by Ethic Committee of The First Affiliated Hospital, Sun Yat sen University and based on the ethical principles for medical research involving human subjects of the Helsinki Declaration. The animal experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol for animal experiments was approved by the Institutional Animal Care and Use Committee of The First Affiliated Hospital, Sun Yat sen University. The animal experiments were conducted based on minimised animal number and the least pains on experimental animals.

Sample collection

The synovial tissues were collected from 30 RA patients who had received arthroscopy biopsy or total joint arthroplasty in The First Affiliated Hospital, Sun Yat sen University from January 2016 to December 2017. The study

group considered of 17 male and 13 female patients (age: 41–62 years; mean age: 52.77±6.04; course of disease: 3–8 years). Diagnosis of all patients was clinically confirmed by arthroscopy and conformed to the classification of American College of Rheumatology in 1987. Meanwhile, peripheral blood samples of the RA patients were collected for later experiment. During routine arthroscopy or open arthroscopic surgery, the synovial tissues were obtained from nine age-matched patients with traumatic joint as control, including four males and five females. Detailed patient information is shown in Supplementary Table S1 online. The tissues were immediately preserved in the liquid nitrogen for subsequent studies.

Dual luciferase reporter assay

The wild type (WT) sequence of STAT1 mRNA 3'untranslated region (3'-UTR) and mutant (MUT) form, in which the miR-124 binding sites were mutated were synthesised and inserted into the pmiR-RB-REPORTTM vector. The luciferase reporter plasmids WT and MUT were co-transfected with NC mimic or miR-124 mimic into HEK293T cells, respectively. 48 h after transfection, cells were lysed and the luciferase detection kit (RG005, Beyotime Biotechnology Co., Ltd., Shanghai, China) was applied to measure the luciferase activity.

Animal treatment

Thirty DBA/1 mice (aged 8 weeks; weighing 160 - 180 g; purchased from Animal Experiment Center of Southern Medical University, Guangdong, China) were randomly assigned into the experimental group (30 mice) and control group (ten mice). The mice were acclimated for one week in a second-level clean animal house with a temperature of 22–24°C, a humidity of 50–60%, and a 12 h light/dark cycle. During this process, the mice had free access to food and drinking water. Calf cartilage type II collagen acetic acid solution (0.5 mg/mL) was fully emulsified in cold Freund's complete adjuvant at a ratio of 1:1, and a 200 μL portion of the emulsified liquid was intracutaneously injected into the left hind pelma, the fundus of stomach, and the back of each mice. After seven days, the same injection was conducted on the right hind pelma, the root of the tail and the back. On days 0, 14, and 21, the thickness of toe was measured by a Vernier caliper. From days 10 to 21, the arthritis index (AI) was evaluated according to the observed swelling of joints. The mice in the control group experienced the same treatment, with normal saline replacing the calf cartilage type II collagen acetic acid solution. An AI value over four indicated the successful establishment of the mouse model. A total of ten mice in the collagen-induced arthritis (CIA) group were observed in comparison with mice in the control group. Then 20 CIA modelled mice were assigned into sh-NC and sh-HDAC1 groups (ten mice each group). Transfected cells were made into cell suspension containing 1 x 10^7 cells/mL and a 100 μL volume was administered to mice by tail vein injection at 14 days after the operation.

Arthritis score of mice with RA

From day 21 onwards, a Vernier caliper was used to measure the thickness of the ipsilateral hind sole of all mice once a week until the 10^{th} week. Meanwhile, from day 21, the swelling of joints was evaluated twice a week. Each evaluation was repeated three times and the results were averaged. In addition, the lesion degree of each joint was assessed by a 5-level scoring method. The highest score of each limb was 4 points, thus giving a maximum of 16 points as follows: 0 point, no swelling; 1 point, slight swelling of small toe joint; 2 points, swelling of toe and vola pedis joints; 3 points, swelling of limbs and claws under ankle joints; 4 points, swelling of all limbs and claws including the ankle joint, and loss of function. After the mice were euthanised by anesthesia overdose, the synovial tissues were extracted from the articular cavity of bilateral knee joints and preserved in formalin solution for histopathological examination.

Haematoxylin-eosin (HE) staining

The synovial tissues of CIA mice were fixed, embedded in paraffin and sectioned into 4 μm-thick slices. Next, the slices were dewaxed by xylene, and hydrated by gradient ethanol and distilled water. Then the slices were stained by haematoxylin for 5 min, and differentiated by hydrochloric acid ethanol for 30 s. Afterwards, the slices were stained in eosin solution for 2 min, dehydrated, cleared and mounted for observation and photographing under an inverted microscope (XSP-8CA, Shanghai Optical Instrument Co., Ltd., Shanghai, China).

Isolation and culture of FLS

The synovial tissues of CIA modelled mice were isolated and cut into 1 x 1 x 1 mm blocks in Dulbecco's modified Eagle's medium (DMEM) (Gibco Company, Grand Island, NY, USA) under aseptic conditions. Next, the tissue blocks were cultured in DMEM containing 20% (v/v) thermal inactivation fetal bovine serum (FBS) (Gibco Company, Grand Island, NY, USA) at 37°C in 5%CO_2. The culture medium was replaced every two days. After one week, cells were passaged, when cell confluence had reached 90%. Cells at passage 3–6 were used in this study.

Then, FLS from CIA mice was cultured in DMEM supplemented with 10% (v/v) FBS, 100 U/mL penicillin, 100 mg/mL (Beyotime Institute of Biotechnology, Shanghai, China) and 10% glucose at 37°C and with 5% CO_2. FLS from CIA mice in logarithmic growth phase was transfected with the plasmids of short-interfering (si)-HDAC1, miR-124 mimic, miR-124 inhibitor, overexpressed (oe)-MARCKS, si-HDAC1 and their relative negative control (NC) alone or in combination using Lipofectamine 2000 or treated with Colivelin [Janus Kinase (JAK)/signal transducer and activator of transcription (STAT) activator]. The above plasmids were purchased from Dharmacon company (Lafayette, CO, USA).

5-Ethynyl-2´-deoxyuridine (EdU) assay

Cells were plated into a 96-well plate at the density of 1.6 x 10^5 cells/well and cultured for 48 h. Each well was then treated with 50 mM EdU at 37°C for 4 h. Later, cells were fixed with 4% for-

Table I. Primer sequence for RT-qPCR.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
miR-124 (human)	GCGCTAAGGCACGCGGT	CAGTGCAGGGTCCGAGGT
miR-124 (mouse)	GCTAAGGCACGCGGTG	GTGCAGGGTCCGAGGT
HDAC1 (human)	CATCTCCTCAGCATTGGCTT	TATTATGGACAAGGCCACCC
HDAC1 (mouse)	ACAGCCACTCGACTGCTCT	GATGCCTCACAAGCTGACAAA
MARCKS (human)	AGCCCGGTAGAGAAGGAGG	TTGGGCGAAGAAGTCGAGGA
MARCKS (mouse)	AGCCCGGTAGAGAAGGAGG	TTGGGCGAAGAAGTCGAGGA
JAK (human)	CTTTGCCCTGTATGACGAGAAC	ACCTCATCCGGTAGTGGAGC
JAK (mouse)	ACCGCCTACATCTCGGAACT	GCAGCTCACCTTCCTTGATCTT
STAT1 (human)	CAGCTTGACTCAAAATTCCTGGA	TGAAGATTACGCTTGCTTTTCCT
STAT1 (mouse)	TCACAGTGGTTCGAGCTTCAG	CGAGACATCATAGGCAGCGTG
GAPDH (human)	CCCCTGGCCAAGGTCATCCATGACAACTTT	GGCCATGAGGTCCACCACCCTGTTGCTGTA
GAPDH (mouse)	AATGGATTTGGACGCATTGGT	TTTGCACTGGTACGTGTTGAT

RT-qPCR: reverse transcription quantitative polymerase chain reaction; miR-124: microRNA-124; HDAC1: histone deacetylase 1; MARCKS: Myristoylatde Alanine rich C Kinase Substrate; JAK: Janus Kinase; STAT: signal transducer and activator of transcription; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

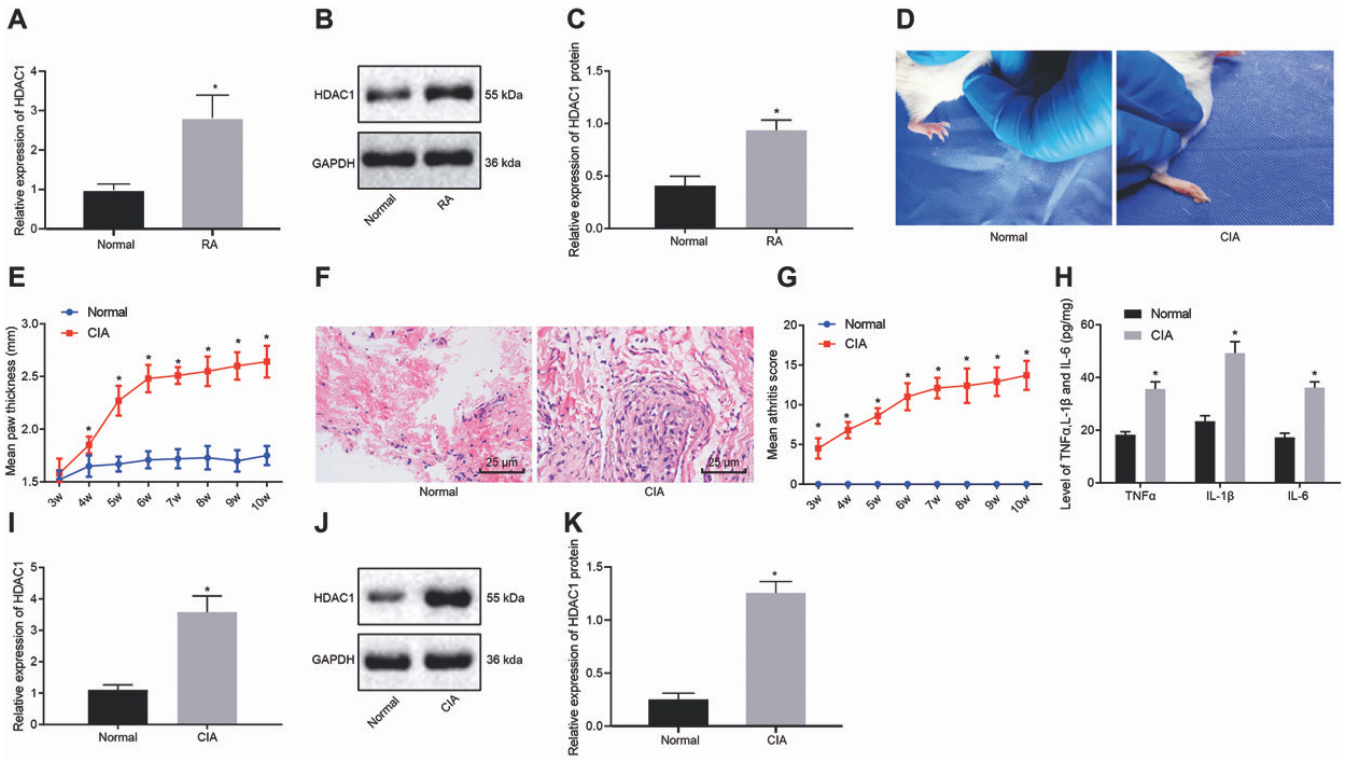

Fig. 1. HDAC1 is expressed at a high level in RA.
A: RT-qPCR analysis of HDAC1 expression in normal synovial tissues (n=9) and RA synovial tissues (n=30).
B-**C**: Western blot analysis of HDAC1 expression in normal synovial tissues and RA synovial tissues B), the band intensity is assessed C).
D: Representative image of joint swelling in normal mice (n=10) and CIA mice (n=10).
E: Thickness of sole in normal mice and CIA mice.
F: HE staining image of synovial tissues in normal mice and CIA mice (400 ×).
G: RA score of normal mice and CIA mice.
H: ELISA of the expression of TNF-α, IL-1β and IL-6 in normal mice and CIA mice.
I: RT-qPCR analysis of HDAC1 expression in synovial tissues of normal mice and CIA mice.
J-**K**: Western blot analysis of HDAC1 expression in synovial tissues of normal mice and CIA mice J), the band intensity is assessed K).
*p<0.05, compared with normal synovial tissues or normal mice. The above data are measurement data and expressed as mean ± standard deviation. Data between two groups are compared by unpaired t-test. n=10.

maldehyde for 15 min and permeabilised by 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 20 min. Then, each well was incubated with 100 μL Apollo® mixture for 30 min, stained with 100 μL Hoechst33342 dye liquor for 30 min, and finally observed and photographed under a fluorescence microscope (CX31, Olympus, Tokyo, Japan). Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA) was used to calculate the number of EdU positive cells (red cells). EdU rate = the number of EdU positive cells /the number of total cells.

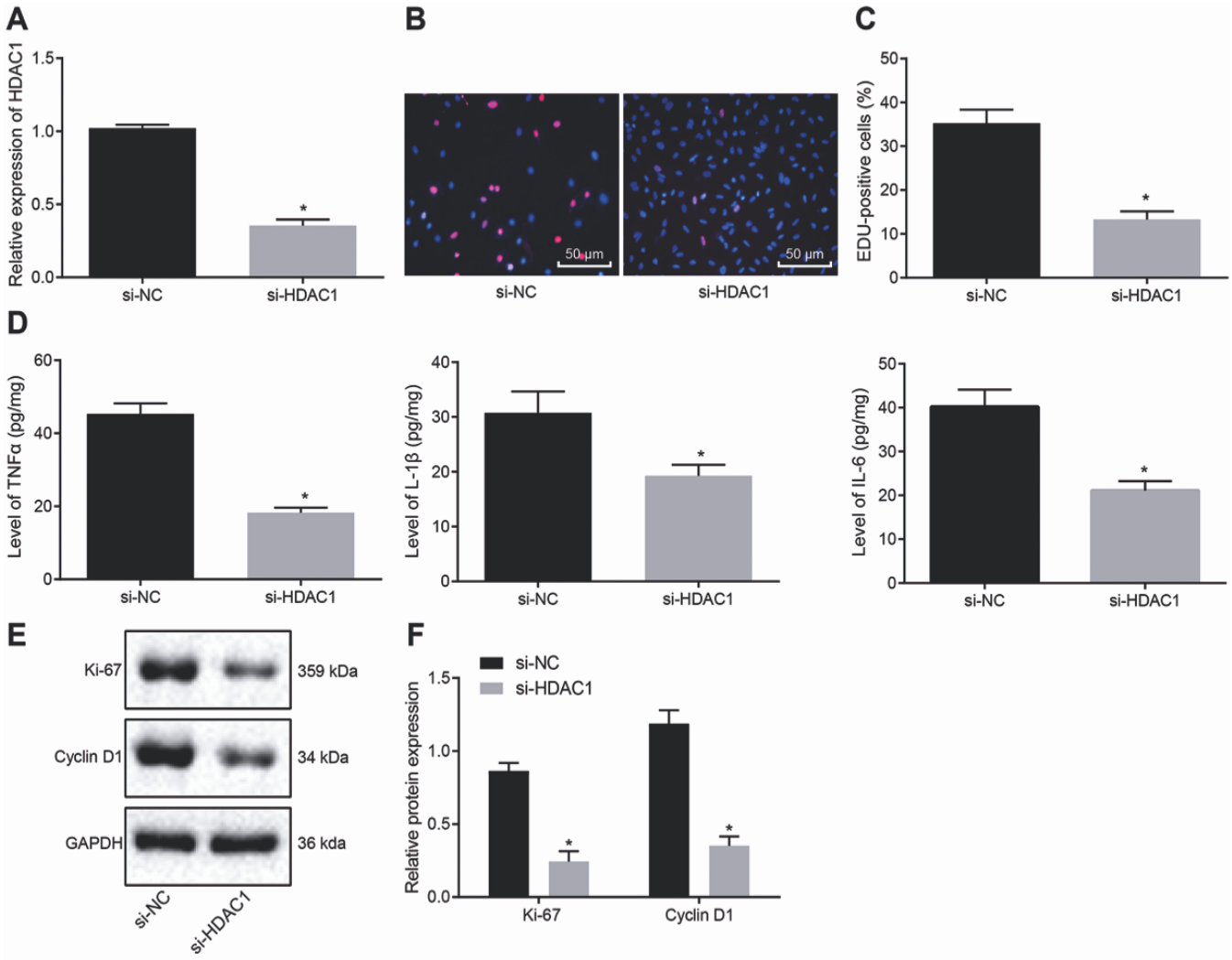

Fig. 2. Knock down of HDAC1 represses synovial cell hyperplasia and synovial inflammation in CIA mice. FLS of CIA mice were transfected with si-HDAC1 plasmid.
A: RT-qPCR of HDAC1 expression in CIA mice.
B-**C**: EdU assay to detect FLA proliferation in CIA mice (200 ×).
D: ELISA of the expression of TNF-α, IL-1β and IL-6 in the supernatant of FLS of CIA mice.
E-**F**: Western blot analysis of expression of proliferation-related factors Cyclin D1 and Ki67 in CIA mice **E**). The band intensity is assessed **F**).
**p*<0.05, compared with the treatment of si-NC plasmid. The above data are measurement data and expressed as mean ± standard deviation.
Data between two groups are compared by unpaired *t*-test. n=10.

Enzyme-linked immunoassay (ELISA)
The supernatant of cells was collected and the expression of interleukin (IL)-1β, IL-6 and TNF-α were determined following the manuals of IL-1β, IL-6 and TNF-α ELISA kits (RapidBio, Tucson, AZ, USA). A microplate reader (SpectraMax M5, Molecular Devices, Abbott Park, IL, USA) was utilised to measure the optical density (OD) value at 450 nm.

Chromatin immunoprecipitation (ChIP)
ChIP was done using ChIP detection kit (PierceTM Agarose ChIP, ThermoFisher Scientific, Waltham, MA, USA). Cells (1.5 x 10^7) were crosslinked in 1% formaldehyde, lysed and sonicated by Bioruptor Plus (Diagenode, Seraing, Ougrée, Belgium) for 90 s. Next, the chromatin was incubated with primary rabbit anti H3 (ab1791, 1:1000, Abcam Inc., Cambridge, UK) overnight at 4°C. The chromatin extract was incubated in a spinner containing 20 μL ChIP protein A//G Plus agarose beads at 4°C for 3 h and centrifuged at 14000 x g for 15 s. The conjugated agarose beads were washed, and the precipitated protein-DNA complex was eluted from those beads at 65°C. At last, the protein-DNA complex was de-crosslinked by incubated with NaCl and protease K at 65°C for 1.5 h. The purified DNA was subjected to RT-qPCR using GoTaq qPCR Master Mix (Promega Corporation, Madison, WI, USA).

RT-qRCR
The total RNA was extracted using TRIzol (15596026, Invitrogen Inc., Carlsbad, CA, USA). Next, RNA was reversely transcribed into complementary DNA using PrimeScript RT reagent Kit (RR047A, Takara, Tokyo, Japan) and TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems,

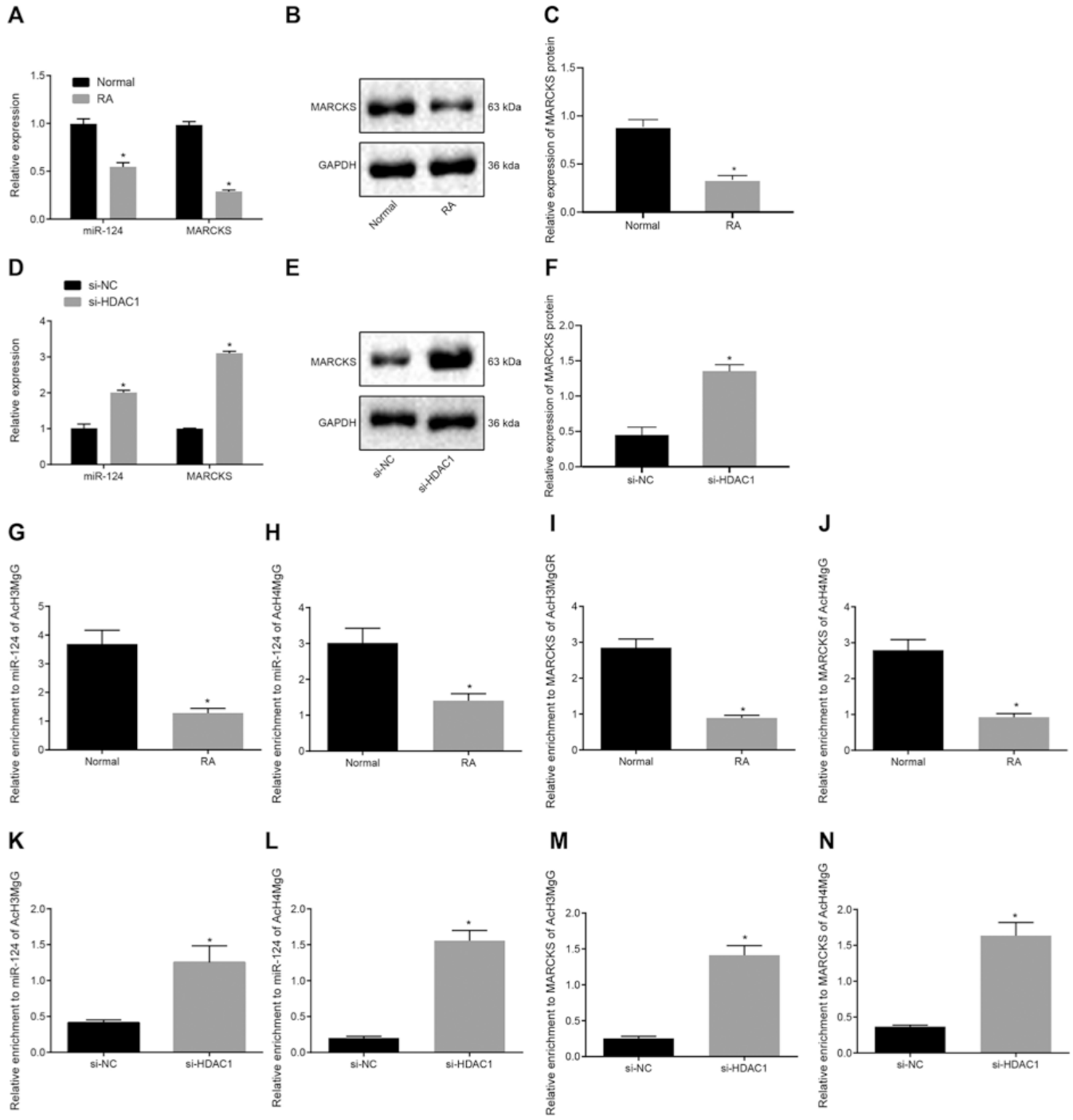

Fig. 3. Deficiency of HDAC1 enhances the expression of miR-124 and MARCKS by increasing H3 and H4 acetylation of miR-124 and MARCKS promoter region.

A: RT-qPCR of the expression of miR-124 and MARCKS in normal synovial tissues (n=9) and synovial tissues of RA patients (n=30).

B-**C**: Western blot analysis of the expression of MARCKS in normal synovial tissues and synovial tissues of RA patients **B**), the band intensity is assessed **C**). **p*<0.05, compared with normal synovial tissues. FLS is isolated from CIA mice and transfected with si-HDAC1 plasmid.

D: RT-qPCR of the expression of miR-124 and MARCKS in FLS of CIA mice transfected with si-HDAC1 plasmid.

E-**F**: Western blot analysis of the expression of MARCKS in FLS of CIA mice transfected with si-HDAC1 plasmid **E**), the band intensity is assessed **F**). **p*<0.05, compared with the treatment of si-NC plasmid.

G-**H**: H3 **G**) and H4 **H**) acetylation of miR-124 promoter region in normal synovial tissues and RA synovial tissues.

I-**J**: H3 **I**) and H4 **J**) acetylation of MARCKS promoter region in normal synovial tissues and RA synovial tissues. **p*<0.05, compared with normal synovial tissues.

K-**L**: H3 K) and H4 L) acetylation of miR-124 promoter region in FLS of CIA mice transfected with si-HDAC1 plasmid.

M-**N**: H3 **M**) and H4 **N**) acetylation of MARCKS promoter region in FLS of CIA mice transfected with si-HDAC1 plasmid. **p*<0.05, compared with FLS of CIA mice transfected with si-NC plasmid. The above data are measurement data and expressed as mean ± standard deviation.

Data between two groups are compared by unpaired *t*-test. n=10.

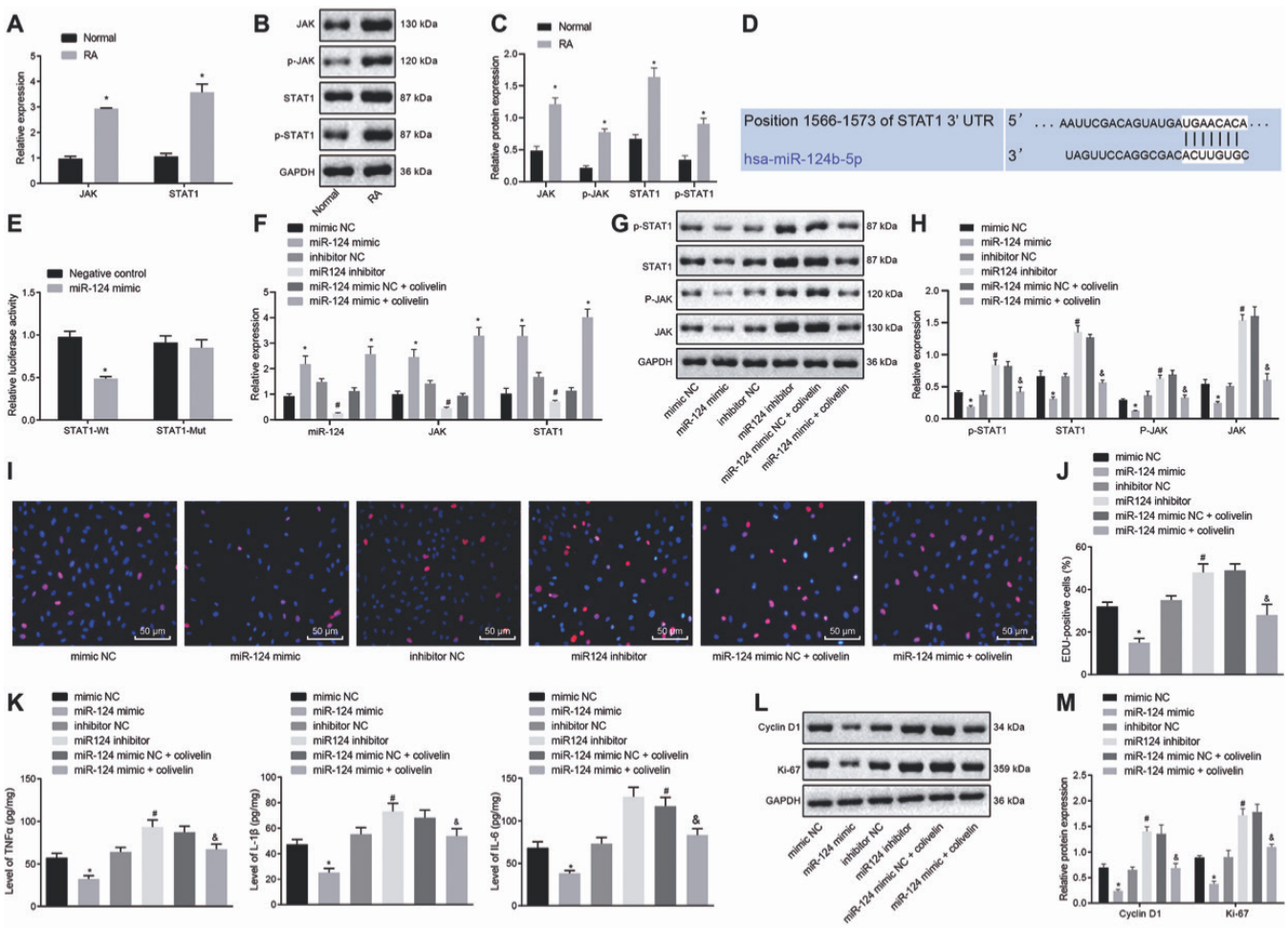

Fig. 4. miR-124 inhibits synovial cell hyperplasia and synovial inflammation in CIA mice by repressing the JAK/STAT signaling pathway.
A: RT-qPCR of JAK and STAT1 expression in normal synovial tissues (n=9) and synovial tissues of RA patients (n=30).
B-**C**: Western blot analysis of JAK and STAT1 expression and the extent of their phosphorylation in normal synovial tissues and synovial tissues of RA patients **B**), the band intensity is assessed **C**). *p<0.05 compared with normal synovial tissues.
D: Binding site between miR-124 and STAT1 predicted by an online prediction website.
E: Dual luciferase reporter assay to verify the target relationship between miR-124 and STAT1. *p<0.05 compared with the treatment of mimic NC. FLS of CIA mice is treated with miR-124 mimic, miR-124 inhibitor or combined miR-124 mimic and Colivelin.
F: RT-qPCR of miR-124, JAK and STAT1 expression in FLS.
G-**H**: Western blot analysis of JAK and STAT1 expression and the extent of their phosphorylation in FLS of CIA mice **G**). The band intensity is assessed **H**).
I-**J**: EdU image I) and quantitative analysis J) of proliferation of FLS from CIA mice (200 ×).
K, ELISA of TNF-α, IL-1 and IL-6 expression in FLS of CIA mice.
L-**M**: Western blot analysis of Cyclin D1 and Ki67 expression and the extent of their phosphorylation in FLS of CIA mice **L**). The band intensity is assessed **M**). *p<0.05 compared with the treatment of mimic NC; #p<0.05 compared with the treatment of inhibitor NC; & p<0.05 compared with the treatment of miR-124 mimic NC + Colivelin. The above data are measurement data and expressed as mean ± standard deviation.
Data between two groups are compared by unpaired t-test. n=10.

Carlsbad, CA, USA). The 7500-type fluorescent quantitative PCR instrument (ABI Company, Oyster Bay, NY, USA) was utilised for real-time fluorescent quantitative PCR (SYBR®Premix Ex Taq™ II, Takara, Tokyo, Japan) following the protocols in the manuals of EasyScript First-Strand cDNA Synthesis SuperMix (AE301-02, Beijing TransGen Biotech Co., Ltd., Beijing, China). The relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ method. Primers were synthesised by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China) (Table I).

Western blot analysis

Cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (P0013B, Beyotime Biotechnology Co., Ltd., Shanghai, China) containing phenylmethanesulfonyl fluoride (PMSF) and phosphatase inhibitor (Hoffmann-La Roche Ltd, Basel, Switzerland). Then, 50 μg protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred on a nitrocellulose membrane. After blocked by 5% skimmed milk for 1.5 h, the membrane was probed with primary rabbit antibodies against HDAC1 (ab7028, 1:2000), MARCKS (ab51100, 1:2000), JAK (ab108596, 1:5000), extent of JAK phosphorylation (ab32101, 1:1000), STAT1 (ab109320, 1:10000), phosphorylated STAT1 (ab30645, 1:1000), Cyclin D1 (ab134175, 1:10000), Ki67 (ab16667,1:1000) and GAPDH (ab9485, 1:2500) overnight at 4°C. The

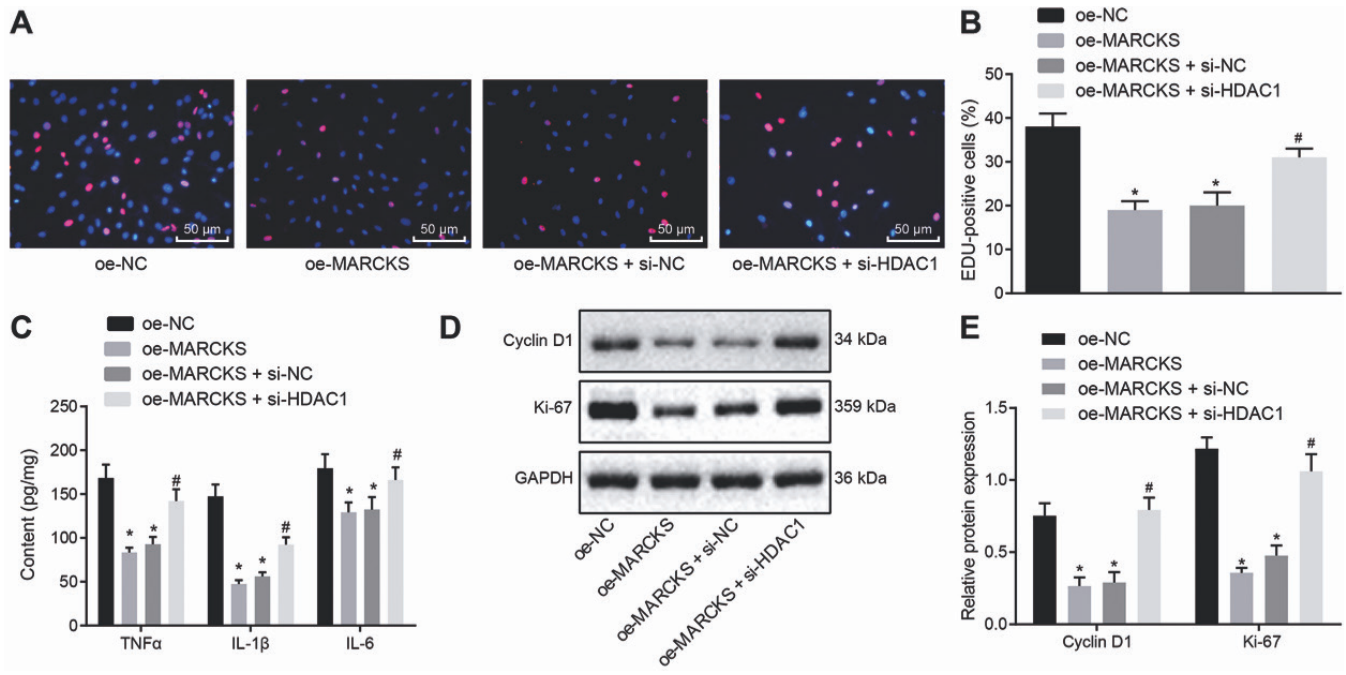

Fig. 5. Silencing HDAC1 induces inhibition of synovial cell hyperplasia and synovial inflammation in CIA mice by upregulating MARCKS. FLS from CIA mice is transfected with oe-MARCKS or oe-MARCKS + si-HDAC1.
A-**B**: Representative images A) and quantitative analysis B) of EdU to assess the proliferation of FLS from CIA mice (200 ×).
C: ELISA to assess the expression of TNF-α, IL-1β and IL-6 in FLS of CIA mice.
D-**E**: Western blot analysis to determine Cyclin D1 and Ki67 expression B). The band intensity is assessed **E**). **p*<0.05 compared with the treatment of oe-NC; #*p*<0.05 compared with the treatment of oe-MARCKS + si-NC. The above data are measurement data and expressed as mean ± standard deviation. Data among multiple groups are analysed by one-way ANOVA with Tukey's *post hoc* test. The experiment was repeated three times.

next day, the membrane was probed with HRP-labelled secondary goat anti-rabbit IgG (ab205718, 1:5000) for 1 h. The above antibodies were purchased from Abcam Inc. (Cambridge, UK). At last, the membrane was visualised by enhanced chemiluminescence solution and photographed by SmartView Pro 2000 (UVCI-2100, Major Science, Sea Gull Way Saratoga, CA, USA). Quanity One software was applied for analysis of protein bands.

Statistical analysis

The statistical analysis was conducted using SPSS 21.0 statistical software (IBM Corp., Armonk, NY, USA). Measurement data were expressed as mean ± standard deviation. When data showed normal distribution and with homogeneity, data between two unmatched groups were compared by unpaired *t*-test. Measurement data among multiple groups were checked by one-way analysis of variance (ANOVA) with Tukey's *post hoc* test. Data comparison among multiple groups at different time points was conducted using repeated measurement ANOVA with Bonferroni's *post hoc* test. A $p<0.05$ demonstrated statistical significance.

Results

HDAC1 is highly expressed in RA

HDAC1 expression in normal synovial tissues and RA synovial tissues was detected by RT-qPCR and western blot analysis (Fig. 1A-C). It was observed that HDAC1 expression was higher in RA synovial tissues than in normal synovial tissues. Moreover, HDAC1 was found to be upregulated in RA patients with positive or negative rheumatoid factor (RF) (Supplementary Fig. 1).

Then the CIA mouse model was established and evaluated in terms of joint swelling and inflammatory infiltration of synovial tissues (Fig. 1D-G). In normal mice, the thickness of sole was normal, and joint swelling and inflammatory infiltration of synovial tissues were absent. In CIA mice, the thickness of sole was increased, and severe swelling and inflammatory infiltration were detected in the joints and synovial tissue, suggesting that CIA mouse model was successfully established. Then, FLS was isolated from synovial tissues and analysed using ELISA, RT-qPCR and Western blot analysis (Fig. 1H-K). These analyses revealed that the expression of HDAC1, TNF-α, IL-1β and IL-6 was increased in synovial tissues of CIA mice ($p<0.05$). Therefore, HDAC1 was expressed highly in the synovial tissues from both RA patients and CIA mouse.

Silencing HDAC1 inhibits synovial cell hyperplasia and synovial inflammation in RA

To study the effect of HDAC1 on RA, HDAC1 expression was silenced in FLS of CIA mice by si-HDAC1. The silencing efficiency was confirmed by RT-qPCR ($p<0.05$) (Fig. 2A). Then EdU assay, ELISA and Western blot analysis detected that silencing HDAC1 resulted in decreased cell proliferation, reduced expression of TNF-α, IL-1β and IL-6 as well as lower expression of proliferation-related factors Cyclin D1 and Ki67 in FLS of CIA mice ($p<0.05$) (Fig. 2B-E). Hence, silencing HDAC1 could inhibit synovial cell hyperplasia and synovial inflammation in CIA mice.

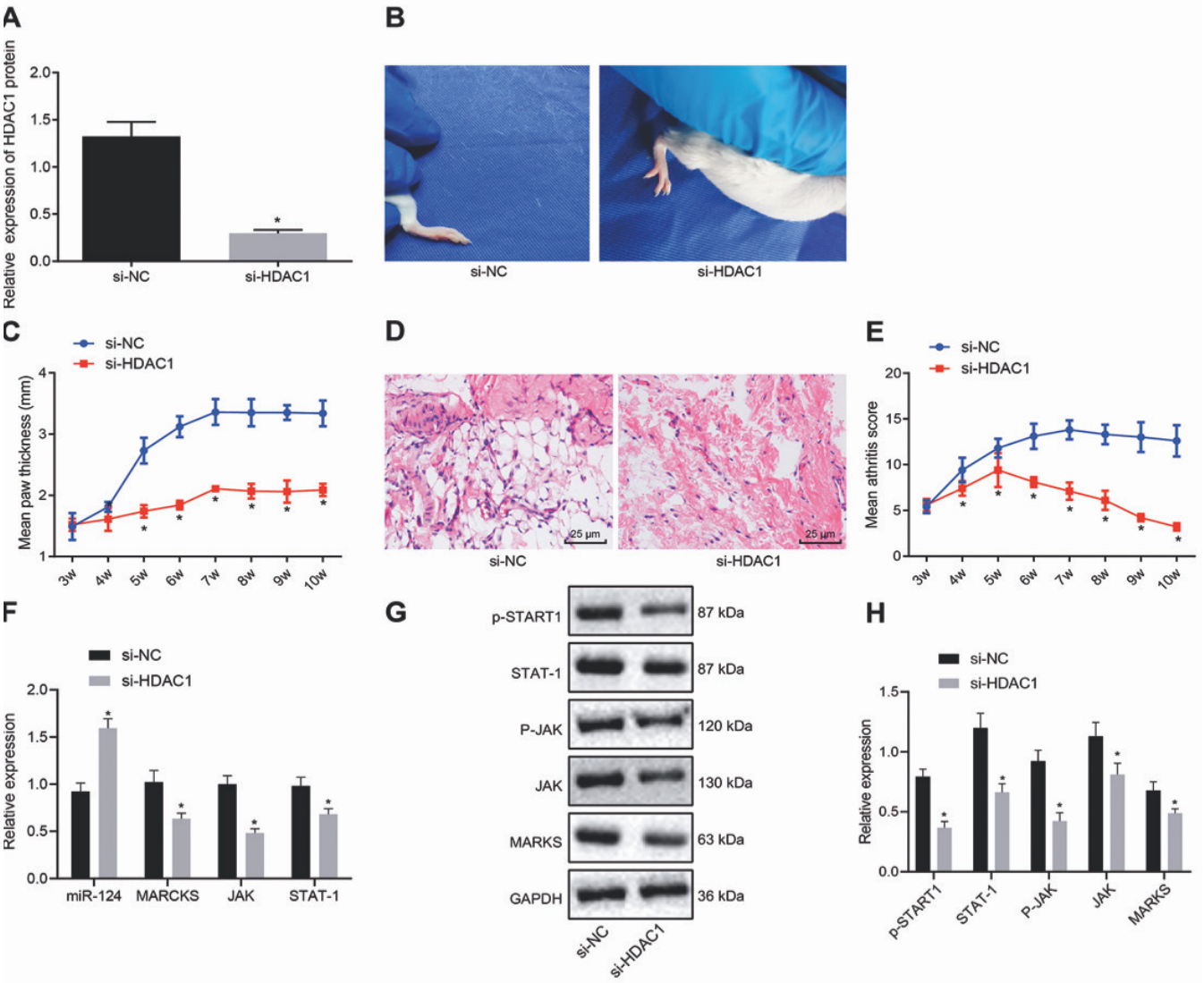

Fig. 6. HDAC1 deficiency provokes inhibition of synovial hyperplasia and synovial inflammation in CIA mice. The CIA mice are treated with si-HDAC1.
A: Expression of HDAC1 in CIA mice.
B: Representative images of the swelling joints in CIA mice.
C: Thickness of soles in CIA mice.
D-**E**: Representative images **D**) and quantitative analysis **E**) of HE staining of synovial tissues in CIA mice (400 ×).
F: RT-qPCR of the expression of miR-124, MARCKS, JAK and STAT1 in FLS of CIA mice.
G-**H**: Western blot analysis of MARCKS, JAK and STAT1 and the extent of JAK and STAT1 phosphorylation in FLS of CIA mice **G**). The band intensity is assessed **H**).
*$p<0.05$ compared with the treatment of si-NC. The above data are measurement data and expressed as mean ± standard deviation. Data between two groups are checked by unpaired *t*-test, and data among multiple groups are analysed by repeated measures ANOVA with Bonferroni's *post hoc* test. n=10.

Silencing HDAC1 promotes the expression of miR-124 and MARCKS

RT-qPCR and Western blot analysis were performed to assess miR-124 and MARCKS expression in synovial tissues of RA patients (Fig. 3A-C). Results showed that miR-124 and MARCKS expression was downregulated in synovial tissues of RA patients ($p<0.05$), which could be rescued after HDAC1 was silenced ($p<0.05$) (Fig. 3D-F).

Then to uncover the regulatory mechanism of HDAC1 on miR-124 and MARCKS, ChIP was utilised to assess H3 and H4 acetylation of miR-124 and MARCKS promoter region in normal human and RA synovial tissues (Fig. 3G-J). In RA synovial tissues, H3 and H4 acetylation of miR-124 and MARCKS promoter region was decreased ($p<0.05$). Besides, silencing HDAC1 led to elevated H3 and H4 acetylation of miR-124 and MARCKS promoter region in FLS of CIA mice ($p<0.05$) (Fig. 3K-N), suggesting that higher HDAC1 expression in RA suppressed H3 and H4 acetylation of miR-124 and MARCKS promoter region, thus downregulating expression of miR-124 and MARCKS in RA. Thus, knocking down HDAC1 upregulated miR-124 and MARCKS expression by promoting H3 and H4 acetylation of miR-124 and MARCKS promoter region.

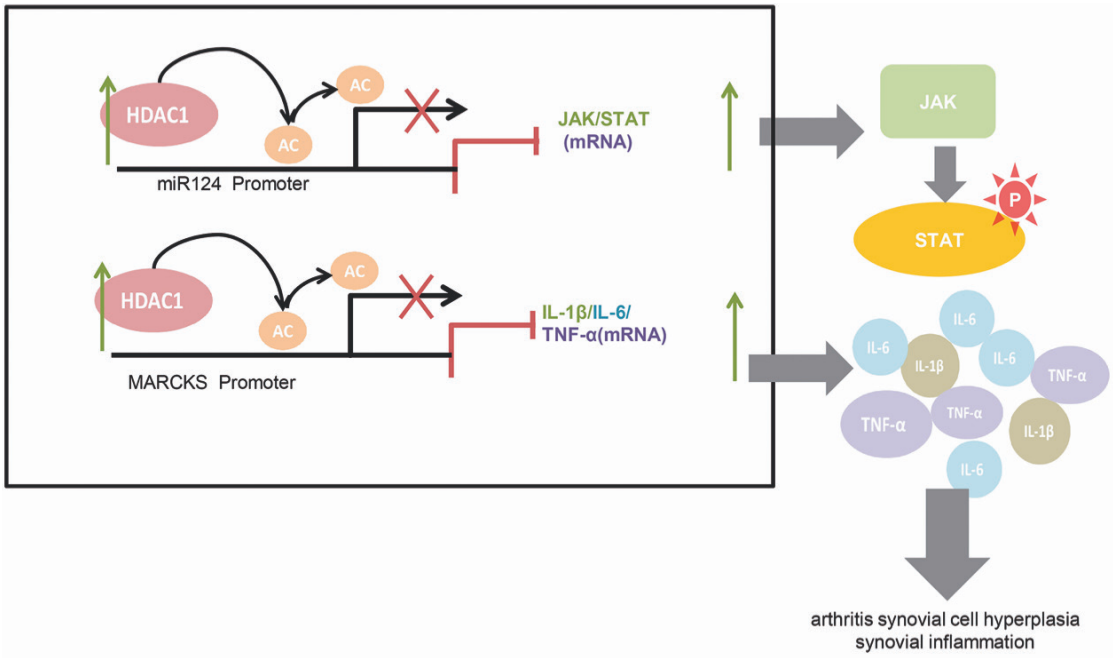

Fig. 7. HDAC1 promotes synovial cell hyperplasia and synovial inflammation by downregulating miR-124 and MARCKS in CIA mice. HDAC1 impairs miR-124 and MARCKS acetylation by decreasing H3 and H4 acetylation in their promoter regions to inhibit the expression of miR-124 and MARCKS in RA and activate JAK/STAT signalling pathway, thus worsening synovial cell hyperplasia and synovial inflammation in CIA modeled mice.

miR-124 represses synovial cell hyperplasia and synovial inflammation in CIA mice through inactivation of JAK/STAT signalling pathway

Subsequently, EdU assay (Fig. 4I-J), ELISA (Fig. 4K) and Western blot analysis (Fig. 4L-M) were conducted to evaluate the effects of miR-124 on synovial cell hyperplasia and synovial inflammation in CIA mice. Results showed that miR-124 mimic repressed the proliferation of FLS from CIA mice and downregulated the expression of TNF-α, IL-1, IL-6, Cyclin D1 and Ki67 ($p<0.05$), proving that miR-124 could inhibit synovial cell hyperplasia and synovial inflammation in CIA mice.

Scrutiny of the online prediction website predicted that there was a binding site between miR-124 and STAT1 (Fig. 4D), which was then verified by dual luciferase reporter assay (Fig. 4E). In the presence of miR-124 mimic, the luciferase activity of STAT1-WT was decreased ($p<0.05$), while that of STAT1-MUT was unaltered ($p>0.05$), thus revealing the target relationship between miR-124 and STAT1. Therefore, miR-124 may be able to regulate JAK/STAT pathway.

Next, FLS of CIA mice was transfected with miR-124 mimic. RT-qPCR and Western blot analysis (Fig. 4F-H) showed that miR-124 mimic decreased the phosphorylation levels of JAK and STAT1 ($p<0.05$), confirming that miR-124 can regulate JAK/STAT pathway. RT-qPCR and Western blot analysis further revealed that the phosphorylation levels of JAK and STAT1 were upregulated in synovial tissues of RA patients ($p<0.05$) (Fig. 4A-C), suggesting the activation of JAK/STAT pathway in RA. Furthermore, the JAK/STAT pathway activator Colivelin could rescue the effects of miR-124 mimic on synovial cell hyperplasia and synovial inflammation ($p<0.05$) (Fig. 4L-M), demonstrating that miR-124 could repress synovial cell hyperplasia and synovial inflammation in CIA mice by blocking the JAK/STAT signalling pathway.

Silencing HDAC1 inhibits synovial cell hyperplasia and synovial inflammation in RA by elevating MARCKS

EdU assay (Fig. 5A-B), ELISA (Fig. 5C) and Western blot analysis (Fig. 5D-E) were performed to assess the effects of HDAC1 and MARCKS on synovial cell hyperplasia and synovial inflammation in CIA mice. Results demonstrated that overexpressing MARCKS decreased FLS proliferation, expression of TNF-α, IL-1β, IL-6, Cyclin D1 and Ki67 ($p<0.05$). Moreover, silencing HDAC1 decreased FLS proliferation, expression of TNF-α, IL-1β, IL-6, Cyclin D1 and Ki67 ($p<0.05$), which could be rescued after inhibiting MARCKS. Hence, silencing HDAC1 repressed synovial cell hyperplasia and synovial inflammation in CIA mice by increasing MARCKS.

Deficiency of HDAC1 suppresses synovial hyperplasia and synovial inflammation in vivo

Finally, we performed a study *in vivo* to explore the effect of HDAC1 on synovial hyperplasia and synovial inflammation in CIA model mice (Fig. 6A-E). The CIA mice had conspicuous swelling in the sole, along with severe swelling and deformity and inflammatory infiltration in limb joints and synovial tissues ($p<0.05$). Yet CIA mice treated with si-HDAC1 exhibited decreased HDAC1 expression and lesser thickness of the sole, along with alleviated joint swelling ($p<0.05$). According to RT-qPCR and Western blot analysis, the expression of MARCKS and miR-124 was increased, while the phosphorylation levels of JAK and STAT1 were decreased in CIA mice treated with si-HDAC1 ($p<0.05$) (Fig. 6F-H). Taken together, HDAC1 silencing impaired synovial hyperplasia and synovial inflammation in mice with RA.

Discussion

RA is often accompanied with severe synovial inflammation in joints, which

can even lead to crippling joint destruction (18). A treat-to-target therapeutic method with immediate control and monitoring, along with timely diagnosis, can relieve sufferings in patients with RA (5). Recently, the discovery of the role of HDAC in RA presents new hope for the better treatment of RA and juvenile idiopathic arthritis (19). Besides, miRNAs have been identified as novel participants in the development of RA and other autoimmune diseases (11). Thus, the present study assessed the functions of HDAC1 and miR-124 on synovial cell hyperplasia and synovial inflammation in RA. Collectively, results of our investigation showed that silencing HDAC1 alleviated synovial cell hyperplasia and synovial inflammation by promoting miR-124 and MARCKS expression in RA.

The initial finding of our study was that HDAC1 was expressed highly and miR-124 and MARCKS were expressed poorly in synovial tissues of RA. Consistent with that finding, a previous study showed that HDAC1 was expressed at a higher level in RA synovial fibroblasts when compared with osteoarthritic synovial fibroblasts (8). Kawabata *et al*. also found that HDAC1 exhibited elevated expression and activity in synovial tissue of RA (9). Meanwhile, miR-124 exhibits a decreased expression in patients with RA (12). Epigenetic changes are probably not restricted to fibroblast-like synoviocytes in RA and/or RA models. Rather, the epigenetic changes might involve several subsets of cells in arthritic joints, which could occur first both in haematopoietic and/or mesenchymal stem cells from RA synovium and/or subchondral bone marrow. Others report that CIA model mice with a T cell-specific deletion of HDAC1 (HDAC1-cKO) are resistant to the development of CIA, whereas the antibody response to collagen type II was undisturbed. Furthermore, human synovium for RA patients showed enhanced expression of HDAC1 in CD4+ $CCR6^{-}$ T cells (20), and the expression of miR-124a is downregulated in RA synoviocytes compared to those seen in osteoarthritis (21).

Besides, our study proved that silencing HDAC1 elevated the expression of miR-124 and MARCKS. The post-transcriptional inhibition of HDAC1 on gene expression is associated with increased miRNA expression mediated by an increase in the affinity of DGCR8 to primary miRNA transcripts (10). For instance, depletion of HDAC1 abrogates reduction of miR-124 expression induced by platelet-derived growth factor (22). HDAC1 enhances MARCKS expression through regulating the H3 and H4 acetylation of MARCKS promoter region (23).

Another important finding of our study was that miR-124 ameliorated synovial cell hyperplasia and synovial inflammation by repressing the JAK/STAT signalling pathway. Similar to our present findings, miR-124a has been revealed to lower the proliferation of synoviocytes in RA (21). miR-124 also results in the inhibition of adjuvant-induced arthritis in rats by repressing bone or cartilage destruction, leucocyte infiltration and synoviocyte proliferation (13). More over, miR-124 has been demonstrated to regulate the expression of STAT1 in colorectal cancer (24), which is consistent with our study. However, only a minority of patients treated by JAK/STAT inhibitors experienced full remission in human clinical trials, likely due to the inherent molecular heterogeneity of RA, which is not a homogeneous disorder.

The most intriguing finding in our study was that silencing HDAC1 induced inhibition of synovial cell hyperplasia and synovial inflammation by upregulating MARCKS, in response to decreased expression of TNF-α, IL-1β, IL-6, Cyclin D1 and Ki67. The factors TNF-α, IL-1β and IL-6 are proinflammatory cytokines that facilitate the inflammation in RA (25, 26). Cyclin D1 is a major modulator of cell cycle (27), and Ki67 is a nuclear protein associated with cell cycle and proliferation (28). High expression of Cyclin D1 and Ki67 is found in most cases of synovial sarcoma (29). In line with our results, inhibition of HDAC1 interferes with inflammatory activation of synovial tissues and macrophages in RA patients (30). Also, silencing HDAC1 and HDAC2 leads to a reduction in cell proliferation and an increase of cell apoptosis in synovial fibroblasts of RA (31). Besides, Angiolilli *et al*. revealed that HDAC3 depletion inhibited the expression of inflammatory genes in FLS of RA (32). In addition, Lee *et al*. expound that inhibition of HDAC6 by treatment with Tubastatin A impaired joint destruction and synovial inflammation in a mouse model with arthritis induced by collagen antibody (33).

In conclusion, downregulation of HDAC1 suppresses synovial cell hyperplasia and synovial inflammation by elevating MARCKS and miR-124, which can inactivate JAK/STAT signalling pathway (Fig. 7). Consequently, these findings suggest HDAC1 as a potential therapeutic target for RA patients.

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Ethics approval and consent to participate

Written informed consent was obtained from all patients prior to the study. Study protocols were approved by Ethic Committee of The First Affiliated Hospital, Sun Yat sen University and based on the ethical principles for medical research involving human subjects of the Helsinki Declaration. The animal experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol of animal experiments was approved by the Institutional Animal Care and Use Committee of The First Affiliated Hospital, Sun Yat sen University. The animal experiments were conducted based on minimised animal number and the least pain on experimental animals.

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