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SIRT1 is transcriptionally repressed by YY1 and suppresses ferroptosis in rheumatoid arthritis

Yuwei Zhan†, Zhou Yang†, Feng Zhan, Yanyan Huang and Shudian Lin*

Abstract

Background Sirtuin 1 (SIRT1) is reported downregulated in rheumatoid arthritis (RA), and the protective effects of SIRT1 on tissue damage and organ failure may be related to cellular ferroptosis. However, the exact mechanism by which SIRT1 regulates RA remains unclear.

Methods Quantitative real-time PCR (qPCR) and western blot assays were performed to explore the expressions of SIRT1 and Yin Yang 1 (YY1). CCK-8 assay was used for cytoactive detection. The interaction between SIRT1 and YY1 was validated by dual-luciferase reporter gene assay and chromatin immunoprecipitation (ChIP). DCFH-DA assay and iron assay were applied to detect the reactive oxygen species (ROS) and iron ion levels.

Results In the serum of RA patients, SIRT1 was downregulated, but YY1 was upregulated. In LPS-induced synoviocytes, SIRT1 could increase cell viability and decrease ROS and iron levels. Mechanistically, YY1 downregulated the expression of SIRT1 by inhibiting its transcription. YY1 overexpression partly revised the effects of SIRT1 on ferroptosis in synoviocytes.

Conclusion SIRT1 is transcriptionally repressed by YY1 and inhibits the ferroptosis of synoviocytes induced by LPS, so as to relieve the pathological process of RA. Therefore, SIRT1 might be a new diagnosis and therapeutic target of RA.

Highlights

1. Combining SIRT1 with synoviocytes ferroptosis in rheumatoid arthritis for the first time.
2. The transcription factor YY1 combined to the SIRT1 promoter in synovial cells and inhibited its expression and functional roles.
3. The inhibition of SIRT1 with YY1 decreased the ferroptosis in synoviocytes.

Keywords Rheumatoid arthritis, Ferroptosis, SIRT1, YY1

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by the erosion of adjacent cartilage and bone caused by chronic joint inflammation. Approximately 0.5–1% of adults in China suffer from RA, placing a heavy burden on economy and health [1]. Researches indicated that the main causes of RA are genetic and environmental factors [2, 3]. However, the exact mechanism of the pathogenesis and progression of RA remains unknown. Synovitis is recognized as the major characteristic of RA [4]. The normal synovium is mainly composed of fibroblast-like synoviocytes, which is the nutrient provider of articular cartilage and the protector of joint structures or adjacent tissues [4]. In RA, the number of synoviocytes conspicuously increased and could cause joint destruction by producing proinflammatory cytokines [5]. Therefore, it is of great significance to find an effective therapeutic agent for synovitis and clarify its mechanism of action for the treatment of RA.

Lipid peroxidation and reactive oxygen species (ROS) were necessary in synovitis development [6], which have been proved contributing to the progressive disease course. Ferroptosis is a new kind of non-apoptotic and iron-dependent cell death, which is featured by iron accumulation and lipid peroxidation to produce ROS resulting in cell death [7]. Some recent findings have indicated that ferroptosis may also be related to the occurrence and development of inflammatory arthritis, including RA [8]. Enhanced lipid peroxidation and decreased glutathione peroxidase (GPX, an anti-oxidant agent) were found in synoviocytes from patients with RA [9, 10]. Abnormal iron metabolism is another contributor of ferroptosis and iron deposits were determined in RA [11, 12]. Moreover, ferroptosis is a crucial process for synovium injury in RA and regulation in the ferroptosis of synovial cells is beneficial to RA treatment [12].

Sirtuin 1 (SIRT1), a nicotinamide adenine dinucleotide-dependent protein deacetylases, manages the key part of lipid metabolism [13]. It was reported that SIRT1 expressed decreasingly in RA synovial vessels [14]. Our previous study proved that SIRT1 overexpression possessed anti-RA activity [15]. Furthermore, the activation of SIRT1 can restrain excess iron-induced ferroptosis evidenced by decrease of lipid ROS levels and increase GPX4 expression [16]. Additionally, the ablation of intestinal SIRT1 ameliorated dysfunctional iron metabolism, increased hepatic glutathione contents, and attenuated lipid peroxidation [17]. However, in the progression of RA, the regulation of SIRT1 in ferroptosis remains unclear.

Based on these previous studies, we aimed to investigate whether SIRT1 could regulate the ferroptosis of synovial cells in RA in vitro, increasing the understanding of RA pathogenesis.

Methods

Clinical sample collection

Serum samples from healthy volunteers (female 9 and male 11, average age 42.7 ± 6.3 year) and RA patients (female 12 and male 8, average age 47.5 ± 5.6 year) who received joint replacement of the knee joint were collected in the Hainan General Hospital (20 samples for each group). All RA patients fulfilled the American College of Rheumatology criteria for classification of disease [18]. The healthy control specimens were from patients with joint trauma undergoing joint replacement surgery, who were free from autoimmune or inflammatory diseases. Participants signed the informed consent forms and the Ethics Committee of Hainan General Hospital approved the procotol (approval number: 2012233).

Cell culture and treatment

Synoviocytes (HUM-CELL-0060, Wuhan PriCells Biomedical Technology, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, South Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO, Grand Island, NY, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (HyClone) and were incubated in a 5% CO_2 incubator at 37 °C. For RA cell model treatment, synoviocytes were treated with 1 μg/mL lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO, USA) for 24 h.

Cell transfection

The full-length cDNA of YY1 and SIRT1 was amplified and integrated into the pcDNA-3.1 expression vector (Invitrogen, Carlsbad, CA, USA). Cell transfection was carried out according to the standard protocol using Lipofectamine 3000 (Invitrogen). After 48 h, the cells were used for subsequent experiments.

Cell counting kit-8 (CCK-8) assay

The cell viability was assessed by a CCK-8 assay (Beyotime, Jiangsu, China). The cells were seeded into a 96-well plate and then cultured at 37℃ for 48 h. Next, 10 μL of CCK-8 reagent was added, and the cells were incubated at 37℃ for an additional 2 h. The optical density (OD) of the solution was then measured at 450 nm to assess cell viability.

Glutathione (GSH)/oxidized GSH (GSSG) detection

Anticoagulant treated blood samples were centrifuged at 1000 × g for 10 min at 4 °C. Then, the top plasma layer was transferred to a new tube, followed by addition with 1/4 vol of 5% SSA. The samples were added in a 96-well plate and GSH/GSSG ratio was detected using a GSH/GSSG Detection Assay kit (#ab138881; Abcam, Cambridge, MA, USA), following the manufacturer's protocols.

Iron level measurement

The intracellular ferrous iron level (Fe^{2+}) were detected using the iron assay kit (#ab83366, Abcam). Synoviocytes seeded in 24-well plate were washed with cold PBS twice and then lysed with cell lysis buffer for 2 h, followed by addition with the iron reducer into the collected supernatants. Finally, iron probe was added for 1 h, and the content was immediately measured on a colorimetric microplate reader (OD 593 nm).

Detection of ROS level

After relevant stimulation and/or treatment, cells were cultivated in a 24-well plate at 37 °C in serum-free medium for 6 h. Cells were then rinsed using PBS and stained with 10 μM DCFH-DA (Sigma-Aldrich) for 20 min in dark. Finally, these cells were washed with PBS and the fluorescence was observed with a confocal laser scanning microscope.

RNA extraction and quantitative real-time (qPCR)

TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to obtain total RNA from the synoviocytes or clinical samples. A NanoDropTM 2000 spectrophotometer (Thermo Fisher Scientific) was utilized to assess the RNA quality. The RNA samples were used to synthesize cDNA by using a Primescript RT reagent kit (Takara, Dalian, China). qPCR was then performed according to the directions of the SYBRTM Green master mix (TaKaRa, Tokyo, Japan). The primers used in this study were listed in Table 1.

Table 1 Primer sequences for qPCR

Primer name	(5′-3′) Primer sequences
YY1-Forward	5′-TACCTGGCATTGACCTCTC-3′
YY1-Reverse	5′-GGCCGAGTTATCCCTGA-3′
SIRT1-Forward	5′-TAGACACGCTGGAACAGGTTGC-3′
SIRT1-Reverse	5′-CTCCTCGTACAGCTTCACAGTC-3′
GAPDH-Forward	5′-CTCCTCCACCTTTGACGCT-3′
GAPDH-Reverse	5′-GGGTCTCTCTCTTCCTCTTGTG-3′

Western blot

The cells were collected and lysed in RIPA buffer (Beyotime) to extract total proteins. Equal amounts of protein (30 μg) were separated on a 10% SDS-PAGE gel, and then transferred to a PVDF membrane (Invitrogen). The membrane was blocked with 5% skim milk powder in tris-buffered saline tween (TBST). The membrane was then incubated overnight at 4℃ with the following primary antibodies: SIRT1 (#ab110304, Abcam), YY1 (#ab227269, Abcam). Next, the membrane was washed 3 times in TBST for 5 min each and then incubated with HRP-conjugated secondary antibody for 2 h at room temperature. After washing, the protein bands were analyzed by an ECL detection kit (Beyotime).

Dual-luciferase reporter assay

Synoviocytes were seeded into a 24-well plate, and the cell density had increased to 60–70% per well on the second day. Two groups were set up as pcDNA3.1 and pcDNA-YY1 (dual-luciferase reporter plasmid was purchased from HonorGene, China). After 48 h, luciferase activity was detected by a dual-luciferase reporter kit (Promega, Madison, WI, USA) and expressed as relative activity.

Chromatin immunoprecipitation (ChIP) assay

The binding of YY1 to the SIRT1 promoter was examined using ChIP assay with pierce magnetic ChIP kit (Thermo Fisher Scientific), according to the manufacturer's protocol. Cells were fixed with formaldehyde (1%) for 10 min at room temperature. YY1 antibody (#ab227269, Abcam) was utilized to generate immunoprecipitants, whereas an IgG antibody (#sc-69786, Santa Cruz Biotechnology, CA, USA) was used as the blank control group. The recuperated DNA fragments were evaluated via qPCR. The relative level of SIRT1 promoter was normalized to the average level of the IgG group.

Statistical analysis

Statistical analysis was performed with SPSS 22.0 software and GraphPad Prism 8.0. The data were expressed as the mean ± SD and assessed normality by Shapiro–Wilk test, followed by analyzed homogeneity of variance utilizing Bartlett's test. After analysis, all the data were consistent with normal distribution and homogeneity of variance. Then, the unpaired student's *t*-test was used for statistical analysis between two groups, and one-way analysis of variance followed by Tukey's test was used for comparison between multiple groups of data. The correlations were analyzed using Pearson's correlation coefficients. $P < 0.05$ was defined as indicating statistical significance.

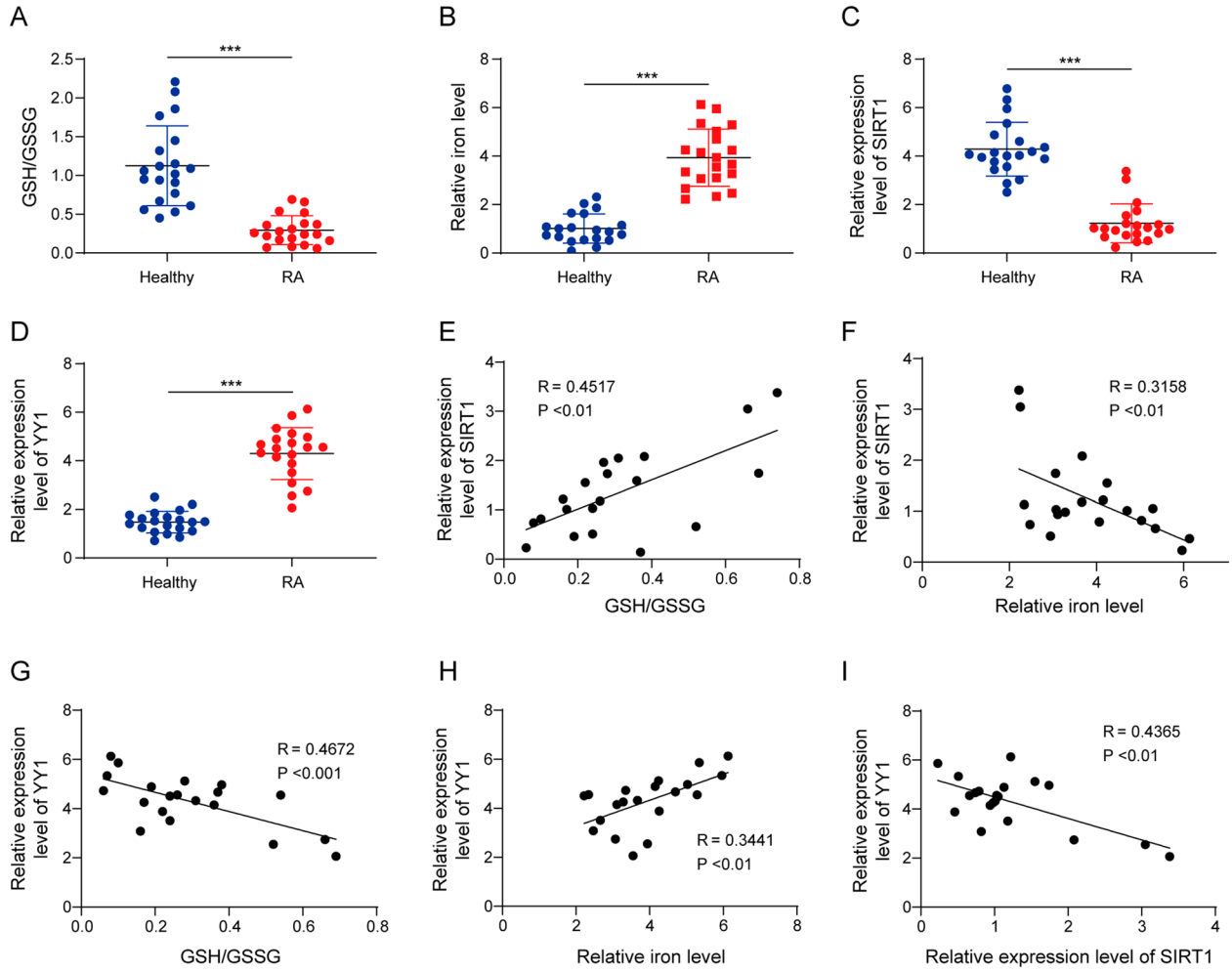

Fig. 1 RA was associated with ferroptosis and the differential expressions of YY1 and SIRT1. **A** GSH/GSSG in the serum of healthy volunteers and RA patients were examined by GSH/GSSG assay. **B** Iron levels in the serum of healthy volunteers and RA patients were examined by iron assay. **C** SIRT1 expression in the serum of healthy volunteers and RA patients was examined by qPCR. **D** YY1 expression in the serum of healthy volunteers and RA patients was examined by qPCR. **E**, **F** The correlation between SIRT1 and GSH/GSSG, iron level in RA. **G**, **H** The correlation between YY1 and GSH/GSSG, iron level in RA. **I** The correlation between YY1 and SIRT1 in RA. N = 20, mean ± SD, ***$p < 0.001$

Results

RA was associated with ferroptosis and the differential expressions of YY1 and SIRT1

We collected 20 clinical blood samples from healthy volunteers and patients with RA. Compared with the healthy group, the serum GSH/GSSG level in RA group was significantly decreased (Fig. 1A). However, the serum iron content of the RA group increased significantly (Fig. 1B). Additionally, qPCR was performed to confirm SIRT1 and YY1 expression in RA patients. Serum SIRT1 was found to be downregulated in RA patients (Fig. 1C), and YY1 was upregulated (Fig. 1D). Furthermore, SIRT1 expression was positively correlated with the level of GSH/GSSG (Fig. 1E) and negatively correlated with iron level (Fig. 1F) or YY1 expression (Fig. 1I) in RA. YY1 expression was negatively correlated with GSH/GSSG level (Fig. 1G) and positively correlated with iron level (Fig. 1H). Overall, the ferroptosis may be involved in the pathogenetic process of RA and the SIRT1 could be the crucial molecule in regulating ferroptosis.

The effects of LPS on ferroptosis, SIRT1 and YY1 expressions in synoviocytes

In order to simulate RA in vitro model, we used LPS to induce synovial cells and explore the occurrence of ferroptosis. Compared with the control group, cell viability of synovial cells induced by LPS was significantly reduced (Fig. 2A). ROS accumulation was related to ferroptosis, and LPS treatment significantly increased cellular ROS levels (Fig. 2B). Iron content can directly reflect the

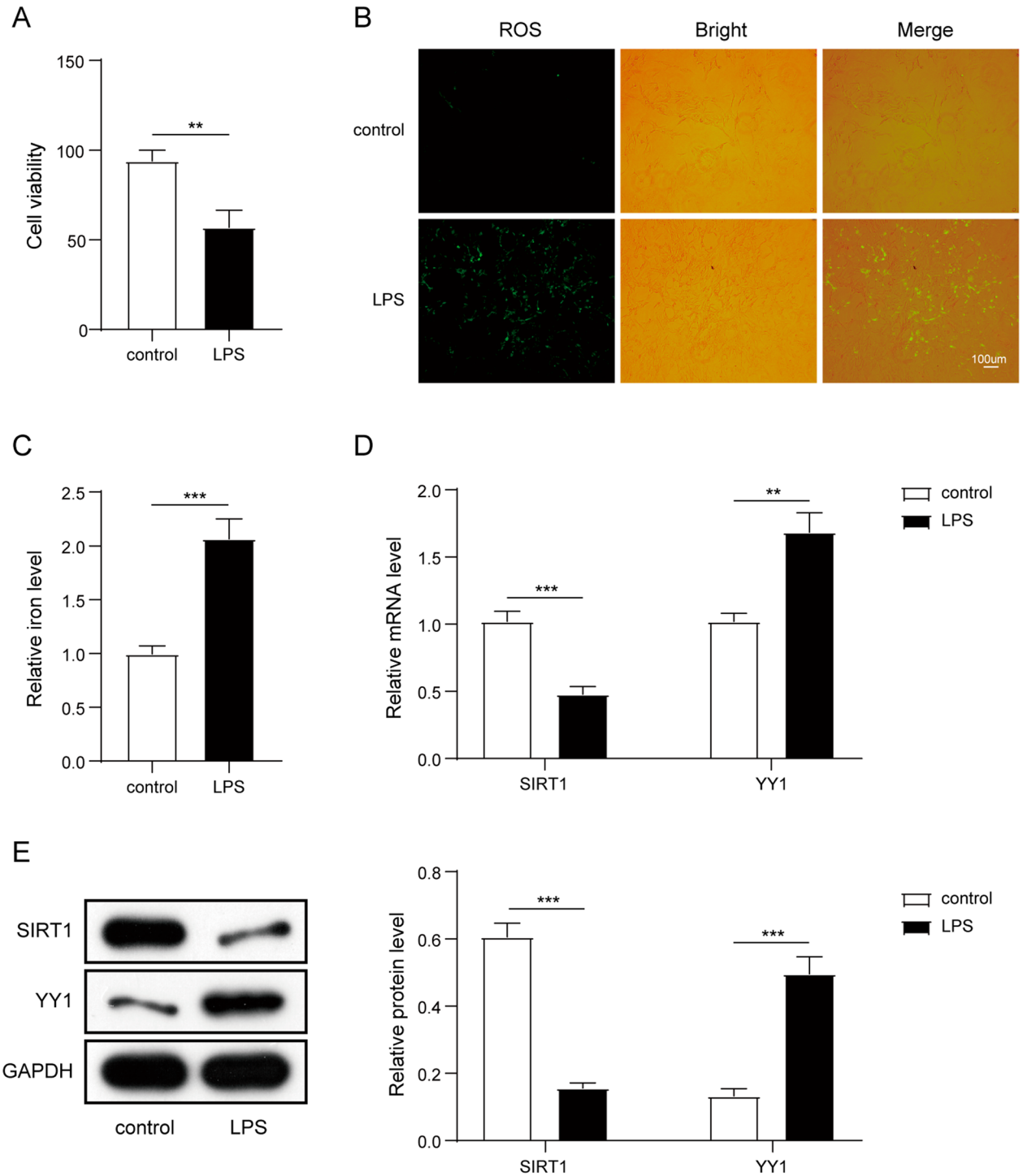

Fig. 2 SIRT1 was downregulated while ferroptosis and YY1 was upregulated in LPS-induced synoviocytes. **A** CCK-8 assay assessed viability. **B** ROS production was detected by DCFH-DA fluorescent probe. **C** Iron level in the synoviocytes was examined by iron assay kit. **D** The mRNA expressions of YY1 and SIRT1 in the synoviocytes were examined by qPCR. **E** The protein levels of YY1 and SIRT1 were detected by western blot. Experiments were carried out in triplicate, mean ± SD, $^{**}p < 0.01$; $^{***}p < 0.001$

ferroptosis in cells. As expected, the iron content in the LPS induced cells was increased significantly (Fig. 2C). Additionally, SIRT1 mRNA level was found to be downregulated, and YY1 mRNA level was upregulated in LPS induced synoviocytes (Fig. 2D). Meanwhile, the protein level of SIRT1 in synoviocytes induced by LPS was significantly reduced, and the protein level of YY1 was significantly increased (Fig. 2E). Therefore, these results were consistent with the clinical level which the ferroptosis might be the key to RA.

Overexpression of SIRT1 inhibited LPS-induced ferroptosis in synovial cells

To explore the effect of SIRT1 on synovial cell function, we overexpressed SITR1 in synovial cells by transfecting with recombinant pcDNA3.1 plasmid. Compared with the control group, after LPS induction, the expression of SIRT1 was significantly downregulated; while transfection of pcDNA-SIRT1 significantly increased its mRNA and protein levels (Fig. 3A–B), suggesting the SIRT1 overexpression vector has been successfully transfected into synovial cells. The CCK-8 assay showed that the cytoactive in the LPS group was significantly reduced

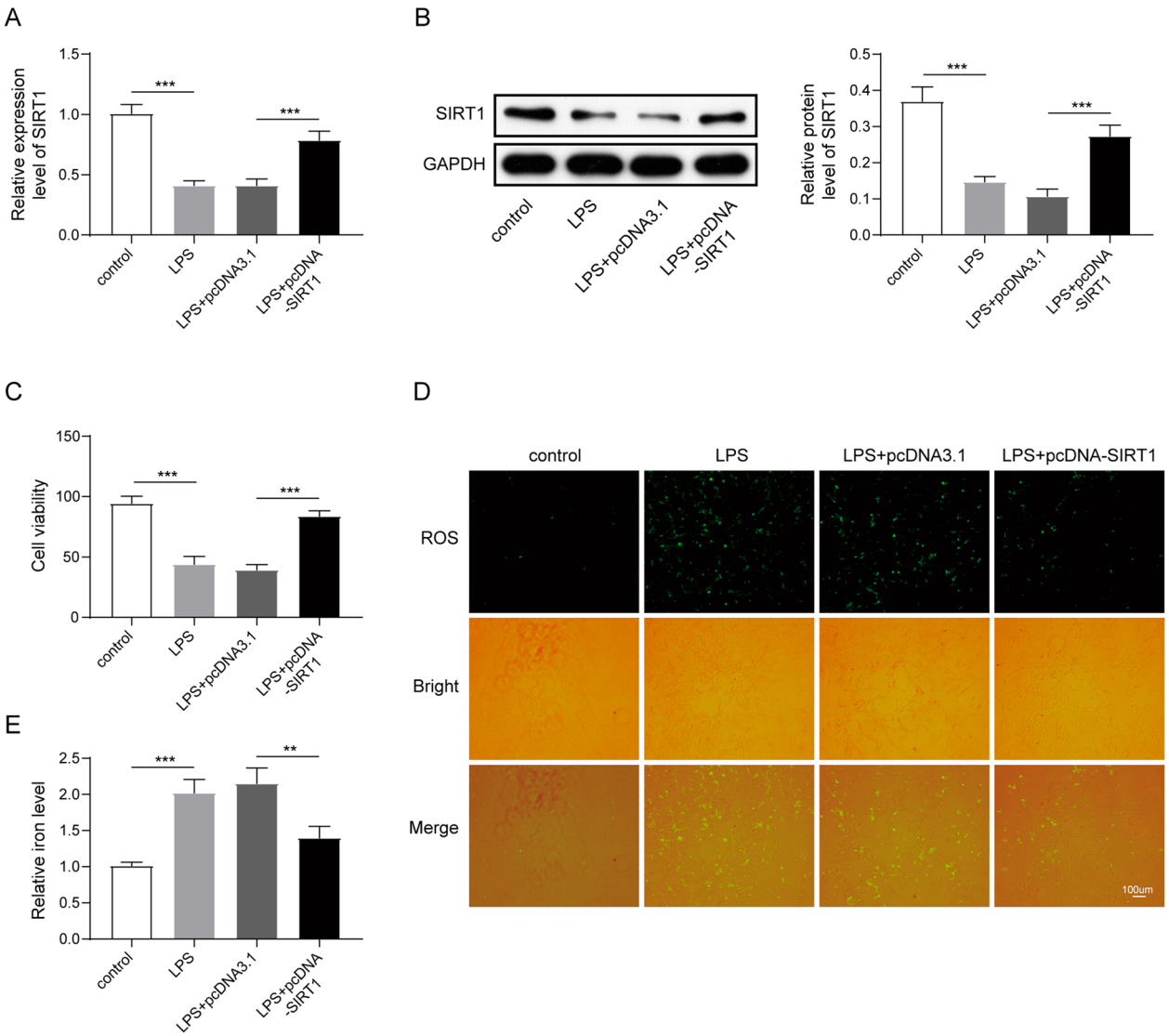

Fig. 3 Overexpression of SIRT1 inhibited LPS-induced ferroptosis of synovial cells. Synoviocytes were transfected with pcDNA-3.1 or pcDNA-SIRT1. **A** The mRNA expression of SIRT1 was examined by qPCR. **B** The protein level of SIRT1 was examined by western blot. **C** Cell viability was assessed by CCK-8 assay. **D** ROS level was detected by DCFH-DA fluorescent probe. **E** Iron level in the synoviocytes was examined by iron assay kit. Experiments were carried out in triplicate, mean ± SD, $^{**}p < 0.01$; $^{***}p < 0.001$

compared with the control group. However, overexpression of SIRT1 significantly increased cell viability (Fig. 3C). Furthermore, LPS significantly increased cellular ROS levels, and overexpression of SIRT1 significantly reduced ROS production (Fig. 3D). Compared with the control group, the iron content in LPS group was significantly increased, while overexpression of SIRT1 significantly reduced the iron content (Fig. 3E). The above results indicated that overexpression of SIRT1 could inhibit LPS-induced ferroptosis in synovial cells.

YY1 downregulated the expression of SIRT1 by inhibiting its transcription

Studies have revealed that the differential expression of genes may be regulated by transcription factors [19, 20]. Accordingly, we suspected that the low expression of SIRT1 in RA may be related to transcriptional regulation. Therefore, we searched the JASPAR database and found that there was a potential binding site between YY1 and SIRT1 promoter region (Fig. 4A). ChIP assay detected a high affinity for the binding of YY1 to the promoter of SIRT1 (Fig. 4B). The dual-luciferase reporter assay indicated that compared with the pcDNA3.1 group, overexpression of YY1 significantly reduced the luciferase activity of the wild type but had no significant effect on the mutant type (Fig. 4C). The qPCR and western blot detection confirmed that after overexpression of YY1, the mRNA and protein levels of YY1 were increased, while the expression of SIRT1 was decreased (Fig. 4D–E). Taken together, these findings indicated that the decrease in SIRT1 expression could be attributed to the to the transcriptional repression of YY1.

YY1/SIRT1 axis promoted the ferroptosis of LPS induced synoviocytes

To further verify the function of YY1 in SIRT1's effects on LPS-induced synovial cells, we conducted rescue experiments in this section. Firstly, human synovial cells were transfected with SIRT1 and YY1 overexpression plasmids before LPS induction, and cell viability was detected by CCK-8. SIRT1 overexpression significantly enhanced the cytoactive restrained by LPS, while overexpression of YY1 partly reversed this effect (Fig. 5A). In addition, overexpression of SIRT1 significantly reduced LPS-induced cellular ROS production, but overexpression of YY1 partially abolished with this change (Fig. 5B). Similarly, overexpression of SIRT1 reduced the iron content induced by LPS, while overexpression of YY1 significantly increased the iron content (Fig. 5C). The above results demonstrated that YY1 inhibited SIRT1 by transcription repression and increased the ferroptosis of synovial cells induced by LPS.

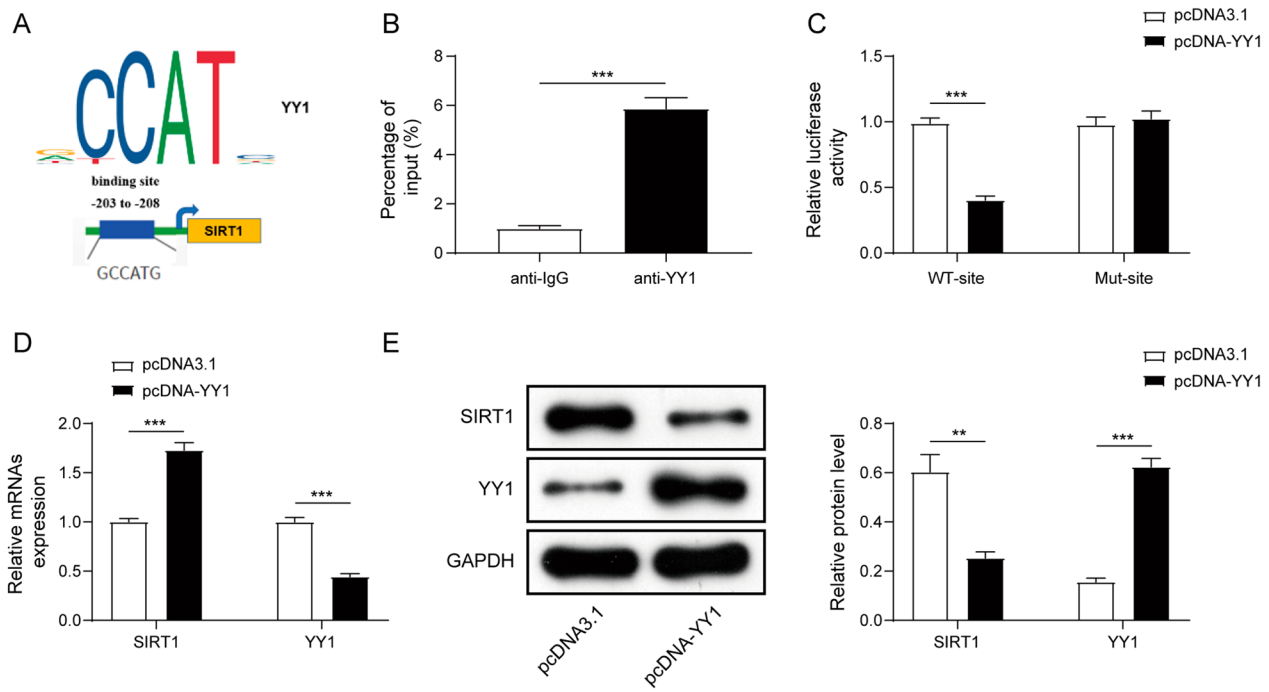

Fig. 4 YY1 downregulated the expression of SIRT1 by inhibiting its transcription. Synoviocytes were transfected with pcDNA-3.1 or pcDNA-SIRT1. **A** JASPAR software predicted the potential binding site between YY1 and SIRT1. **B** ChIP detected the interaction between YY1 and SIRT1. **C** Dual-luciferase reporter assay detected the combination of YY1 and SIRT1. **D** The mRNA expression of SIRT1 was examined by qPCR. **E** The protein level of SIRT1 was examined by western blot. Experiments were carried out in triplicate, mean ± SD, $^{**}p < 0.01$; $^{***}p < 0.001$

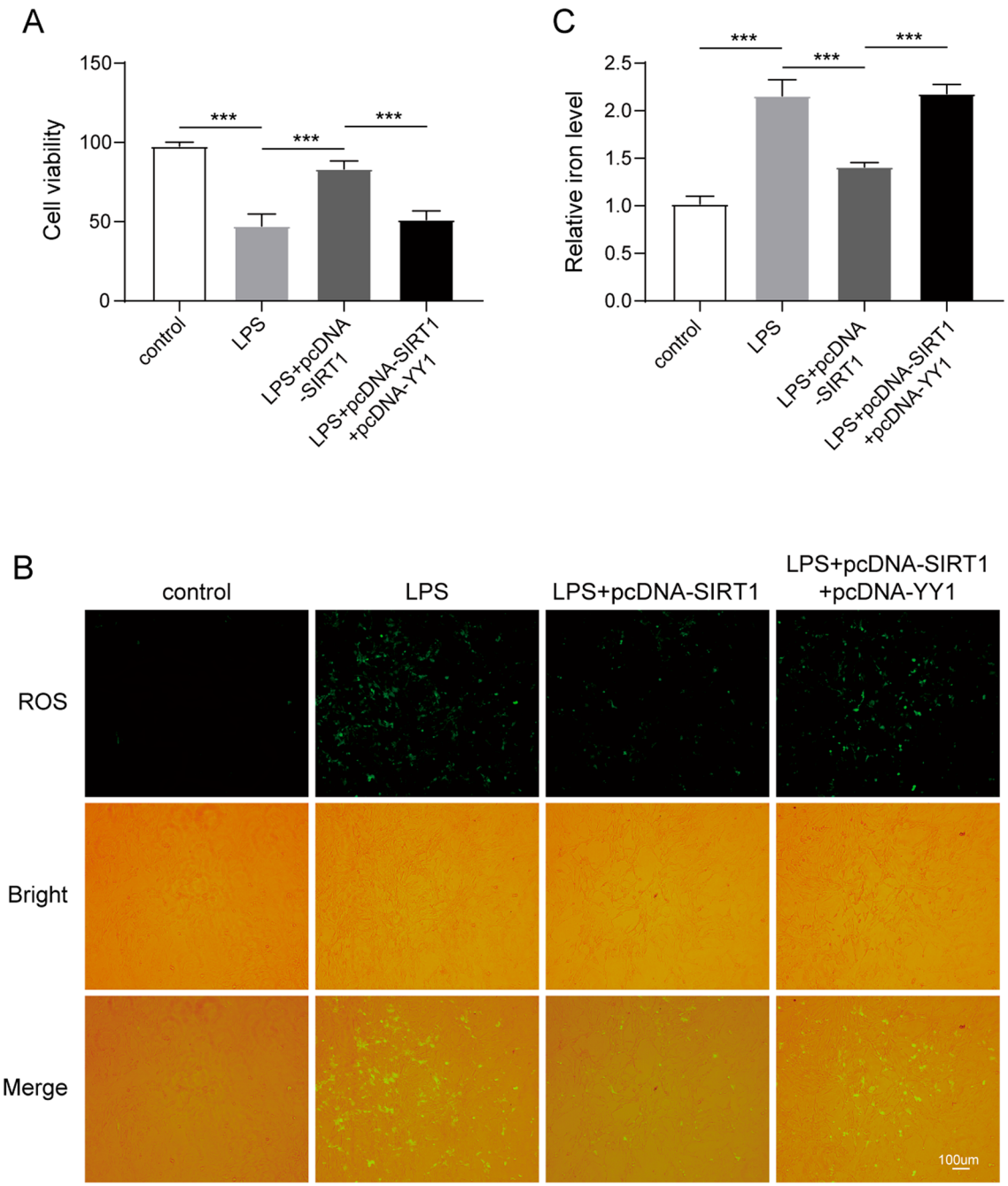

Fig. 5 YY1 promoted LPS-induced ferroptosis of synoviocytes through transcriptional inhibition of SIRT1. Synoviocytes were transfected with pcDNA-3.1, pcDNA-SIRT1 or pcDNA-YY1. **A** Cell viability was assessed by CCK-8 assay. **B** ROS production was detected by DCFH-DA fluorescent probe. **C** Iron level in the synoviocytes was examined by iron assay kit. Experiments were carried out in triplicate, mean ± SD, ***$p < 0.001$

Discussion

Ferroptosis is an iron-dependent form of nonapoptotic cell death firstly discovered by Dixon and colleagues in 2012 [21]. In recent years, research of ferroptosis continuously emerging in various disease. Ferroptosis is considered to be an important factor in myocardial ischemia/reperfusion injury [22], renal cell carcinoma [23], cerebral infarction [24] and so on. Moreover, ROS accompanied with ferroptosis may function as a reciprocal with cell death that interplays with RA [25]. Excessive accumulation of ROS could damage numerous cell types, which is related to the decrease of GSH content

[26]. Lower GSH levels and GSH to oxidized GSH ratio (GSH/GSSG), as measures of redox balance, have previously been reported in the cells for survival inhibition and ferroptosis [27]. In our research, the lower GSH/GSSG and higher iron content were detected in serum samples from RA patients, which reflected that the ferroptosis in RA patients were enhanced. Meanwhile, SIRT1 was found to be downregulated in RA patients, and YY1 was upregulated. Further correlation analysis showed that YY1 was positively correlated with ferroptosis, and SIRT1 was negatively correlated with ferroptosis. In our previous study, we confirmed that SITR1 overexpression restrained the proliferation and inflammation of RA-fibroblast-like synoviocytes [15]. Hussain et al. also demonstrated that significant downregulation of mitochondrial SIRT1 was related with increased risk of arthritis and can be used as an indicator of clinical prognosis [28]. However, the underlying mechanisms of the action of SIRT1 in RA need deeper investigation.

Although the functions of human sirtuins have not yet been determined, Pasquereau et al. found that the increased inflammation in adjuvant-induced arthritis rats compared to healthy control was accompanied by an increased SIRT1 activity in both PBMCs and liver [29]. However, SIRT1 expression in RA endothelial cells and synovial vessels was declined. Conditional SIRT1 deletion in endothelial cells delayed the dissolution of experimental methyl-bovine serum albumin-(mBSA)-induced arthritis [14]. Additionally, SIRT1 was found to be downregulated in RA patients and LPS-induced synovial cells. Li et al. revealed that SIRT1 restrained the invasive and inflammatory responses of RA-fibroblast-like synoviocytes by inhibiting the NF-κB pathway [30]. In addition, upregulation of SIRT1 induced by resveratrol in RA-fibroblast-like synoviocytes may significantly reverse the invasion of these cells and attenuate joint inflammation [31]. Luo et al. found that the inhibition of ferroptosis by activating the GPX4 pathway may be exploited as a new therapeutic strategy for RA [12]. In this study, the overexpression of SIRT1 inhibited LPS-induced ferroptosis of synoviocytes presenting as reduced ROS production and iron ion level. Thus, synoviocyte ferroptosis might be a new target of SIRT1 in RA.

YY1 is a widely distributed transcription factor belonging to Gli-Kruppel zinc finger proteins and is involved in the inhibition and activation of various gene promoters. YY1 can activate or repress gene promoters by directing histone deacetylases and histone acetyltransferases to the promoter. Therefore, histone modification may also be closely related to YY1. Lin et al. demonstrated that the regulation of YY1 by miR-124-3p facilitate Th17 cell pathogenicity by interaction with T-bet in RA [32]. Wang et al. demonstrated that lncRNA NEAT1 targets fibroblast-like synoviocytes in RA via the miR-410-3p/YY1 axis [33]. Inhibition of YY1 reduced the neutrophil infiltration by inhibiting IL-8 production via the PI3K-Akt-mTOR signaling pathway in RA [34]. In the present study, YY1 was verified to have a high affinity for binding to SIRT1 promoter region. YY1 downregulates the expression of SIRT1 by inhibiting its transcription, thereby prompting the ferroptosis of LPS induced synoviocytes. Therefore, YY1 can be a novel therapeutic target for treatment of RA.

Furthermore, there are also some other limitations in this study: (i) iron chelations were not used for further validation on the present results; (ii) the regulatory effect of SIRT1 on ferroptosis was not verified in RA animal models; (iii) it is unclear whether there is a significant difference in the relevant indicators between the serum samples from RA patients undergoing joint replacement and RA patients without joint replacement. These would be the research directions in our future studies.

Conclusions

In this study, we firstly revealed the functional role and mechanism of SIRT1 in regulating ferroptosis during RA progression. Overall, SIRT1 is transcriptionally suppressed by YY1 and modulates the ferroptosis of synovial cells induced by LPS. These research findings would provide reliable theoretical support for further understanding the pathogenesis of RA and developing new treatment strategies.

Abbreviations

RA	Rheumatoid arthritis
SIRT1	Sirtuin 1
YY1	Yin Yang 1
ChIP	Chromatin immunoprecipitation
ROS	Reactive oxygen species
LPS	Lipopolysaccharide
qPCR	Quantitative real-time PCR
GPX4	Glutathione peroxidase 4
CCK-8	Cell counting kit-8
GSH	Glutathione
GSSG	Oxidized GSH
TBST	Tris-buffered saline tween

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Author contributions

SL guaranteed the integrity of the entire study; YZ designed the study and literature research; ZY defined the intellectual content; FZ performed experiment; YH collected the data; Shudian Lin analyzed the data; SL wrote the main manuscript and prepared figures. All authors reviewed the manuscript. All authors read and approved by the final manuscript.

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Availability of data and materials
The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability
Not applicable.

Declarations

Ethics approval and consent to participate
Participants signed the informed consent forms and the Ethics Committee of Hainan General Hospital approved the procotol (approval number: 2021233).

Consent for publication
Not Applicable.

Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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