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Original Article

# HIF-1α dependent RhoA as a novel therapeutic target to regulate rheumatoid arthritis fibroblast-like synoviocytes migration in vitro and in vivo

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ABSTRACT

*Objective:* The purpose of this work is to investigate how the Rho family of GTPases A (RhoA) mediates the pathogenesis of rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS).
*Methods:* The expression of RhoA in the synovial tissues of RA and Healthy people (Control) was detected using immunohistochemistry methods. The expression of RhoA and hypoxia-inducible factor-1α (HIF-1α) is inhibited by small interfering RNAs (siRNAs). The inhibition effect on RA-FLS migration was further investigated. The protein expression level of HIF-1α, RhoA, focal adhesion kinase (FAK), and myosin light chain (MLC) was also analysed using western blotting (WB). DBA1 mice were immunised with the mixture of bovine type II collagen and Freund's adjuvant to establish collagen induced arthritis (CIA) mouse model. Lip-siRhoA is administered through joint injection every two days. Micro-computed tomography (micro-CT) was used to detect mouse ankle joint destruction and evaluate the bone loss of the periarticular side. Destruction of the ankle articular cartilage was tested by histology. Expressions of P-RhoA, P-FAK and P-MLC in the ankle joint was detected by immunohistochemistry assay.
*Results:* The expression level of RhoA in the synovial tissues of RA patients was significantly higher than that in control group. Hypoxia was able to up-regulate the expression of RhoA. Whereas, HIF-1α siRNA (siHIF-1α) could down-regulate the expression of RhoA. Additionally, both of siHIF-1α and RhoA siRNA (siRhoA) delivered by liposome (Lip-siHIF-1α and Lip-siRhoA) were found to suppress FAK and MLC phosphorylation in vitro. In CIA mouse model, Lip-siRhoA was demonstrated to ameliorate the destruction of ankle joint and reduce the severity of ankle joint cartilage damage by micro-CT and histological staining, respectively. Therefore, inhibition of FLS cell migration can protect articular bone from destruction. Furthermore, the expression of P-RhoA, P-FAK and P-MLC was evaluated and found to be down-regulated by Lip-siRhoA in vivo.
*Conclusion:* The results demonstrated that under hypoxic environment, HIF-1α dependent RhoA pathway played an important role on cytoskeleton remodelling and RA-FLS migration. Through down-regulating RhoA expression, it could effectively treat RA in vitro and in vivo.
*The translational potential of this article:* Our study provides new evidence for the potential clinical application of RhoA as a candidate for the treatment of RA.

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## 1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic destructive arthropathy which primarily manifests as abnormal immune cell activation, joint destruction, and systemic lesions [1,2]. RA fibroblast-like synoviocytes (RA-FLS) migration and invasion constitute the primary cause of joint cartilage destruction [3] and synovial pannus formation [4]. RA-FLS migration was to be an important factor of RA deterioration, while the mechanism of joint migration of activated RA-FLS in vivo is still unknown. The exploration of its mechanism will deepen our comprehending of the pathology and it could be adopted as a potential therapeutic target of RA.

Hypoxia microenvironment was a major hallmark of RA synovial tissues and it was able to promote RA-FLS migration and invasion [5–7]. RA-FLS up-regulated or down-regulated the expression of hypoxia-inducible factors (HIFs) to initiate their internal organization and transcription programs to adapt to a hypoxic environment after sensing a change in oxygen levels at microenvironment [8,9]. HIF-1α was a member of hypoxia-inducible factors (HIFs) family, which was confirmed to highly express in RA-FLS and was closely related to the degree of arthritis activity, synovial hyperplasia and angiogenesis [10, 11]. However, it is still unclear that how HIF-1α regulate the RA-FLS migration and invasion.

A critical step of cell migration is cytoskeleton reprogramming, during which the GTPase members of the Rho family play a core role by controlling the molecular switches of morphogenesis and motion [12]. The Rho family is a family of GTP-dependent proteins, in which the most common classical members such as Rho family of GTPases A (RhoA), cell division cycle 42 (CDC42) and Rac Family Small GTPase 1 (Rac1) are important signalling molecules in cell migration [13]. It has been proved that HIF-1α induced breast cancer cells migration by regulating RhoA and modulate the cytoskeleton [14]. Inspired by these findings, we hypothesis that hypoxia-induced RA-FLS migration is attributable to HIFs which regulate the Rho family proteins and lead to cytoskeleton remodelling.

In this work, we found that RhoA was highly expressed in RA-FLS and its mechanism for the first time . Under hypoxic microenvironment (3% $O_2$), RhoA dependent HIF-1α was highly expressed and further induced the migration of RA-FLS. Its mechanism was demonstrated that RhoA regulated the changes of RA-FLS cytoskeleton by promoting the phosphorylation of focal adhesion kinase (FAK) and myosin light chain (MLC). Inspired by these results, RhoA small interfering RNA (siRNA) was synthesized and delivered by liposome (Lip-siRhoA) to treat RA-FLS in vitro and in Collagen-induced arthritis (CIA), showing that down-regulation of RhoA could inhibit RA-FLS migration and alleviate the RA joint destruction.

## 2. Materials and methods

### 2.1. RA patient recruitment and synovial tissue specimen analysis

RA patients (n = 8, 3M: 5F) and Healthy control group (n = 6, 3M: 3F) were recruited from the Rheumatology Department, University of Chinese Academy of Sciences Shenzhen Hospital (Shenzhen, Guangdong). RA patients fulfilled the standards of the American College of Rheumatology. All patients gave fully informed written consent approved by the institutional ethics committee (SIAT-IRB-191115-H0401), and the present study was conducted in accordance with the Declaration of Helsinki. Synovial tissue specimens from patients with inflammatory arthritis were obtained at arthroscopy under local anaesthesia using a Wolf 2.7-mm needle arthroscope (Storz, Tuttlingen, Germany), as previously described [15]. Biopsy specimens were either embedded in formaldehyde solution or snap-frozen in liquid nitrogen for immunohistochemical and protein analysis.

### 2.2. Culture of RA-FLS

Primary RA-FLS were isolated from RA biopsies by digestion with 1 mg/mL collagenase type 1 (Sigma–Aldrich, St. Louis, USA). RA-FLS were cultured in RPMI 1640 (Hyclone, Logan, USA) containing 10% FBS (BRL Life Technologies, New York, USA) and 1% penicillin/streptomycin solution (Hyclone). RA-FLS were cultured under normoxic conditions and 3% $O_2$ (reflecting the joint environment in vivo) [4,5]. Dissociated cells were grown to confluence and used between passages 4 and 8.

### 2.3. Immunofluorescence assay

RA-FLS were fixed with 4% paraformaldehyde (Sigma), permeabilized with 0.1% Triton X-100 (Sigma) for 10 min, and blocked with 5% non-fat dry milk with gentle agitation for 30 min. For focal adhesion staining of pFAKY397 (Abcam), antibodies were used. Actin filaments and nuclear DNA were stained using Alexa-Fluor 488-conjugated phalloidin (Abcam) and 300 nM 4′,6-diamidino-2-phenylindole (DAPI) (Sigma).

### 2.4. Real-time quantitative PCR (RT-qPCR) analyses

RT-qPCR was performed in a LightCycler 96 system (Bio-Rad, Hercules, CA, USA). The reaction conditions employed were as follows: 50 °C for 2 min and 95 °C for 10 min under predenaturation conditions and 40 cycles at 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 30 s. The RhoA primer sequences used were as follows: forward, 5′- CCATCATCCTGGTTGGGAAT -3’; reverse 5′-CCATGTACCCAAAAGCGC-3’. The GAPDH primer sequences were as follows: forward, 5′- GCACCGTCAAGGCTGAG

AAC-3’; reverse, 5′- TGGTGAAGACGCCAGTGGA-3’.

### 2.5. Gene silencing by RNA interference

RA-FLS were cultured and transfected in 6-well plates; 5 μL small interfering RNA (siRNA) duplexes (HIF-1α, RhoA, or Scramble) were diluted with 200 μL Opti-MEM (Thermo Fisher Scientific, Waltham, MS, USA) serum-free medium; 5 μL Lipofectamine 3000 (Thermo Fisher Scientific) was diluted with 200 uL Opti-MEM serum-free medium, and mixed gently. Incubation was carried out at room temperature for 20 min in the dark, following which the contents were added to the well containing 1590 μL medium and cells; incubation was then carried out for a further 24 h. Scrambled control (a nonsense siRNA of the target sequence) and the following siRNA duplexes were sourced from GenePharma (Shanghai, China). The siRNA duplexes for HIF-1α (5′-CGAGGAAGAACUAUGAACATT-3′, 5′-UGUUCAUAGUUCUUCCU

CGTT-3′); RhoA (5′-CAGCCCUGAUAGUUUAGAATT-3′, 5′-UUCUAAACUAUC AGGGCUGTT-3′).

### 2.6. Western blotting (WB) analysis

The total protein of synovial tissue lysates and RA-FLS were extract using RIPA lysis buffer (Thermo Fisher Scientific; #8900). Protein samples were resolved on SDS-PAGE and transferred onto PVDF membranes (Millipore). Membranes were blocked in wash buffer containing 5% non-fat dry milk with gentle agitation for 1 h at room temperature. The membranes were then incubated with Antibody Dilution-diluted primary antibodies, as follows. HIF-1α antibody (1:500, GeneTex, GTX127309); RhoA antibody (1:500, Abcam, ab54835); phosphorylated (P)-RhoA antibody (1:500, Abcam, ab41435); FAK antibody (1:500, Abcam, ab40794); P-FAK antibody (1:500, Abcam, ab81298); MLC antibody (1:1000, CST, #3672); P-MLC antibody (1:1000, CST, #3675); GAPDH antibody (1:2000, Abcam, ab8245), at 4 °C overnight with gentle agitation. After washing thrice on the shaker for 10 min each time, the

membranes were incubated at 1:1000 dilution of Goat anti-rabbit IgG (HRP) (1:2000, Abcam, ab6721) or anti-mouse IgG (HRP) (1:2000, Abcam, ab6728) for 2 h. Following three final 15-min washes, the enhanced chemiluminescence (ECL) detection reagent (Millipore) was placed on the membranes for 5 min before they were exposed to Hyper film ECL.

### 2.7. Cell migration assays

To perform scratch assays, RA-FLS that had been transiently transfected with siRNA or scrambled control siRNA (Scr) were plated in 6-well cell culture plates, and maintained in RPMI 1640 for 4 h prior to being serum starved with RPMI 1640 [1% Fetal Bovine Serum (FBS)] overnight. A scratch was made across the cell layer using a sterile pipette tip. After washing twice with serum-free medium, full RPMI 1640 (5% FBS) was added and the cells were incubated under conditions of normoxia or 3% hypoxia for 24 h. The RA-FLS scratch pattern was photographed a phase-contrast microscope at 100 × magnification.

### 2.8. Collagen-induced arthritis (CIA) and experimental group

Immunisation grade bovine type II collagen (20022; Chondrex; dissolved in 0.05 M acetic acid) was mixed and emulsified with isovolumic Incomplete Freund's adjuvant (IFA; 7002; Chondrex). Then, 100 μl of the CII-IFA emulsification solution was intra-dermally injected into the tail of each mouse. The injection site was about 1 cm away from the root of the mouse tail. 21 days after the first immunization, another 50 μl of the CII-IFA emulsification solution was intra-dermally injected as a booster immunization into the opposite side of the tail of each mouse. The score ranged from 0 to 4 per paw [16]. After arthritis onset, mouse were divided into the following 3 groups (n = 6): Saline group (Saline); and Lip-siRhoA group. Lip-siRhoA group was injected with 5 nmol/20g of siRhoA in the joint every two days. Saline group joints were injected with 20ul of saline.

### 2.9. Micro-CT

After three weeks of treatment, micro-CT (Sky-Scan, Bruker, Belgium) analyses were performed on the ankle joint. The scanning parameters of the ankle joints were 18 mm resolution, a voltage of 60 kV, and a current of 400 μA with a 0.1 mm filter. Reconstruction and analysis were performed automatically. Each sample was scanned with a Hounsfield unit supplied with a micro-CT machine. All image data were analysed and reconstructed by professional software. Ankle joint bone radiological destruction was scored on a scale from 0 to 3: 0, no damage; 1, minor; 2, moderate; 3, severe [17].

### 2.10. Histological analysis

Ankle joints were fixed for 24 h in 4% buffered formalin (Sigma–Aldrich). The formalin was washed with water, and the tissue was decalcified (Sigma–Aldrich) and embedded in paraffin. Ankle joints tissues were then sliced into 5 μm thick sections. H&E (Beyotime, Beijing, China) and Safranine O-Fast Green (Solarbio, Beijing, China) staining were performed according to standard protocols. After staining, observe the changes of pathological tissues under an optical microscope. H&E scored [18] from 0 to 4, 0 = normal joint synovial tissue and bone structure; 1 = synovial cell hypertrophy and inflammatory cell erosion of synovial tissue; 2 = cartilage destruction and pannus present; 3 = most articular cartilage and subchondral bone are destroyed; 4 = joint adhesions and stiffness and accompanying disability. Safranine O-Fast Green staining was scored [19] from 0 to 3, where 0 = normal, 1 = slight cartilage erosion, 2 = moderate cartilage erosion, and 3 = cartilage erosion or bone destruction.

### 2.11. Statistical analysis

SPSS 17.0 software was used for analysis. One-Way ANOVA of variance was used for multiple group analysis. Student's t tests were used to analyse the data from two groups. Results were expressed as mean ± standard error of the mean (SEM) and $p < 0.05$ were considered significant and $p < 0.01$ were considered highly significant.

## 3. Results

### 3.1. High expression of RhoA in the synovial tissues of RA patients

The expression level of RhoA in RA synovial fibroblasts was analysed by a bioinformatics method from the published single cell sequencing data and transcriptome sequencing database. The results of single cell sequencing of synovial tissue samples from RA patients and CIA models were obtained from ImmPort database [20]. Visual reanalysis is carried out by using Broad Institute Single Cell Portal. Lining Fibroblasts is marked by Proteoglycan 4 (PRG4), and Sublining Fibroblasts is marked by Cluster of Differentiation 90 (CD90). The scatter data is visualized by t-distributed stochastic neighbor embedding, and the expression unit of RhoA is Log (TPN+1), that is, the transcript of Log2 per million reads. The results showed that RhoA was highly expressed in the synovial fibroblasts from RA patients (Fig. 1A and B) and CIA models (Fig. 1C and D). It was also found that RhoA in synovial tissue of RA patient was significantly higher than that of healthy people (Fig. 1E).

The synovial tissue of RA patients and Normal (Control group) were respective subjected to immunohistochemical analysis (Fig. 1F), which showed that the RhoA expression in the synovial tissues of RA patients were significantly higher than that of the Normal group (Fig. 1G). Furthermore, the expression of RhoA as well as HIF-1α of RA patient synovial tissues was detected by WB and was found to be higher than that of Normal group (Fig. 1H). These data demonstrated that RhoA and HIF-1α are highly expressed in synovium tissue from both RA patients and CIA model.

### 3.2. HIF-1α dependent hypoxia-induced expression of RhoA

RhoA was reported to be highly expressed in tumor cells under hypoxia environment, which was related to the tumor cells migration [21]. Inspired by this find, the expression of RhoA on RA-FLS was investigated in hypoxic environment. After exposing RA-FLS to hypoxia (3% $O_2$) condition for 12 h, their RhoA expression level were significantly higher than that in normoxia condition (Fig. 2A and B). HIF-1α as a hallmark of hypoxia was demonstrated to be highly expressed in hypoxia environment (Fig. 2B). As seen in the representative fluorescence images, HIF-1α and P-RhoA were confirmed much more in RA-FLS in hypoxia, in comparison to that in normoxia (Fig. 2C).

To further elucidate the relationship of HIF-1α and RhoA, we synthesized HIF-1α and RhoA specific small interfering RNA (siRNA), and then respectively encapsulated them with liposome to be Lip-siHIF-1α and Lip-siRhoA. Negative siRNA with no function was used as control scramble (Scr). The uptake of RA-FLS of Lip-siHIF-1α and Lip-siRhoA was first evaluated using confocal microscopy, showing that the liposome nanoparticles could high-effectively enter cells compared to naked small interfering RNAs (siRNAs) (Fig. 2D). This result suggested liposome could be a good carrier to deliver HIF-1α and RhoA siRNAs. After incubation of Lip-siHIF-1α and Scr under normoxic and hypoxic conditions, the expression of RhoA mRNA was firstly detected using RT-qPCR. It was found that the expression of RhoA mRNA increased more intensely under hypoxia than that under normoxia, while which was significantly suppressed at the presence of Lip-siHIF-1α (Fig. 2E), indicating that the down-regulation of HIF-1α could inhibit the expression of RhoA mRNA. At the same time, the down-regulation of HIF-1α was also demonstrated to inhibit the protein expression of RhoA (Fig. 2F). These results suggested that the up-regulation of RhoA was mediated by hypoxia-induced

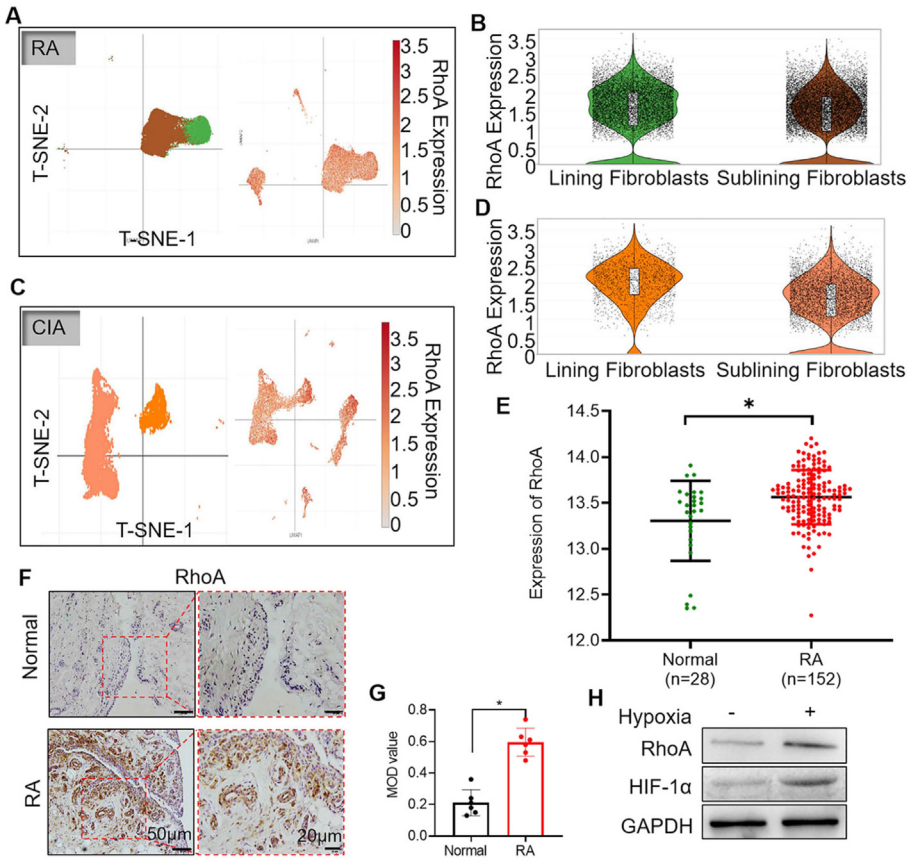


**Fig. 1.** Expression of RhoA in synovial tissues extracted from RA patients. The expression of RhoA in RA (A and B) and CIA (C and D) synovial fibroblasts was analyzed by single cell data (data from Nature. 2020; 528: 259–264) (E) The expression of RhoA in synovial tissue of RA and Normal (transcriptome data from NCBI) (F) Representative photomicrographs showing the localisation of RhoA in synovial tissue sections from RA patients and Normal group (G) Quantification of RhoA in the synovial of RA synovium versus Normal synovium (n = 6) (H) Representative western blot showing RhoA and HIF-1α protein in Normal group versus RA synovial tissue. GAPDH was used as loading control. Bars show the mean ± SEM. *p < 0.05, versus control patients.

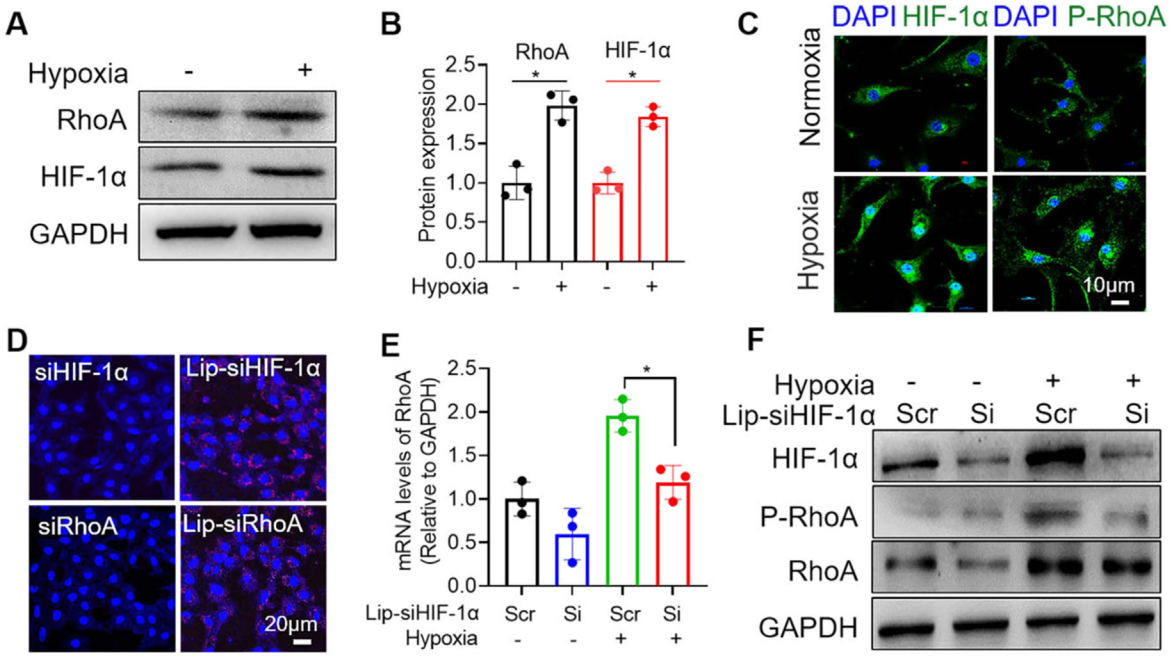


**Fig. 2.** The expression of RhoA in RA-FLS increased under hypoxic environment and was dependent on HIF-1α (A) Representative western blot of RhoA and HIF-1α under conditions of normoxia, and hypoxia (B) showed the relative quantification of protein (*p < 0.05 versus normoxic baseline n = 3) (C) Immunofluorescent analysis of RA-FLS under normoxia or hypoxia for 12 h nuclear translocation of HIF-1α and P-RhoA were observed by immunofluorescence (Scale bar = 10 μm) (D) RA-FLS uptake of siRhoA and Lip-siRhoA (Scale bar = 20 μm) (E) mRNA expression of RhoA in RA-FLS following transient transfection with scramble control (Scr) or siHIF-1α and under conditions of normoxia or hypoxia. Bars show the mean ± SEM (n = 3), *p < 0.05, data were normalized to GAPDH (F) Representative western blots of HIF-1α, pRhoA and RhoA in RA-FLS following transient transfection with scramble control (Scr) and siHIF-1α under normoxia or hypoxia for 12 h.

expression of HIF-1α.

### 3.3. RhoA promoted RA-FLS migration and cytoskeleton remodelling

RhoA was reported to play an important role in tumor cell migration that induced tumor metastasis [22]. With the similar effect, during the progress of RA, RA-FLS could destroy joint bone and articular cartilage mainly because of their migration in joint. Therefore, the influence of RhoA on RA-FLS migration was investigated. As shown in Fig. 3A and B, under normoxic condition, RA-FLS displayed negligible migration, while under hypoxic condition, RA-FLS showed much more migration, indicating hypoxia environment could promote RA-FLS migration. After incubation with Lip-siRhoA for 24h, RA-FLS migration was significant suppressed, demonstrating silence of RhoA expression could inhibit the RA-FLS migration. To study its mechanism, the cell migration related polymerized actin protein (F-actin) was stained by FITC-conjugated phalloidin. As shown in Fig. 3C, F-actin of RA-FLS expressed much more under hypoxic environment for 12 h than normoxic environment. After treatment with Lip-siRhoA, the expression of F-actin was suppressed significantly. The cell migration related protein P-FAK was also down-regulated by Lip-siRhoA by immunofluorescence staining (Fig. 3D). WB was further used to detect the RhoA, FAK, P-FAK, MLC, and

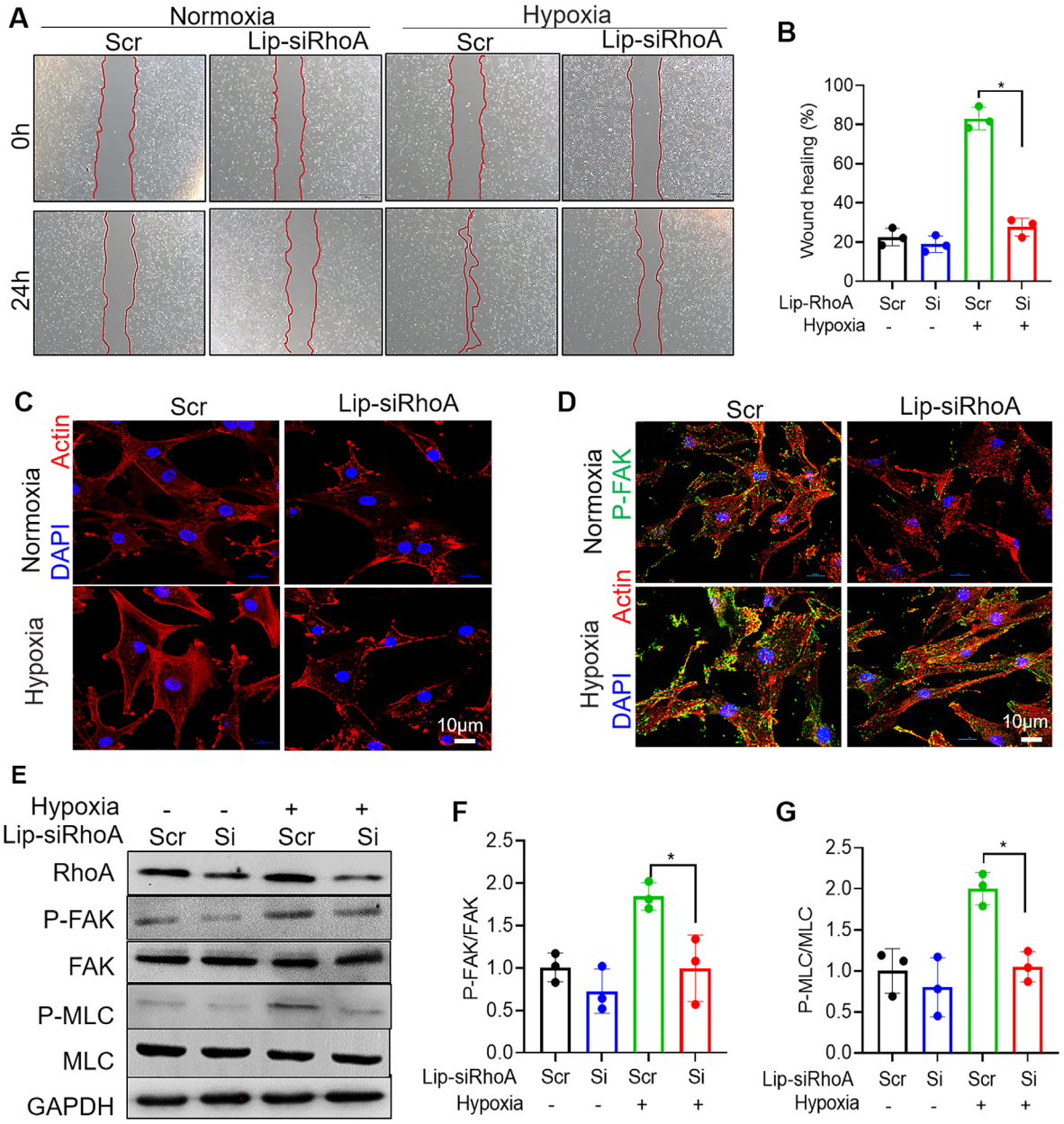


**Fig. 3.** RhoA regulates FAK and MLC phosphorylation and cytoskeletal remodeling (A) Representative images of RA-FLS wound repopulation following transient transfection with scramble control (Scr) or siRhoA and exposure to conditions of normoxia or hypoxia (n = 3). % of wound healing in 24 h (n = 3) *p < 0.05 (B) % of wound healing in 24 h (n = 3) (C) RA-FLS were following transient transfection with scramble control (Scr) or siRhoA and exposure to conditions of normoxia or hypoxia for 12 h and stained with FITC conjugated phalloidin (red) to detect F-actin (stress fibers) and with DAPI to detect nuclei (blue) (bars = 20 μm) (D) RA-FLS were following transient transfection with scramble control (Scr) or siRhoA and exposed to normoxia or hypoxia for 12 h and were stained with FITC-phalloidin (F-actin; red), anti-pFAKT397 (focal adhesions; green), and DAPI (nuclei; blue) (bars = 10 μm) (E) Representative western blots of RhoA, pFAK, FAK, pMLC and MLC in RA-FLS following transient transfection with scramble control (Scr) and siRhoA under normoxia or hypoxia for 8 h (F) and (G) Densitometry quantification of P-FAK/FAK (F) and P-MLC/MLC (G), bars show the mean ± SEM (n = 3, *p < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

P-MLC proteins of RA-FLS before and after treatment with Lip-siRhoA under hypoxia or not. The results showed that hypoxia could promote the expression of P-FAK and P-MLC protein, leading to the increment of P-FAK/FAK, and P-MLC/MLC. In comparison, after treatment with Lip-siRhoA, P-FAK/FAK and P-MLC/MLC decreased to the level of Scr group under hypoxia (Fig. 3E–G). As demonstrated above, RhoA was dependent on HIF-1α. Therefore, we used Lip-siHIF-1α to treat RA-FLS and showed the similar effect of Lip-siRhoA on RA-FLS (Figs. S1–3). These results suggested that RhoA could be a potential therapeutic target for RA.

### 3.4. *In vivo RA therapy of Lip-siRhoA*

Encouraged by the prominent effect of siRhoA on RA-FLS migration in vitro, it was further used for in vivo RA therapy. The CIA mouse model was first established and then was treated by Lip-siRhoA (Fig. 4A). Through three weeks' treatment, the paw thickness of RA mice significantly decreased, which was closed to that of healthy mice (Fig. 4B and C). The classic clinical score showed that the symptom of RA such as redness and swelling was alleviated obviously compared to that of saline treated RA mice (Fig. 4D). Previous studies have shown that the migration of FLS can lead to cartilage destruction [3,4], so here we evaluated whether inhibition of RhoA expression can protect articular bone destruction. The integrity of the articular cartilage was examined by micro-CT imaging. As shown in Fig. 4E, bone damage was clearly visible in the saline treated RA group, while it was repaired after treatment by Lip-siRhoA (Fig. 4E and F). Quantitative analysis of bone volume to tissue volume (BV/TV) and trabecular number (Tb.N) showed significant improvement in Lip-siRhoA group (Fig. 4G and H).

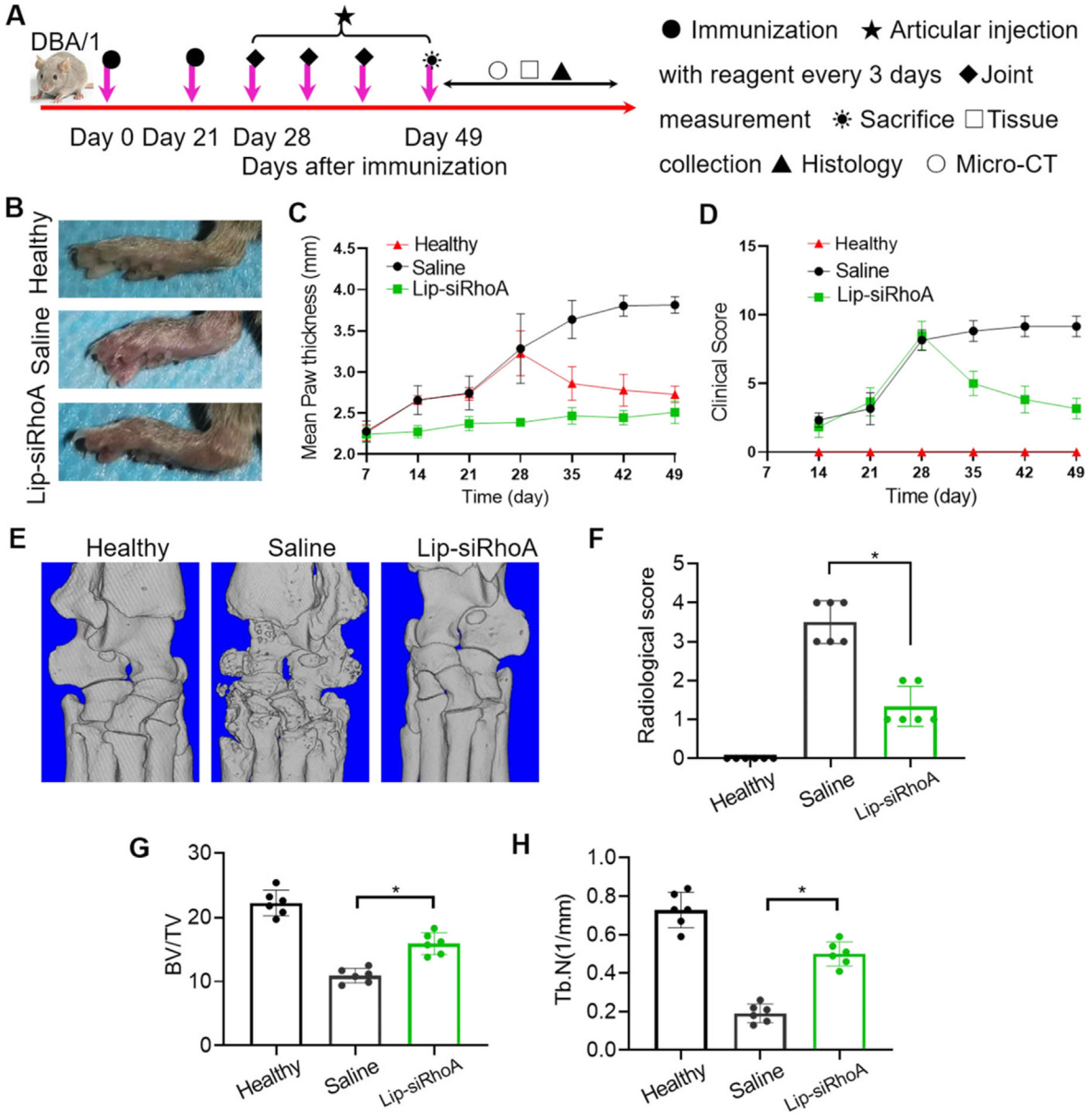


**Fig. 4.** The effect of Lip-siRNA on CIA (A) The experimental schedule of CIA therapy and diagnosis. Inject 5nmol/20g of siRhoA into the joint every two days (B) Paw photos of CIA mouse were taken 21 days after treatment (C) Paw thickness in CIA mouse treated with Healthy, Saline or Lip-siRhoA ($n = 6$,*$P < 0.05$) (D) Arthritis scores were monitored once every 7 days ($n = 6$,*$P < 0.05$) (E) Representative 3D reconstructions of micro-CT images of the ankle joint (F) Radiological score ($n = 6$,*$P < 0.05$) (G) Ratio of bone volume to tissue volume (BV/TV), (*$P<0.05$, $n = 6$) (H) Trabecular number (Tb. N), (*$P<0.05$, $n = 6$).

### 3.5. Histological analyses

The excised joint tissues were proceed to histological analysis. Haematoxylin and eosin (HE) staining showed that the ankle joints of CIA mice in saline treated group had extensive damage and rough surfaces, along with abnormal synovial tissue hyperplasia and substantial inflammatory cell infiltration, compared to that of mice in normal group (Fig. 5A). After three weeks of treatment with Lip-siRhoA, the symptom of CIA mice seen in HE images were obviously alleviated (Fig. 5A and B). The articular cartilage of CIA mice in saline treated group was stained by Safranine O-Fast Green and showed thicker than that of normal mice. Treatment of Lip-siRhoA significantly ameliorated the cartilage hyperplasia of CIA mice ankle joint (Fig. 5C and D). Immunohistochemistry staining showed that P-RhoA, P-FAK and P-MLC expression was significantly down-regulated in synovial tissues after treatment with Lip-siRhoA (Fig. 5E). In order to detect pannus formation in synovial tissue, we labeled the like blood vessels of synovial tissue with cluster of differentiation 31 (CD31) antibody. The results showed that the pannus decreased significantly after Lip-siRhoA treatment (Fig. S4).

## 4. Discussion

RA is an autoimmune disease with complex pathophysiological mechanisms. There is no known cure for RA because the pathophysiology is still being investigated. Our research group investigated the pathophysiology and management of RA [2,10,23]. One of the typical traits that fosters active RA is a hypoxic joint microenvironment, but its molecular mechanism is still unclear [24]. Here, we discovered that a hypoxic milieu can encourage RhoA expression in RA-FLS cells, facilitating their migration. These research offer valuable information for examining the pathophysiology of RA and developing new therapeutic targets.

The obtained results indicated the steps involved in hypoxia, which induced RA-FLS migration. Hypoxia initialized the stabilization of HIFs, which in turn promoted the formation of actin polymerization/stress fibres and adhesion plaque mediated by the regulation of FAK and MLC phosphorylation by a Rho family protein, RhoA. The consequent cytoskeleton remodelling eventually led to cell migration. Here, we found that the expression of RhoA in the synovial tissues of RA patients than that in healthy group. Synovial tissues are mainly composed of RA-FLS, which are major effector cells involved in the RA pathological process. In vitro experiment, it was discovered that a hypoxic environment could promote RA-FLS migration [24]. Moreover, phosphorylation of FAK and MLC is also involved in cell migration [25,26]. FAK is a tyrosine kinase that controls cell adhesion. A lack of FAK results in increased cell adhesion and weakened migration ability [27,28]. The force transferred to the adhesion sites originates via the interaction between myosin II and actin filaments attached to these sites. The activity of myosin II is regulated by the phosphorylation of MLC [29]. Similar to the observation made in the experiments involving tumour cells [30,31], it was determined that HIF-1α regulated FAK and MLC phosphorylation in a hypoxic environment in RA-FLS, resulting in cytoskeleton remodelling.

A prior study revealed a high degree of correlation between hypoxia and Rho family proteins in metastatic cancer cells [32]. In melanoma cells, hypoxia-induced migration is a result of the up-regulation of RhoA by HIF-1α [33]. It has also been reported that hypoxia induces RA-FLS migration via Signal transducer and activator of transcription 3 (STAT3), Notch1, and other signalling pathways [34,35]. However, there is a lack of data on Rho family signals which are most important for RA-FLS migration. In this study, we found that a hypoxic environment (3% $O_2$) in RA-FLS could augment their mRNA and protein expression of RhoA. In addition, knocking down HIF-1α using siRNA could lower the expression of RhoA induced by hypoxia. These results supported that HIF-1α regulated cytoskeleton change via altering RhoA levels in the hypoxic environment to control cells migration.

Earlier studies had shown that RhoA regulated FAK and MLC phosphorylation, thereby causing remodeling of the cytoskeleton [36,37]. Our study demonstrated that knocking down RhoA using siRNA in the hypoxic environment reduced RA-FLS migration, concomitantly weakening FAK and MLC phosphorylation. Additionally, it was suggested that HIF-1α affected cytoskeleton remodelling in the hypoxic environment via modulation of the RhoA signalling pathways, resulting in cell migration.

## 5. Conclusion

RhoA have been shown to be highly expressed in the synovial tissues of RA patients and CIA model. RhoA expression is upregulated in FLS during hypoxia, and RhoA expression inhibition can prevent the hypoxic-induced migration of FLS. Based on the findings, we developed a potential therapy strategy that using a RhoA siRNA and encapsulating with

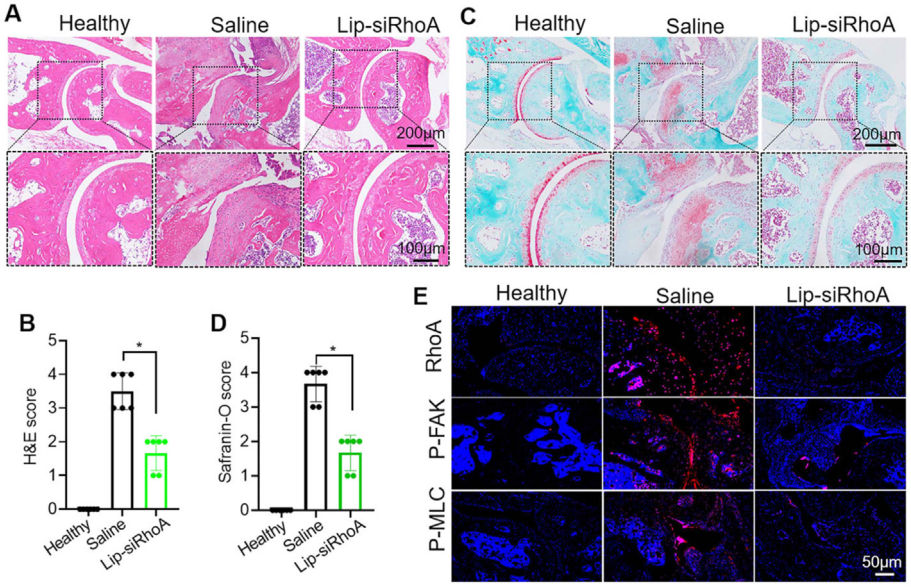


**Fig. 5.** Histological staining (A) H&E stains the ankle joint (B) H&E semi-quantitative score (*P < 0.05, n = 6) (C) Safranine O-Fast Green staining (D) Cartilage destruction scores (*P < 0.05, n = 6) (E) Representative microscopy images of synovial tissues in which P-RhoA (red), P-FAK (red), P-MLC (red) and DAPI (blue) are visualised by immunofluorescence staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

a liposome to treat RA-FLS in vitro and in vivo. Lip-siRhoA can effectively prevent the degradation of joint bone, according to micro-CT and histological research.

## Patient consent

Obtained.

## Authorship

*Category 1*

Conception and design of study: Benguo Wang,Peng Zhang, Jingqin Chen, Jianhai Chen; acquisition of data: Jianhai Chen, Jianwei Tan, Jian Li, Qijing Wang;analysis and/or interpretation of data: Jianhai Chen, Wenxiang Chen, Liqing Ke, Anqiao Wang.

*Category 2*

Drafting the manuscript: Jianhai Chen, Jianwei Tan, Jian Li; revising the manuscript critically for important intellectual content: Jingqin Chen, Peng Zhang, Benguo Wang, Sien Lin, Gang Li.

*Category 3*

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## Declaration of competing interest

The authors have declared no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2023.05.004.

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