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Foxc1 promotes the proliferation of fibroblast-like synoviocytes in rheumatoid arthritis via PI3K/AKT signalling pathway

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ABSTRACT

Forkhead box c1 (Foxc1) is a vital member of the Fox family of transcription factors which play important roles in numerous biological processes including metabolism, differentiation, proliferation, apoptosis, migration, invasion and longevity. However, up to date, the role of Foxc1 in the development of Rheumatoid Arthritis (RA) has not been fully elucidated. In the present study, the markedly higher expression of Foxc1 was observed in fibroblast-like synoviocytes (FLSs) of RA compared to control. Besides, we found that Foxc1 had a co-localization with THY1 (a marker for fibroblast-like synoviocytes). Moreover, during the process of TNF-α-induced inflammatory response model, Foxc1 was progressively accumulated in FLSs which was in parallel with MMP-1, MMP-13. Consistently, cell inflammatory response was distinctly hindered by small interfering RNA. Even more importantly, we discovered that Foxc1 promoted cell proliferation by upregulation PI3K/AKT signaling, which was inflammation-dependent. In summary, these data implied that Foxc1 might regulates fibroblast-like synoviocytes proliferation by reducing PI3K/AKT signaling pathway and all above findings provide novel therapeutic effects in the treatment for RA patients.

1. Introduction

Rheumatoid Arthritis (RA) is a chronic and systemic disease which is characterized by recruitment of inflammatory cells and accumulation of cytokines, leading to infiltration by macrophages and T cell synovial lining hyperplasia, neo-angiogenesis, pannus formation and destruction of cartilage and bone (Henry et al., 2013). There are various cell types participating in the chronic inflammatory process of RA pathogenesis, including T cells, B cells, fibroblast-like synoviocytes (FLSs) and macrophages. Among them, the FLSs are the most important cells responsible for initiating and driving the inflammatory process as well as the invasive nature of the rheumatoid synovium (Feldmann et al., 1996). Recent studies have demonstrated that abnormal activation of FLSs in the RA inflammatory environment may have unique morphology and share many characteristics with tumour-like cells. These cells finally become insensitive to cell-cell contact inhibition and acquired enhanced migration, invasion and angiogenesis potentiality. FLSs, which were stimulated by RA inflammatory infiltration, were considered to be responsible for the abnormal proliferation and pathogenesis of RA (Zhu et al., 2011). More, FLSs promote chronic inflammation response through secretion of pro-inflammatory cytokines, chemokines and matrix degrading enzymes, such as TNF-α, IL-1β and MMPs. All these inflammation cytokines deeply maintain and enlarg the inflammatory and joint destruction in RA (Neumann et al., 2010). Although RA synoviocytes and those transformed cells are shared with the same characteristics, the pathogenesis of proliferation was still poor understood (Lee et al., 2014).

Forkhead boxc1 (FOXC1) is a member of Forkhead box family transcription factors which play important roles in multiple pathophysiological biological processes such as metabolism, differentiation, proliferation, apoptosis, migration, invasion and longevity (Nishimura et al., 1998). Previous studies have indicated that Foxc1 has participated in the development of various types of malignancy. For example, in nasopharyngeal carcinoma, the expression level of Foxc1 was enhanced, and Foxc1 plays a key role in epithelial–mesenchymal transition (EMT) with the upregulation of Vimentin, fibronectin and N-cadherin expression (Ou-Yang et al., 2015). Over-expression of Foxc1 promotes tumor metastasis and indicates poor prognosis in hepatocellular carcinoma, non-small cell lung cancer and pancreatic ductal adenocarcinoma patients (Wang et al., 2013; Wei et al., 2013; Xia et al., 2013). Therefore, considering the fact that activated FLSs in the RA inflammatory environment may have unique morphology and share many characteristics with tumour-like cells (Lefevre et al., 2015), we hypothesized that Foxc1 may also play an important role in the RA

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inflammation process.

In this investigation, we confirmed the markedly upregulated expression of Foxc1 in the synovium of RA patients compared with healthy controls, as well as its co-localization with FLSs. Besides, the role of Foxc1 in the expression of inflammatory cytokines and MMPs in response to TNF-α treatment had also been further studied (Chang et al., 2014; Yamazaki et al., 2003). Furthermore, we also verified that Foxc1 could promote the proliferation of RA-FLSs via inactivation of PI3K/AKT signaling pathway. Taken together, our findings suggest that Foxc1 could be a useful therapeutic target in RA disease.

2. Materials and methods

2.1. Patients and RASFs

Our cohort of RA patients included five females and five males. Samples were obtained from patients who underwent total knee replacement surgery. This study was conducted RA patients, fulfilling the 1987 American College of Rheumatology classification criteria for rheumatoid arthritis. In our current study, the Disease Activity Score (DAS) is the major scoring system for evaluating disease activity of rheumatoid arthritis (RA) and RA patients whose DAS score is higher than 3.2 were recruited in the study (Prevoo et al., 1995; Radstake et al., 2004). As control, ten cases of synovial tissue were obtained from healthy patients who underwent arthroscopic procedures, and patients who have a history of RA disease were excluded. The average age of the control group was 45 ± 5 years old, and the RA patients were 46 ± 6 years old. The study was approved by the institutional medical ethics committee of the Affiliated Hospital of Nantong University. Written, informed agreement from each participant was obtained before surgery.

2.2. Synovial cell culture and stimulated

Synovial tissue which dislodged fat, blood vessels and fibrous tissue was wished 3 times with the phosphate-buffered solution (PBS), and continued to be shredded repeatedly into approximately $1mm^3$ pieces, bottling to flasks with the spacing of about 5 mm tiling uniformly distributed. An appropriate amount of DMEM (Hyclone, Thermo Fisher scientific, Waltham, MA, USA) culture medium with 10% fetal bovine serum (FBS; Hyclone, Thermo Fisher scientific) was addded in these flasks and placed uprightly into the 5% CO_2 thermostatic incubator at 37℃ for tissue adherence. Then, the flat is laid slowly after 4 h for continued culture; the cell culture medium is changed for average 3 days. A trypsin-EDTA solution is adopted to remove the monolayer (coverage > 80%) and pass 3–5 times for experimental use. If the typical spindle-shaped, fibroblast-like appearance of these cells was presented and anti-CD55 staining was positive. We considered them as type B fibroblast -like synovial cells. Such characteristics were enriched with over 90% of cells in passage 3. All FLSs were adopted in the fifth passage and treated with TNF-α (Human, Sigma Chemical Co., S. Louis, MO, USA) at the indicated time and concentration. The same treatment was performed in SW982 cells.

2.3. Western blot analysis

Cells were harvested by a lysis buffer (50 mM Tris − HCL (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1%Triton X-100, 0.1 mg/ml PMSF, and 2 mg/ml leupetin). Then, subjected these samples to SDS-PAGE and transferred to nitrocellulose membranes (Millipore). The specific antibodies were Foxc1 (1:500; Santa Cruz, USA), MMP-1 (1:500; Santa Cruz, USA), MMP-13 (1:500; Santa Cruz, USA), GAPDH (1:1000; Santa Cruz, USA), PCNA (1:1000; Santa Cruz, USA), PI3K (1:1000; Cell signaling, Cruz, USA), p-PI3K (1:1000; Cell signaling, Cruz, USA), AKT (1:1000; Cell signaling, Cruz, USA), p-AKT (1:1000; Cell signaling, Cruz, USA), GSK-3β (1:1000; Cell signaling, Cruz, USA), p-GSK-3β (1:1000;Cell signaling, Cruz, USA), and secondary antibodies according to protocols of the manufacturer. Eventually, the membrane with an enhanced chemiluminescence kit was detected.

2.4. Immunohistochemistry

Tissue sections which came from patients' pathological specimen were cut into 5 μm in a cryostat microtome and stored at −20 ℃ for further use. Before following experiments, put all sections into an autoclave with 10 mM citrate buffer (pH 6.0) and heated to 121 ℃ for 20 min to retrieve the antigen, and then the endogenous peroxidase activity was blocked by soaking buffered saline (PBS) (pH 7.2), incubating the sections with the primary antibody against Foxc1 (1:500; Santa Cruz, USA) at the room temperature for 2 h. Besides, the peroxidase-anti-peroxidase method (DAKO, Hamburg, Germany) was adopted to process all the slides. After rinsing in PBS, the peroxidase reaction was imagined by incubating the sections with the liquid mixture DAB (0.02% diaminobenzidine tetrahydrochloride, 0.01% phosphate buffer solution and 3% H_2O_2). After rinsing in water, the sections were redyed and dehydrated with hematoxylin, when the cover was slipped.

2.5. Immunofluorescent histochemical staining analysis

Blocked the sections with 10% normal serum-blocking solution species, samely applying to the secondary antibodies, containing 3% (w/v) BSA, 0.1% Triton X-100 and 0.05%Tween 20 for 2 h at room temperature when excluding unspecific staining. Then the treated-section was incubated with an specific antibody for Foxc1 and the FLSs marker: thymocyte differentiation antigen 1 (THY1) (rabbit anti-THY1, 1:100, Santa Cruz Biotechnology) overnight at 4 ℃, followed by a mixture of fluorescein isothiocyanate (FITC)-and tetramethylrhodamine (TRITC)-conjugated secondary antibodies (Jackson ImmunoResearch) for 2 h at 4 ℃. Finally, the stained sections were examined with a Leica fluorescence microscope (Leica, DM 5000B; Leica CTR 5000; Germany).

2.6. SiRNA and transfection

The specific SiRNA used in the proposed study was designed and synthesized by Santa Cruz Biotechnology. Transient transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions (Tao et al., 2012), and cells were incubated at 37 ℃ for 6 h in DMEM without any serum or antibodies. After 6 h, the transfection mixtures were changed to 10% FBS-containing DMEM, and cells were cultured continuously for 48 h before being stimulated with TNF-α for 30 min.

2.7. Cell counting Kit-8 (CCK-8) assay

Briefly, $\sim 2.5 \times 10^3$ RA-FLSs (100 μl) in the logarithmic growth phase were plated in the 96-well plate. By following the same method, cells which were transfected with Foxc1-SiRNA and NC-SiRNA were incubated for 24, 48 and 72 h. Then 10 μl of the CCK-8 solution were added into each well and continued to be incubated at 37 ℃ for another 3 h. The absorbance at 450 nm was measured to evaluate the viability of cells.

2.8. Colony formation assay

To explore the proliferation ability of RA-FLSs, colony formation assay was performed. Cells in the logaithmic phase after transfections were changed into a single-cell suspension for 48 h. The cell suspension including 500 cells was seeded in the 6-well plate and cultured for two weeks to allow colony formation. After incubation for indicated time, the plates were washed with phosphate buffered saline (PBS) twice softly; later, the cell was fixed by methanol for 15 min and stained in Giemsa (HarveyBio, Beijing, China) for 20 min. The cells were air-dried