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Original Article

Insulin-like growth factor binding protein 2 regulates cell proliferation and migration in the adjuvant arthritis synovial cells through ERK signaling pathway

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Abstract: To probe the role of Insulin-like growth factor binding protein 2 (IGFBP2) in regulating cell proliferation, invasion, migration and apoptosis of fibroblast-like synoviocytes (FLS) from rheumatoid arthritis (RA) patients, the mRNA and protein expression were measured by qRT-PCR, Western blot and immunofluorescence assay first. Then, siRNA against IGFBP2 was used to inhibit IGFBP2 expression in RA-FLS. The proliferation of RA-FLS was detected by CCK8 assay, invasion and migration *in vitro* were detected by transwell chamber assay and wound healing assay, the apoptosis of RA-FLS was observed by flow cytometry. Increased IGFBP2 was discovered in RA-FLS compared to FLS. In RA-FLS, the level of IGFBP2 was down-regulated by siRNA, siRNA IGFBP2 decreased the proliferation, invasion and migration of RA-FLS, and increased the apoptosis rate. Furthermore, siRNA IGFBP2 restrained the phosphorylation of ERK. IGFBP2 regulated the cell proliferation, invasion, migration and apoptosis of RA-FLS, which was mediated by the ERK signaling pathway. Our research suggested that targeting IGFBP2 might be a promising therapy for RA.

Keywords: Rheumatoid arthritis, fibroblast-like synoviocytes, IGFBP2, ERK

Introduction

Rheumatoid arthritis (RA) is considered an autoimmune disease characterized by chronic synovial joint inflammation, and closely related to the immune response of lymphocytes, plasma cells, macrophage infiltration and lymphoid follicles [1, 2]. RA is a painful disease that caused the infiltration of inflammation cells, fibrin deposition, synovial hyperplasia and progressive destruction of cartilage and bone, osteoporosis or the systemic bone loss associated with RA increases the risk for fragility fractures, which can affect quality of life dramatically in RA patients [3]. The prevalence of this disease is approximately 1% of the population worldwide [4]. Current treatments for RA including drug therapy, immunotherapy, surgical treatment and psychotherapy, although they can reduce pain, they also have side effects such as increased risk of infection and not work for everyone [5].

Due to the diversity of pathogenic pathways in RA, the therapy of RA remains unmet medical need. Fibroblast-like synoviocytes (FLS) are a main constituent of the synovial hyperplasia, play a critical role in the immunopathogenesis of RA [6]. It has been widely accept that RA-FLS keep their ability of increased proliferation, anchorage-independent growth and invasion. In addition, RA-FLS could produce substantial levels of pro-inflammatory cytokines and chemokines, such as IL-6, IL-1β, IL-17, TNF-a and MCP-1 [7-9]. It was reported that RA-FLS also expresses a variety of matrix metalloproteinases (MMPs), small ubiquitin-related modifier-1 (SUMO), vascular endothelial growth factor (VEGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which leading to promote cell proliferation and apoptotic resistance [10, 11].

The insulin-like growth factor (IGF) signaling pathway has been proved to be involved in the

RA progression. Human insulin-like growth factors binding proteins (IGFBPs) are indirectly binding to IGF also produced in IGF-1 stimulated osteoblast proliferation and differentiation [12, 13]. Previous studies showed that overexpression of IGFBP2 might be involved in the progression and development of different kinds of cancer, for instance, gliomas, ovarian cancer, prostatic cancer and gastric cancer [14, 15]. According to the result of DNA microarray, IGFBP2 is up-regulated in the RA patients when compared with the healthy people [16, 17].

However, there is very little data on the role and expression of IGFBP2 plays during the RA progression. In the present study, we have detect the expression and activity of IGFBP2 in RA-FLS cells, the role of IGFBP2 was predicted with the proliferation and migration of RA-FLS cells in the development of RA.

Materials and methods

Cell lines and culture

RA-FLS cells (NO. 408RA-05a) and FLS cells (NO. 408-05a) were purchased from Cell Applications (California, USA). All the cells were cultured routinely in DMEM high glucose medium (Gibco, New York, USA) supplemented with 10% FBS (Gibco, New York, USA), streptomycin (50 units/mL) and penicillin (100 units/mL) at 37°C in a humidified incubator containing v/v 5% CO_2.

CCk-8 assays

First, FLS cells and RA-FLS cells were transfected with negative control siRNA (NC siRNA) or IGFBP2 siRNA, respectively. Second, 2000 cells/well were seeded in triplicate in 96-well plates. Cell viability was measured by the Cell Counting kit-8 (CCK-8, Beyotime, China) kit, 10 μl of CCk-8 stock solution were added to each well for 3 hours incubation at 37°C. The absorbance of samples at 450 nm was measured by Universal Microplate Spectrophotometer (Thermo Fisher Scientific, mμltiscan MK3).

Flow-cytometric analysis of cell apoptosis

Firstly, RA-FLS cells were transfected NC siRNA or IGFBP2 siRNA as described above and plated in six well plates. After 48 h cultivation, cells were collected and washed with PBS. Then, cells were incubated with Annexin V-fluorescein isothiocyanate and propidium iodide (Sigma-Aldrich) for 20 min in the dark, respectively. Finally, according to the manufacturer's instructions, the cells were analyzed by flow cytomrtry (Beckman Coulter, USA).

QRT-PCR analyses

Total RNAs was extracted from cultured cells with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The extracted RNA samples were determined by the 260/280 nm ratio. PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa) was used for qRT-PCR. The primers for IGFBP2: 5'-TGTTCGTCATGGGTGTGAAC-3', 5'-ATGGCATGGACTGTGGTCAT-3' and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5'-TGTTCGTCATGGGTGTGAAC-3', 5'-ATGGCATGGACTGTGGTCAT-3', The relative expression of IGFBP2 was normalized to the expression of GAPDH by using the $2^{-\Delta\Delta}$Ct method. All samples were performed in triplicate.

Cell invasion assay

According to the manufacturer's instructions [18], the cell invasion assay was performed in membrane invasion 24-well culture system chambers (Coster) with a matrigel layer (BD Biosicence). RA-FLS cells transfected with NC siRNA or IGFBP2 siRNA were seeded in each upper chamber, and incubated for 48 h at 37°C in DMEM with 10% FBS. The cells that invaded the magtrigel layer were fixed with 4% paraformaidehyde, stained with crystallized purple, and counted with Upright Metallurgical Microscope (OLYMPUS CX41).

Wound healing assay

Cell migration ability was measured by using scratch wound-healing assay, after the RA-FLS cells transfected with NC siRNA or IGFBP2 siRNA were grown to 90% confluence in a 6-well plate, a micro pipette tip was used to scratch a wound. The cellular debris was washed with serum-free medium for three times. The wound area was photographed under Upright Metallurgical Microscope (OLYMPUS CX41) at 0 h, 48 h.

Western blot analysis

Western blot analysis of cultured RA-FLS cells was performed as previously described [19]. Whole cell lysates were extracted from cultured

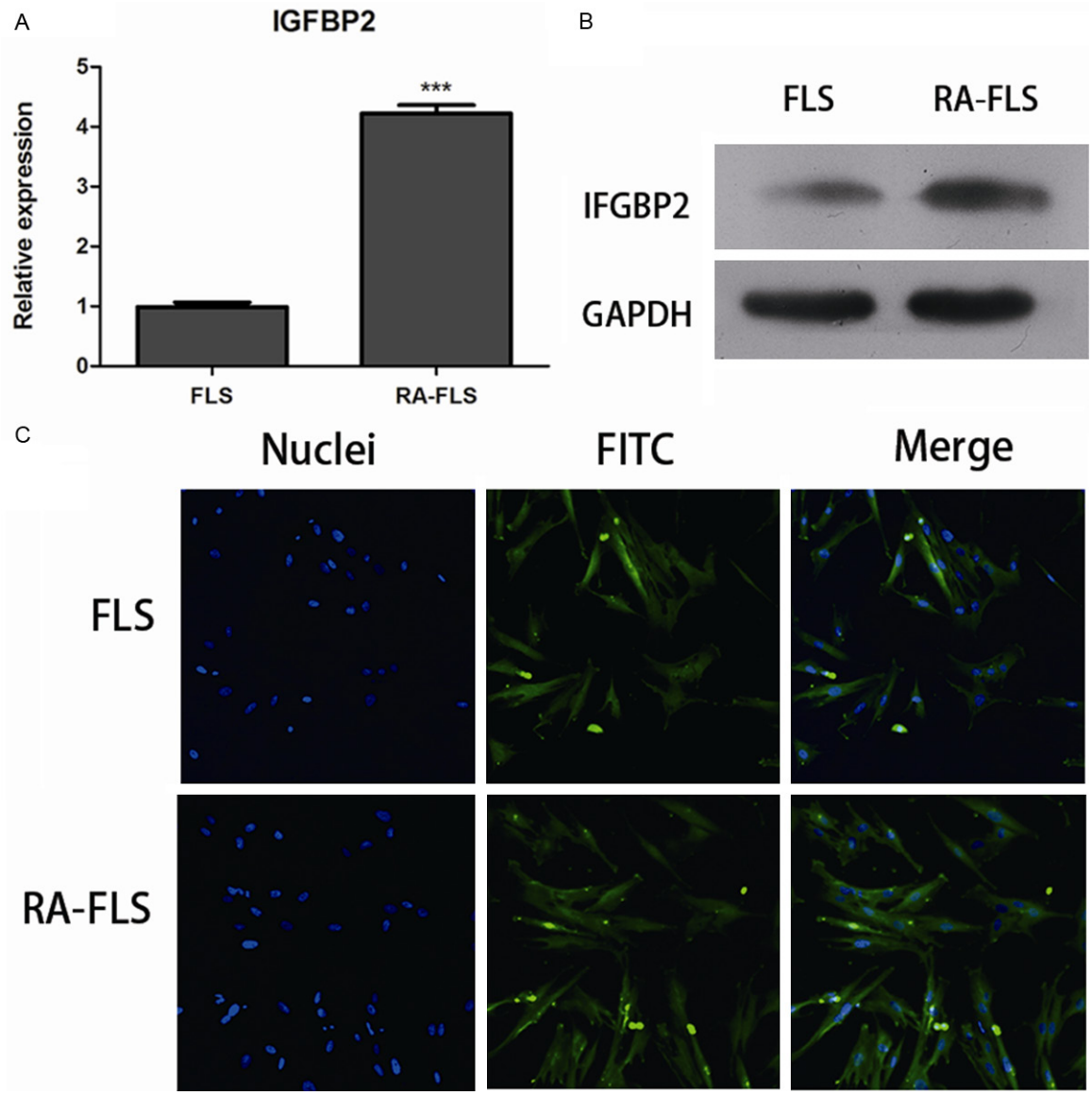

Figure 1. The expression of IGFBP2 in FLS cells and RA-FLS cells. A. qRT-PCR assay was used to detect the mRNA expression of IGFBP2 in FLS cells and RA-FLS cells. ****P<0.001* vs FLS. B. Western blot detected the protein levels of IGFBP2. C. Immunostaining analyzed the expression of IGFBP2 in FLS cells and RA-FLS cells.

RA-FLS cells in the ice-cold RIPA buffer (Beyotime Biotechnology, China), the total protein concentration were determined by BCA Protein Quantitation Kit (Beyotime Biotechnology, China). Briefly, 30 μg protein/lane of the cell lysates were separated on 10% SDS-PAGE and electransferred to PVCF membrane (Bio-Rad). The membranes were blocked with 5% nonfat milk for 1 hour at 4°C. Then, the membranes were incubated with Rabbit polyclonal to IGFBP2 (1:100, NO. ab91404, Abcam) on ice overnight. Next day, mouse momoclonal [6C5] to GAPDH (1:500, NO. ab8245, Abcam) was used to incubate the membranes for 2 hours. The protein bands were visualized by enhanced-chemiluminescence (ECL kit) reagents (Pierce), and imaged using ChemiDoc XRS (BIO-RAD, USA). GAPDH used as an internal control to confirm equal protein loading.

Immunofluorescence

Immunofluorescence analysis was performed as described previously [20]. Firstly, the transfected RA-FLS cells were plated in six-well tissue culture plate for 24 h. Next, the cells were fixed with 4% paraformaldehyde for 30 min and 0.1% Triton X-100 for 10 min. After washing, the

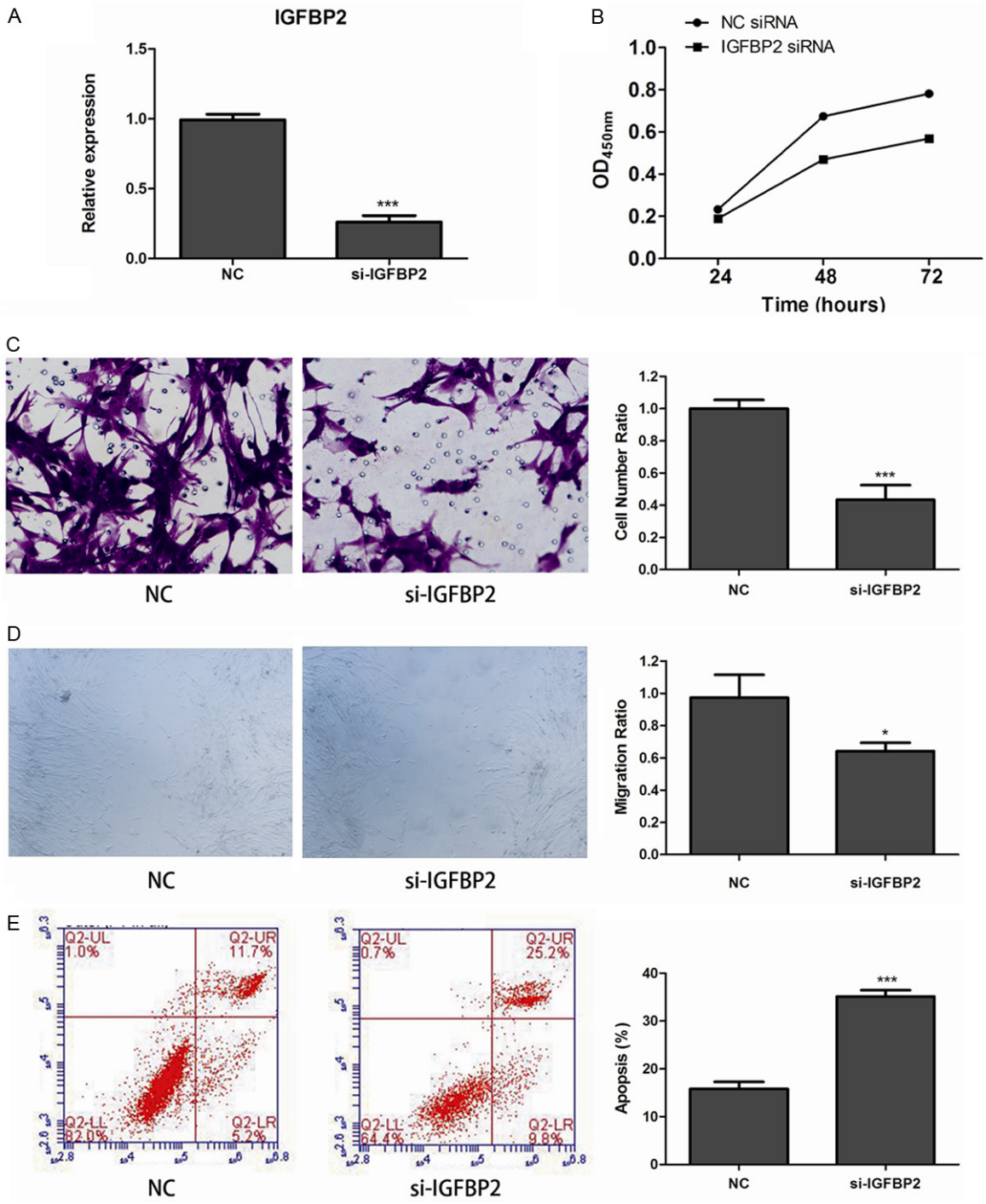

Figure 2. Effects of IGFBP2 siRNA on proliferation, invasion and apoptosis *in vitro*. A. Effects of IGFBP2 siRNA on the mRNA level of IGFBP2. ****P<0.001* vs NC siRNA. B. CCK8 assay investigated the cell viability in IGFBP2 siRNA transfected RA-FLS. C. Transwell assay detected the cell invasion ability in IGFBP2 siRNA transfected RA-FLS. The invasive cells were quantified. ****P<0.001* vs NC siRNA. D. Wound healing assay used to measure the migration rate of IGFBP2 siRNA transfected RA-FLS. The migrated cells were quantified. **P<0.05* vs NC siRNA. E. Flow cytometry detected the cell apoptosis in IGFBP2 siRNA transfected RA-FLS. The cell apoptosis proportion were quantified. **P<0.05* vs NC siRNA.

cells were blocked with 3% BSA at 37°C. The cells were stained with the anti-IGFBP2 antibody (ab91404; Abcam) in a wet box for overnight at 4°C. Followed by, incubated with Goat

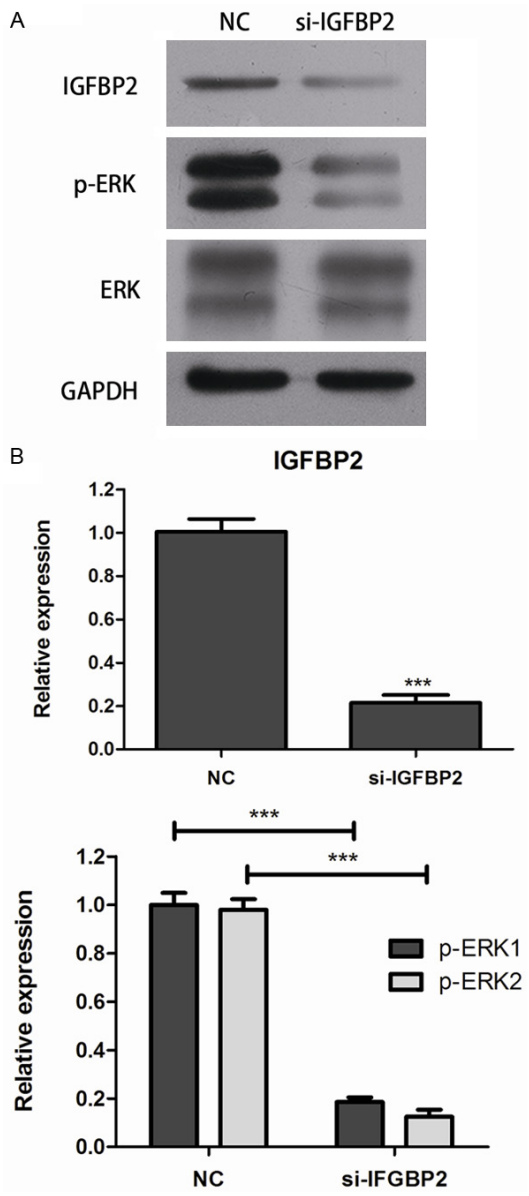

Figure 3. Effects of IGFBP2 siRNA on ERK signaling pathway. A. Western blot detected the effects of IGFBP2 siRNA on ERK activation in RA-FLS. B. The protein expression of IGFBP2 and ERK were quantified. ****P<0.001* vs NC siRNA.

Anti-Mouse IgG H&L (FITC) (1:1000, abcam) for 1 h in the dark at 37°C. Nuclei were counterstained with DAPI (1 μg/mL) for 15 min. Images of the cells were obtained using an Olympus confocal microscope (Olympus America, USA).

Statistical analysis

Data were expressed as mean ± standard deviation (SD). The findings were performed using one-way analysis for statistical analysis. *P* value of <0.05 was considered statistically significant. All the experiments in this study were performed in three or more trials.

Results

The expression of IGFBP2 in FLS cells and RA-FLS cells

In order to investigate the function of IGFBP2 in rheumatoid arthritis, firstly, we evaluated the expression levels of IGFBP2 in FLS cells and RA-FLS cells. As shown in **Figure 1A** and **1B**, the results of qRT-PCR and western blot showed that the mRNA and protein expression of IGFBP2 remarkably increased in RA-FLS cells when compared with FLS cells. Furthermore, the results of immunofluorescence also proved that the IGFBP2 expression level increased in RA-FLS cells than in FLS cells (**Figure 1C**). Therefore, these results indicated that IGFBP2 may play an important role in rheumatoid arthritis.

Effects of IGFBP2 on proliferation, invasion and apoptosis in vitro

In order to study the mechanisms underlying the effects of IGFBP2 on proliferation, invasion and apoptosis of RA-FLS cells, IGFBP2 siRNA was transfected into RA-FLS cells to down-regulate the IGFBP2 expression, NC siRNA was served as negative control. As shown in **Figure 2A**, efficiency of transfection was verified by significantly decrease IGFBP2 expression which was determined by qRT-PCR. We found exogenous inhibition of IGFBP2 could inhibit the proliferation and invasion of RA-FLS cells by CCK-8 assay (**Figure 2B**). In addition, IGFBP2 siRNA could alleviate the invasive capacity and migratory ability of RA-FLS cells when compared to NC (**Figure 2C** and **2D**). We also found RA-FLS cells transfected with IGFBP2 siRNA had a significant higher rate of apoptosis by flow cytometry (**Figure 2E**).

Effects of IGFBP2 on ERK signal pathway in RA-FLS cells

ERK (extracellular signal-regulated kinase) pathway is associated with cell growth and apoptosis of RA-FLS cells. To investigate the contribution of IGFBP2 siRNA in regulating cellular signaling, we detected the signaling of ERK pathway with Western blot. As shown in **Figure 3A** and **3B**, the phosphor-ERK expression was

downregulated by IGFBP2 siRNA, and no significant changes were observed in total-ERK.

Discussion

RA is a chronic disease, and a special cell population are more susceptible to rheumatoid synovium which known as activated RA-FLS. Ample evidence indicated that synovial hyperplasia is a symbol of RA. We focused on FLS for a host of reasons, most notably because they contribute a key regulatory cell in RA and produce major pro-inflammatory and joint damage [21].

In the present study, we first confirmed IGFBP2 highly expressed in RA-FLS when compared to FLS in the mRNA and protein expression level (**Figure 1**). According to the previous studies, IGFBP2 displayed an increase expression in case of lung epithelial damage, IGFBP2 gene knockout mice significantly decreased cell transfer and the invasiveness of tumor angiogenesis [22, 23]. The researches demonstrate that increasing IGFBP2 plays an important role in the growth of breast cancer tumor cells, apoptosis and the angiogenesis of tumor, through the IGFBP2/IGF-1/IGF1R signaling pathway both *in vitro* and *in vivo* [24]. Activity of IGFBP2 is associated with metabolic dysfunction including diabetes, diabetes and insulin resistance, it was proposed that IGFBP2 could modulate the hepatic metabolic dysfunction through regulating IGF-1 homeostasis. In this study, IGFBP2 siRNA was used to down-regulate IGFBP2 expression on RA-FLS, and we demonstrate that IGFBP2 siRNA could conduct an inhibition of proliferation, induction of apoptosis, anti-invasion and anti-migration role in RA-FLS (**Figure 2**).

According to the research, IGFBP2 has an Arg-Gly-Asp (RGD) cell adhesion element that can attach to integrin receptors, ultimately, activate extracellular signal-regulated kinases (ERKs) in order [25]. Increasing IGFBP2 levels has been shown to activate the ERK signaling pathway, could stimulate proliferation in glioblastoma cells, breast cancer cells and ovarian cancer cells [26-28]. On the contrary, PD98059 and U1026 as the inhibitors of the ERK pathway abrogated IGFBP2-induced activation [26]. Silencing IGFBP2 with lentivirus-based shRNA inhibit IGF1-induced phosphorylation of ERK in PC-3 cells, decreased activation of AKT and ERK pathway in human leukemia cells [29]. ERK signaling is closely related to cellular growth, differentiation and proliferation in cancer-related cellular processes. In this study, silencing IGFBP2 with siRNA was shown to inhibit the phosphorylation of ERK in RA-FLS (**Figure 3**), and it was essential for IGFBP2-induced proliferation, invasion, migration and apoptosis in RA-FLS. Therefore, IGFBP2 were mainly exerted its functions through the ERK signaling pathway.

Conclusions

In summary, IGFBP2 showed a higher mRNA and protein expression level in RA-FLS than that in FLS. Silencing IGFBP2 was shown to decrease proliferation, invasion, migration and apoptosis via ERK signaling pathway in RA-FLS. Therefore, silencing IGFBP2 levers may represent a new targeted therapy for the treatment of rheumatoid arthritis.

Disclosure of conflict of interest

None.

Authors' contribution

Xinliang Wang performed the experimental work. Yanan Li participated in experimental design and coordination. Lin Chen and Zhiqi Hou contributed to analysis and interpretation of data. The article was written by Xinliang Wang. All authors have read and approved the final manuscript.

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References

[1] Ai R, Hammaker D, Boyle DL, Morgan R, Walsh AM, Fan S, Firestein GS and Wang W. Joint-specific DNA methylation and transcriptome signatures in rheumatoid arthritis identify distinct pathogenic processes. Nat Commun 2016; 7: 11849.

[2] Bhattacharjee M, Balakrishnan L, Renuse S, Advani J, Goel R, Sathe G, Keshava Prasad TS,

Nair B, Jois R, Shankar S and Pandey A. Synovial fluid proteome in rheumatoid arthritis. Clin Proteomics 2016; 13: 12.
[3] Hruskova V, Jandova R, Vernerova L, Mann H, Pecha O, Prajzlerova K, Pavelka K, Vencovsky J, Filkova M and Senolt L. MicroRNA-125b: association with disease activity and the treatment response of patients with early rheumatoid arthritis. Arthritis Res Ther 2016; 18: 124.
[4] Khalifa O, Pers YM, Ferreira R, Senechal A, Jorgensen C, Apparailly F and Duroux-Richard I. X-linked miRNAs associated with gender differences in rheumatoid arthritis. Int J Mol Sci 2016; 17.
[5] Kalden JR. Emerging therapies for rheumatoid arthritis. Rheumatol Ther 2016; 3: 31-42.
[6] Park SH, Kim KW and Kim JC. The role of insulin-like growth factor binding protein 2 (IGFBP2) in the regulation of corneal fibroblast differentiation. Invest Ophthalmol Vis Sci 2015; 56: 7293-7302.
[7] Bartok B and Firestein GS. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. Immunol Rev 2010; 233: 233-255.
[8] Wei Z, Wang F, Song J, Lu Q, Zhao P, Xia Y, Chou G, Wang Z and Dai Y. Norisoboldine inhibits the production of interleukin-6 in fibroblast-like synoviocytes from adjuvant arthritis rats through PKC/MAPK/NF-kappaB-p65/CREB pathways. J Cell Biochem 2012; 113: 2785-2795.
[9] Park YJ, Yoo SA, Kim GR, Cho CS and Kim WU. Urinary interleukin-6 as a predictor of radiographic progression in rheumatoid arthritis: A 3-year evaluation. Sci Rep 2016; 6: 35242.
[10] Li F, Li X, Kou L, Li Y, Meng F and Ma F. SUMO-conjugating enzyme UBC9 promotes proliferation and migration of fibroblast-like synoviocytes in rheumatoid arthritis. Inflammation 2014; 37: 1134-1141.
[11] Connolly M, Mullan RH, McCormick J, Matthews C, Sullivan O, Kennedy A, FitzGerald O, Poole AR, Bresnihan B, Veale DJ and Fearon U. Acute-phase serum amyloid A regulates tumor necrosis factor alpha and matrix turnover and predicts disease progression in patients with inflammatory arthritis before and after biologic therapy. Arthritis Rheum 2012; 64: 1035-1045.
[12] Szeremeta A, Jura-Poltorak A, Komosinska-Vassev K, Zon-Giebel A, Kapolka D and Olczyk K. The association between insulin-like growth factor 1 (IGF-1), IGF-binding proteins (IGFBPs), and the carboxyterminal propeptide of type I procollagen (PICP) in pre- and postmenopausal women with rheumatoid arthritis. Scand J Rheumatol 2016; 1-9.
[13] Xi G, Rosen CJ and Clemmons DR. IGF-I and IGFBP-2 stimulate AMPK activation and autophagy, which are required for osteoblast differentiation. Endocrinology 2016; 157: 268-281.
[14] Catsburg C, Gunter MJ, Tinker L, Chlebowski RT, Pollak M, Strickler HD, Cote ML, Page DL and Rohan TE. Serum IGFBP-2 and risk of atypical hyperplasia of the breast. J Cancer Epidemiol 2015; 2015: 203284.
[15] Zhu H, Yun F, Shi X and Wang D. Inhibition of IGFBP-2 improves the sensitivity of bladder cancer cells to cisplatin via upregulating the expression of maspin. Int J Mol Med 2015; 36: 595-601.
[16] Guiot J, Bondue B, Henket M, Corhay JL and Louis R. Raised serum levels of IGFBP-1 and IGFBP-2 in idiopathic pulmonary fibrosis. BMC Pulm Med 2016; 16: 86.
[17] Kang HS, Cho HC, Lee JH, Oh GT, Koo SH, Park BH, Lee IK, Choi HS, Song DK and Im SS. Metformin stimulates IGFBP-2 gene expression through PPARalpha in diabetic states. Sci Rep 2016; 6: 23665.
[18] Zhang X, Jiang P, Shuai L, Chen K, Li Z, Zhang Y, Jiang Y and Li X. miR-589-5p inhibits MAP3K8 and suppresses CD90+ cancer stem cells in hepatocellular carcinoma. J Exp Clin Cancer Res 2016; 35: 176.
[19] Qin C, Qiu K, Sun W, Jiao N, Zhang X, Che L, Zhao H, Shen H and Yin J. A proteomic adaptation of small intestinal mucosa in response to dietary protein limitation. Sci Rep 2016; 6: 36888.
[20] Jiang W, Fu Y, Yang F, Yang Y, Liu T, Zheng W, Zeng L and Chen T. Gracilaria lemaneiformis polysaccharide as integrin-targeting surface decorator of selenium nanoparticles to achieve enhanced anticancer efficacy. ACS Appl Mater Interfaces 2014; 6: 13738-13748.
[21] Shang CH, Zhang QQ and Zhou JH. Oridonin inhibits cell proliferation and induces apoptosis in rheumatoid arthritis fibroblast-like synoviocytes. Inflammation 2016; 39: 873-880.
[22] Yazawa T, Sato H, Shimoyamada H, Okudela K, Woo T, Tajiri M, Ogura T, Ogawa N, Suzuki T, Mitsui H, Ishii J, Miyata C, Sakaeda M, Goto K, Kashiwagi K, Masuda M, Takahashi T and Kitamura H. Neuroendocrine cancer-specific up-regulating mechanism of insulin-like growth factor binding protein-2 in small cell lung cancer. Am J Pathol 2009; 175: 976-987.
[23] Das SK, Bhutia SK, Azab B, Kegelman TP, Peachy L, Santhekadur PK, Dasgupta S, Dash R, Dent P, Grant S, Emdad L, Pellecchia M, Sarkar D and Fisher PB. MDA-9/syntenin and IGFBP-2 promote angiogenesis in human melanoma. Cancer Res 2013; 73: 844-854.
[24] Li Z, Wu Z, Ren G, Zhao Y and Liu D. Expression patterns of insulin-like growth factor system members and their correlations with growth and carcass traits in Landrace and Lantang

pigs during postnatal development. Mol Biol Rep 2013; 40: 3569-3576.
[25] Myers AL, Lin L, Nancarrow DJ, Wang Z, Ferrer-Torres D, Thomas DG, Orringer MB, Lin J, Reddy RM, Beer DG and Chang AC. IGFBP2 modulates the chemoresistant phenotype in esophageal adenocarcinoma. Oncotarget 2015; 6: 25897-25916.
[26] Han S, Li Z, Master LM, Master ZW and Wu A. Exogenous IGFBP-2 promotes proliferation, invasion, and chemoresistance to temozolomide in glioma cells via the integrin beta1-ERK pathway. Br J Cancer 2014; 111: 1400-1409.
[27] Foulstone EJ, Zeng L, Perks CM and Holly JM. Insulin-like growth factor binding protein 2 (IGFBP-2) promotes growth and survival of breast epithelial cells: novel regulation of the estrogen receptor. Endocrinology 2013; 154: 1780-1793.
[28] Lee EJ, Mircean C, Shmulevich I, Wang H, Liu J, Niemisto A, Kavanagh JJ, Lee JH and Zhang W. Insulin-like growth factor binding protein 2 promotes ovarian cancer cell invasion. Mol Cancer 2005; 4: 7.
[29] Chen X, Zheng J, Zou Y, Song C, Hu X and Zhang CC. IGF binding protein 2 is a cell-autonomous factor supporting survival and migration of acute leukemia cells. J Hematol Oncol 2013; 6: 72.