Tropical Journal of Pharmaceutical Research October 2020; 19 (10): 2067-2072
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v19i10.X7

Original Research Article

Inhibition of miR-665 alleviates lipopolysaccharide-induced inflammation via up-regulation of SOCS7 in chondrogenic ATDC5 cells

Qiaoyi Ning[1,2], Yiting He[3]*, Wukai Ma[2], Fang Tang[2], Ying Huang[2], Xueming Yao[2]
[1]The 2nd Clinical School, Guangzhou University of Chinese Medicine, Guangzhou City, Guangdong Province 510120, [2]Department of Rheumatology and Immunology, The 2nd hospital affiliated to Guizhou University of Chinese Traditional Medicine, Guiyang City, Guizhou Province 550003, [3]Department of New Drug Development, the 2nd Clinical School, Guangzhou University of Chinese Medicine, Guangzhou City, Guangdong Province 510120, China

For correspondence:* ***Email:* *YitingHedjk@163.com;* ***Tel:*** *+86-20-81887233-31011*

Sent for review: 13 May 2020 *Revised accepted: 24 September 2020*

Abstract

Purpose: *To examine the effect and mechanism of action of miR-665 in osteoarthritis.*
Methods: *An in vitro inflammatory injury model of osteoarthritis was established using chondrogenic ATDC5 cells with lipopolysaccharide (LPS) treatment. The expression levels of inflammatory cytokines were determined by enzyme-linked immunosorbent assays (ELISAs) and by quantitative real-time polymerase chain reaction (qRT-PCR). A binding target for miR-665 was predicted using TargetScan and then evaluated using a dual-luciferase reporter assay.*
Results: *Treatment with LPS significantly up-regulated the inflammatory cytokine expressions of interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α), in ATDC5 cells ($p < 0.01$), and the expression of miRNA-665 was significantly increased in LPS-treated ATDC5 cells ($p < 0.01$). Knockdown of miR-665 down-regulated the expression levels of these inflammatory cytokines. Suppressor of cytokine signaling 7 (SOCS7) was identified as a target of miR-665. Data from qRT-PCR and western-blot analyses indicated that SOCS7 expression was promoted by miR-665 inhibition and inhibited by miR-665 over-expression. LPS treatment significantly decreased the expression of SOCS7 protein in ATDC5 cells ($p < 0.01$), and over-expression of SOCS7 attenuated the LPS-stimulated inflammatory injury. In addition, over-expression of miR-655 enhanced the inflammatory injury and reversed the protective effect of SOCS7 against LPS-stimulated inflammation.*
Conclusion: *Inhibition of miR-665 alleviated LPS-stimulated inflammatory injury in ATDC5 cells via the up-regulation of SOCS7, suggesting a potential therapeutic target for osteoarthritis.*

Keywords: *MiR-665, Lipopolysaccharide, Inflammation, SOCS7, Chondrogenic, ATDC5*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

The clinical manifestations of osteoarthritis, including recurrent joint pain and the progressive worsening of joint-movement disorders, has a large impact on the health of the elderly around the world [1]. Chondrocytes are regarded as the main biomechanical elements leading to osteoarthritis [2], and osteoarthritis is strongly affected by chondrocyte inflammation [2]. Although joint-replacement therapy may be

recommended at later stages of osteoarthritis [2], new therapeutic targets are urgently needed to inhibit chondrocyte inflammation. Research has indicated that microRNAs (miRNAs) play a critical role in the regulation of osteogenesis, and affect the catabolism and anabolism of bone and cartilage [3]. A newly discovered inflammatory regulator, miRNA-665, has been shown to reduce inflammation and apoptosis during intestinal ischemia/reperfusion [4] and is now known to be associated with osteogenic differentiation of adipogenic stem cells [5]. However, its effects on inflammation in osteoarthritis have yet to be reported. Suppressor of cytokine signaling (SOCS) proteins, significant physiological regulators of cytokine responses, have demonstrated significant implications for osteoarthritis pathology [6]. For example, SOCS1 has been shown to negatively regulate the inflammatory response in osteoarthritis cartilage [7]. However, whether SOCS proteins are involved in miR-665 regulation of osteoarthritis has not been determined.

In the present study, we first determined the expression level of miR-665 in lipopolysaccharide- (LPS) induced chondrogenic ATDC5 cells, and then investigated both the effect of miR-665 on inflammation in ATDC5 cells and the underlying mechanism. The results suggest that miR-665 is a potential therapeutic target for osteoarthritis.

EXPERIMENTAL

Cell culture

Chondrogenic ATDC5 cells were cultured in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Rockford, IL, USA) supplemented (for cell growth) with 10% fetal bovine serum (HyClone, Logan, UT, USA) in a 37°C incubator with a 5% CO_2 atmosphere. At 80% confluency, cells were treated with LPS (10 µg/ml, Sigma-Aldrich, St. Louis, MO, USA) for 5 h before subsequent experiments.

Cell transfections

For the over-expression of SOCS7, its full-length sequence was introduced into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). A miRNA-665 mimic, an inhibitor, and negative-control constructs were synthesized by GenePharma (Shanghai, China). Chondrogenic ATDC5 cells, with or without LPS treatment, were seeded (1×10^6 cells per well) and then transfected with the miR-665 mimic, inhibitor (both at 100nM), pcDNA-SOCS7 (300 µg), or the corresponding negative controls using Lipofectamine 2000. Two days later, transfection efficiency was evaluated.

Assessment of inflammatory cytokines

ELISA kits (R & D Systems, Minneapolis, MN, USA) were used to detect levels of IL-1β, IL-6 and TNF-α in the culture supernatant of ATDC5.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA or miRNAs from ATDC5 cells were isolated and then reverse-transcribed into cDNA. SYBR Green Master Mix (Roche, Mannheim, Germany) was used for the qRT-PCR analyses with endogenous controls (GAPDH or U6). The primer sequences are shown in Table 1.

Table 1: The forward (F) and reverse (R) primer sequences used in the study.

ID	Sequence (5'- 3')
GAPDH F	5'-CAAAGCCAGAGTCCTTCAGA-3'
GAPDH R	5'-GATGGTCTTGGTCCTTAGCC-3'
IL-6 F	5'-GACAACTTTGGCATTGTGG-3'
IL-6 R	5'-ATGCAGGGATGATGTTCTG-3'
IL-1β F	5'-AGCAGCTTTCGACAGTGAGG-3'
IL-1β R	5'-CTCCACGGGCAAGACATAGG-3'
TNF-α F	5'-GAACTGGCAGAAGAGGCACT-3'
TNF-α R	5'-GGTCTGGGCCATAGAACTGA-3'
miR-665 F	5'-ACCAGGAGGCTGAGGCCCCT-3'
miR-665 R	5'-AACGAGACGACGACAGACTTT-3'
SOCS7 F	5'-CCAGCTCCAGGAGACTTAACA-3'
SOCS7 R	5'-CTGGTACGAGACAGCTCTGAT-3'
U6 F	5'-CTCGCTTCGGCAGCACA-3'
U6 R	5'-AACGCTTCACGAATTTGCGT-3'

Dual-luciferase reporter assay

Both wild type and mutant SOCS7 sequences were subcloned into the pmirGLO luciferase-reporter vector (Promega, Madison, Wisconsin, USA).

Chondrogenic ATDC5 cells were seeded (3×10^4/well), and then co-transfected with the luciferase-reporter vectors along with miR-665 inhibitor, or negative control. After 48 h, luciferase activity was assayed using the Lucifer Reporter Assay System (Promega).

Western-blot analysis

Cells were lysed using RIPA buffer (Beyotime Institute of Biotechnology, Beijing, China). Lysates (30 µg) were separated using SDS-PAGE, and then transferred to PVDF membranes. The PVDF membranes were blocked using 5% fat-free milk, and then incubated overnight at 4°C with primary

antibodies against SOCS7 (1:1500; Abcam, Cambridge, MA, USA) and β-actin (1:3000). Following incubation with an HRP-labeled secondary antibody (1:5000; Abcam), protein signals were visualized and quantified using Quantity One 4.6.2 software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All results were expressed as means ± SEMs. Using GraphPad Prism 7.0 software, the data were evaluated using a one-way analysis of variance. $p < 0.05$ was considered statistically significant.

RESULTS

LPS-induced inflammatory response, and up-regulation of miR-665 expression in ATDC5 cells

Data from the qRT-PCR analyses (Figure 1 A, left panel) indicated that LPS treatment (10 μg/ml) significantly up-regulated the mRNA expression levels of pro-inflammatory factors (IL-1β, IL-6, and TNF-α) in ATDC5 cells. This LPS-induced inflammatory response in ATDC5 cells was also confirmed by ELISA, as demonstrated by the up-regulation of pro-inflammatory factors (Figure 1 A, right panel). Furthermore, miR-665 was up-regulated in LPS-stimulated ATDC5 cells (Figure 1 B), suggesting a possible regulatory role for miR-665 in osteoarthritis.

Inhibition by miR-665 alleviated the LPS-stimulated inflammatory response

First, the transfection efficiency of the miR-665 inhibitor was confirmed (Figure 2 A), and then both the qRT-PCR and ELISA analyses indicated that the miR-665 inhibitor dramatically down-regulated the pro-inflammatory factors in LPS-induced ATDC5 cells (Figure 2 B).

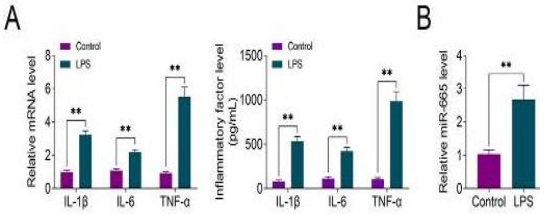

Figure 1: LPS-induced inflammatory response, and up-regulation of miR-665 expression in ATDC5 cells. (A) Effect of LPS on mRNA and protein expressions of IL-1β, IL-6 and TNF-α in ATDC5 cells determined by qRT-PCR (left) and ELISA (right). (B) Effect of LPS on miR-665 expression in ATDC5 cells determined by qRT-PCR; $^{**}p < 0.01$

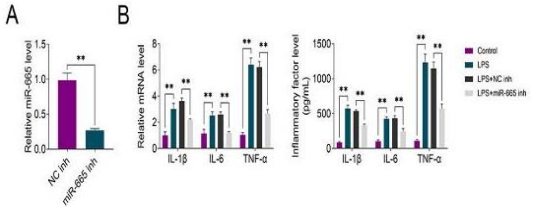

Figure 2: Inhibition of miR-665 alleviated the LPS-stimulated inflammatory response. (A) Transfection efficiency of the miR-665 inhibitor in LPS-induced ATDC5 cells. (B) Effect of the miR-665 inhibitor on mRNA and protein expressions of IL-1β, IL-6 and TNF-α in ATDC5 cells assessed by qRT-PCR (left) and ELISA (right); $^{**}p < 0.01$

SOCS7 as a binding target for miR-665

Using a TargetScan analysis, SOCS7 was identified as a potential binding target for miR-665 (Figure 3 A). Application of the miR-665 inhibitor decreased the luciferase activity of the wild type SOCS7 reporter vector, while the same inhibitor had no significant effect on the mutant SOCS7 reporter (Figure 3 B), confirming the binding ability between miR-665 and SOCS7. Furthermore, SOCS7 mRNA expression was up-regulated in ATDC5 cells transfected with the miR-665 inhibitor (Figure 3 C).

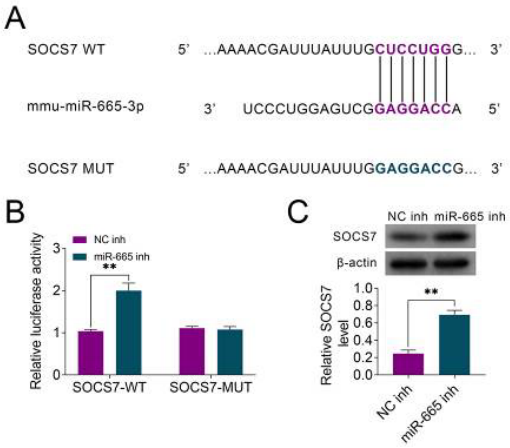

Figure 3: SOCS7 was identified as a target for miR-665 binding. (A) Potential binding site for miR-665 in SOCS7, and the corresponding mutant binding site. (B) Effect of miR-665 inhibition on luciferase activity in wild type and mutant SOCS7 luciferase-reporter vectors in ATDC5 cells determined by a dual-luciferase reporter assay. (C) Effect of the miR-665 inhibitor on SOCS7 mRNA and protein expressions in ATDC5 cells determined by qRT-PCR; $^{**}p < 0.01$

Over-expression of SOCS7 alleviated the LPS-stimulated inflammatory response

In ATDC5 cells, LPS induced a significant decrease of SOCS7 (Figure 4 A), while over-

expression of SOCS7 reversed this LPS-induced SOCS7 suppression (Figure 4 A). In addition, over-expression of SOCS7 reduced the LPS-stimulated increase in mRNA and protein levels of pro-inflammatory factors (Figure 4 B).

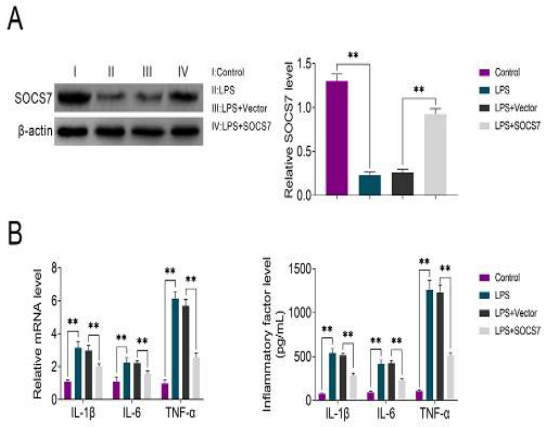

Figure 4: Over-expression of SOCS7 alleviated the LPS-stimulated inflammatory response. (A) Effect of LPS on SOCS7 protein expression in ATDC5 cells determined by western-blot analysis. (B) Effect of LPS and SOCS7 on mRNA and protein expressions of IL-1β, IL-6, and TNF-α in ATDC5 cells determined by qRT-PCR and ELISA analyses; $^{**}p < 0.01$

MiR-665 promoted the inflammatory response by down-regulating SOCS7

First, the transfection efficiency of the miR-665 mimic was determined (Figure 5 A), and then both the qRT-PCR and ELISA analyses revealed that SOCS7 over-expression counteracted the effect of the miR-665 mimic on the expression levels of the pro-inflammatory factors in LPS-treated ATDC5 cells (Figure 5 B).

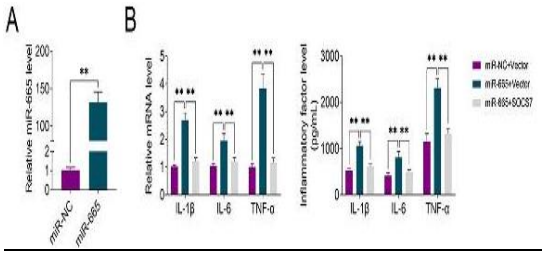

Figure 5: MiR-665 promoted an inflammatory response via down-regulation of SOCS7. (A) The transfection efficiency of the miR-665 mimic in LPS-treated ATDC5 cells. (B) Effect of the miR-665 mimic and SOCS7 on mRNA and protein expressions of IL-1β, IL-6, and TNF-α in ATDC5 cells determined by qRT-PCR (left) and ELISA (right) analyses. $^{**}p < 0.01$

DISCUSSION

To date, 16 miRNAs have been found to be dysregulated in patients with osteoarthritis compared to people with normal cartilage [8]. Here, we evaluated the effect and underlying mechanism of miR-665 in a model of osteoarthritis.

Although osteoarthritis has traditionally been regarded as a non-inflammatory disease, inflammatory responses have been shown to contribute to its pathogenesis [9]. Inflammatory responses in articular cartilage promote the secretion of pro-inflammatory factors from chondrocytes, and result in the degeneration of articular cartilage and the development of osteoarthritis [10].

Lipopolysaccharide is known, both *in vitro* and *in vivo*, to stimulate inflammatory responses via increases in pro-inflammatory factors [11]. In addition, the chondrogenic cell line, ATDC5, has long been used as an *in vitro* model for chondrogenesis [12]. Recently, an LPS-stimulated chondrocyte model was developed for investigating the pathogenesis of osteoarthritis [13]. In the present study, LPS-treated ATDC5 cells have been established as a model for testing new drugs against osteoarthritis.

Previous research has shown that LPS treatment can promote the secretion of pro-inflammatory factors such as IL-1β, IL-6, and TNF-α [11]. Here, we demonstrate that the expressions of pro-inflammatory factors were also increased in LPS-treated ATDC5 cells, suggesting that the treatment represents an inflammatory injury to these cells. IL-1β functions as an important regulator of the inflammatory response, promoting chondrocyte apoptosis, and articular cartilage damage during the development of osteoarthritis [14].

Interleukin 6 mediates the proliferation and activation of T- and B-cells in the inflammatory process [15], and tumor necrosis factor-α also acts as a trigger for the inflammatory response [11]. Inhibition of these pro-inflammatory factors has been shown to be promising for ameliorating inflammatory injuries in osteoarthritis [11].

Moreover, it has been reported that miR-665 was up-regulated in LPS-stimulated ATDC5 cells, indicating that miR-665 participates in osteoarthritis regulation. Consistent with previous research [4], we have demonstrated a loss-of-function assay showing that miR-665 inhibition decreased the secretion of pro-inflammatory

factors, effectively relieving osteoarthritis inflammatory injury.

Although we have demonstrated an anti-inflammatory effect of miR-665 inhibition in LPS-treated ATDC5 cells, an underlying mechanism is still unclear. We identified SOCS7 as a binding target for miR-665 in this study, and as a member of the SOCS protein family regulating cytokine responses, SOCS7 has been implicated in allergic inflammation of mast cells via thymic stromal lymphopoietin [16], in insulin resistance [17], and over-expression of SOCS7 attenuated the inflammation in mice with unilateral urethral obstruction [18]. This study has shown that LPS stimulated a decrease in SOCS7, and that over-expression of SOCS7 relieved inflammation by decreasing the expression of pro-inflammatory factors.

Moreover, the anti-inflammatory effect of SOCS7 was reversed by an miR-665 mimic, revealing the critical role of the miR-665/SOCS7 axis for mediating LPS-stimulated inflammatory injury in ATDC5 cells. However, a previous study has shown that nuclear factor-κB, SRY-related high-mobility-group box 9-related, and other signaling pathways are also associated with miRNA regulation in osteoarthritis pathogenesis [19], so the signaling pathways associated with regulation of the miR-665/SOCS7 axis in osteoarthritis also need further investigation.

CONCLUSION

MicroRNA-665 exhibits a pronounced pro-inflammatory effect in LPS-induced inflammation in ATDC5 cells; inhibition of miR-665 is manifested as an anti-inflammatory effect in this model of osteoarthritis via regulation of SOCS7. These findings reveal that the miR-665/SOCS7 axis may be a new potential therapeutic target for osteoarthritis.

DECLARATIONS

Acknowledgement

This work was supported by National Science Foundation of China Project (Grant nos. 81760740 and 81760866), Guizhou Provincial Science and Technology Project (Grant no. 2017GZ67175), Qiankehe Foundation (Grant no. {2017}1006), Qiankehe Platforms Talents [2017]5735-09), Science and Technology Project of Guiyang City, (Grant no. Zhuke contract [2017-4]24) and Guizhou Provincial Administration of Traditional Chinese Medicine Project (Grant no. QZZY-2016-025).

Competing interests

The authors state that there are no conflicts of interest to disclose.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. QYN and YTH conceived and designed the experiments. WKM and FT analyzed and interpreted the results of the experiments. YH and XMY performed the experiments.

Open Access

REFERENCES

1. *Xiang Y, Li Y, Ling Z, Cheng Y. Structural analysis and insight into novel MMP-13 inhibitors from natural chemiome as disease-modifying osteoarthritis drugs. Trop J Pharm Res 2018; 17(11): 2255-2262.*
2. *Jiang R, Hao P, Yu G, Liu C, Yu C, Huang Y, Wang Y. Kaempferol protects chondrogenic ATDC5 cells against inflammatory injury triggered by lipopolysaccharide through down-regulating miR-146a. Int Immunopharmacol 2019; 69: 373-381.*
3. *Yu XM, Meng HY, Yuan XL, Wang Y, Guo QY, Peng J, Wang AY, Lu SB. MicroRNAs' Involvement in Osteoarthritis and the Prospects for Treatments. Evidence-based complementary and alternative medicine: eCAM 2015; 2015: 236179.*
4. *Li Z, Wang G, Feng D, Zu G, Li Y, Shi X, Zhao Y, Jing H, Ning S, Le W et al. Targeting the miR-665-3p-ATG4B-autophagy axis relieves inflammation and apoptosis in intestinal ischemia/reperfusion. Cell Death Dis 2018; 9(5): 483.*
5. *Wu R, Ruan J, Sun Y, Liu M, Sha Z, Fan C, Wu Q. Long non-coding RNA HIF1A-AS2 facilitates adipose-derived stem cells (ASCs) osteogenic differentiation through*

miR-665/IL6 axis via PI3K/Akt signaling pathway. Stem Cell Res Ther 2018; 9(1): 348.

6. *de Andres MC, Imagawa K, Hashimoto K, Gonzalez A, Goldring MB, Roach HI, Oreffo RO. Suppressors of cytokine signalling (SOCS) are reduced in osteoarthritis. Biochem Bioph Res Co 2011; 407(1): 54-59.*
7. *Choi YS, Park JK, Kang EH, Lee YK, Kim TK, Chung JH, Zimmerer JM, Carson WE, Song YW, Lee YJ. Cytokine signaling-1 suppressor is inducible by IL-1beta and inhibits the catabolic effects of IL-1beta in chondrocytes: its implication in the paradoxical joint-protective role of IL-1beta. Arthritis Res Ther 2013; 15(6): R191.*
8. *Iliopoulos D, Malizos KN, Oikonomou P, Tsezou A. Integrative microRNA and proteomic approaches identify novel osteoarthritis genes and their collaborative metabolic and inflammatory networks. PloS one 2008; 3(11): e3740.*
9. *Lund S, Christensen KV, Hedtjärn M, Mortensen AL, Hagberg H, Falsig J, Hasseldam H, Schrattenholz A, Pörzgen P, Leist M. The dynamics of the LPS triggered inflammatory response of murine microglia under different culture and in vivo conditions. J Neuroimmunol 2006; 180(1-2): 71-87.*
10. *Saklatvala J. Inflammatory signaling in cartilage: MAPK and NF-kappaB pathways in chondrocytes and the use of inhibitors for research into pathogenesis and therapy of osteoarthritis. Curr Drug Targets 2007; 8(2): 305-313.*
11. *Liu M, Gu M, Xu D, Lv Q, Zhang W, Wu Y: Protective effects of Toll-like receptor 4 inhibitor eritoran on renal ischemia-reperfusion injury. Transplant Proc 2010; 42(5): 1539-1544.*
12. *Yao Y, Wang Y. ATDC5: an excellent in vitro model cell line for skeletal development. J Cell Biochem 2013; 114(6): 1223-1229.*
13. *Chang CH, Hsu YM, Chen YC, Lin FH, Sadhasivam S, Loo ST, Savitha S. Anti-inflammatory effects of hydrophilic and lipophilic statins with hyaluronic acid against LPS-induced inflammation in porcine articular chondrocytes. J Orthop Res 2014; 32(4): 557-565.*
14. *Ying X, Peng L, Chen H, Shen Y, Yu K, Cheng S. Cordycepin prevented IL-beta-induced expression of inflammatory mediators in human osteoarthritis chondrocytes. Int Orthop 2014; 38(7): 1519-1526.*
15. *Ataie-Kachoie P, Pourgholami MH, Richardson DR, Morris DL. Gene of the month: Interleukin 6 (IL-6). J Clin Pathol 2014; 67(11): 932-937.*
16. *Knisz J, Banks A, McKeag L, Metcalfe DD, Rothman PB, Brown JM. Loss of SOCS7 in mice results in severe cutaneous disease and increased mast cell activation. Clin Immunol 2009; 132(2): 277-284.*
17. *Banks AS, Li J, McKeag L, Hribal ML, Kashiwada M, Accili D, Rothman PB. Deletion of SOCS7 leads to enhanced insulin action and enlarged islets of Langerhans. J Clin Invest 2005; 115(9): 2462-2471.*
18. *Yang R, Xu X, Li H, Chen J, Xiang X, Dong Z, Zhang D. p53 induces miR199a-3p to suppress SOCS7 for STAT3 activation and renal fibrosis in UUO. Sci Rep 2017; 7: 43409.*
19. *Xu B, Li YY, Ma J, Pei FX. Roles of microRNA and signaling pathway in osteoarthritis pathogenesis. J Zhejiang Univ B 2016; 17(3): 200-208.*