Original Article

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Estradiol and proinflammatory cytokines stimulate ISG20 expression in synovial fibroblasts of patients with osteoarthritis

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Summary **Interferon stimulated gene 20-kDa (ISG20) has been implicated in the pathology of osteoarthritis (OA) and it has been separately found to be responsive to estrogen stimulation. OA disproportionately affects women, and especially older women, suggesting some role of reproductive hormones in its pathology. The current study characterized the expression of ISG20 following stimulation with estradiol (E2) and proinflammatory cytokines in OA synovial fibroblasts (OASFs). E2 and the proinflammatory cytokines interleukin-6 (IL-6), lipopolysaccharide (LPS), and tumor necrosis factor α (TNF-α) were used to stimulate OASFs *in vitro*. The expression of ISG20 before and after stimulation was detected using quantitative real-time polymerase chain reaction (RT-qPCR) and Western blotting. E2 and proinflammatory cytokine (IL-6, LPS and TNF-α) stimulation significantly induced the expression of ISG20 both at the messenger RNA (mRNA) and protein level. Moreover, the induction was time- and dose-dependent. Small interfering RNA (siRNA) was transfected into OASFs, and expression of the inflammatory factors interleukin-1α (IL-1α), IL-6, and interleukin-10 (IL-10) was detected using RT-qPCR. Silencing ISG20 with siRNA inhibited the expression of IL-1α, IL-6, and IL-10. Thus, expression of ISG20 was regulated by estradiol and proinflammatory factors, while ISG20 in turn regulated the expression of other inflammatory factors. These data support the contention that ISG20 plays a role in the inflammatory process of OA.**

Keywords: Osteoarthritis, ISG20, Estradiol, proinflammatory factor, inflammation

1. Introduction

Osteoarthritis (OA) is a severe, chronic, progressive inflammatory disease of the joints, and particularly the knee. Affected joints are painful and have restricted function, which could lead to long-term disability (*1*). The pathogenesis of OA has yet to be fully understood, but is thought to be associated with sex, increasing age, trauma, wound healing, cartilage metabolic abnormalities, and immune abnormalities (*2,3*). Indeed, OA disproportionately affects women, and its prevalence is higher among older individuals. Women over the age of 50 years have a higher incidence of OA, so the menopause-related changes in hormone levels are hypothesized to contribute to arthritis (*4*). As the global population continues to age, understanding the pathogenesis of OA will become increasingly important.

Interferons (IFNs) comprise a family of secretory proteins chiefly characterized by their ability to induce cellular antiviral proteins (*5*). The interferon-stimulated genes (ISGs) produce proteins that act as antiviral effectors. One of these, interferon stimulated gene 20-kDa (ISG20), is an RNA exonuclease, and its expression can be induced by IFN types I (IFNα and IFNβ) and II (IFNγ) in various cell lines. ISG20 can cleave single-stranded RNA and DNA and it plays a key role in mediating the antiviral activity of IFN (*6-*

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9). Expression of ISG20, also called HEM45 (HeLa estrogen-modulated, band 45), in human cervical cancer cells increases when stimulated with estrogen (*10*). Thus, ISG20 appears to be responsive to hormonal signals.

Recent evidence has also implicated ISG20 in the pathology of OA. Indeed, the messenger RNA (mRNA) level of ISG20 is lower in the synovial tissues of patients with OA than that in patients with rheumatoid arthritis (RA) (*11*). A recent study by the current authors found that inflammatory mediators such as lipopolysaccharide (LPS), interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α) can stimulate ISG20 production in OA synovial fibroblasts (OASFs), indicating that ISG20 may act as a "sensor" in OASFs to exacerbate inflammation. However, the mechanism responsible for this function has yet to be identified. In order to determine how ISG20 contributes to the inflammation of OASFs, the current study characterized the expression and pathogenic signaling pathway of ISG20 in RASFs during inflammation. Since estradiol (E2) and proinflammatory factors play important roles in OA, this study also assessed the expression of ISG20 in response to stimulation with E2 and proinflammatory cytokines. These findings should identify the role of ISG20 in the pathogenesis of OA.

2. Materials and Methods

2.1. *Synovial tissues*

Synovial tissues (STs) were provided by Shandong Provincial Hospital. Tissues were collected during knee replacement surgery from patients with OA. All participants provided written informed consent to participate in this study, and the study plan was approved by the ethics committee of the Shandong Academy of Medical Sciences.

2.2. *Cell culture and treatment*

Synovial tissue was macerated and incubated with type II collagenase (1 mg/mL, Sigma-Aldrich) in Dulbecco's modified Eagle medium (DMEM, HyClone, Thermo Scientific) for 6 h at 37°C in 5% CO_2 (Thermo Scientific). The tissue was treated with 0.25% trypsin (Solabio) diluted in a phosphate-buffered saline (PBS) solution at a volume equivalent to the DMEM. Cells were filtered and cultured overnight in DMEM, supplemented with 10% fetal bovine serum (FBS, HyClone, Thermo Scientific), penicillin (100 IU/mL), and streptomycin (100 μg/mL, Gibco) for three passages. OASFs from passages 4-6 that tested negative for CD14, CD3, CD19, and CD56 expression according to flow cytometry were used is this study.

OASFs were cultured for 18 h at a density of 2-4 × 10^4/well in DMEM supplemented with 2% FBS. Then cells were cultured in the presence of E2, IL-6, LPS, or TNF-α. In order to avoid inclusion of other substances, E2 was diluted with certified charcoal-stripped FBS (BI, 04-201-1A) and serum without phenol red (MACGENE, CM15020).

2.3. *Inhibition of ISG20 expression with small interfering RNA (siRNA)*

Cultured OASFs were transfected with siRNA at 200 nmol/L using a HiPerFect transfection reagent (QIAGEN, Germany) according to the manufacturer's protocol. The cells were harvested for analysis 24 h after transfection. The sequence of siISG20 was 5'-GGCTACACAATCTACGACA-3'; a scrambled siRNA (5'-GGCTACACAATCTACGACA-3') was used as the negative control.

2.4. *Quantitative real-time PCR (RT-qPCR)*

Total RNA was extracted from cultured cells and human tissues using a TRIzol Reagent (Invitrogen) according to the manufacturers' protocol. RNA was reverse-transcribed using a ReverTra Ace qPCR RT Kit (Toyobo). RT-qPCR was conducted using a LightCycler 480 (Roche) with the following amplification protocol: denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 1 min, and extension at 72°C for 1s. Primers for RT-qPCR were also designed in accordance with the consensus sequence. GAPDH was used as an internal loading control. The sequences of primers were as follows: GAPDH 5'-GCACCGTCAAGGCTGAGAAC-3' (forward) and 5'-TGGTGAAGACGCCAGTGGA-3' (reverse); ISG20 5′- TGTTCTGGATGCTCTTGTGC-3′ (forward) and 5′- GCACTGAAAGAGGACATGAGC-3′ (reverse); ESR1 5′- GTCGCCTCTAACCTCGGG-3′ (forward) and 5′- GCTTTGGTGTGGAGGGTCAT -3′ (reverse). All primers were synthesized by BioSune (Shanghai, China). Relative messenger RNA (mRNA) levels were measured using the $2^{-\Delta \text{cycle threshold}}$ ($2^{-\Delta CT}$) method.

2.5. *Western blotting*

Whole cell lysates were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride membrane (Amersham Biosciences, Little Chalfont, UK). Western blotting was performed using anti-ISG20 (1:1000, ABCAM, ab154393). Tubulin (1:1000, ABCAM, ab7291) was used as a loading control for nuclear and cytoplasmic proteins.

2.6. Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences, version 17.0 (SPSS, USA). Data from cytological experiments were analyzed with the Student's *t*-test or chi-square test. $P < 0.05$ was

considered statistically significant in all calculations.

3. Results

3.1. *E2 can stimulate the expression of ISG20 in OASFs*

Women over the age of 50 have a higher incidence of OA, which suggests that changes in reproductive hormone levels may promote arthritis. In particular, E2 secretion decreases at menopause (*12*). To investigate whether ISG20 is responsive to E2, RT-qPCR and Western blotting were used to examine the expression of ISG20 in OASFs after stimulation with estradiol (E2). *ISG20* expression increased significantly after stimulation with 10^{-8} mol/L of E2 for 6 h ($p < 0.01$) (Figure 1A and 1B). Western blotting (Figure 1C) verified that the protein product was consistent with increased *ISG20* expression according to RT-qPCR.

3.2. *ISG20 is sensitive to extra-cellular stimulation with IL-6, TNF-α, and LPS*

To understand the pathological role of ISG20 in OA inflammation, the current study first investigated whether ISG20 expression is responsive to various inflammatory factors implicated in OA. Indeed, IL-6 (I), LPS (II), and TNF-α (III) treatments induced *ISG20* expression in OASFs to varying degrees (Figure 2A, 2B, and 2C). Moreover, the expression of *ISG20* increased in a dose-dependent manner in response to stimulation.

3.3. *The role of ISG20 in regulating inflammatory factors in OASFs*

Since the expression of ISG20 increased significantly after stimulating OASFs with E2 and pro-inflammatory cytokines, the current authors hypothesized that ISG20 may participate in inflammatory processes in OA. To test this hypothesis, siRNA was used to knock down expression of *ISG20*. After verifying the efficiency of the knockdown, RT-qPCR was used to detect its effect on expression of inflammatory factors interleukin-1α (IL-1α), IL-6, and interleukin-10 (IL-10), which play an important role in the inflammatory phenomenon of osteoarthritis (*12*). Results indicated that transfection of 200 nmol/L of siRNA-ISG20 for 24 hours significantly down-regulated the level of *ISG20* mRNA expression compared to the control group (Figure 3A, $p < 0.05$). Moreover, RT-qPCR results indicated that knockdown of *ISG20* in OASFs promoted lower levels of IL-1α, IL-6, and IL-10 expression (Figure 3B and 3C).

4. Discussion

The incidence of OA in women increases abruptly after menopause and is accompanied by a decrease in E2 secretion (*13*). E2 is the main estrogen in women who are premenopausal and postmenopausal. The estrogen level in joint fluid correlates with the level in blood, and E2 levels in joint fluid are similarly correlated with estrogen levels in serum from women with OA (*14*). These findings suggest that levels of hormones such as E2 change, increasing the incidence of OA. The current study found that an appropriate concentration of E2 can stimulate ISG20 expression in OASFs. This is consistent with results of studies indicating that E2 stimulation upregulates ISG20 expression in human cervical cancer cells and BD5, MDA-L3, MCF-7, and HepG2 cells (*10*).

Nonetheless, the function of E2 in OA-related

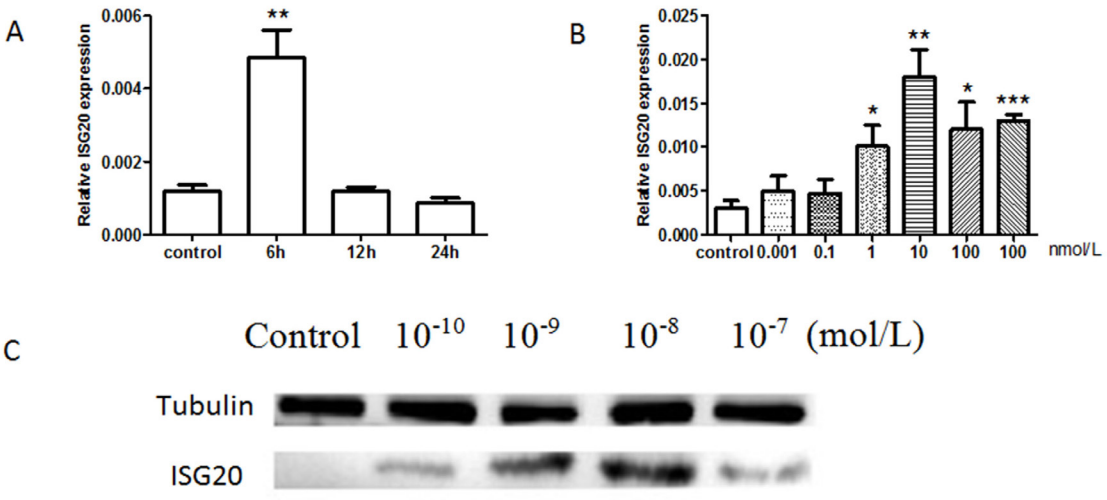

Figure 1. Effect of E2 on ISG20 in OASFs. (A) RT-qPCR was used to detect the effect of 10^{-8} mol/L of E2 on ISG20 in OASFs at different times; **(B)** RT-qPCR was used to detect ISG20 after OASFs were stimulated with E2 at serial concentrations for 6 h. The *p* value in A and B represents each of the times/doses compared to the control. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; **(C)** Western blotting and RT-qPCR were used to detect ISG20 after OASFs were stimulated 2with E2 at serial concentrations for 6 h.

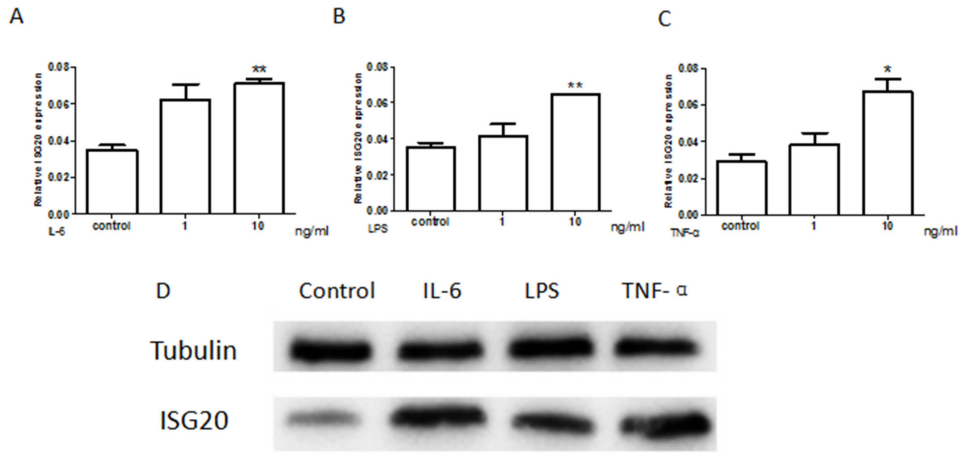

Figure 2. Proinflammatory factors can stimulate ISG20 in OASFs. OASFs were treated with10 ng/mL of IL-6 **(A)**, TNF-α **(B)**, or LPS **(C)** for 24 h in the presence or absence of a vehicle control. The expression of ISG20 was detected with RT-qPCR and Western blotting **(D)**. The *p* value in **A**, **B**, and **C** represents each of the doses compared to the control. All results are presented as the mean ± s.e.m. of three independent experiments performed in triplicate.$^{*}p < 0.05$, $^{**}p < 0.01$.

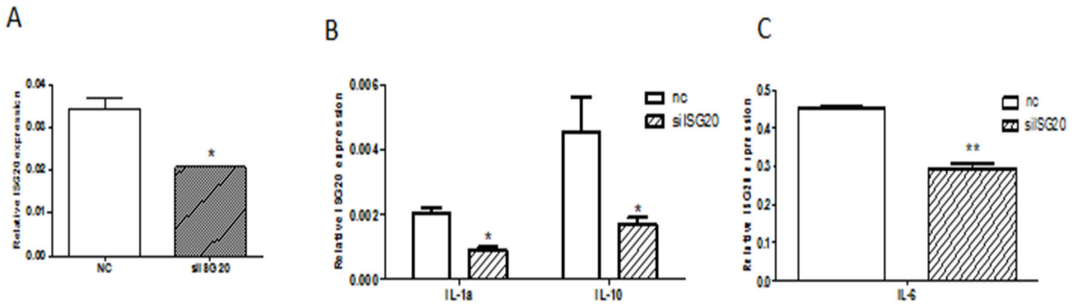

Figure 3. ISG20 is required for the production of inflammatory factors in OASFs. RT-qPCR was used to detect the efficiency of siRNA ISG20 **(A)** and the expression of inflammatory factorsIL-1α, IL-10, and IL-6 after the interference **(B** and **C)**. $^{*}p < 0.05$, $^{**}p < 0.01$.

inflammation is still debated. Martín-Millán and Castaneda hypothesize that estrogen plays a dual role as both an anti-inflammatory and a pro-inflammatory agent in the pathogenesis of OA (*15*). In contrast, de Klerk *et al*. suggest that there is no convincing evidence of a link between estrogen and OA (*16*). However, the current finding that ISG20 expression could be induced in OASFs by E2 and OA-related proinflammatory factors supports previous contentions that E2 may be pro-inflammatory in OA.

Inflammation is a common symptom of OA and is characterized by the presence of immune cells and the secretion of cytokines. Inflammatory factors are highly expressed in OA synovial tissue compared to normal synovial tissue (*3*). The current findings indicate that inflammatory factors IL-6, LPS, and TNF-α can promote *ISG20* mRNA expression. At the same time, knocked down expression of ISG20 with specifically targeted siRNA promotes the down-regulation of inflammatory cytokines such as IL-6, IL-1α, and IL-10. Thus, ISG20 may play a role in promoting inflammation in OASFs.

In summary, the current study found that ISG20 can be regulated by estradiol and proinflammatory factors such as IL-6, LPS, and TNF-α, and that, in turn, ISG20 can regulate the expression of the inflammatory cytokines IL-6, IL-1α, and IL-10 in OASFs. These findings help to understand the pathogenesis of OA, and particularly that among older women, and may lead to new therapeutic targets.

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