

## Original Article

# Downregulation of HSF2 inhibits inflammation in human intestinal epithelial cells by regulating IL-6/STAT3 signaling pathway

Anning Yin<sup>1</sup>, Yuanping Sun<sup>2</sup>

<sup>1</sup>Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan, Hubei Province, China;

<sup>2</sup>Department of Neurology, Huangshi Central Hospital, Huangshi, Hubei Province, China

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**Abstract:** Heat shock transcription factor 2 (HSF2) is up-regulated in inflammatory bowel disease (IBD). However, the role and mechanism of HSF2 in human intestinal epithelial cells remains unclear. Human intestinal epithelial T84 cells were divided into control group, lipopolysaccharide (LPS, 1 µg/ml) group and HSF2 siRNA +LPS group. HSF2 expression was analyzed by real-time PCR and Western blot. MTT assay was used to analyze cell proliferation. Interleukin-1 (IL-1), IL-6 and IL-10 levels were analyzed by ELISA. Real-time PCR was used to measure the expression of β-catenin and E-cadherin. The expressions of Occludin, Claudin-1 and STAT3 signaling pathway-associated molecules were detected by Western blot. In LPS-induced inflammatory model group, HSF2 expression was increased, cell proliferation was inhibited, IL-1 and IL-6 levels were increased, IL-10 level was decreased, and the expression of β-catenin and E-cadherin was decreased. The difference was significant compared with control group (P<0.05). In LPS group, the expression of Occludin and Claudin-1 was decreased, and p-STAT3 expression was increased. Compared with LPS group, HSF2 siRNA transfection into T84 cells significantly down-regulated HSF2 expression, promoted cell proliferation, decreased IL-1 and IL-6 expression, increased IL-10 expression, increased expression of β-catenin and E-cadherin (P<0.05). In addition, HSF2 siRNA transfection increased the expression of Occludin and Claudin-1 and decreased p-STAT3 expression. Down-regulation of HSF2 inhibits inflammatory factors, promotes anti-inflammatory factors secretion, and regulates intestinal barrier function by regulating IL-6/STAT3 signaling pathway.

**Keywords:** Heat shock transcription factor 2, inflammatory bowel disease, inflammatory factor, tight junction protein, IL-6/STAT3 signaling pathway

## Introduction

Inflammatory bowel disease (IBD) is a bowel disease with unknown etiology. It has genetic susceptibility and is characterized by abnormal function of the intestinal mucosal immune system, abnormal immune tolerance of the intestinal flora, chronic diarrhea and blood in the stool, leading to intestinal tissue damage, seriously affecting the quality of life of patients [1, 2]. IBD clinically has two important features, recurrent gastrointestinal tract and recurrent inflammation [3, 4]. The two typical diseases of IBD are ulcerative colitis (UC) and Crohn's disease [4]. The etiology and pathogenesis of IBD remain unclear and may be related to environmental, genetic, infection, and immune factors [5, 6]. Up to 4 million patients worldwide are

affected by IBD diseases, and IBD patients are increased year by year due to environmental changes and immune factors [7]. During the progression of IBD, imbalance of inflammatory factors, as well as steady-state loss of the immune system, abnormal immune regulation, persistent intestinal infection, and intestinal mucosal barrier defects, might cause the progression of IBD gastrointestinal inflammation into tumors, such as colitis-related gastrointestinal cancer [8, 9]. But so far, the pathogenesis of IBD has not been fully elucidated, which has brought great difficulties to the treatment. Therefore, identifying and clarifying the key molecular targets of IBD will play an important role in exploring new therapeutic methods and analyzing molecular mechanisms [10].

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**Table 1.** Primer sequences for real-time PCR

Gene	Forward 5' sequence	Reverse 5' sequence
GAPDH	AGTACCAGTCTGTTGCTGG	TAATAGACCCGGATGCTCTGGT
HSF2	CCTTAGTAGACCTCTCTCTG	TGTATAGATGTGTGTGGTAG
βGTATAGAT	AGATGTGACCAGATC	GTAGATGCTCTCTACCAGT
E-cadherin	CCTCTATAGTGTCGG	AGATGCTACGTCGGATA

Heat shock factor (HSF) is a stress-related gene family that is widely expressed in eukaryotes with 4 members, HSF1, HSF2, HSF3 and HSF4, among those, HSF3 is expressed only in birds and the others, HSF1, HSF2, and HSF4 have a high homology in mammals [11, 12]. HSF has a variety of biological functions, and it has different regulatory effects on biological processes such as biological embryo development, repair loss, and cell apoptosis and proliferation [13]. However, HSF expression is significantly increased during stress such as inflammation, and may be involved in pathological processes including cardiovascular disease, acidosis, and tumorigenesis [14, 15]. However, the expression profile and role of HSF in IBD remains poorly understood. Some studies have found that heat shock factor 2 (HSF2) is up-regulated in the intestinal mucosa of patients with IBD [16]. However, the role and mechanism of HSF2 in human intestinal epithelial cells has not been reported.

### Materials and methods

#### Reagents and equipment

The human intestinal epithelial T84 cell line was preserved in our laboratory and stored in liquid nitrogen. LPS was purchased from Sigma-Aldrich (USA). DMEM medium, fetal bovine serum (FBS), and cyan chain double antibody were purchased from Hyclone (USA). Dimethyl sulfoxide (DMSO) and MTT powder were purchased from Gibco. Trypsin-EDTA digest was purchased from Sigma-Aldrich. Rabbit anti-human HSF2 monoclonal antibody, rabbit anti-human Occludin monoclonal antibody and Claudin-1 monoclonal antibody, rabbit anti-human STAT3 and p-STAT3 monoclonal antibody, mouse anti-rabbit horseradish peroxidase (HRP) labeled IgG secondary antibody were purchased from Cell Signaling Technology. The RNA extraction kit and the reverse transcription kit were purchased from Axygen (USA). IL-1, IL-6 and IL-10 ELISA kits were purchased from R&D (USA). The HSF2 siRNA sequence was synthesized by Gema (Shanghai). The

lipo2000 reagent was purchased from Invitrogen (USA). The Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad Corporation (USA). The ABI 7700 Fast Quantitative PCR Reactor was purchased from ABI (USA). The ultra-clean workbench was purchased from Suzhou Sutai Purification Equipment Engineering Co., Ltd. The DNA amplification instrument was purchased from the PE Gene Amp PCR System 2400 (USA).

#### Cell culture and grouping

Human intestinal epithelial T84 cell line was resuscitated from liquid nitrogen, and cultured in DMEM containing 10% FBS and 1% cyan chain antibody. The cultured 2-8 generation logarithmic growth phase T84 cells were used for experiments and randomly divided into 3 groups: control group (normal cell culture), LPS group (1 μP/ml LPS was added to prepare inflammation model), and HSF2 siRNA group (HSF2 siRNA was transfected into T84 cells based on the LPS-induced inflammation model).

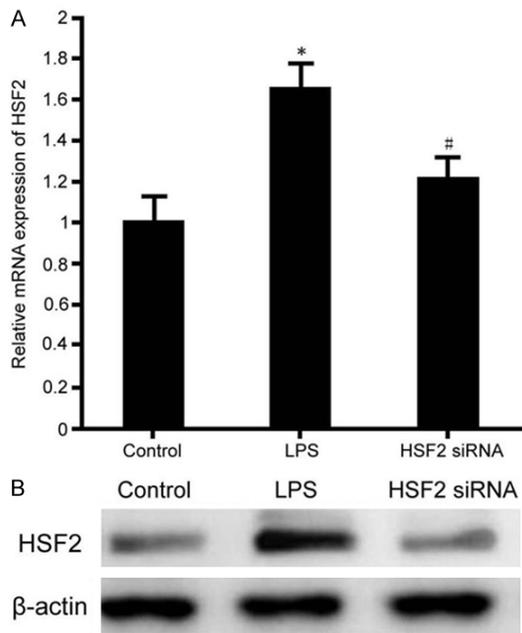
#### Cell transfection of HSF2 siRNA

The HSF2 siRNA sequence was transfected into T84 cells. The HSF2 siRNA sequence is 5'-GTCCGTTCAAACCTATCCAT-3'. When cultured cells reached a confluence of 70-80% and density of 65 × 10<sup>4</sup> in a 6-well plate, HSF2 siRNA liposome was separately added into 200 μl of serum-free DMEM medium, and mixed well for 15 min incubation at room temperature. The mixed lipo2000 was separately mixed and incubated for 30 min at room temperature. The cultured cell serum was removed; PBS was gently rinsed, followed by addition of 1.6 ml serum-free DMEM medium and further culture in a 5% CO<sub>2</sub> incubator at 37 °C of 1.6 ml serum-free DMEM medium separately mixed was replaced and cultured for 48 hours followed by relevant analysis.

#### Real-time PCR

Trizol reagent was used to isolate RNA which was reversely transcribed into cDNA according to the kit instructions. The primers were designed according to each gene sequence by PrimerPremier 6.0 and synthesized by Shanghai Yingjun Biotechnology Co., Ltd. (Table 1).

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**Figure 1.** The expression of HSF2 in LPS-induced inflammatory intestinal epithelial cells. Total RNA or protein was isolated from cells in different groups followed by measuring the mRNA expression of HSF2 by real-time PCR (A) or protein expression of HSF2 by western blot (B). Compared with control group, \* $P < 0.05$ ; Compared with LPS group, # $P < 0.05$ .

Real-time PCR was then performed with the reaction conditions as follows: 56°C 1 min, 92°C 30 S, 58°C 45 S, 72°C 35 S, with a total of 35 cycles. Data were collected using the PCR reactor software and GAPDH was used as a reference. Based on the standard CT value, a standard curve was drawn and then the semi-quantitative analysis was carried out using  $2^{-\text{tot}}$  method.

### Western blot

RIPA lysis buffer containing protease inhibitor was added to extract protein followed by subsequent incubation on ice for 15-30 min. After centrifugation at 10,000 g for 15 min at 4°C, the supernatant was transferred to a new Eppendorf (Ep) tube, and the protein was quantified using BCA assay and stored at -20°C. The protein was quantified using BCA assay and transferred to a PVDF membrane, blocked with 5% skim milk powder for 2 h and incubated with primary antibody with different dilutions at 4°C overnight. After washing with PBST, the membrane was incubated with 1:2000 goat anti-rabbit secondary antibody for 30 min incubation under dark. Protein was

visualized after addition of enhanced chemiluminescence. The data were analyzed using protein image processing system software and Quantity one software. The experiment was repeated four times ( $n=4$ ) and subjected to statistical analysis.

### MTT assay

Cells were inoculated into the 96-well culture plate with 10% fetal bovine serum DMEM culture medium at a cell number of  $5 \times 10^3$ , and the supernatant was discarded after 24 h of culture. After 48 h, 20  $\mu\text{l}$  of sterile MTT was added with 3 replicate wells in each treatment group followed by continuous culture for 4 h and subsequent removal of the supernatant and addition of 150  $\mu\text{l}$ /well of DMSO for 10 min. After the purple crystals were fully dissolved, the absorbance (A) value was detected at 570 nm to calculate the proliferation. The experiment was repeated three more times.

### Analysis of IL-1, IL-6 and IL-10 levels by ELISA

IL-1, IL-6 and IL-10 secretion in the supernatant of each group was analyzed using ELISA kit. According to the instructions, the standard curve was prepared separately, and the sample was added. Three replicates of each sample were added, and the enzyme-labeled antibody, the color developer and the stop solution were sequentially added, and the optical density value was detected at 450 nm by a microplate reader. The measurement was carried out within 15 min after addition of the stop solution.

### Statistical analysis

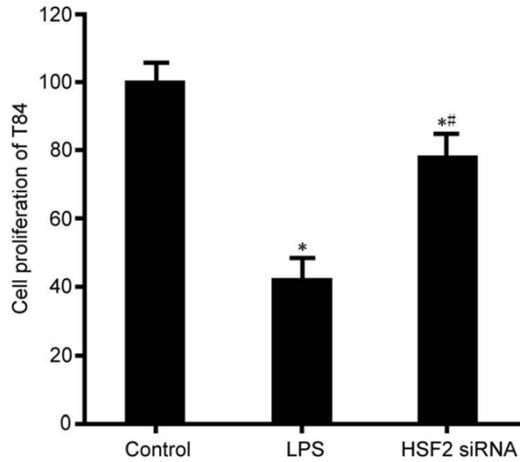
All data were shown as mean  $\pm$  SD. The two groups were compared by student t-test. Comparisons of the differences among groups were assessed by analysis of variance (ANOVA).  $P < 0.05$  was considered statistically significant.

## Results

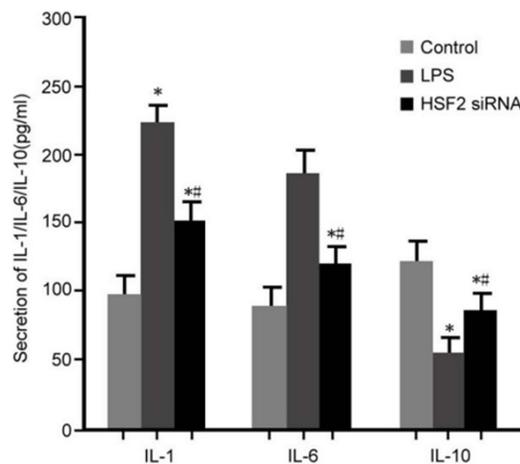
### Expression of HSF2 in LPS-induced inflammatory human intestinal epithelial cells

The expression of HSF2 was significantly increased in LPS-induced inflammatory human

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**Figure 2.** Effect of HSF2 downregulation on cell proliferation. Cell proliferation in different treatment groups was measured by MTT assay. Compared with control group, \* $P < 0.05$ ; Compared with LPS group, # $P < 0.05$ .

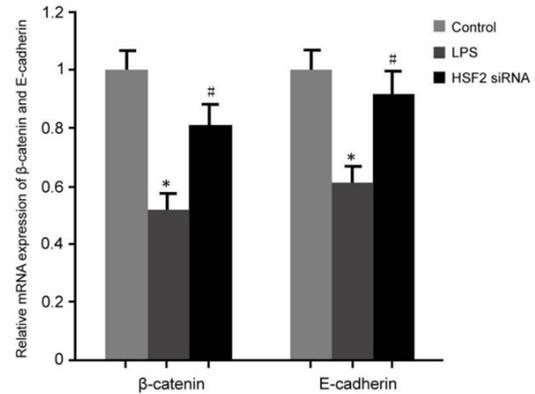


**Figure 3.** Effect of HSF2 downregulation on the secretion of IL-1/IL-6/IL-10. After different treatment, the secretion of IL-1/IL-6/IL-10 was measured by ELISA. Compared with control group, \* $P < 0.05$ ; Compared with LPS group, # $P < 0.05$ .

intestinal epithelial cell model compared with that in control group ( $P < 0.05$ ). HSF2 siRNA transfection significantly down-regulated the expression of HSF2 in the HSF2 siRNA + LPS group, compared with the LPS group ( $P < 0.05$ ) (Figure 1).

### Effect of HSF2 down-regulation on the proliferation of inflammatory human intestinal epithelial cells

LPS treatment significantly decreased cell proliferation compared with control group ( $P < 0.05$ ).



**Figure 4.** Effect of HSF2 downregulation on the mRNA expression of  $\beta$  on the mRNA expression of Total RNA was extracted after HSF2 transfection followed by analysis of the mRNA expression of  $\beta$  mRNA expression of ted after HSF2 transfection compared with control group, \* $P < 0.05$ ; Compared with LPS group, # $P < 0.05$ .

HSF2 siRNA transfection promoted cell proliferation of LPS-induced inflammatory human intestinal epithelial cells as compared with LPS group ( $P < 0.05$ ) (Figure 2).

### Effect of HSF2 on the secretion of IL-1, IL-6 and IL-10 in inflammatory human intestinal epithelial cells induced by LPS

ELISA analysis showed that LPS significantly increased inflammatory factors IL-1 and IL-6 secretion in cell supernatant and inhibited the secretion of anti-inflammatory factor IL-10 compared with the control group ( $P < 0.05$ ). HSF2 siRNA was transfected into LPS-induced inflammatory human intestinal epithelial cells, which significantly inhibited the secretion of IL-1 and IL-6 but promoted the secretion of IL-10 compared with LPS group ( $P < 0.05$ ) (Figure 3).

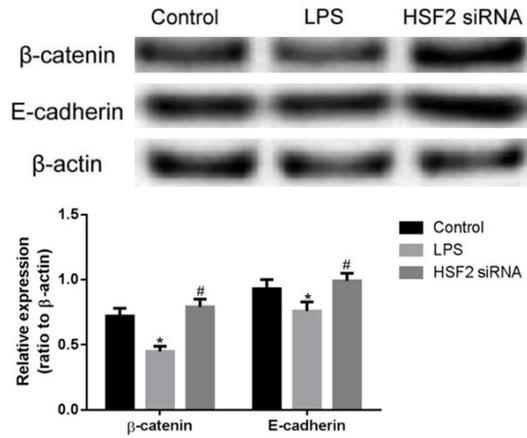
### Effect of HSF2 down-regulation on the expression of -6 but promoted the secre

Compared with the control group, LPS treatment significantly inhibited secretion of IL-10 compared with LPS group (Pnt antiRNA transfection rescued cued roup, LPS treatment expressions in LPS-induced inflammatory human intestinal epithelial cells significantly, as compared with LPS group ( $P < 0.05$ ) (Figure 4).

### Effect of HSF2 down-regulation on LPS-induced expression of tight junction proteins

LPS inhibited the expression of tight junction proteins Occludin and Claudin-1. HSF2 siRNA

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**Figure 5.** Effect of HSF2 downregulation on the protein expression of β on the protein expression of β. Total protein was extracted after HSF2 transfection followed by analysis of the protein expression of β protein expression of cted after HSF2 transfection Compared with control group, \*P<0.05; Compared with LPS group, #P<0.05.

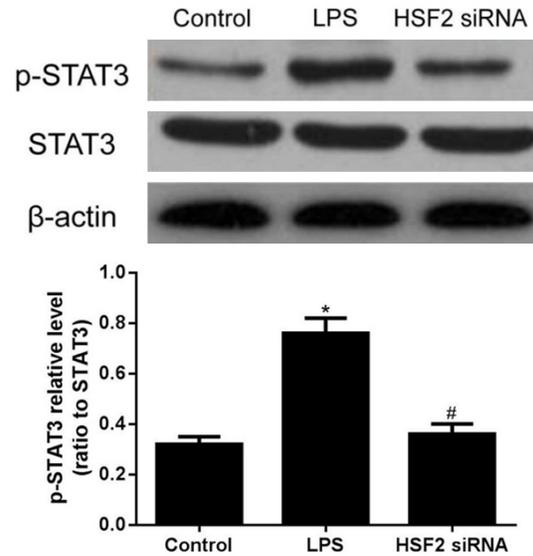
transfection promoted these expressions in LPS-induced inflammatory human intestinal epithelial cells (Figure 5).

### Effect of HSF2 down-regulation on the expression of STAT3

Western blot analysis demonstrated that LPS promoted p-STAT3 expression; whereas HSF2 siRNA transfection down-regulated p-STAT3 expression (Figure 6).

### Discussion

As a chronic recurrent disease of the gastrointestinal tract, IBD can cause serious damages to the intestinal epithelium due to chronic long-term inflammation, leading to clinical symptoms such as abdominal pain, repeated diarrhea, and bloody stools, which seriously affect the quality of life of patients, and can induce tumorigenesis in severe cases [17]. IBD is a multifactorial disease, and increased secretion of inflammatory factors such as IL-1 and IL-6, and decreased secretion of anti-inflammatory factor IL-10, cause the development of inflammation, which stimulates leukocytes in the blood circulation to move and infiltrate into the intestinal mucosa and subsequent promotion of local inflammatory response, so the imbalance between pro-inflammatory cytokines and anti-inflammatory factors plays a key role in the process of inflammatory injury of intesti-



**Figure 6.** Effect of HSF2 downregulation on the protein expression of p-STAT3 and STAT3. Total protein was extracted after HSF2 transfection followed by analysis of the expression of p-STAT3 and STAT3 by western blot. Compared with control group, \*P<0.05; Compared with LPS group, #P<0.05.

nal mucosa [18, 19]. LPS participates in the induction of intestinal and systemic inflammation [20]. Therefore, this study used LPS to treat intestinal epithelial cells to establish an inflammatory model, and analyzed the effects of HSF2 on LPS-induced inflammatory human intestinal epithelial cells and related mechanisms.

This study demonstrates that HSF2 down-regulation in LPS-induced intestinal epithelial inflammatory models can significantly promote intestinal epithelial cell proliferation, inhibit the secretion of IL-1, IL-6 and other inflammatory factors, and promote the secretion of IL-10, which in turn delays intestinal epithelial cell damage and promotes cell proliferation through regulating the balance of inflammatory cytokines and anti-inflammatory factors. A previous study has found that increased permeability of the intestinal mucosal barrier promotes the development of IBD, and the tight junction of intestinal epithelial cells helps to ensure the integrity of the cell barrier and is therefore closely related to IBD [21]. Loss of tight junctions in the intestine leads to increased permeability, which promotes antigen entry into the intestine, and triggers intestinal tract [22]. The cell tight junction proteins Occludin and Claudin-1 are important parts of intracellular

tight junctions and promote direct cell epithelial connections [23]. Cell adhesion molecules such as  $\beta$ -catenin and E-cadherin mediate cell-to-cell or cell-to-extracellular matrix contact and adhesion, which facilitates cell tight junctions, cell proliferation and differentiation [24]. In this study, we showed that in LPS-induced intestinal epithelial cell inflammation models, cell tight junction proteins Occludin and Claudin-1, as well as well as LPS-induced intestinal epithelium expression were decreased; while downregulation of HSF2 promoted the expression of Occludin and Claudin-1 as well as well as f Occludin and Claudin-1 as well as -1 as wepithelial cell inflammation models. The IL-6/STAT3 signaling pathway participates in the development of various diseases, and its activation regulates development of IBD [25]. Consistent with this, our study demonstrated that IL-6/STAT3 signaling pathway was activated in LPS-induced intestinal epithelial cell inflammation model as shown by increased expression of p-STAT3 and IL-6; whereas HSF2 down-regulation in LPS-induced inflammatory intestinal epithelial cells inhibited p-STAT3 expression and IL-6 secretion. These results suggest that down-regulation of HSF2 expression in LPS-induced inflammatory intestinal epithelial cells can improve intestinal barrier function and reverse LPS-induced intestinal barrier damage regulation through regulating IL-6/STAT3 signaling pathway and the expression of tight junction proteins and adhesion molecules. In future, it is necessary to further analyze the expression and regulation of HSF2 in clinical IBD patients, aiming to provide reference for the mechanism of clinical IBD and the selection of therapeutic targets.

### Conclusion

Down-regulation of HSF2 inhibits the secretion of inflammatory cytokines, promotes the secretion of anti-inflammatory factors through regulating IL-6/STAT3 signaling pathway, and improves the intestinal barrier function through modulating tight junction proteins and cell adhesion molecules expression.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Yuanping Sun, Department of Neurology, Huangshi Central

Hospital, No. 141 Tianjin Road, Huangshi 435000, Hubei Province, China. Tel: +86-0714-6233931; Fax: +86-0714-6233931; E-mail: congmiyo6638@163.com

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