Original article

Knockdown of IRF3 inhibits extracellular matrix expression in keloid fibroblasts

Yi Zhang, Li Zhang, Xiao-hua Lin, Zhi-ming Li, Qi-yu Zhang

*Department of Dermatology, The First Affiliated Hospital of WenZhou Medical University, WenZhou 325000, China
**Department of Hepatic Surgery, The First Affiliated Hospital of WenZhou Medical University, WenZhou 325000, China

Abstract

Knockdown of IRF3 inhibits extracellular matrix expression in keloid fibroblasts

**Article Info**

Article history:
Received 16 November 2016
Received in revised form 23 January 2017
Accepted 25 January 2017

Keywords:
Interferon regulatory factor 3 (IRF3)
Keloid fibroblasts (KFs)
Extracellular matrix (ECM)
TGF-β1/Smad pathway

**1. Introduction**

Keloid is a pathologic fibro-proliferative disorder and is characterized by hyper-proliferation of fibroblasts and excess extracellular matrix (ECM) deposition. Interferon regulatory factor 3 (IRF3) is a member of the interferon-regulatory factor (IRF) family and has been shown to play a critical modulator in the progression of fibrosis. However, the function of IRF3 in dermal fibrosis remains unclear. Thus, in this study, we investigated the effects of IRF3 on keloid-derived fibroblasts (KFs) proliferation and ECM expression, and explored the underlying mechanism. Our results indicated that the expression of IRF3 was highly expressed in human keloid tissues. Down-regulation of IRF3 significantly inhibited KF proliferation and the expression of type I collagen and α-smooth muscle actin (α-SMA), as well as suppressed the expression of TGF-β receptor 1 and II in TGF-β1-stimulated KFs. Furthermore, down-regulation of IRF3 suppressed the phosphorylation levels of Smad2 and Smad3 in human KFs induced by TGF-β1. Taken together, our data showed that down-regulation of IRF3 inhibited the proliferation and ECM expression in KFs via suppressing the TGF-β1/Smad signaling pathway. Thus, our findings suggest that IRF3 may represent a promising target for treatment of the keloid disease.

© 2017 Elsevier Masson SAS. All rights reserved.

1. **Introduction**

Keloid is a pathologic fibro-proliferative disorder and is characterized by excess extracellular matrix (ECM) deposition and hyper-proliferation of fibroblasts [1]. It severely impairs the quality of life through causing cosmetic and functional deformities, discomfort and psychological stress [2]. Although the pathogenesis of keloid formation still remains unclear, mounting evidence has demonstrated that transforming growth factor β1 (TGF-β1), one of the strongest pro-fibrotic cytokines, stimulates the synthesis of collagen, and also differentiates fibroblasts into myofibroblasts, exacerbating the formation of keloid [3–5]. Therefore, inhibition of the TGF-β1 signaling pathway is a therapeutic strategy for the treatment of keloids.

Members of the interferon regulatory factor (IRF) family are transcription factors involved in the regulation of a variety of biological processes [6]. Interferon regulatory factor 3 (IRF3) is a member of the IRF family and has been shown to regulate cell proliferation, apoptosis, inflammation, innate immune response and insulin resistance [7–9]. Moreover, several studies showed that IRF3 plays a critical modulator in the progression of fibrosis [10,11]. A study by Ni et al. confirmed that down-regulation of IRF3 significantly suppressed TGF-β1-induced hepatic stellate cells (HSCs) proliferation and increased its apoptosis, as well as reduced the expression level of type I collagen and α-smooth muscle actin (α-SMA) in activated HSCs [12]. However, the function of IRF3 in dermal fibrosis remains unclear. Thus, in this study, we investigated the effects of IRF3 on keloid-derived fibroblasts (KFs) proliferation and ECM expression, and explored the underlying mechanism.

2. **Materials and methods**

2.1. **Tissue specimens**

Fresh tissue specimens were obtained from ten patients with keloids and eight healthy patients with non-pathological scar during Department of Dermatology, the First Affiliated Hospital of WenZhou Medical University (China). The samples were immediately stored in liquid nitrogen in preparation for use. The study was approved by the Ethics Committee of the First Affiliated Hospital of WenZhou Medical University, and written informed consent was obtained from all subjects.

http://dx.doi.org/10.1016/j.biopha.2017.01.142
0753-3322 © 2017 Elsevier Masson SAS. All rights reserved.
2.2. Cell culture and treatment

Human dermal fibroblasts were obtained by skin biopsy from the keloids and non-pathological scars. Fibroblasts were cultured in Dulbecco modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/ml penicillin and 0.1 g/ml streptomycin (Sigma, St. Louis, MO, USA). All cell culture protocols were performed at 37°C in a humidified 5% CO2 environment. All studies used cells from passage number three to six. KFs were treated with human recombinant TGF-β1 (10 ng/ml; Sigma, St. Louis, MO, USA) for 24 h.

2.3. RNA interference and transfection

Small interfering RNAs targeting IRF3 (siRNAs-IRF3) and its negative scrambled siRNA (scramble) were synthesized by Ribó Biotech (GuangZhou, China). The target sequences for IRF3 were as follows: siRNA1, 5’-GGAGUGAUUGCUACGUGA-3’; siRNA2, 5’-CCCUUCAUUGUAGAUCUGATT-3’. For transfection, KFs were seeded to 50–60% confluence in DMEM and grown for 24 h, and then transfected with siRNAs-IRF3 or scramble using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

2.4. Cell proliferation assay

Cell proliferation was evaluated using the WST-1 assay according to the manufacturer’s protocol. Briefly, KFs at a density of 1 × 10³ cells/well were plated in 96-well plates and cultured for 24 h after transfection. Then, 10 μl of WST-1 substrate was added to each well and incubated for 3 h at 37 °C. Optical density was determined at a wavelength of 570 nm using microplate reader (Bio-Rad, Hercules, CA, USA).

2.5. Real-time reverse transcription PCR (real-time RT-PCR)

Total RNA was isolated from tissue specimens or KFs using the Favorgen RNA extraction kit (Favorgen Biotech) according to the manufacturer’s protocol. Three μg of total RNA was reverse-transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). RT-PCR was performed using an ABI Prism 7500 System (Applied Biosystems) with the SYBR Green Supermix (Invitrogen, Carlsbad, CA, USA). The sequences of primers were as follows: IRF3 forward 5’-AGAGGCTGTGATGTTCAAGGTT-3’ and reverse 5’-AGTGGTGCGCTGCTTGAAATTG-3’; β-actin forward 5’-GATCTGCTCTCTGCTGAGT-3’ and reverse 5’-ACTCTGGTTCGCTCAGTCC-3’. The relative expression level was calculated by using 2-ΔΔCt cycle threshold method.

Fig 1. Down-regulation of IRF3 inhibits TGF-β1-induced KFs proliferation. A, The mRNA expression of IRF3 in keloid tissues was determined using real-time RT-PCR, *P < 0.05 compared with normal skin tissues. B, The protein expression of IRF3 in keloid tissues was determined using western blot, *P < 0.05 compared with normal skin tissues. C, KFs were incubated with siRNAs-IRF3 or scramble for 48 h. The transfection efficiency was confirmed using western blot, *P < 0.05 compared with scramble. D, KFs were transfected with siRNA1-IRF3 or scramble, and then treated with TGF-β1 (10 ng/ml) for 24 h. Cell proliferation was evaluated using the WST-1 assay. All experiments were repeated at least three times. *P < 0.05 compared with scramble; #P < 0.05 compared with TGF-β1 + scramble.
2.6. Western blot

Tissue specimens or KFs were lysed in lysis buffer (50 mM Tris-HCl, pH 8.0; 150 mM protease and phosphatase inhibitor mixtures). Equal protein amounts of cell lysates were separated on 10% SDS-polyacrylamide gels and electro-botted onto nitrocellulose membranes (Millipore, Boston, MA, USA). The immunoblot was incubated for 1 h with blocking solution (5% skim milk) at room temperature, and then incubated with specific primary antibodies [anti-IRF3, anti-type I collagen, anti-α-SMA, anti-TGF-β receptor I (TGF-β R1), anti-TGF-β RII, anti-p-Smad2, anti-Smad2, anti-p-Smad3, anti-p-Smad3 and anti-GAPDH] (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 4 °C overnight. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Finally, bound proteins were visualized by enhanced chemiluminescence (ECL) detection system (Amersham, Little Chalfont, UK). The optical densities of the bands were quantified using a Gel-Pro Analyzer v4.0 (Media Cybernetics, Rockville, MD, USA).

2.7. Statistical analysis

Data are presented as the means ± standard deviation (SD) of duplicate experiments carried out for at least three separate runs. A one way ANOVA followed by a Student’s t-test was used to determine if the results were statistically significant. P < 0.05 was considered to be statistically significant.

3. Results

3.1. Down-regulation of IRF3 inhibits TGF-β1-induced KFs proliferation

First, we detected the expression of IRF3 in human keloid tissues using real-time RT-PCR and western blot. The results demonstrated that the expression levels of IRF3 at both mRNA and protein in keloid tissues were significantly increased compared to that of control tissues (Fig. 1A and B). Furthermore, to examine the effect of IRF3 on KF proliferation, we knocked down the expression of IRF3 in KFs using siRNAs. The results of western blot analysis showed that the protein expression level of IRF3 was greatly down-regulated in KFs after transfection of siRNA1-IRF3 or siRNA2-IRF3, respectively (Fig. 1C). The WST-1 assay indicated that TGF-β1 treatment significantly promoted KF proliferation. At the same time, siRNA1-IRF3 obviously suppressed KF proliferation induced by TGF-β1. Similarly, siRNA2-IRF3 also significantly inhibited TGF-β1-induced KF proliferation. (Fig. 1D).

3.2. Down-regulation of IRF3 inhibits the expression of ECM in human KFs

Keloid scarring is characterized by excessive production of ECM components. Thus, we examined the effect of IRF3 on ECM expression in TGF-β1-stimulated KFs. As shown in Fig. 2, TGF-β1 treatment significantly up-regulated the protein expression levels of type I collagen and α-SMA in KFs. Meanwhile, siRNA1-IRF3 or siRNA2-IRF3 dramatically suppressed the expression of ECM components in KFs induced by TGF-β1, respectively.

3.3. Down-regulation of IRF3 inhibits the expression of TGF-β receptor I and II in human KFs

Next, we investigated the effect of IRF3 on TGF-β R1 and RII expression in TGF-β1-stimulated KFs. The results of western blot analysis demonstrated that the protein expression levels of TGF-β
the phosphorylation levels of Smad2 and Smad3 in human KFs induced by TGF-β1.

IRF3 has been shown to regulate cardiac fibrosis. Silencing IRF3 significantly suppressed the development of cardiac fibrosis and decreased the left ventricle end-diastolic dimension in an angiotensin II-infusion mouse model [10]. In this study, we observed that the expression levels of IRF3 at both mRNA and protein in keloid tissues were increased compared to that of control tissues, which suggested that IRF3 may serve as a potential biomarker in the formation of keloid.

Keloid is a pathologic fibro-proliferative disorder and is characterized by hyper-proliferation of fibroblasts and excessive accumulation of ECM. The induction of KF proliferation is stimulated by a variety of cytokines, including TGF-β1 [13–15]. Several studies demonstrated that TGF-β1 was upregulated in keloid tissue, which induced KF proliferation during the progression of keloid [16–18]. Consistent with the results of previous studies, herein, we observed that TGF-β1 treatment significantly promoted KF proliferation. Meanwhile, down-regulation of IRF3 obviously suppressed KF proliferation induced by TGF-β1. In the development of keloid, massive proliferation of fibroblasts promotes collagen secretion and leads to a large number collagen deposition [19]. In this study, we found that down-regulation of IRF3 significantly inhibited the expression of type I collagen and α-SMA in TGF-β1-stimulated KFs. These data suggest that si-IRF3 exhibits inhibitory effects in KFs through decreasing KF proliferation and the expression of ECM proteins.

Growing body of evidence suggests that the TGF-β/Smad signaling pathway plays an important role in the development of keloids [20–22]. It was found that reported that the expression levels of TGF-β RI and TGF-β RII in KFs were higher than that of normal dermal fibroblasts [23]. Similarly, herein, we observed that TGF-β1 treatment significantly up-regulated the expression of TGF-β RI and TGF-β RII in KFs, however, knockdown of IRF3 greatly inhibited the TGF-β1-induced TGF-β RI and TGF-β RII expression. Moreover, Smads may have critical functions in the formation of keloids. Within the R-Smad family, Smad3 mediates collagen production in dermal fibroblasts stimulated by TGF-β [24]. A study by Wang et al. confirmed that inhibition of Smad3 in KFs leads to reduced expression of types I and III procollagen [25]. Herein, we found that TGF-β1 treatment markedly enhanced the phosphorylated levels of Smad2 and Smad3 in KFs. However, down-regulation of IRF3 resulted in a decrease of p-Smad2 and p-Smad3 in TGF-β1-stimulated KFs. These data suggest that down-regulation of IRF3 inhibited the proliferation and ECM expression in KFs via suppressing the TGF-β1/Smad signaling pathway.

In conclusion, our data showed that down-regulation of IRF3 inhibited the proliferation and ECM expression in KFs via suppressing the TGF-β1/Smad signaling pathway. Thus, our findings suggest that IRF3 may represent a promising target for treatment of the keloid disease.

5. Disclosure

The authors report no conflicts of interest in this work.
Acknowledgments

This research was funded by Health Bureau of Zhejiang Province (grant number: 2015KYB244) and the most important subject of higher education in Education Department of Zhejiang Province (grant number: 2008–255).

References