MiR-155-5p modulates inflammatory phenotype of activated oral lichen-planus-associated-fibroblasts by targeting SOCS1

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Research Article

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Abstract

Micro (MiR)-155-5p plays a vital role in inflammation and immune responses, but the function in oral lichen planus (OLP) remains unclear. Recent research has revealed that OLP associated-fibroblasts (OLP AFs) contribute to the inflammatory process with activated phenotype. This study was carried out to assess the role of miR-155-5p in the pro-inflammatory process of activated OLP AFs. Normal mucosal fibroblasts (NFs) and OLP AFs were isolated from oral mucosa of healthy controls and OLP patients respectively. We detected the expression of miR-155-5p and FAP-α in NFs and OLP AFs by real-time RT-PCR and calculated their correlation. Enzyme-linked immunosorbent assay was used to detect cytokine production. Immunohistochemistry and western blotting assay were used to detect suppressor of cytokine signaling 1 (SOCS1) expression. Dual-luciferase reporter assay was conducted to explore the interaction between miR-155-5p and SOCS1. MiR-155-5p and FAP-α were significantly increased in OLP AFs, and positively correlated. The expression of SOCS1 is decreased in the epithelial layer of OLP tissues and OLP AFs. Overexpression of miR-155-5p augmented interleukin-6 (IL-6) and interleukin-8 (IL-8) release. The knockdown of miR-155-5p in OLP AFs decreased IL-6 and IL-8 release. Furthermore, miR-155-5p inhibits SOCS1 expression by directly targeting its 3'-UTR in NFs and OLP AFs. SOCS1 silencing augmented IL-6 and IL-8 production in NFs. Here, we suggest that miR-155-5p regulates the secretion of IL-6 and IL-8 by downregulating the expression of SOCS1 in activated OLP AFs. Our results provide novel insights into the pathogenesis of OLP and a new target for OLP future therapy.

Introduction

Oral lichen planus (OLP) is a common chronic inflammatory disease of oral mucosa, mainly affecting the buccal mucosa and tongue [1]. According to clinical manifestation, OLP is mainly divided into two types: erosive type OLP (EOLP) and non-erosive type OLP (NEOLP). Moreover, OLP is listed as a potential oral malignant disease by the World Health Organization (WHO) [2].

Cytokines are small peptide proteins that can be synthesized and secreted by various immune cells and non-immune cells including keratinocytes, fibroblasts, epithelial cells, etc [3]. A wide range of studies have shown that the levels of pro-inflammatory cytokines are upregulated in lesions, saliva, serum and peripheral blood mononuclear cells from patients with OLP, such as interleukin 6 (IL-6) and interleukin 8 (IL-8) [4–6]. Although the etiology and pathogenesis of OLP are not fully understood, abundant evidence suggest that inflammation-related cytokines participant in the development of the disease. Fibroblasts are known to be activated and develop epigenetically imprinted, site and disease specific phenotypes especially in the inflammatory environment [7]. Previous studies have demonstrated that fibroblasts participate in the inflammatory process and affect immune cells in the process of chronic inflammation and autoimmunity diseases, including rheumatoid arthritis, systemic sclerosis, and ulcerative colitis [8–12]. Recent research has revealed that a subset of fibroblasts cultured from OLP lesions acquire an active phenotype and secrete pro-inflammatory cytokines in response to microorganismal infection *in vitro* [13], However, the research of OLP AFs' pro-inflammatory function is limited.

MicroRNAs (miRNAs/miRs) are a class of 20-22 nucleotide length non-coding RNAs that regulate gene expression by post-transcriptionally binding to the 3' -untranslated region of the target mRNA, resulting in mRNA degradation or translation inhibition [14]. Furthermore, numerous reports have focused on miRNAs involved in the regulation of immune responses and inflammatory processes by affecting associated signaling pathways. Meanwhile, miRNAs act as potential biomarkers for the diagnosis and prognosis of many diseases [15, 16]. Compelling evidence indicates that abnormal miRNA expression leads to inflammation and immune diseases. MiRNAs were reported to be significantly dysregulated in mucosal tissues in patients with OLP and healthy controls, especially miR-155-5p [17]. MiR-155-5p is a typical multifunctional miRNA that participates in hematopoiesis, inflammation and immune responses [18, 19]. In addition, miR-155-5p was identified to be linked with OLP-associated cytokines and target genes that encode proteins associated with inflammation [20, 21]. However, the cell origin of miR-155-5p in OLP mucosal lesions and its role in OLP local inflammation are still unknown.

Here, we aimed to detect the levels of miR-155-5p in NFs and OLP AFs, and assess the relationship between miR-155-5p expression and the level of activated fibroblasts' marker. We speculated that miR-155-5p could participate in the pro-inflammatory response of OLP AFs. To confirm our hypothesis, we investigated the target gene that may contribute to the pro-inflammatory function of miR-155-5p. These results may provide a better understanding in the potential mechanisms of miR-155-5p in OLP AFs.

Materials And Methods

Ethics Statement

This study was approved by the Ethical Committee of the Affiliated Hospital of Stomatology, Nanjing Medical University (permission number PJ2016–034–001) and the Institutional Review Board of Nanjing Medical University (permission number 2014–132). Participants signed informed consent voluntarily and we conducted all procedures according to the Declaration of Helsinki.

Study Subjects

We collected tissue samples from 15 healthy controls and 30 OLP patients (15 NEOLP patients and 15 EOLP patients) for culturing fibroblasts. All participants were recruited from the Department of Oral Medicine of the Affiliated Hospital of Stomatology at Nanjing Medical University from November 2019 to May 2021. The OLP diagnosis was established based on clinical and histopathological characteristics according to the criteria made by the WHO in 1978 and van der Meij et al. in 2003 [22]. Normal tissue samples were collected from gingival tissues around the extracted teeth, and tissue samples of OLP groups were collected from buccal mucosa. The subjects had neither any systemic disorders nor any soft tissue lesions. Meanwhile, they had not received any treatment within 6 months. Smokers and severe alcoholics were excluded. The clinical features were displayed in Table 1. Fifteen tissue samples were collected from healthy controls, NEOLP group and EOLP group respectively for IHC following the above requirements. The clinical features were displayed in Table2.

Primary Culture

Fresh specimens were washed three times in sterile phosphate-buffered saline (PBS) (Gibco) containing 5% penicillin-streptomycin-glutamine (100X) (Gibco) and minced into fragments. These fragments were cultured in DMEM medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotics, at 37 °C with 5% CO2. OLP AFs and normal mucosal fibroblasts (NFs) were used for experiments at 3-6 generations.

Immunofluorescence

Cells were seeded on cover slips at approximately 70 % confluence overnight. For immunofluorescence processing, they were fixed and permeabilized, and then stained with primary antibody overnight at 4 °C after blocking with 3% BSA for 30 min at room temperature. Cells were then incubated with secondary antibodyies for 60 min in dark at room temperature. The nuclei were counterstained with DAPI (C1005, Beyotime, China) for 5 min and coverslips were imaged and mounted under a fluorescent microscope. Antibodies are listed in Table S2.

Real-time RT-PCR

Total RNA of the NFs or OLP AFs was extracted using Trizol Reagent (Invitrogen, USA). Next, cDNA was obtained using a PrimeScript RT reagent kit (Takara, Japan). Real-time RT-PCR was performed using TB Green Premix Ex Taq II (Takara). U6 and GAPDH were used as internal controls for miR-155-5p and FAP-α relatively. The 2-ΔΔCt method was used for relative quantification. Primers are listed in Table S1.

miRNA and siRNA (Small interfering RNAs) transfection

All the compounds were purchased from RiboBio (Guangzhou, China). Following the manufacturer's protocol of the riboFECT™ CP Transfection Kit (RiboBio), miR-155-5p mimic (50 nmol l^{-1}), miR-155-5p inhibitor (125 nmol l^{-1}) and negative control (NC) were transfected into control NFs and OLP AFs respectively. Si-SOCS1 (siRNA#1 and siRNA#2) (100 nmol l^{-1}) and siRNA NC were transfected into NFs. Associated fibroblasts were used for the specified experiments at 48-72 h after transfection.

Western blotting assay

Protein samples were extracted with RIPA buffer (Beyotime, China) containing 10 mM protease inhibitor PMSF (Beyotime) on ice. Next, the protein was separated by 12.5% SDS-PAGE and transferred to a PVDF membrane (Millipore, USA). Afterwards, the membranes were incubated with 5% non-fat milk, followed corresponding primary antibody overnight at 4 °C and corresponding secondary antibodies for 2 h at 37°C. The blots were examined using an ECL system (NCM Biotech, China) and quantified by Image J (National Institutes of Health, USA). Antibodies are listed in Table S2.

Immunohistochemistry (IHC)

The tissue samples were standardly fixed and embedded. The mucosal sections were repaired with sodium citrate, and then blocked with 1% BSA/PBS at 37˚C for 30 min. According to the manufacturer's protocols, add anti-SOCS1 primary antibody and incubate overnight at 4 °C. Follow with anti-goat-HRP and culture at 37°C for 30 minutes. After that, add DAB (MXB Biotechnologies) chromogenic reagent, counterstain with hematoxylin, and dehydration. The strength of IHC staining was quantified by ImageJ. Antibodies are listed in Table S2.

Enzyme-linked immunosorbent assay (ELISA)

Fibroblasts from relevant groups were seeded and placed in serum-free Dulbecco's modified Eagle's medium without antibiotics after 24h. Those cells were transfected as described above for additional 48-72 h in culture medium. Then medium was then refreshed. The levels of IL-6 and IL-8 in supernatant were measured by using ELISA kits (Multi Sciences) after 24h.

miRNA target prediction

Through TargetScan (www.targetscan.org), a bioinformatics prediction program employed to predict miRNA targets, we identified the potential target of miR-155-5p.

Dual-Luciferase Reporter Assay

According to the manufacturer’s instructions, the pLUC firefly luciferase vectors contained wild type or mutant SOCS1 sequence were co-transfected into HEK 293T cells together with firefly luciferase plasmid pGL3 (RiboBio) and miR-155-5p transfection reagent. Dual-Luciferase Reporter Assay System (Vazyme, China) measured the luciferase activity after 48 h transfection.

Statistical analysis

Data are expressed as a mean ± standard deviation of three independent experiments. Statistical analysis was performed using the GraphPad Prism 8.0.2 (San Diego, CA, USA). The Student’s t test was used for statistical analysis with 2 groups. A one-way analysis of variance was performed to detect the data comparison of more than two groups. Pearson’s correlation coefficient was applied for detecting the correlations among miR-155-5p and FAP-α. $p < 0.05$ was considered significant.

Results

Primary cultured NFs and OLP AFs are of fibroblast origin *in vitro*

Primary cultured fibroblasts were isolated from oral mucosa of healthy controls and OLP groups. As shown in Fig.1, immunofluorescence revealed vimentin was expressed in primary cultured fibroblasts, while no keratin was detected. These data indicate that the cultured fibroblasts are of fibroblast origin, but not epithelial cell origin. The cultured fibroblasts were used for subsequent experiments.

MiR-155-5p and FAP-α expression levels are upregulated and positively correlated in OLP AFs

The expression levels of miR-155-5p and FAP-αwere detected in fibroblasts by real-time RT-PCR. MiR-155-5p and FAP-α were significantly upregulated in NEOLP AFs and EOLP AFs compared with NFs (Fig. 2a, p=0.0106, p<0.001; Fig. 2b, p=0.0041, p<0.001), whereas no significant difference was observed between the NEOLP group and EOLPs group. Furthermore, the miR-155-5p expression was found to be significantly related with the FAP-α expression using the correlation analysis (Fig. 2c, p< 0.001, r= 0.8145).

MiR-155-5p promotes cytokine production in NFs and OLP AFs

The release of IL-6 and IL-8 in supernatants of OLP AFs were significantly higher than that in NFs (Fig. 3a, p=0.0072, p<0.001; Fig. 3b, p=0.0042, p<0.001), while no differences were found between the NEOLP group and the EOLP group. In order to investigate the role of miR-155-5p in the cytokine production of OLP AFs, miR-155-5p mimic and inhibitor were transfected into NFs and OLP AFs respectively. MiR-155-5p overexpression in NFs augmented IL-6 and IL-8 release (Fig. 3c, p=0.0420; Fig. 3d, p=0.0062). Moreover, knockdown of miR-155-5p in OLP AFs reduced IL-6 and IL-8 release (Fig. 3e, p=0.0368; Fig. 3f, p=0.0232). These findings reveal that miR-155-5p can promote the cytokine secretion in OLP AFs.

SOCS1 was identified as a potential target of miR-155-5p in OLP.

To understand the potential mechanisms of miR-155-5p involved in the inflammatory behavior of OLP AFs, TargetScan prediction program (www.targetscan.org) was used to select potential target genes of miR-155-5p (Fig. 4a). We identified SOCS1 as an underlying miR-155-5p target in OLP AFs. Moreover, IHC showed that the staining intensity of SOCS1 in epithelial layer of healthy controls was stronger than that in the OLP group (Fig. 4b, p<0.001), but there was no significant difference between the NEOLP group and the EOLP group. In addition, the expression of SOCS1 decreased in NEOLP AFs and EOLP AFs than in NFs (Fig. 4c; Fig. 4d, p=0.0106, p=0.0095). No difference of SOCS1 expression was detected between NEOLP AFs and EOLP AFs. Taken together, these results suggest that SOCS1 is a potential target of miR-155-5p in OLP.

MiR-155-5p inhibits SOCS1 expression by directly targeting its 3'-UTR

To determine the interaction between miR-155-5p and SOCS1, wild-type and mutant pmiR-RB-SOCS1^{TM}-3' UTR vectors incorporating miR-155-5p binding site were constructed for dual-luciferase reporter assay. After co-transfected with the wild-type vector and miR-155 mimic, luciferase activity was significantly decreased (Fig. 5a, p=0.0041), whereas no difference was found after transfected with the mutant vector, indicating that miR-155-5p could directly bind to SOCS1 mRNA. Furthermore, western blotting assay detected that SOCS1 expression is decreased with transfection of miR-155-5p mimic in NFs (Fig. 5b, p<0.001), but is increased with transfection of miR-155-5p inhibitor in OLP AFs (Fig. 5c, p=0.0204). These results suggest that miR-155-5p directly regulates SOCS1 in OLP AFs.

SOCS1 inhibits pro-inflammatory cytokine release in NFs

SOCS1 is known as an inflammatory feedback inhibitor. Western blotting assay detected that SOCS1 expression was decreased in OLP AFs (Fig. 6a, b, p=0.0203, p=0.0018). To explore whether SOCS1 could regulate the cytokine release, we knocked down the expression of SOCS1 in NFs and determined the effects. A significant increase of IL-6 and IL-8 release was detected after SOCS1 knockdown, supporting the hypothesis that SOCS1 influence the cytokine production of fibroblasts in oral mucosa (Fig. 6c, p=0.0280, p=0.0037; Fig. 6d, p=0.0147, p=0.0201).

Discussion

Previous studies demonstrate that OLP AFs can acquire activated phenotype and participate in local inflammatory and angiogenic processes. However, studies on OLP-AFs are still scarce [23]. Fibroblasts synthesize the extracellular matrix of connective tissue and play an essential role in wound healing and fibrosis [7, 24]. Fibroblast heterogeneity can be influenced by specific tissue microenvironment [25, 26]. Moreover, activated fibroblasts are reported to involve in inflammation and cancer processes, including the production of pro-inflammatory cytokines and the ability of regulating leukocyte recruitment or retention in tissues [27, 28].

FAP-α is a homodimeric integral membrane gelatinase belonging to the serine protease family [29]. Abnormal expression of FAP-α can be used as a marker of fibroblast activation, but it can hardly be detected in healthy tissues [28]. Additionally, FAP-α is thought to be involved in the control of fibroblast growth and epithelial-mesenchymal interactions during development, tissue repair, and epithelial carcinogenesis [30–32]. Furthermore, recent studies have shown that FAPα + fibroblast population is essential to the pathogenesis of autoimmune diseases such as Sjogren's syndrome, rheumatoid arthritis and Crohn's disease [28, 33, 34]. Interestingly, we found the expression of FAP-α in OLP AFs was significantly increased compared with NFs by real-time RT-PCR. However, there was no significant difference of FAP-α expression between the NEOLP and EOLP groups, which was consistent with previous reports [13, 23]. This suggest that a subset of OLP-AFs acquire active phenotypes and may be involved in the pathogenesis of OLP.

The expression of miR-155-5p in OLP AFs was significantly higher than that in NFs, but no difference was found between NEOLP and EOLP group. Elevated expression of miR-155-5p has been observed in the lesions and peripheral blood mononuclear cells of patients with OLP [20]. Here we report the abnormal expression of miR-155-5p in OLP AFs for the first time.

MiR-155-5p is encoded with an exon of the B cell integration cluster gene and is closely associated with inflammation, tumor development and immune regulation [35, 36]. The expression of miR-155-5p is positively correlated with cytokine release [18, 37, 38]. ELISA results revealed that the secretion of IL-6 and IL-8 by OLP AFs was higher than NFs, whereas the level in NEOLP group had no difference with the EOLP group. Furthermore, miR-155-5p overexpression in NFs increased IL-6 and IL-8 secretion, whereas knockdown the expression of miR-155-5p in OLP AFs resulted in decreased secretion of IL-6 and IL-8. Our

results suggest that abnormal expression of miR-155-5p in activated OLP AFs plays a role in the secretion of pro-inflammatory cytokines. Interestingly, miR-155-5p expression was found to be correlated with FAP-α expression in OLP AFs. This implicate that the activated phenotype of OLP AFs may be linked with miR-155-5p. In addition, we speculate that OLP AFs can be one of the cell sources of miR-155-5p dysregulation and co-participate in the formation of local inflammatory microenvironment in OLP lesion.

In this study, we found that miR-155-5p was closely related to pro-inflammatory behavior in OLP AFs. Thus, validation of target genes was focused on candidate genes known to be involved in inflammatory processes. Here, we identified SOCS1 as a direct target of miR-155-5p. SOCS1 is a physiological negative regulator of cytokines which is essential to maintain immune homeostasis and subvert inflammation [39–41]. The miR-155/SOCS1 axis exerts an important role in immune system regulation by mediating several signaling pathways [20, 42]. SOCS1 directly binds to the activation ring of JAK and inhibits the activation of the JAK/STAT pathway [43]. SOCS1 inhibits NF-κB by decreasing the stability of p65 in the nucleus [44], thereby inhibiting the release of inflammatory cytokines including IL-6 and IL-8. MiR-155-5p affects the above pathways by regulating SOCS1, and can directly target SOCS1 to positively drive differentiation of Treg/Th17 cells and promote competition and proliferation of Tregs [45]. In addition, SOCS1 expression affects the ratio of Th1 and Th2 cell differentiation [46].

Dual-luciferase reporter assay results proved that SOCS1 had a conservative binding site with miR-155-5p. The results of IHC demonstrated that the expression level of SOCS1 in normal tissues was significantly higher compared with OLP tissues. The expression of SOCS1 in the NFs was higher than that in OLP AFs by western blotting assay, but no differences were found between NEOLP and EOLP groups. SOCS1 may be involved in the function of OLP AFs as a target gene. We found that overexpression of miR-155-5p in NFs resulted in downregulation of SOCS1 expression and knockdown of miR-155-5p in OLP AFs lead to increase of SOCS1 expression. Finally, the knockdown of SOCS1 increased the secretion of IL-6 and IL-8 in NFs. These data prove that miR-155-5p promotes pro-inflammatory cytokine release in OLP AFs by targeting SOCS1.

Conclusion

Our results revealed that a portion of fibroblasts acquire an active phenotype in chronic inflammatory environments and participate in promoting local inflammation through miR-155-5p. Taken together, miR-155-5p promotes local inflammatory response in OLP AFs by targeting SOCS1. Our results provide novel insights into the pathogenesis of OLP and a new target for the therapy of OLP.

Declarations

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Conflicts of interest

The authors have no conflicts of interest to declare.

Availability of data and material

The relative data are included within the article and its additional files.

Code availability

Not applicable.

Authors' contributions

JC and YZ recruited volunteers and collected specimens with help from KL, JX and JC, YZ and KL conducted the experiments. JC and YZ analyzed the data and drafted the manuscript with the help of YW. All authors read and approved the final manuscript.

Ethics approval

This work was approved by the Ethical Committee of Affiliated Hospital of Stomatology, Nanjing Medical University (permission number PJ2016-034-001) and the Institutional Review Board of Nanjing Medical University (permission number 2014-132).

Consent to participate

The patients/participants agreed to participate in this study by signing informed consent.

Consent for publication

Not applicable.

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Tables

Table 1 No difference was found of in age (p=0.2624) and gender (p=0.6596) of each group.

Characteristics	Healthy controls (n = 15)	NEOLP patients (n = 15)	EOLP patients (n = 15)
Age (mean ± SD)	39.13±3.053	43.00±3.147	43.40±2.651
Male/female	6/9	6/9	4/11

Table 2 No difference was found of in age (p=0.1334) and gender (p=0.7520) of each group.

Characteristics	Healthy controls (n = 15)	NEOLP patients (n = 15)	EOLP patients (n = 15)
Age (mean ± SD)	40.27±2.80	42.27±2.583	48.27±3.175
Male/female	6/9	7/8	8/7

Figures

Fig.1

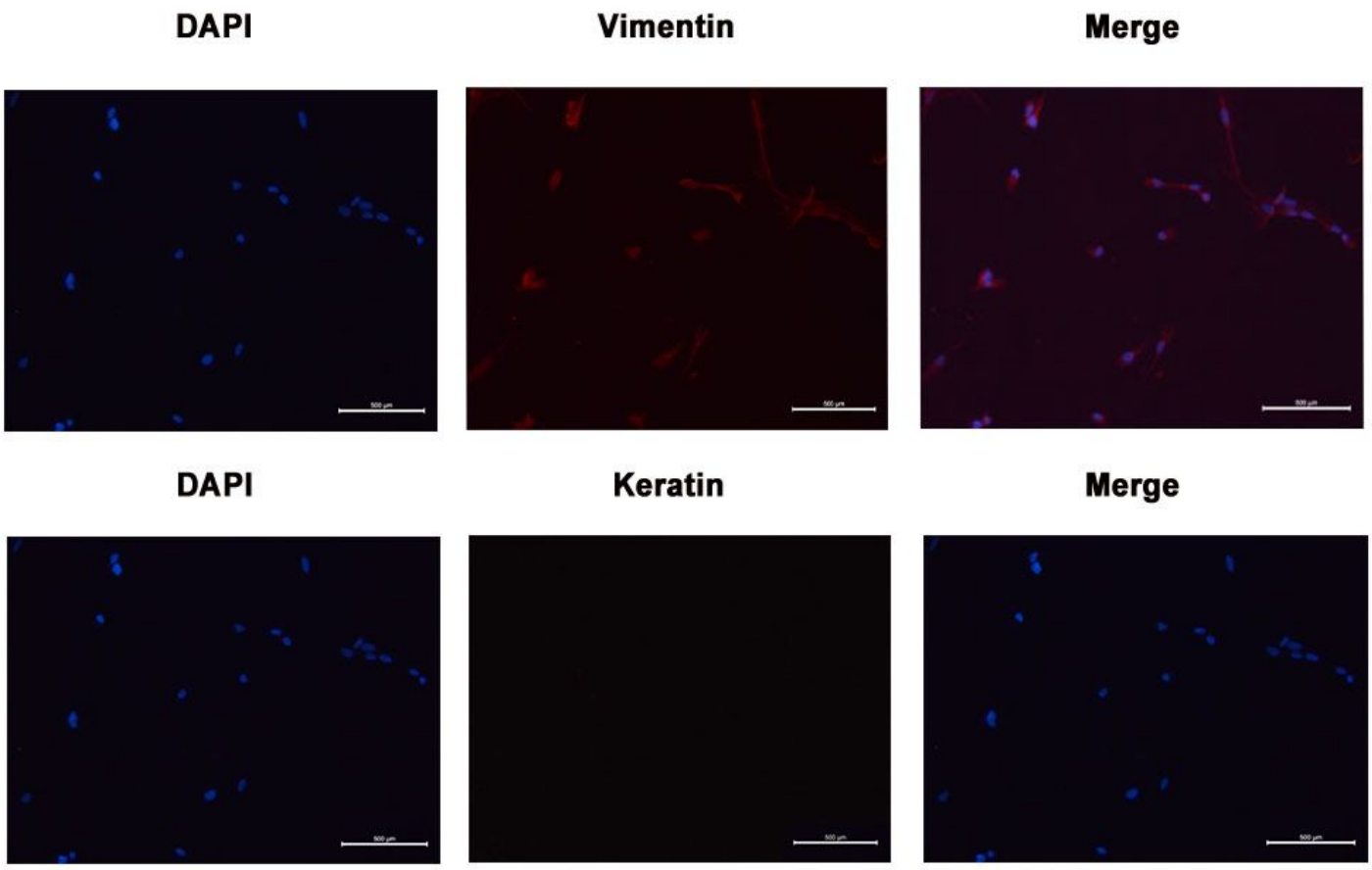

Figure 1

Primary cultured NFs and OLP AFs are of fibroblast origin in vitro. Immunofluorescence for keratin and vimentin in primary cultured cells isolated from healthy controls and OLP.

Fig.2

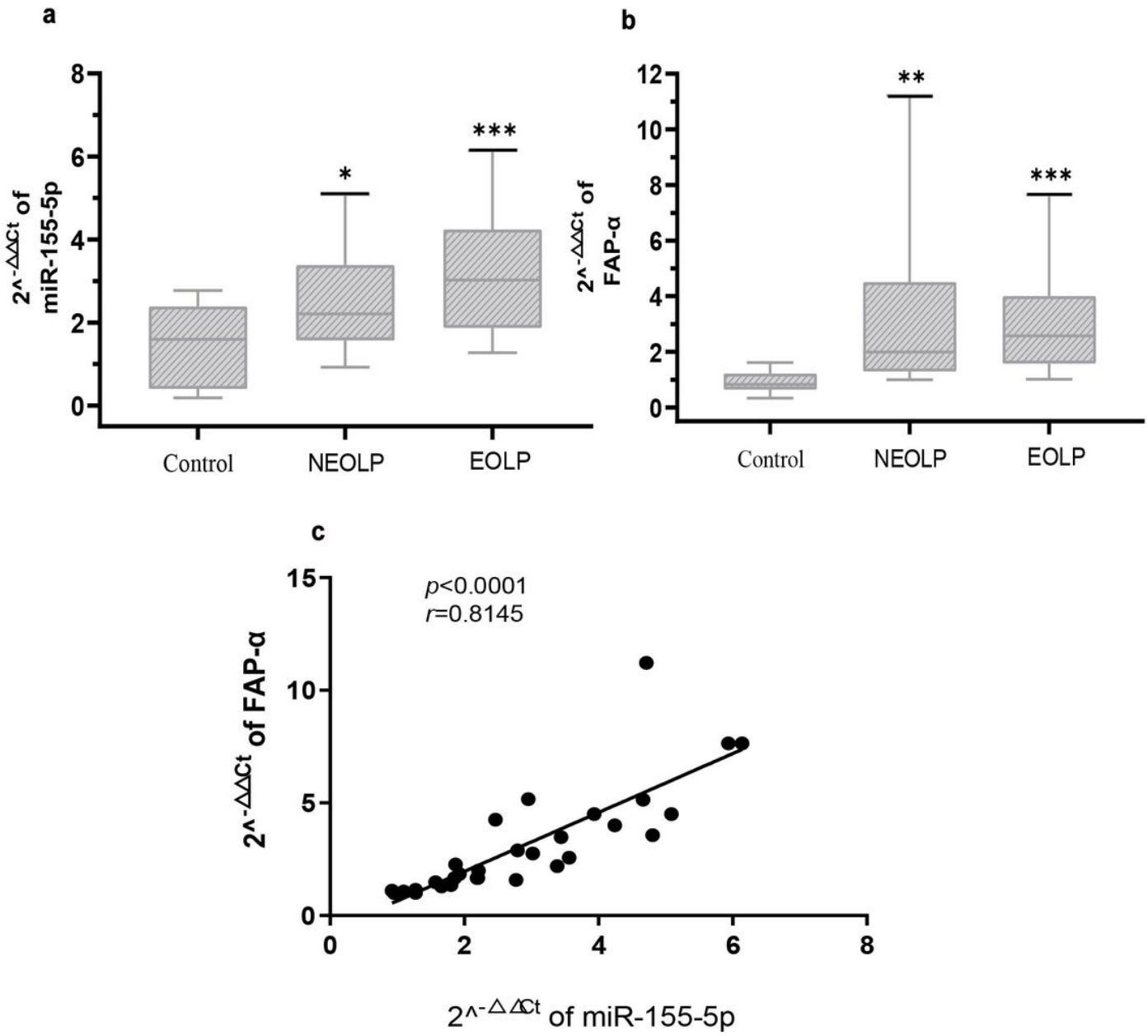

Figure 2

MiR-155-5p and FAP-α expression levels are upregulated and positively correlated in OLP AFs. (a, b) The relative expression of miR-155-5p and FAP-α were detected by real-rime RT-PCR, and results were represented as box plots (box, 25–75% whisker, 10–90% line, median). (c) Pearson's correlation test was applied to observe the correlation between FAP-α mRNA expression and miR-155-5p level. *p < 0.05; **p < 0.01; ***p < 0.001.

Fig.3

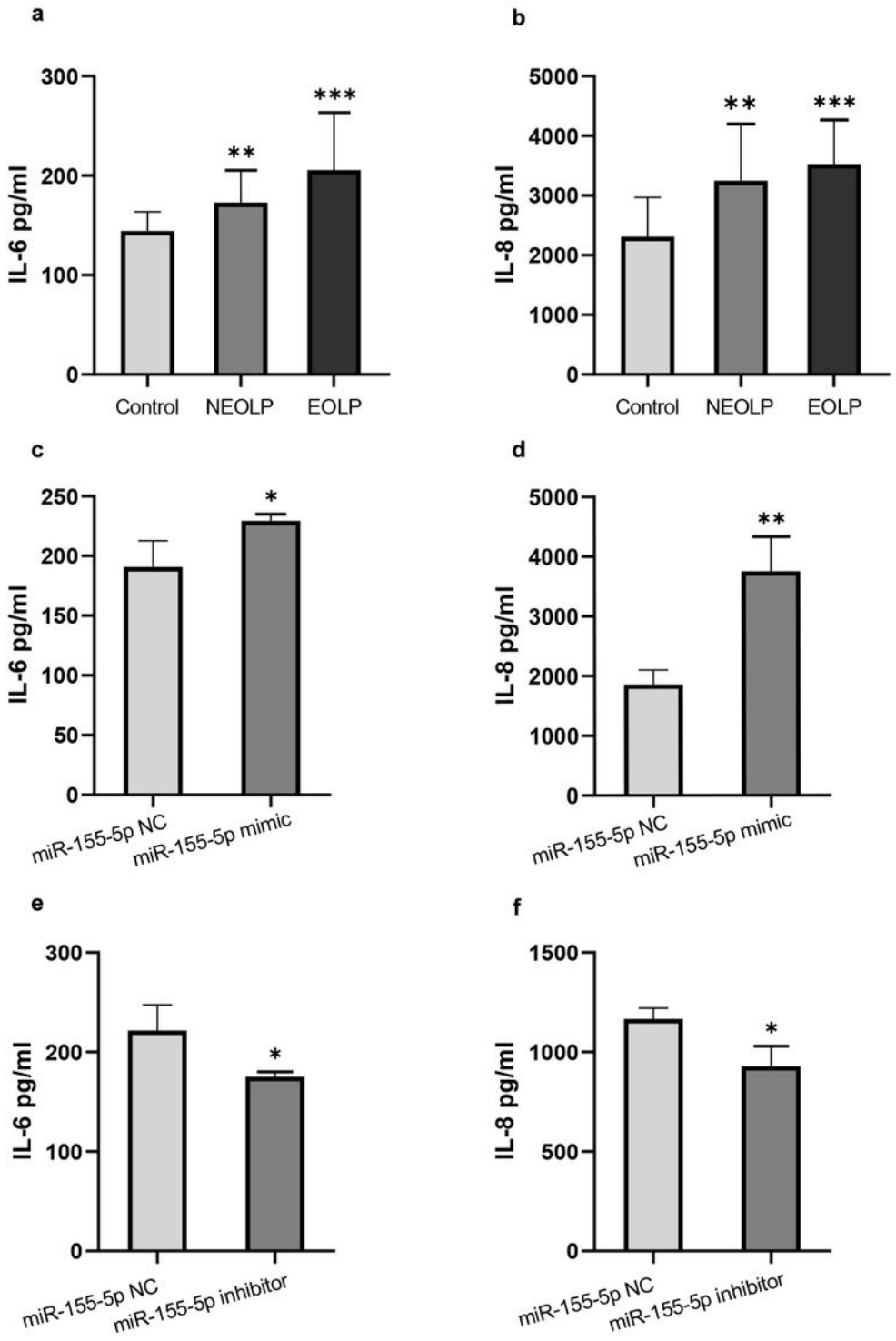

Figure 3

MiR-155-5p promotes cytokine production in NFs and OLP AFs. (a-b) NFs and OLP AFs (from NEOLP and EOLP groups) (n=6 respectively) were incubated in medium and supernatants were collected after 24 h. (c-d) NFs (n=3) were transfected with miR-155-5p mimic and culture media were collected after 48 h. (e-f) OLP AFs (n=3) were transfected with miR-155-5p inhibitor and culture media were collected after 48 h. IL-6 and IL-8 levels in the supernatants were determined by ELISA. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

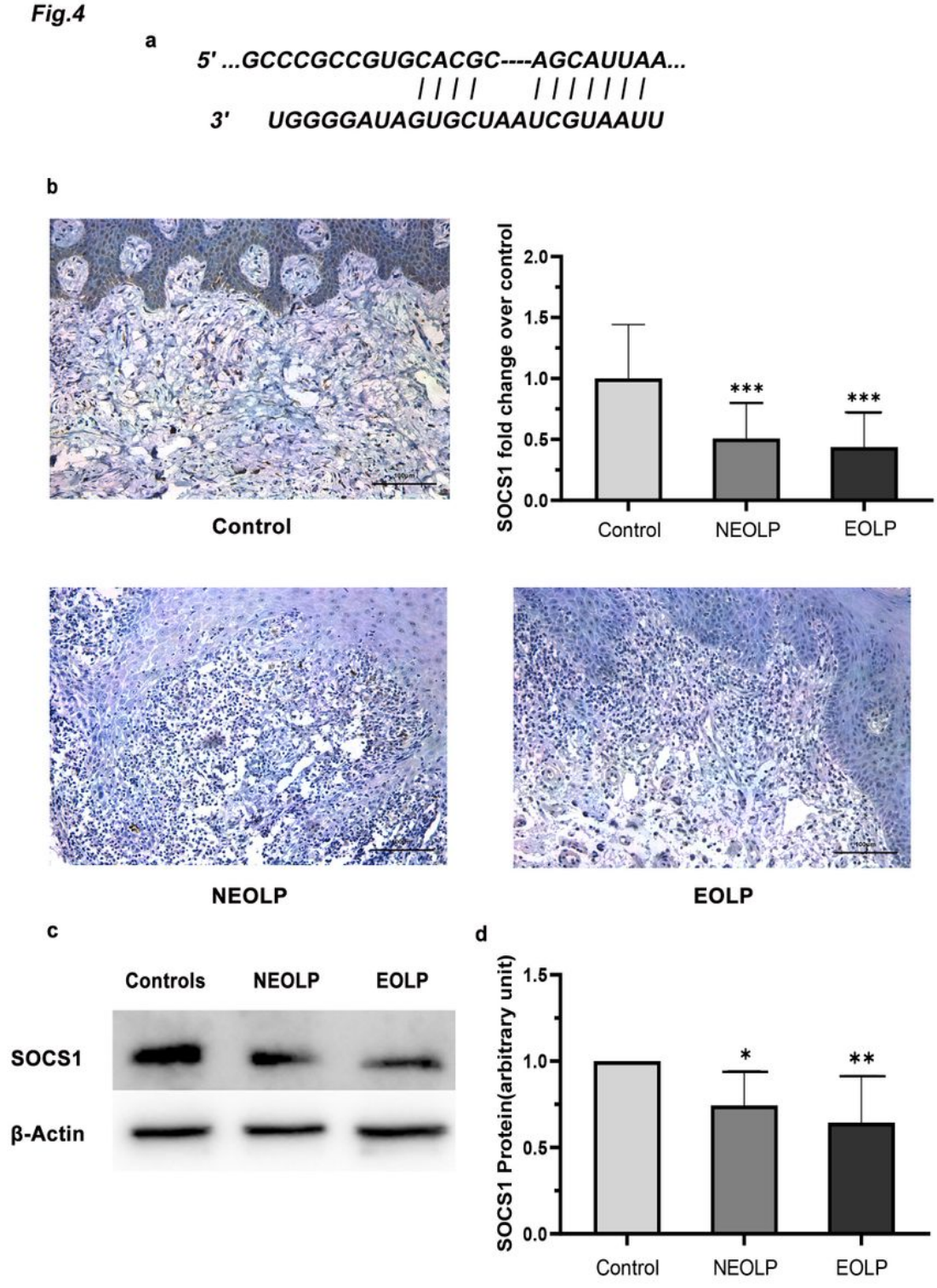

Figure 4

SOCS1 was identified as a potential target of miR-155-5p in OLP. (a) Schematic representation of putative miR-155-5p-binding site in the 3'-untranslated region (3'-UTR) of SOCS1. (b) The images (x200) and quantification analysis of SOCS1 in OLP group and control group was examined using IHC in the epithelial layers (n=15 respectively). (c-d) Western blotting assay was used to explore the protein level of

SOCS1 in NFs and OLP AFs. β-actin was used as an endogenous control (n=6). *p < 0.05; ***p < 0.001. Scale bar:100 μm.

Fig.5

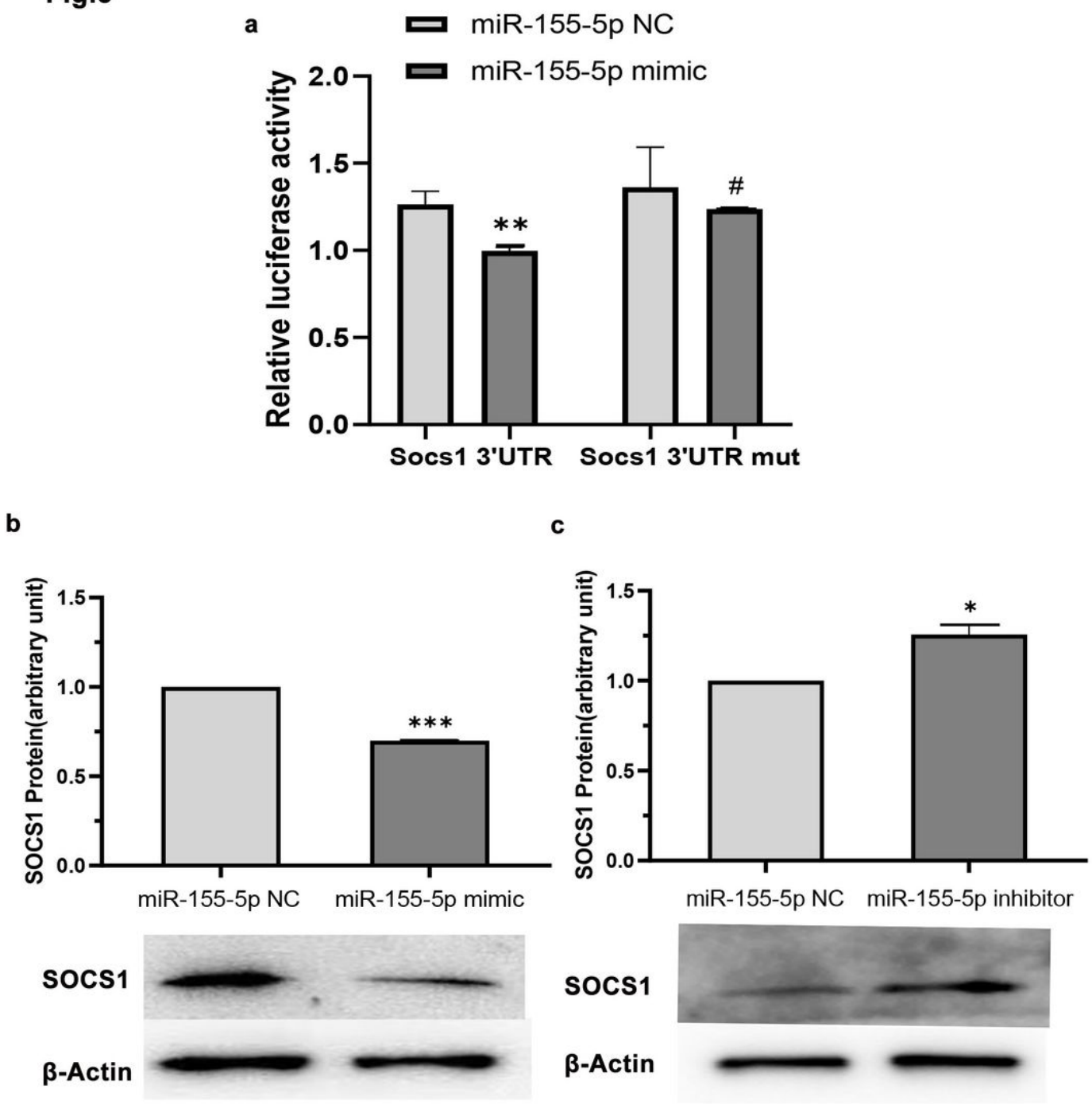

Figure 5

MiR-155-5p inhibits SOCS1 expression by directly targeting its 3'-UTR. (a) Luciferase activity was detected after co-transfected with reporter plasmid (pmiR-RB-SOCS1 3'-UTR wild or pmiR-RB-SOCS1 3'-UTR mut) and miR-155-5p transfection reagent (miR-155-5p mimic or miR-155-5p mimic NC) respectively.

(b) The SOCS1 expression of NFs following miR-155-5p mimic transfection was analyzed by western blotting assay (n=3). (c) The SOCS1 expression of OLP AFs following transfection with miR-155-5p inhibitors or miR-155-5p inhibitors NC was analyzed by western blotting assay (n=3). *p < 0.05; **p < 0.01; ***p < 0.001.

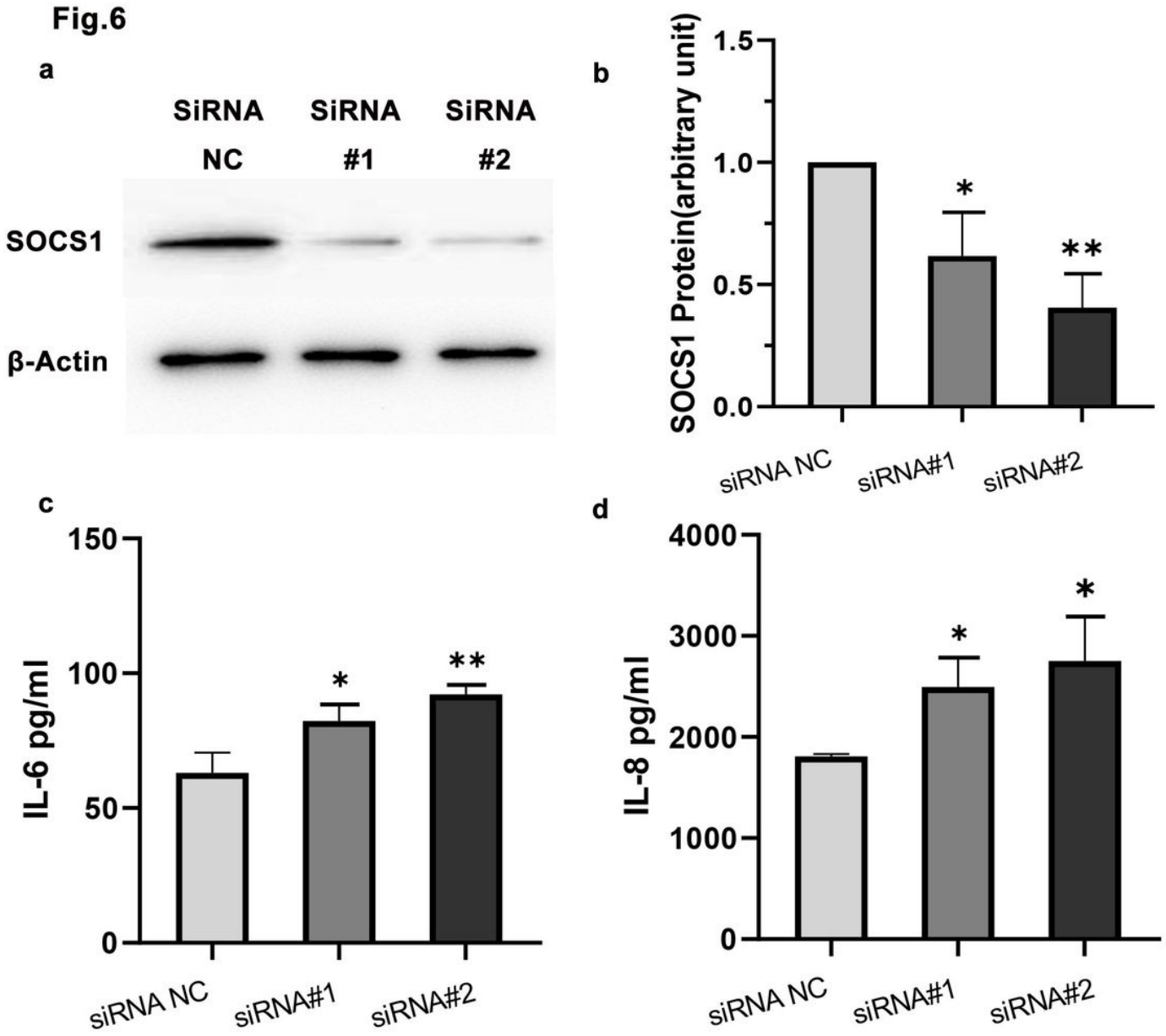

Figure 6

SOCS1 inhibits pro-inflammatory cytokine release in NFs. (a-b) The SOCS1 expression was determined using western blotting assay in NFs transfected with si-SOCS1 (siRNA#1 and siRNA#2) or siRNA NC (n=3). (c-d) IL-6 and IL-8 secretion were determined by ELISA in the supernatants of NFs transfected with si-SOCS1 (siRNA#1 and siRNA#2) or siRNA NC (n=3). *p <0.05; **p <0.01.

Supplementary Files

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- SupplementaryMaterials.docx