SOCS3, Transcriptionally Activated by NR4A1, Induces Apoptosis and Extracellular Matrix Degradation of Vaginal Fibroblasts in Pelvic Organ Prolapse

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INTRODUCTION

Pelvic organ prolapse (POP), a common gynecological disease among females, is characterized by the protrusion of the pelvic organs (vagina, uterus, bladder, and/or rectum) into or out of the vaginal canal. Treatment options for POP include pelvic floor muscle training, vaginal pessaries, and reconstructive pelvic surgery. However, the therapeutic outcomes are often unsatisfactory and relapse is a common occurrence. Therefore, it is imperative to elucidate the pathogenesis of POP to develop effective treatments for this condition.

The most common form of POP is anterior vaginal wall prolapse, commonly known as cystocele. In the vaginal wall, connective tissues provide the supportive strength for the pelvic floor. The connective tissue layer consists of an extracellular matrix (ECM), which obtains its strength from collagen I, collagen III, and elastin. Vaginal fibroblasts control ECM production and remodeling, by generating anabolic proteins, like collagens, and activating catabolic enzymes, such as matrix metalloproteinases (MMPs), thus modifying their surrounding matrix. In patients with POP, fibroblast dysfunction leads to damaged collagen metabolism and an unbalanced disturbed ECM production. Additionally, some studies have confirmed increased fibroblast apoptosis in POP. However, there is a limited number of studies that focus on the underlying mechanism. Hence, enhancing the functions of fibroblasts emerges as a viable approach to impede the progression of POP.

Background: Pelvic organ prolapse (POP) is a common gynecological chronic disorder. Human vaginal fibroblasts (HVFs) that maintain the integrity of vaginal wall tissues are essential for keeping pelvic organs in place. Apoptosis and the degradation of the extracellular matrix in HVFs contribute to the progression of POP. The cytokine signal transduction inhibitor 3 (SOCS3) exerts significant regulatory effects on cell signal transduction pathways, thereby affecting various pathological processes.

Aims: To explore the role and mechanism of SOCS3 on HVFs in the context of POP.

Study Design: In vitro cell lines and human-sample study.

Methods: Anterior vaginal wall tissues were obtained from POP or non-POP patients for the analysis of SOCS3 expression. HVFs were isolated from the vaginal tissues of POP patients, and SOCS3 was either overexpressed or knocked down in HVFs via lentivirus infection. Subsequently, the biological function and mechanism of SOCS3 in HVFs were investigated.

Results: SOCS3 was highly expressed in the vaginal tissues of POP patients compared to non-POP patients. Functionally, the overexpression of SOCS3 suppressed cell viability while promoting cell apoptosis in HVFs. The overexpression of SOCS3 also accelerated extracellular matrix degradation (decreasing collagen I, collagen III, and elastin, and increasing MMP2 and MMP9). In terms of mechanism, NR4A1 transcriptionally activated SOCS3 by binding to its promoter. Furthermore, rescue experiments revealed that SOCS3 knockdown hindered NR4A1 overexpression-induced cell apoptosis and extracellular matrix degradation in HVFs.

Conclusion: SOCS3 mediated the apoptotic and extracellular matrix degradation effects of NR4A1 on HVFs, underlining that the restraining of the SOCS3 expression may be a promising strategy for POP treatment.
The cytokine signal transduction inhibitor 3 (SOCS3), one of the SOCS family proteins, interacts with various activated proteins of the tyrosine kinase signaling pathway. SOCS3 suppresses cell proliferation and its deletion mitigates myocardial apoptosis and fibrosis. Moreover, the downregulation of SOCS3 promotes collagen release and aggravates fibrosis in murine models. Database analysis showed an upregulation in SOCS3 expression in the anterior vaginal wall tissue of patients with POP. Therefore, SOCS3 may participate in the development of POP.

Human nuclear receptor 4A 1 (NR4A1) is an orphan nuclear receptor associated with fibrosis of multiple organs. NR4A1 limits fibrotic diseases by regulating transforming growth factor-β (TGF-β) signaling. Hiwatashi et al. reported that NR4A1 inhibits vocal fold fibrosis, while NR4A1 knockdown promotes collagen I expression in fibroblasts. NR4A1 also participates in the pathological progression of POP by promoting the apoptosis of fibroblasts. Furthermore, 9-AA, an agonist of NR4A1, enhances the expression levels of SOCS3. Based on these published results, we hypothesize that the NR4A1/SOCS3 axis may play a role in the progression of POP.

In this study, we investigated the function and mechanism of SOCS3 in POP. We found that SOCS3 expression was higher in the vaginal tissues of POP patients than those in non-POP patients. SOCS3 overexpression induced cell apoptosis and ECM degradation in human vaginal fibroblasts (HVFs) from patients with POP. Additionally, we detected that SOCS3 was transcriptionally activated by NR4A1, and the effect of SOCS3 on HVFs was regulated by NR4A1. This study provides evidence for the role of SOCS3 in POP.

**MATERIALS AND METHODS**

**Tissue samples collection**

Anterior vaginal wall tissues were collected from POP patients (n = 36, age range: 47-74) and non-POP patients (n = 18, age range: 39-57). All patients participating in the study signed the informed consent. This study was conducted following the Declaration of Helsinki, and approved by the Shengjing Hospital of China Medical University Ethics Committee (2022PS040K).

**Fibroblast culture and identification**

Primary HVFs were isolated from anterior vaginal wall tissues and cultured as described previously. The cells were cultured in Dulbecco’s modified Eagle’s medium (Servicebio, China) supplemented with 10% fetal bovine serum (Sijiqing, China) at 37 °C and 5% CO₂. Cells were passaged and expanded until reaching 60% confluence, and those up to the fourth passage were used for subsequent experiments. Immunofluorescence was performed to identify cells at the fourth passage using markers for vimentin (a marker for fibroblasts), cytokeratin (a marker for epithelial cells), and desmin (a marker for smooth muscle cells).

**Lentivirus infection**

The coding sequences of SOCS3 or NR4A1 were inserted into pLVX-IRES-puro (Fenghbio, China) to generate SOCS3 overexpression lentivirus (LV-SOCS3) or NR4A1 overexpression lentivirus (LV-NR4A1). The map of pLVX-IRES-puro is shown in Figure S2a. Additionally, SOCS3-targeted shRNA was inserted into pLVX-shRNA1 (Fenghbio, China) to establish shSOCS3 lentivirus (LV-shSOCS3). The map of pLVX-shRNA1 is shown in Figure S2b. The SOCS3 shRNA sequence is as follows: 5'-GGCTCGACTGCGTCTCAAGC-3'. LV-SOCS3, LV-NR4A1, LV-shSOCS3, and their negative controls (NC) were subsequently packaged in HEK293T cells. The lentiviral-containing supernatant was harvested and filtered. HVFs were then infected with lentivirus-containing supernatants at a multiplicity of infection of 100 and incubated at 37 °C with 5% CO₂.

**Statistical analysis**

The data were analyzed using GraphPad Prism 8.0 (GraphPad Software, USA). All data were presented as the mean ± standard deviation. T-tests were employed to compare data between two groups, while one-way ANOVA with Bonferroni post-hoc test was used for comparing data involving more than two groups. P < 0.05 was deemed statistically significant.

For detailed methods, please see the Supplementary material.

**RESULTS**

SOCS3 is highly expressed in the vaginal tissues of POP patients

Built on the differential expression analysis of the gene expression omnibus database, we found 265 upregulated and 13 downregulated genes in patients with POP. Subsequently, GO enrichment analysis of differential expression genes (DEGs) revealed an association among POP, ECM, and cell apoptosis (Figure 1a). In these DEGs, SOCS3 knockdown inhibits cell apoptosis and promotes collagen release, suggesting that SOCS3 may be involved in the progression of POP. In addition to the GSE12852 dataset, the high expression of SOCS3 in POP tissues was observed in the GSE53868 dataset (Figure 1b). To verify our conjecture, we collected vaginal tissues from POP and non-POP patients. Then, Masson trichrome staining and TUNEL staining were performed to explore the histomorphological differences. The results of Masson trichrome staining showed decreased collagen (blue) in tissues of POP patients compared with non-POP patients (Figure 1c). TUNEL staining revealed a higher incidence of apoptosis in tissues from POP patients (Figure 1d). These results indicated more prominent collagen degradation and apoptosis in patients with POP, consistent with previous reports and our bioinformatics analysis. To further investigate the role of SOCS3 in POP, we detected SOCS3 mRNA expression in vaginal tissues. The results showed that SOCS3 was significantly upregulated in patients with POP (Figure 1e). Western blot analysis further verified the upregulation of SOCS3 at the protein level (Figure 1f). Moreover, IHC and the histologic score (H-score) were performed to further verify the expression pattern of SOCS3 in POP. The results showed higher SOCS3 expression levels in the POP group compared with the non-POP group (Figure 1g-h). Furthermore, we analyzed the association between the expression levels of SOCS3 and patient characteristics in Table S1;
however, no correlation was observed. These results suggested that SOCS3 was upregulated in the vaginal tissues of patients with POP.

**Morphological identification of HVFs**

Primary HVFs obtained from the vaginal tissues of POP patients were cultured and collected for microscopic observation (Figure S1a). Then, the fourth passage of HVFs was subjected to immunofluorescence staining to identify the phenotype using specific biomarkers (Figure S1b). The results revealed high cytoplasmic expression of vimentin (a marker of fibroblasts) in the fourth passage of HVFs. In contrast, no significant increase in the expression of cytokeratin (a marker of epithelial cells) and desmin (a marker of smooth muscle cells) was observed. The above results illustrated that cells isolated from vaginal tissues were mainly fibroblasts without the contamination of epithelial and smooth muscle cells.

**SOCS3 overexpression inhibits viability and induces apoptosis in HVFs**

Considering the increased expression of SOCS3 in POP, the function of SOCS3 in POP was further investigated. SOCS3 was overexpressed in the fourth passage of HVFs through lentivirus infection, and the gene overexpression was confirmed with RT-qPCR and western blot (Figure 2a, b). Subsequently, the results from the CCK-8 assay showed that SOCS3 overexpression inhibited the cell viability of HVFs (Figure 2c). Additionally, a significantly higher apoptosis rate was observed in SOCS3 overexpression groups compared to the control groups (Figure 2d). The TUNEL assay further verified the above results, with more TUNEL-positive cells observed in SOCS3 overexpression HVFs (Figure 2e). In addition, pro-apoptotic protein Bax and cleaved-caspase-3, as well as anti-apoptotic protein Bcl-2, were detected by western blot. The expression levels of Bax and Bcl-2 were quantified (Figure 2f). The results were presented as the mean ± SD.

**SD,** Standard deviation

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**FIG. 1.** SOCS3 is upregulated in the anterior vaginal wall tissues of patients with pelvic organ prolapse (POP). (a) A volcano plot of differentially expressed genes (DEGs) and GO enrichment analysis of DEGs based on the GSE12852 dataset. (b) The expression level of SOCS3 in anterior vaginal wall tissues and uterosacral ligament tissues of patients with POP from gene expression omnibus (GEO). (c) Collagen deposition in anterior vaginal wall tissues of POP and non-POP patients was detected by Masson trichrome stain. (d) Apoptotic protein expression was detected by TUNEL assay. (e) The mRNA expression of SOCS3 was detected by RT-qPCR. (f) The protein expression of SOCS3 was detected by western blot. (g) SOCS3 expression was detected by immunohistochemistry (IHC), and SOCS3 expression scores were shown in (h). The results were presented as the mean ± SD.

**FIG. 2.** SOCS3 overexpression inhibits viability and induces apoptosis in human vaginal fibroblasts (HVFs). HVFs isolated from patients with POP were infected with SOCS3 overexpression lentivirus (LV-SOCS3) or the negative control (LV-NC), 48 h postinfection, (a) the mRNA level was detected by RT-qPCR. (b) The protein expression level was detected by western blot. (c) Cell viability was detected by CCK-8 assay. (d) Cell apoptosis was detected by flow cytometry. (e) Cell apoptosis was detected by TUNEL staining. (f) The protein expression levels of Bax, Bcl-2, and cleaved-caspase 3. (g) Quantification of the protein levels of Bax, Bcl-2, and cleaved-caspase 3. The results were presented as the mean ± SD.

**SD,** Standard deviation
cleaved-caspase 3 increased, whereas that of Bcl-2 decreased, as observed after SOCS3 overexpression (Figure 2f, g). These results suggested that SOCS3 overexpression inhibited viability and induced apoptosis of HVFs.

**SOCS3 overexpression induces ECM degradation in HVFs**

Previous experiments validated the significance of collagen degradation as a prominent characteristic in patients with POP. Thus, to explore whether SOCS3 participates in the regulation of ECM components, the protein expression of collagen I, collagen III, and elastin was analyzed. The results revealed that SOCS3 overexpression reduced the expression of collagen I, collagen III, and elastin (Figure 3a). Besides, we detected the protein expression of MMP2 and MMP9. Collagen-degrading MMPs are involved in ECM remodeling processes, as shown in Figure 3b, increased expression of MMP2 and MMP9 was observed after SOCS3 overexpression. Moreover, the expression of components related to collagen synthesis pathways, including TGF-β, Smad2/3, and phosphorylated Smad2/3 (p-Smad2/3) was detected. We found that TGF-β1 and p-Smad2/3 showed low expression in the SOCS3 overexpression HVFs, and the expression of Smad2/3 was almost unchanged (Figure 3c, d). Together, all the signs indicated that the upregulation of SOCS3 caused ECM degradation in HVFs.

**SOCS3 is transcriptionally activated by NR4A1 via binding the SOCS3 promoter**

NR4A1 accelerates the pathological process of POP by inducing fibroblast apoptosis. To investigate the relationship between SOCS3 and NR4A1, we constructed NR4A1-overexpressing lentivirus (LV-NR4A1) to infect HVFs. A clear rise of NR4A1 mRNA and protein expression was shown in HVFs (Figure 4a, b). NR4A1 overexpression was accompanied by increased SOCS3 mRNA and protein levels (Figure 4c, d), suggesting that SOCS3 expression might be regulated by NR4A1. Based on the above results, we predicted NR4A1-binding sites on the SOCS3 promoter (Figure 4e), and the interaction between NR4A1 and SOCS3 was further confirmed by the dual luciferase reporter assay. As shown in Figure 4f, when fragment A (-1948, -1585 bp, containing four sites) or fragment B (-1585, -643 bp, containing two sites) was truncated, the luciferase activity was decreased. Moreover, when there was only fragment C (-643 to +36, containing one site), the promoter activity was increased compared with the control vector. These results indicated that the regions from -1948 to -1585 bp, -1585, -643 bp, and -643 to +36 bp contained binding sites of NR4A1. The above results suggest that NR4A1 transcriptionally activates SOCS3 by binding to its promoter.

**NR4A1 overexpression induces cell apoptosis and ECM degradation in HVFs**

To further verify the effects of NR4A1 on POP, we assessed NR4A1 expression in vaginal tissues from POP and non-POP patients. Higher NR4A1 expression at the mRNA level was observed in patients with POP (Figure 5a). IHC staining and H-score further confirmed that NR4A1 expression was higher in patients with POP (Figure 5b, c). However, the expression level of NR4A1 had no association with the characteristics of POP (Table S2). Subsequently, NR4A1 was overexpressed in the HVFs via lentivirus infection. NR4A1 overexpression suppressed cell viability (Figure 5d) and...
promoted cell apoptosis in HVFs (Figure 5e, f). In addition, the protein expression levels of collagen I, collagen III, and elastin were attenuated in NR4A1-overexpression HVFs (Figure 5g), indicating that NR4A1 promoted ECM degradation.

**SOCS3 mediates the effect of NR4A1 on HVFs**

To determine whether SOCS3 mediates the effect of NR4A1 on HVFs, HVFs were co-infected with NR4A1 overexpression lentivirus and shSOCS3 lentivirus. The results showed that SOCS3 knockdown increased cell viability and decreased cell apoptosis in NR4A1 overexpression HVFs (Figure 6a, b). Furthermore, upregulated protein expression of collagen I, collagen III, and elastin was observed when SOCS3 was knocked down (Figure 6c). Together, these results revealed that the function of NR4A1 on HVFs was blocked via SOCS3 knockdown, indicating that NR4A1 affected HVFs by SOCS3-mediated apoptosis and ECM degradation in HVFs.

**The screening of potential genes regulated by SOCS3**

RNA-seq analysis was further performed to detect the possible mechanism of SOCS3 on HVFs. Initially, principal component analysis exhibited significantly different component profiles between SOCS3 overexpression (LV-SOCS3) and control (LV-NC) groups (Figure 7a). DEGs were identified by volcano plot filtering (Figure 7b). We identified 203 downregulated and 914 upregulated DEGs. Then, DEGs were significantly enriched for POP-related GO or KEGG terms, such as ECM organization, programmed cell death, and collagen catabolic process (Figure 7c). Further analysis identified DEGs involved in these processes, presented in a heatmap (Figure 7d). Gene Set Enrichment Analysis (GSEA) confirmed significant enrichment of DEGs involved in the regulation of ECM organization and collagen catabolic process (Figure 7e). These findings suggested that SOCS3 may regulate ECM and collagen-related downstream signaling.

**DISCUSSION**

POP, characterized by a descent of pelvic organs into the vaginal cavity, significantly impairs the quality of life for many women. However, the etiopathogenesis of POP remains poorly understood. Anterior vaginal wall prolapse is the most common form of POP. Vaginal fibroblasts maintain the integrity of vaginal wall tissues, which is important for supporting pelvic organs and preventing POP. Numerous studies have demonstrated that fibroblast apoptosis and ECM loss play critical roles in the progression of POP. In our study, we selected vaginal tissues as experimental subjects to explore the pathogenesis of POP. As expected, the vaginal tissues showed decreased collagen deposition and enhanced apoptosis in patients with POP. Subsequently, through bioinformatics analysis, we observed an elevated expression of SOCS3 in patients with POP, characterized by a descent of pelvic organs into the vaginal cavity, significantly impairs the quality of life for many women. However, the etiopathogenesis of POP remains poorly understood. Anterior vaginal wall prolapse is the most common form of POP. Vaginal fibroblasts maintain the integrity of vaginal wall tissues, which is important for supporting pelvic organs and preventing POP. Numerous studies have demonstrated that fibroblast apoptosis and ECM loss play critical roles in the progression of POP. In our study, we selected vaginal tissues as experimental subjects to explore the pathogenesis of POP. As expected, the vaginal tissues showed decreased collagen deposition and enhanced apoptosis in patients with POP.
In this study, we confirmed that SOCS3 expression was higher in the vaginal tissues of patients with POP, suggesting that increased SOCS3 expression may participate in the progression of POP. Recent studies have reported that SOCS3 inhibits cell proliferation and promotes cell apoptosis. In our study, we also observed that SOCS3 overexpression inhibited viability and induced apoptosis in HVFs. Additionally, collagen I, collagen III, and elastin are the main ingredients of ECM. The strength and resilience of vaginal tissues are determined by collagens and elastin, respectively. Dees et al. reported that the deletion of SOCS3 promotes collagen release. In light of this finding, we sought to investigate whether SOCS3 regulates the ECM in HVFs. Our results demonstrated that SOCS3 overexpression led to decreased expression levels of collagen I, collagen III, and elastin. Moreover, MMPs are involved in the degradation of ECM and suppression of collagen deposition in POP. Some studies have reported that SOCS3 knockdown results in the downregulation of MMP2 and MMP9. Consistent with previous research, our study showed that upregulation of SOCS3 elevated the expression of MMP2 and MMP9. Besides, our studies further found that SOCS3 also downregulated TGF-β1 and p-Smad2/3 protein expression. TGF-β1 is a crucial regulator in fibrosis-related diseases, which induces myofibroblast transformation and regulates ECM production. It promotes ECM deposition by enhancing collagen synthesis and suppressing collagen deposition in POP. Through activating transcriptional regulators of the Smad pathway, the p-Smad2/3 produced upon TGF-β1 activation is responsible for regulating collagen and fibronectin gene expression. A previous study has reported that increased SOCS3 expression inhibits TGF-β1 expression. TGF-β1 blocks the loss of ECM by suppressing MMP2 and MMP9 expression via the TGF-β1/Smad3 signaling pathway in the uterosacral ligament of patients with POP. Our findings revealed that SOCS3 increased the expression of p-Smad3 and p-Smad2. Therefore, we hypothesized that SOCS3 may enhance the expression of MMP2 and MMP9 by inhibiting TGF-β1/Smad2/3, thereby promoting ECM degradation. Nevertheless, these possible mechanisms and detailed interactions among them require further exploration. In summary, SOCS3 overexpression induced apoptosis and ECM degradation of HVFs.

The abnormal expression of SOCS3 is often attributed to the transcriptional activation of its promoter. For example, the transcriptional activation of the farnesoid X receptor enhances SOCS3 expression. In this study, we confirmed that NR4A1 overexpression enhanced the levels of SOCS3, and we identified the NR4A1 binding sites on the SOCS3 promoter. Thus, SOCS3 might be transcriptionally...
regulated by NR4A1. NR4A1, belonging to the orphan nuclear hormone receptors of the Nur77 family, is a transcription factor involved in the cell cycle, apoptosis, and tumorigenesis. Similarly, Zeng et al. demonstrated that NR4A1 induces apoptosis by linking the actin cytoskeleton in parametrial ligament fibroblasts of POP. Here, we found that NR4A1 was highly expressed in the vaginal tissues of POP patients. NR4A1 overexpression promoted cell apoptosis and ECM degradation in HVFs. More importantly, SOCS3 knockdown reversed the negative effects of NR4A1 on HVFs, suggesting that NR4A1 affected the function of HVFs by transcriptionally activating SOCS3 (Figure 8).

Furthermore, the RNA-seq analysis was performed to investigate the potential downstream genes that may be regulated by SOCS3. When SOCS3 was overexpressed, DEGs were significantly enriched in ECM organization, programmed cell death, and collagen catabolic process, which was consistent with our findings. SOCS3 overexpression upregulated the expression of various genes, such as HTRA1 and CTSS. The upregulated expression of HTRA1 has been reported to inhibit cell proliferation and induce cell apoptosis. Additionally, reducing the expression of CTSS increases ECM deposition. These downstream factors may play a role in the POP process. However, follow-up experiments are needed to further study the functions of these factors.

The dysfunction of SOCS3 may contribute to several disorders. The activation of SOCS3 leads to insulin resistance and retinal endothelial cell apoptosis in the diabetic retina. SOCS3 deletion suppresses myocardial apoptosis, which results in the block of left ventricular remodeling after myocardial infarction. Moreover, hepatocyte steatosis was accelerated by promoting SOCS3 expression. Selective inhibition of SOCS3 may attenuate some of these consequences, suggesting the potential role of SOCS3 inhibitors as a promising therapy for disease treatment. For example, PARy agonist ROZ alleviated steatosis by decreasing SOCS3 expression. Similarly, the inhibition of SOCS3 expression by ruxolitinib attenuates atherosclerosis. Besides, microRNAs have been identified as regulators of SOCS3 expression. Repression of microRNA-122 reduces SOCS3 expression by promoter methylation. These methods of inhibiting SOCS3 expression may offer new clinical avenues for the treatment of POP.

In conclusion, we found no significant association between the expression of SOCS3 or NR4A1 and the characteristics of patients with POP. We speculated that this may be due to the insufficient number of samples. In the future, we will continue to collect clinical samples to clear the relationship between the expression levels of SOCS3 or NR4A1 and the characteristics of patients with POP.

In conclusion, this study provides evidence that SOCS3, transcriptionally activated by NR4A1, promotes the progression of POP by inducing HVF apoptosis and ECM degradation. Thus, the NR4A1/SOCS3 axis emerges as a promising therapeutic target for the treatment of POP.

Data Sharing Statement: The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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