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

MATERIALS AND METHODS

Immunohistochemistry (IHC)

After routine dehydration and paraffin embedding, slices of tissues were dewaxed, and performed antigen retrieval and catalase incubation. Slides were blocked with 1% bovine serum albumin (BSA, Sangon, China) for 15 min. Subsequently, slides were incubated overnight with primary antibodies SOCS3 (ABclonal, A0694, 1:100 dilution) or NR4A1 (ABclonal, A6676, 1:100 dilution) overnight at 4 °C. Then, samples were incubated with horse radish peroxidase (HRP)-conjugated goat IgG (ThermoFisher, #31460, 1:500 dilution) for 60 min at 37 °C. The slides were developed color with DAB chromogen (Maixin, China). Finally, the slides were counterstained using hematoxylin for 3 min, washed, dehydrated, and mounted. The images were photographed by a BX53 microscope (Olympus, Tokyo, Japan). The expression level of SOCS3 or NR4A1 was assessed via H-score according to previously reported.[1]

Masson staining

The levels of collagen deposition of anterior vaginal wall tissues in POP and non-POP patients were detected by Masson staining. The tissues embedded in paraffin were deparaffinized and dyed with Masson trichrome stain (Sinopharm, Shanghai, China). The images were visualized.

TUNEL staining

Fixed samples were permeabilized with 0.1% Triton X-100 (Beyotime, China) and incubated in the TUNEL reaction mix (Wanleibio, China) at 37 °C for 60 min. After counterstaining with DAPI (Aladdin, China), slides were imaged with a BX53 fluorescence microscope (Olympus, Japan).

Immunofluorescence

The cells were fixed with 4% paraformaldehyde and incubated with 0.1% Triton X-100 for permeabilizing cells. After blocking cells with 1% BSA for 15 min, cell slides were incubated with anti-vimentin antibody (ABclonal, A19607, 1:200 dilution), anti-Cytokeratin antibody (ABclonal, A0247, 1:200 dilution) or anti-Desmin antibody (ABclonal, A3736, 1:200 dilution) overnight at 4 °C. The following day, cell slides were incubated for 60 min with Cy3-labeled goat anti-rabbit IgG (Invitrogen, A27039, 1:200 dilution). Subsequently, slides were counterstained with DAPI (Aladdin, China) for imaging by a BX53 fluorescence microscope (Olympus, Tokyo, Japan).

Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from fibroblasts, and cDNA was synthesized using BeyoRT II M-MLV reverse transcriptase (Beyotime, China) according to the manufacturer's protocol. The RT-qPCR was performed with SYBR Green (Solarbio, China) using Exicycler™ 96 Real-Time Quantitative Thermal Block (Bioneer, Daejeon, Korea). The sequences of the primers included the SOCS3-specific forward 5'-ACCCACAGCAAGTTTCCC-3' and reverse 5'-GCACTGCGTTCACCACC-3', and NR4A1-specific forward 5'-CGCAAGTGGGCGGAGAA-3' and reverse 5'-CCAGGCGGAGGATGAAGAG-3'. Data analysis used the $2^{-\Delta\Delta Ct}$ method.

Western blot

The total protein was extracted from fibroblasts and tissues. Protein samples were separated by SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF, Millipore, USA) membranes. Membranes were blocked with 5% skim milk (Yili, China) for 60 min followed by incubation with primary antibodies at 4 °C overnight. After washing with TBST buffer, the secondary antibody of HRP-labeled goat anti-rabbit IgG (Beyotime, A0208, 1:5,000 dilution) was added into membranes, and incubation was performed at 37 °C for 45 min. Digital images of specific protein bands were captured after incubating with ECL chromogenic substrate (Beyotime, China) and the optical density was quantified by Gel-Pro-Analyzer. Mouse anti-β-actin (Santa Cruz, sc-47778, 1:1,000 dilution) was used as an internal control. The primary antibodies and concentrations were following: SOCS3 (Abclonal, A0694, 1:500 dilution), NR4A1 (Abclonal, A6676, 1:1,000 dilution), Bcl-2 (Abclonal, A19693, 1:1,000 dilution), bax (Abclonal, A19684, 1:1,000 dilution), cleaved caspase 3 (CST, #9661, 1:1,000 dilution), collagen I (Abclonal, A5786, 1:1,000 dilution), collagen III (CST, #66,887, 1:1,000 dilution), elastin (Abclonal, A2723, 1:1,000 dilution), MMP2 (Proteintech, 10373-2-AP, 1:500 dilution), and MMP9 (Proteintech, 10375-2-AP, 1:1,000 dilution), TGF-β1 (Abclonal, A2124, 1:500 dilution), p-Smad2/3 (CST, #8828, 1:1,000 dilution), Smad2/3 (CST, #8685, 1:400 dilution),.

CCK-8 assay

Cell viability was analyzed by CCK-8 assay (Keygen, China). Fibroblasts (3 x 10^3 cells/well) were seeded into 96-well plates. After lentivirus infection or co-infection for 48 h, 10 µl CCK-8 solution was added into each well and then incubated at 37 °C for 2 h. The absorbance at 450 nm was measured with a microplate reader (800Ts, BIOTEK, VT, USA).

Apoptotic assay

The cell apoptosis was evaluated with an Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Keygen, China). Fibroblasts were harvested 48 h after infection via centrifugation (150 g, 5 min) and washed twice. Then fibroblasts were resuspended in a Binding Buffer and incubated with Annexin V-FITC and Propidium Iodide (PI) for 15 min. Cell apoptosis was carried out on a Novocyte flow cytometer (Agilent, CA, USA).

Dual-luciferase reporter assay

Luciferase assay was executed with a Dual-Luciferase Reporter assay kit (Keygen, China) according to the manufacturer's protocol. The promoter sequences of SOCS3 were inserted into the luciferase reporter vector (pGL3-Basic). HEK293T cells were co-transfected with NR4A1 expression plasmids and SOCS3 promoter plasmids. Cells were harvested and lysed 48 h after transfection. The relative firefly luciferase activity was normalized by the Renilla luciferase activity. Luciferase signals were detected on a microplate reader (BIOTEK, VT, USA).

TABLE S1. SOCS3 Expression in Clinical and Pathological Characteristics of 36 POP Patients.

		SOCS3 expression		
Parameters	n	High (n)	Low (n)	*P* value
Age				
< 60	16	7	9	
≥ 60	20	11	9	0.5023
BMI				
< 25	27	12	15	
≥ 25	9	6	3	0.2482
Parity				
1~2	27	13	14	
≥ 3	9	5	4	0.7003
POP manifestation				
Isolated cystocele	10	6	4	
Mixed prolapse	26	12	14	0.4568
POP stage				
III	31	17	14	
IV	5	1	4	0.1482
Menopausal status				
Premenopausal	6	3	3	
Postmenopausal	30	15	15	> 0.9999

TABLE S2. NR4A1 Expression in Clinical and Pathological Characteristics of 36 POP Patients.

		NR4A1 expression		
Parameters	n	High (n)	Low (n)	*P* Value
Age				
< 60	16	8	8	
≥ 60	20	10	10	>0.9999
BMI				
< 25	27	12	15	
≥ 25	9	6	3	0.2482
Parity				
1~2	27	16	11	
≥ 3	9	2	7	0.0543
POP manifestation				
Isolated cystocele	10	6	4	
Mixed prolapse	26	12	14	0.4568
POP stage				
III	31	17	14	
IV	5	1	4	0.1482
Menopausal status				
Premenopausal	6	2	4	
Postmenopausal	30	16	14	0.3711

BMI: Body mass index

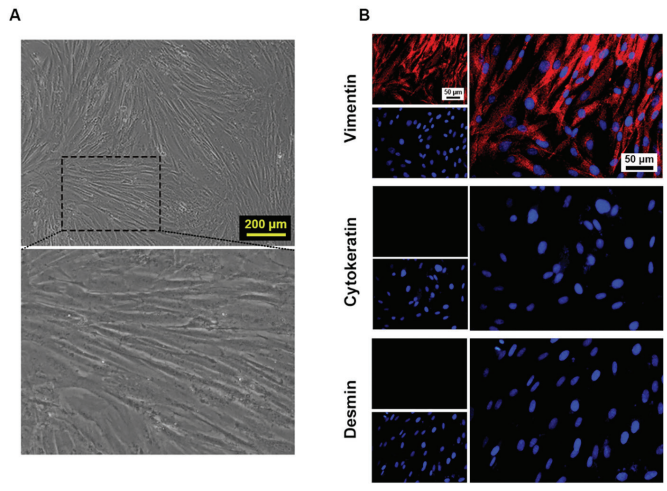

FIG. S1. Morphological and immunocytochemical identification of vaginal fibroblasts. **(a)** The images of primary cultured fibroblasts at passage 4. **(b)** The immunofluorescence staining images of vimentin, cytokeratin, and desmin in fibroblasts. DAPI represents the nucleus.

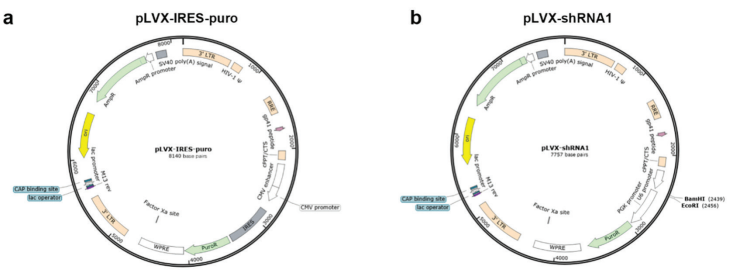

FIG. S2. (a) The map of pLVX-IRES-puro. **(b)** The map of pLVX-shRNA1.

REFERENCES

1. Hua Q, Sun Z, Liu Y et al. KLK8 promotes the proliferation and metastasis of colorectal cancer via the activation of EMT associated with PAR1. *Cell Death Dis*. 2021;12:860. **[CrossRef]**