MATERIALS AND METHODS

Immunohistochemistry (IHC)

After routine deparaffinization and paraffin embedding, slices of tissues were dewaxed, and performed antigen retrieval and anti-rabbit IgG antibody (Abclonal, A6676, 1:100 dilution) incubation for 15 min. Subsequently, slides were incubated overnight with primary antibodies (SOCS3, Abclonal, A6676, 1:100 dilution overnight at 4°C; TGF-β1, 1:500 dilution; Collagen III, CST, #66,887, 1:1,000 dilution) and secondary antibodies (H-270, 1:1,000 dilution) were used as an internal control. The absorbance at 450 nm was measured with a microplate reader (BIO-TEK, VT, USA).

Immunofluorescence

The cells were fixed with 4% paraformaldehyde and incubated with 0.1% Triton X-100 for permeabilization. After blocking with 1% BSA for 15 min, cells were incubated with anti-Desmin antibody (Abclonal, A3736, 1:200 dilution), anti-α-SMA antibody (Abclonal, A6157, 1:200 dilution), and anti-E-cadherin antibody (Abclonal, A7376, 1:200 dilution) overnight at 4°C, followed by anti-rabbit IgG secondary antibodies (1:200 dilution). The nuclei were stained 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 BeyoFast II M-MV reverse transcriptase (Beyotime, China) according to the manufacturer’s protocol. RT-qPCR was performed with SYBR Green (Solarbio, China) and incubated in the TUNEL reaction mix (Roche, Switzerland) for 2 h. The absorbance at 450 nm was measured with a microplate reader (BIO-TEK, VT, USA). The expression levels of target genes were calculated using the 2^(-ΔΔCt) 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 (anti-rabbit IgG-HRP, Abclonal, A21280, 1:1,000 dilution) was added into membranes, and incubation was performed at 25 °C for 60 min. Digital images of specific protein bands were captured after incubation with ECL chromogenic substrate (Beyotime, China) and enhanced chemiluminescence (ECL) was used as described before. The primary antibodies and concentrations were following: SOCS3 (Abclonal, A6676, 1:100 dilution), NR4A1 (Abclonal, A6676, 1:100 dilution), MAPK (Proteintech, 10373-2-AP, 1:1,000 dilution), and GAPDH (Abclonal, A6485, 1:1,000 dilution) was used as an internal control. The absorbance at 450 nm was measured with a microplate reader (BIO-TEK, VTU, USA).

Immunohistochemistry (IHC)

The slides were blocked with 1% bovine serum albumin (BSA, Sangon, China) for 30 min at room temperature. After blocking with 1% BSA for 15 min, cells were incubated with anti-Desmin antibody (Abclonal, A3736, 1:200 dilution), anti-α-SMA antibody (Abclonal, A6157, 1:200 dilution), and anti-E-cadherin antibody (Abclonal, A7376, 1:200 dilution) overnight at 4°C, followed by anti-rabbit IgG secondary antibodies (1:200 dilution). The nuclei were stained with DAPI (Aladdin, China) for imaging by a BX53 fluorescence microscope (Olympus, Tokyo, Japan).

Cell viability assay

Cell viability was analyzed by CCK-8 assay (KeyGen, China). Fibroblasts (5 × 10^3 cells/well) were seeded into 96-well plates. After lincomycin co-infection for 48 h, 10 μl of 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 (BIO-TEK, VTU, USA).

Apoptosis assay

The cell apoptosis was assayed with an Annexin V-FITC Apoptosis Detection Kit (Beyotime, China). Fibroblasts were harvested after 48 h of treatment with or without SOCS3 overexpression plasmid and 10 μg/ml lincomycin. Fibroblasts were harvested and lysed 48 h after the treatment. The relative fluorescent activity was normalized by the Resilir assay. Cell viability signals were detected on a microplate reader (BIO-TEK, VTU, USA).

REFERENCES