

Secreted protein, acidic and rich in cysteine is crucial for the vascular endothelial growth factor-stimulated fibrotic process in human Tenon's fibroblasts

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Abstract

Background Aberrant post-surgical scarring is responsible for failure of glaucoma filtration surgery (GFS) and is attributed to strong fibrotic process of human Tenon's fibroblasts (HTFs). Vascular endothelial growth factor (VEGF) and secreted protein, acidic and rich in cysteine (SPARC) contribute to angiogenesis and fibrosis. However, whether SPARC can regulate the VEGF-mediated fibrotic process in HTFs has not been clarified. This study aimed to examine how SPARC and VEGF crosstalk to regulate the expression of Collagen-I and matrix metalloproteinase 9 (MMP9) as well as the ERK signalling in HTFs.

Methods Human Tenon's capsule tissues were cultured for preparation of HTFs, which were characterized by immunofluorescence. The effects of VEGF treatment on SPARC, Collagen-I and MMP9 expression and ERK phosphorylation were determined by Western blot, quantitative RT-PCR and immunofluorescence. The proliferation and wound healing induced by VEGF were examined in HTFs and SPARC-silenced HTFs.

Results Following successful passages, immunofluorescent assays indicated that HTFs at passages 3-9 displayed unique characters of fibroblasts with Vimentin, but not keratin, expression. Treatment with VEGF significantly up-regulated SPARC, Collagen-I and MMP9 expression and ERK phosphorylation, and promoted the proliferation and wound healing of HTFs. The stimulatory effects of VEGF were significantly mitigated by SPARC silencing in HTFs.

Conclusions Our data provided novel evidence that SPARC was crucial for the VEGF-stimulated fibrotic process in HTFs and may be a novel target for anti-fibrotic therapies post GFS.

Background

Glaucoma can cause irreversible blindness and it may affect several millions of people in the near future¹. Filtration surgery (FS) for glaucoma is usually effective. However, surgical failure occurs when excessive wound healing happens below the conjunctiva because human Tenon's fibroblasts (HTFs) can activate and proliferate, contributing to fibrotic process. Actually, many cytokines and biochemical factors can activate HTFs and promote their fibrotic process, leading to an aberrant scarring response²⁻⁴. Thus, understanding the pathogenic process in HTFs will be of significance in uncovering new therapeutic target for prevention and intervention of post-FS scarring.

Vascular endothelial growth factor (VEGF) can regulate angiogenesis and fibrosis, and has been considered to be important for post-FS scar formation. Previous studies have shown that VEGF can promote wound healing of HTFs and scar formation in glaucoma patients⁵⁻⁶. In addition, high concentrations of VEGF were detected in the aqueous humor of glaucoma patients and rabbits following FS⁷. Besides, VEGF can be expressed by fibroblasts and stimulate the proliferation of Tenon's fibroblasts, contributing to fibrotic process⁸. However, the molecular mechanisms by which VEGF regulates the fibrotic process in HTFs have not been clarified.

Secreted protein, acidic and rich in cysteine (SPARC) is one of the matricellular proteins that are crucial for scar formation and tissue fibrosis^{9–10}. SPARC can regulate the expression of Collagens and matrix metalloproteinases (MMPs), as well as the extracellular matrix (ECM) remodeling. However, little is known about whether SPARC regulates the VEGF-stimulated fibrotic process in HTFs.

In this study, we prepared and characterized HTFs and tested how VEGF stimulated SPARC, Collagen-I and MMP9 expression and ERK activation as well as the wound healing and proliferation of HTFs. Furthermore, we examined the impact of SPARC silencing on the VEGF-stimulated fibrotic process in HTFs.

Methods

Primary cell culture and quality control

This study was approved by the Ethics Committee of Shanghai General Hospital (Registration number: 2016KY034). All patients signed a written informed consent. Small biopsied Tenon's capsule specimens were isolated from five patients when they underwent a strabismus surgery. After being washed, the tissues were cultured for preparation of HTFs, as described previously¹¹. The HTFs were cultivated in Dulbecco's Modified Eagle's medium (DMEM) containing 10% of fetal bovine serum (FBS) and the cells were passaged every 3–5 days. Subsequently, their morphology and purity were examined by immunofluorescence using rabbit-anti-vimentin (1:400, ab92547, abcam), mouse-anti-keratin (1:50, ab185627, abcam), Alexa Fluor 488-conjugated goat-anti-rabbit or Alexa Fluor 647-conjugated goat-anti-mouse IgG and DAPI staining. HTFs between passage 3 and passage 9 were used for experiments after starving for 24 h.

Transduction

HTFs were cultured overnight and transduced with control lentivirus or lentivirus for expression of SPARC-specific shRNA at a multiplicity of infection (MOI) of 10 in the presence of puromycin (4 µg/ml) for four days to generate HTF-NC and HTF-SPARC-shRNA cells, respectively. The second generation of control lentivirus or lentivirus for SPARC-specific shRNA were generated by transfecting 293T cells with pL_shRNA_mKate2-SPARC-543 and Packaging Mix (cat. no. K4975-00, Invitrogen) using lipofectamine 2000 (cat. no. 11668019, Invitrogen)¹². The efficacy of SPARC silencing was determined by Western blot.

Cell viability assay

Cell viability was measured by MTS assay using a kit, according to the manufacturer's instruction (Promega G3580). Briefly, HTFs (1×10^3 cells/mL) were cultured in 96-well plates and when reaching 80% confluency, the cells were treated in sextuplicate with vehicle PBS or VEGF (25–100 ng/mL, 96–100–20A-2, PeproTech) for 24 h. Individual wells were added with 20 µL of MTS and cultured at 37° for 2 h. The absorbance at 490 nm was measured using a microplate reader (Epoch™ Bio-Tek Instruments,

Winooski, VT, USA). Additional MTS assays were performed in HTF, HTF-NC and HTF-SPARC-shRNA cells using 50 ng/ml of VEGF for stimulation.

Western blot

The impact of VEGF on SPARC and other fibrosis-related protein expression was determined by Western blot. Briefly, HTFs were stimulated with, or without, 25–100 ng/ml of VEGF for 24 h and their cell lysates were prepared. After determination of protein concentrations, cell lysates (50 µg/lane) were analysed by Western blot using primary antibodies against β -actin (1:1000, 60008–1-Ig, Proteintech), SPARC (1:1000, ab207743), Collagen-I (1:1000, ab138942), MMP9 (1:1000, ab76003), ERK1/2 (1:1000; ab184699, abcam) and p-REK1/2 (1:1000, #9101, Cell Signaling Technology) and HRP-goat anti-rabbit IgG (1:5000, #A27024) or HRP-goat anti-mouse IgG (1:5000, #A11357, Thermofisher) as well as an ECL assay kit. The data were analysed by densitometry using ImageJ software. Additional Western blot assays were performed in HTF, HTF-NC and HTF-SPARC-shRNA cells using 50 ng/ml of VEGF for stimulation.

Immunofluorescence

HTFs (1×10^3 cells/mL) were grown on glass coverslips in 24-well plates and when reaching 80% confluency, the cells were treated with VEGF for 24 h. The cells were stained with primary antibodies against SPARC, Collagen-I and MMP–9. After being washed, the cells were incubated with Alexa Fluor–488-conjugated secondary antibodies (1:1000, Invitrogen, Carlsbad, CA, USA), followed by nucleary stained with DAPI. The fluorescent signals were visualized using a confocal microscope (Carl Zeiss). Additional immunofluorescent assays were performed in HTF, HTF-NC and HTF-SPARC-shRNA cells using 50 ng/ml of VEGF for stimulation.

Quantitative RT-PCR

The effect of VEGF on SPARC and other fibrosis-related gene mRNA transcripts were determined quantitative RT-PCR using special reagents including TRIzol, the PrimeScript 1st Strand cDNA Synthesis kit (RR036A, TaKaRa Bio, Dalian, China), the SYBR Premix Ex Taq (RR420, TaKaRaBio) in Vii7 Real-Time PCR System (Applied Biosystems, Life Techonogies, Carlsbad, CA, USA). The primer sequences were forward 5'-GGACTTCGAGCAAGAGATGG–3' and reverse 5'-AGCACTGTGTTGGCGTACAG–3' for β -actin; forward 5'-AGGAAACCGAAGAGGAGG–3' and reverse 5'-GCAAAGAAGTGGCAGGAA–3' for SPARC; forward 5'-GACCTCAAGTGGCACCACCA–3' and

reverse 5'-GTGGTACTGCACCAGGGCAA–3' for MMP9; forward 5'-CCCAGCCACAAAGAGTCTACA–3' and reverse 5'-GTTTCCACACGTCTCGGTCA–3' for collagen-I. The data were analyzed by $2^{-\Delta\Delta Ct}$. Additional quantitative RT-PCR assays were performed in HTF, HTF-NC and HTF-SPARC-shRNA cells using 50 ng/ml of VEGF for stimulation.

Wound healing assay

HTFs were cultured in twelve-well plates. When the cells reached full confluency, they were scratched using a pipette tip. The cells were treated with, or without, 25–100 ng/ml of VEGF and the dynamic process of cell proliferation and migration was monitored longitudinally by photoimaging. The data were expressed as the percentage of wound healing. Additional wound healing assays were performed in HTF, HTF-NC and HTF-SPARC-shRNA cells using 50 ng/ml of VEGF for stimulation.

Statistical analysis

Data are presented as the means \pm standard deviation (SD). The statistical comparisons were performed by Student's t-test or ANOVA using SPSS version 17.0 for Windows (SPSS, USA). A *P*-value of <0.05 was considered statistically significant.

Results

Preparation and characterization of HTFs.

Following culture of the tissue explants for 10 to 14 days, many cells migrated out and the cells were continually passaged. Immunofluorescence revealed that all cells were positive for anti-vimentin, but negative for anti-keratin staining, the hallmarks of fibroblast-like cells (Fig. 1). We observed that their morphology and growth characteristics were not affected by resuscitation and cryopreservation in our experimental conditions.

VEGF up-regulates the expression of fibrotic proteins and promotes the proliferation and migration of HTFs

VEGF can stimulate angiogenesis, inflammation and migration of fibroblasts^{5–6}. To understand the molecular mechanisms underlying the action of VEGF, we examined whether VEGF could modulate the SPARC, Collagen-I, MMP9 expression and ERK1/2 activation in HTFs. Western blot indicated that treatment with 50 ng/ml of VEGF significantly increased the relative levels of SPARC, Collagen-I, MMP9 expression and ERK phosphorylation in HTFs (Fig. 2A). However, treatment with VEGF at a lower or higher dose either did not significantly up-regulate or only moderately increased their expression and phosphorylation in HTFs. Similar patterns of VEGF-enhanced SPARC, Collagen-I and MMP9 expression were observed in HTFs by immunofluorescence (Fig. 2B). Similarly, treatment with VEGF increased the relative levels of SPARC, Collagen-I and MMP9 mRNA transcripts in HTFs (Fig. 2C). Functionally, VEGF treatment significantly promoted the proliferation and wound healing of HTFs (Fig. 2D and E). Hence, VEGF promoted the proliferation and wound healing of HTFs by enhancing fibrotic protein expression and ERK1/2 activation in vitro.

SPARC silencing mitigates the VEGF-induced fibrotic process in HTFs

SPARC can regulate ECM remodelling by modulating collagen expression and MMP activity^{9,10}. To understand the regulatory role of SPARC in VEGF-induced fibrotic process in HTFs, we generated stable

SPARC-silenced HTFs by lentivirus-based shRNA technology. Following stimulation with VEGF (50 ng/ml), we found that SPARC silencing not only decreased the VEGF-stimulated SPARC expression, but also mitigated the VEGF-enhanced Collagen-1 and MMP expression and ERK phosphorylation in HTFs (Fig. 3A-C). Functionally, measurements of wound healing and cell proliferation revealed that following VEGF stimulation, the percentages of wound healing and viability in the SPARC-silenced HTFs were significantly lower than that in the controls (Fig. 3D and E). Such data indicated that SPARC silencing mitigated the VEGF-stimulated wound healing and proliferation in HTFs *in vitro*. Collectively, these findings suggest that VEGF-stimulated fibrotic process in HTFs may partially depend on inducing SPARC expression in our experimental conditions. Meanwhile, our experiment showed that all the aspect mentioned above of NC HTFs were slightly down regulated compared with normal HTFs. Probably, the process of infecting cells with virus would also affect the function of cells to some extent.

Discussion

FS is one of the most effective treatments for glaucoma¹³. Unfortunately, a failure in FS for glaucoma occasionally occurs due to excessive post-surgical wound healing process and scar formation. Previous studies have shown that VEGF₁₂₁ and VEGF₁₈₉ can induce the proliferation and migration of HTFs by activating the ERK signaling^{14,5} and higher levels of VEGF expression in Tenon's tissues are associated with post-FS fibrosis⁸. In this study, we prepared HTFs from human Tenon's capsule tissues and characterized them. We found that almost all cells displayed typical morphology of fibroblasts with Vimentin, but not keratin, expression. Furthermore, we found that VEGF treatment stimulated the proliferation and wound healing of HTFs, accompanied by up-regulating SPARC, Collagen-I and MMP9 expression and ERK1/2 phosphorylation. Such data clearly demonstrated that VEGF promoted the fibrotic process of HTFs and suggest that SPARC may participate the process of VEGF-mediated fibrosis.

SPARC is an ECM protein and its up-regulated expression is associated with fibrosis. Actually, *SPARC*^{-/-} mice exhibit deficient scarring and SPARC deficiency increases successful rates of FS in a mouse model of Glaucoma¹⁵. In this study, we found that treatment with VEGF stimulated SPARC expression in HTFs, which extended previous observations in HMEC-1 cells and HUVECs¹⁶. Furthermore, VEGF also up-regulated Collagen-I and MMP9 expression in HTFs and promoted the proliferation and wound healing of HTFs. More importantly, SPARC silencing significantly mitigated the VEGF-stimulated Collagen-I and MMP9 expression in HTFs and attenuated the VEGF-stimulated proliferation and wound healing of HTFs. Previous studies have shown that SPARC can modulate MMP activity in U87MG glioma cells¹⁷ and altered MMP activity can affect the ECM remodeling and fibroblast proliferation and migration¹⁸⁻¹⁹. Moreover, SPARC can promote cell adhesion²⁰ and SPARC deficiency inhibits cell migration in various types of cells²¹⁻²². It is possible that SPARC modulates MMP activity and wound healing, contributing to the progression of fibrosis²³. Hence, such data further indicated that SPARC participated in the fibrotic process in HTFs.

It is notable that SPARC can bind to collagen and acts as a chaperone to promote its post-translational process^{24, 25}. Furthermore, SPARC can activate both platelet-derived growth factor (PDGF) and fibroblast growth factor-2 (FGF2), which are crucial for Collagen-I expression^{9,26}. VEGF can activate the p38 and ERK signaling in vascular endothelial cells^{14,27}. We found that VEGF treatment enhanced the ERK1/2 phosphorylation in HTFs, consistent with a previous report¹⁴. Interestingly, SPARC silencing significantly mitigated the VEGF-enhanced ERK1/2 activation in HTFs. It is possible that SPARC silencing may minimize PDGF and FGF2 activation, which cross-talks with the VEGF-related ERK1/2 signaling to reduce Collagen-I expression and fibrotic process in HTFs. We are interested in further investigating the precise molecular mechanisms underlying the action of SPARC in regulating Collagen-I and MMP9 expression following VEGF stimulation in HTFs.

Conclusions

Our data indicated that VEGF treatment stimulated the wound healing and proliferation of HTFs by up-regulated SPARC, Collagen-I and MMP9 expression and ERK phosphorylation, which were significantly mitigated by SPARC silencing. Such data suggest that SPARC may be crucial for the VEGF-stimulated fibrotic process in HTFs. Conceivably, SPARC may be a valuable therapeutic target for prevention and invention of FS-related scarring.

List Of Abbreviations

GFS: Glaucoma Filtration Surgery

HTF: Human Tenon's fibroblast

VEGF: Vascular endothelial growth factor

SPARC: Secreted protein, acidic and rich in cysteine

MMP: Matrix metalloproteinase

FS: Filtration surgery

ECM: Extracellular Matrix

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shanghai General Hospital (Registration number: 2016KY034). All patients signed a written informed consent.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Xiang X, Fu Y, and Tang M were primarily responsible for experimental concept and design. Xiang X, Luo L and Chen Y performed the experiments and data acquisition. Xiang X, Ye F and Fu Y performed the data analysis as well as drafting of the manuscript. All authors reviewed and approved the final manuscript.

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Figures

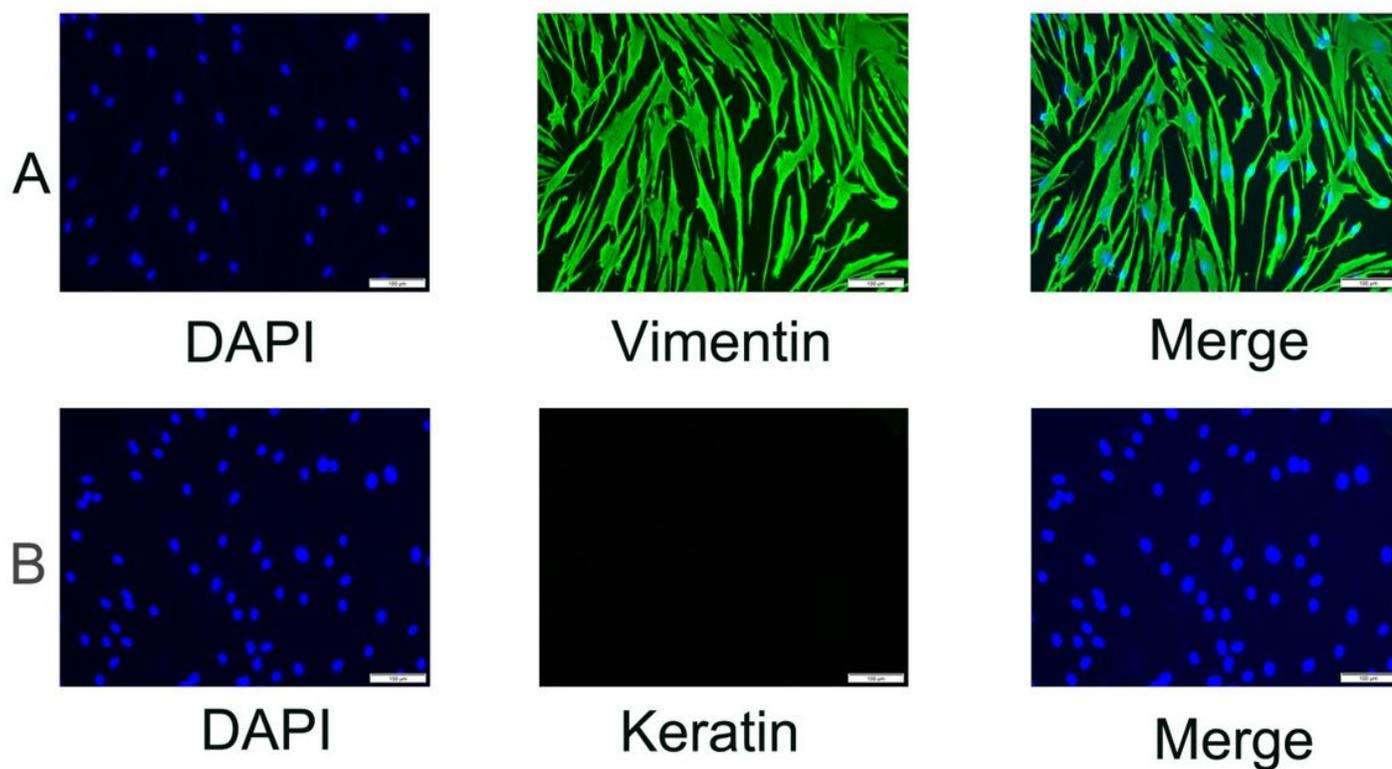


Figure 1

Immunofluorescent characterization of HTFs. Human Tenon's specimens were cultured for 15 days and many cells migrated out. The cells were passaged every 3 days for 3-9 times. The cells were stained with anti-Vimentin or anti-keratin, and after being washed, the cells were stained with Alexa Fluor 488-conjugated goat-anti-rabbit IgG or Alexa Fluor 647-conjugated goat-anti-mouse IgG. Subsequently, the cells were nuclearly stained with DAPI and the fluorescent signals were captured under a confocal microscope. Data are representative images (magnification x 200) from three separated experiments. (A) Immunofluorescent staining of cells with anti-Vimentin. (B) Immunofluorescent staining with anti-keratin. Scale bar: 100 μ m.

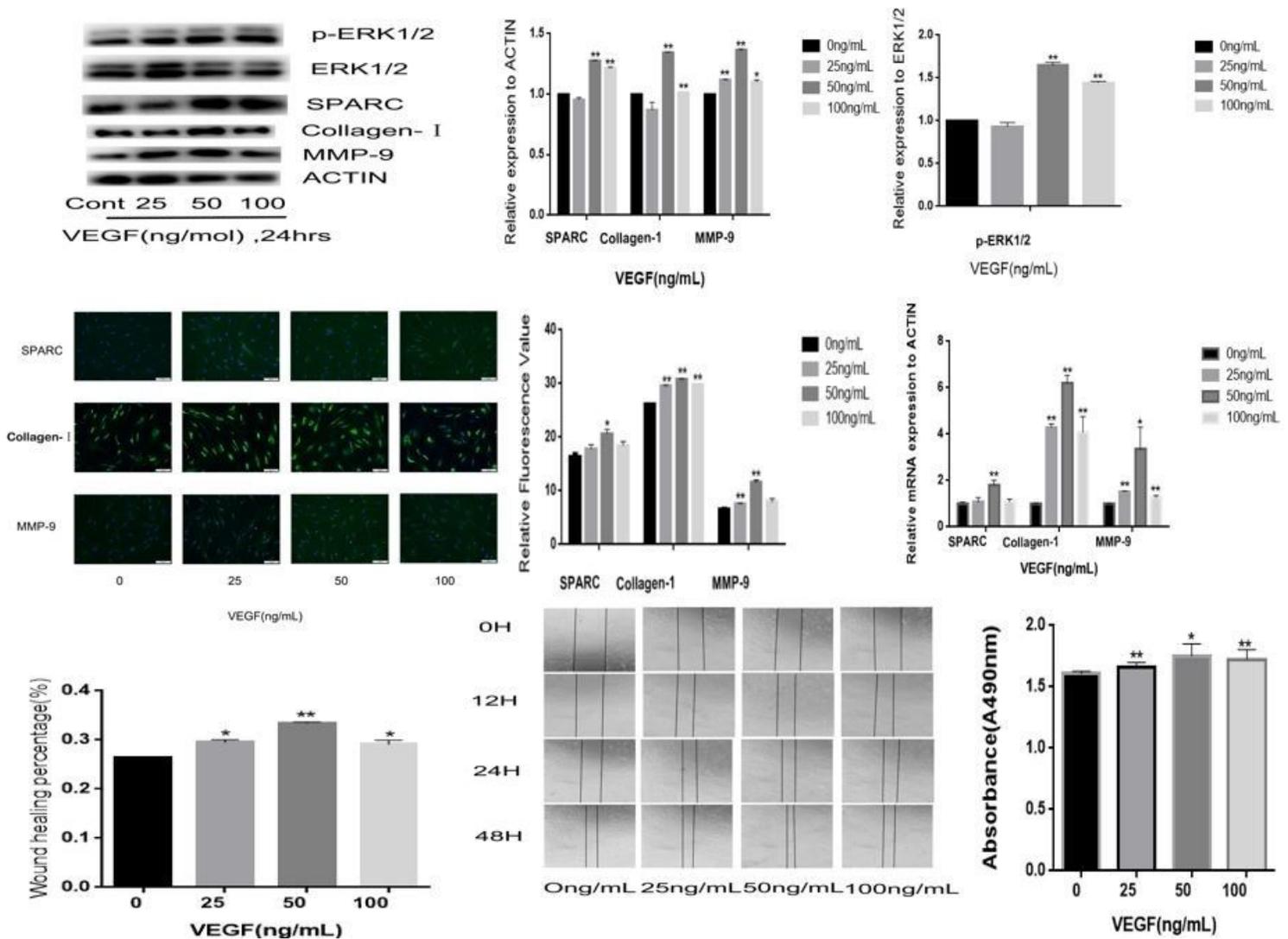


Figure 2

VEGF stimulates SPARC, Collagen-I and MMP9 expression and ERK phosphorylation to promote the proliferation and wound healing in HTFs. HTFs were starved overnight and stimulated in triplicate or sexduplicate with the indicated concentrations of VEGF for 24 h. (A) The relative levels of SPARC, Collagen-I and MMP9 expression and ERK1/2 phosphorylation were determined by Western blot. (B) The expression of SPARC, Collagen-I and MMP9 was characterized by immunofluorescence. (C) The relative levels of SPARC, Collagen-I and MMP9 mRNA transcripts were quantified by quantitative RT-PCR. (D) The wound healing. (E) The viability of HTFs was examined by MTS assays. Data are representative images or expressed as the mean \pm SD of each group of cells from three separate experiments. *P < 0.05; **P < 0.01.

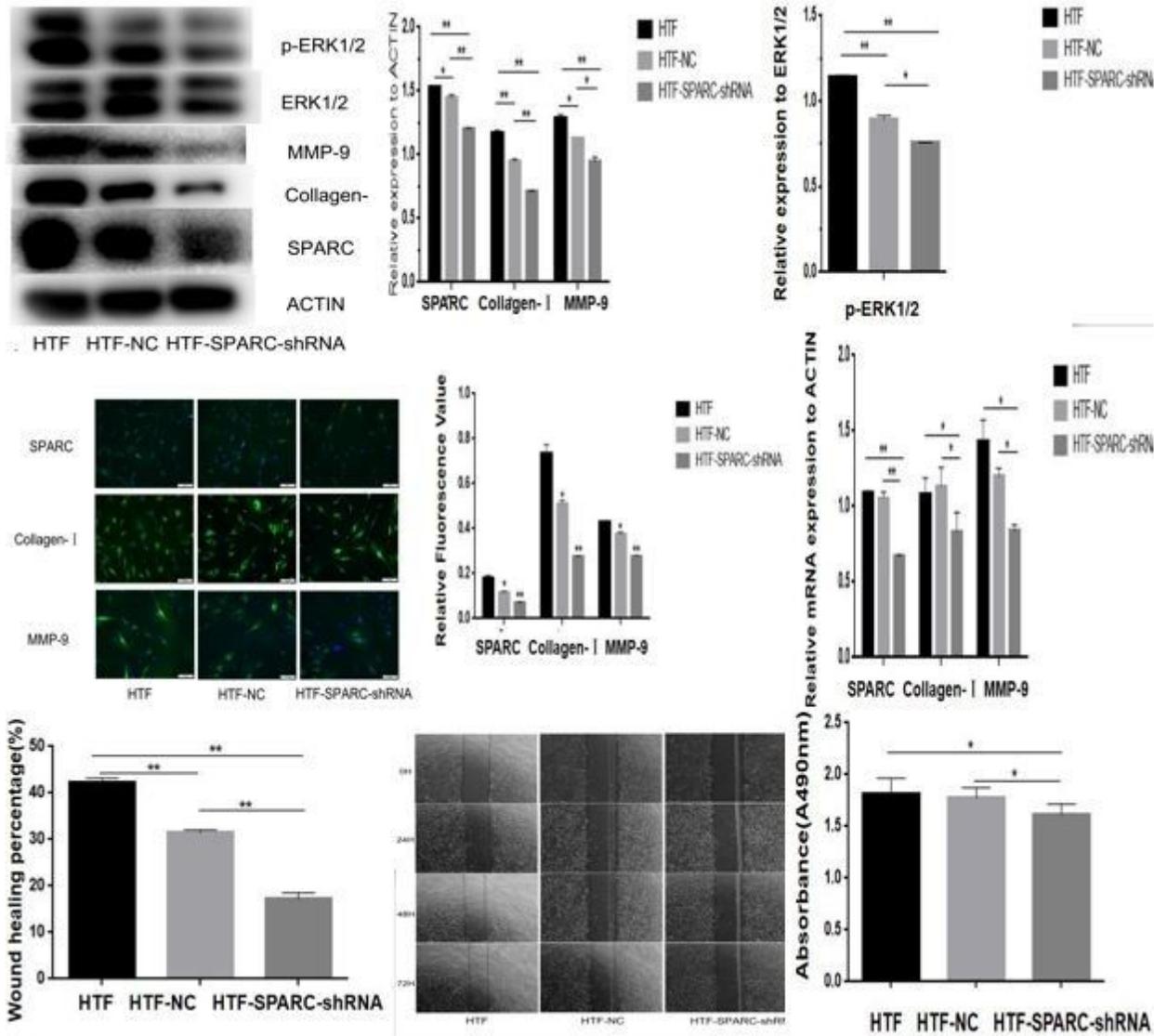


Figure 3

SPARC silencing mitigates the VEGF-stimulated fibrotic process in HTFs. HTFs, control HTF-NC and stable SPARC-silenced HTF-SPARC-shRNA cells were starved overnight and stimulated in triplicate or sexduplicate with 50 ng/ml of VEGF for 24 h. (A) The relative levels of SPARC, Collagen-I and MMP9 expression and ERK1/2 phosphorylation were determined by Western blot. (B) The expression of SPARC, Collagen-I and MMP9 was characterized by immunofluorescence. (C) The relative levels of SPARC, Collagen-I and MMP9 mRNA transcripts were quantified by quantitative RT-PCR. (D) The wound healing. (E) The viability of HTFs was examined by MTS assays. Data are representative images or expressed as the mean \pm SD of each group of cells from three separate experiments. *P < 0.05; **P < 0.01.