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In vitro analyses of the anti-fibrotic effect of SPARC silencing in human Tenon's fibroblasts: comparisons with mitomycin C

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Abstract

Failure of glaucoma filtration surgery (GFS) is commonly attributed to scarring at the surgical site. The human Tenon's fibroblasts (HTFs) are considered the major cell type contributing to the fibrotic response. We previously showed that SPARC (secreted protein, acidic, rich in cysteine) knockout mice had improved surgical success in a murine model of GFS. To understand the mechanisms of SPARC deficiency in delaying subconjunctival fibrosis, we used the gene silencing approach to reduce SPARC expression in HTFs and examined parameters important for wound repair and fibrosis. Mitomycin C-treated HTFs were used for comparison. We demonstrate that SPARC-silenced HTFs showed normal proliferation and negligible cellular necrosis but were impaired in motility and collagen gel contraction. The expression of pro-fibrotic genes including collagen I, MMP-2, MMP-9, MMP-14, IL-8, MCP-1 and TGF-β_2 were also reduced. Importantly, TGF-β_2 failed to induce significant collagen I and fibronectin expressions in the SPARC-silenced HTFs. Together, these data demonstrate that SPARC knockdown in HTFs modulates fibroblast functions important for wound fibrosis and is therefore a promising strategy in the development of anti-scarring therapeutics.

Keywords: SPARC • fibrosis • conjunctiva • glaucoma • mitomycin C

Introduction

Glaucoma is the major leading cause of irreversible blindness worldwide. It is predicted that by 2020, 79.6 million people will have the condition, and greater than 11 million individuals will be bilaterally blind from glaucoma [1]. Elevated intraocular pressure (IOP) is the major modifiable risk factor and its reduction remains the only proven treatment [2, 3]. For glaucoma that is refractory to topical ocular hypotensive medicines, filtration surgery is the most effective method for reducing the IOP to a consistently low level, thereby delaying disease progression in the individual's lifetime.

Scarring at the level of the subconjunctiva and episcleral tissue is a major cause for surgical failure in GFS. The HTF is the main effector cell involved in the scarring response [4–6]. The wound healing response at the operated site is characterized by complex molecular events which include stimulation of cell proliferation [4], enhanced expression of extracellular matrix (ECM) proteins [7] and inflammation [8–10]. Thus, the modulation of some, if not all of these parameters in HTFs, constitutes the principal aim in the research of potential anti-fibrotic adjuncts to GFS.

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To deter excessive scar formation, mitomycin C (MMC) is commonly used during GFS to disrupt the post-operative wound healing and thereby prolong bleb survival. MMC affects HTFs by inhibiting cell proliferation [11], inducing widespread apoptosis [12], increasing susceptibility to T cell-mediated lysis [13], reducing the production of pro-fibrotic genes and proteins and reducing cell migration [14]. At the moment, adjuvant MMC is the 'gold standard' against which other potential anti-fibrotic therapeutics are compared. However, the success rate with MMC application, although improved, is still far from ideal [15]. In addition, the non-specific cytotoxic effects of MMC is associated with a host of sight-threatening side effects and a pathology characterized by extensive cell death, abnormal wound healing responses and the clinical development of cystic, avascular blebs [16, 17]. Hence, the search for alternate, safer and more targeted anti-fibrotic agents and strategies is an on-going endeavour to increase surgical success and the lifespan of GFSs in patients.

Secreted protein acidic and rich in cysteine, also known as osteonectin and BM-40, is a 32 kD calcium-binding matricellular protein involved in modulating cell-ECM interactions without contributing structurally to the ECM [18]. The capacity of SPARC to interact with a plethora of key growth factors and ECM proteins, as well as regulate the expression of enzymes involved in ECM remodelling, such as matrix metalloproteinases (MMPs), strongly supports a fundamental role for SPARC in modulating cellular responses to tissue repair and remodelling *via* modification of ECM organization. We showed recently that SPARC depletion in knockout mice have improved surgical success compared to wild-type mice in a mouse model of GFS [19]. The diminished surgical fibrosis in the SPARC-null conjunctival tissue is associated with reduced expression of collagen I characterized by smaller collagen fibrils as well as compromised maturation and assembly of the ECM after surgery [19]. However, the mechanisms for the anti-fibrotic effect of SPARC deficiency in the subconjunctiva have not been delineated.

In this study, we investigated the wound healing properties of HTFs knocked down for SPARC expression by using small interfering RNA (siRNA). We describe the properties of these cells in assays for proliferation, apoptosis, cell migration, collagen gel contraction and expression of pro-fibrotic genes. We compared these effects against MMC-treated counterparts as the 'gold standard'. Herein, we provide evidence to support SPARC depletion as a potential therapeutic strategy for inhibiting cellular fibrotic events commonly associated with wounding in the subconjunctiva.

Materials and methods

Cell culture

Small biopsy samples containing subconjunctival HTFs were obtained during standard intraocular surgery with informed consent from patients and approval by the institutional ethics committee. The tenets of the Declaration of Helsinki were followed. The human tenon explants were placed on a culture dish with a drop of foetal bovine serum (FBS) for 15 min. before supplementing with additional culture medium consisting of DMEM supplemented with 10% FBS and penicillin–streptomycin (100 U/ml and 100 μg/ml, respectively). The explants were incubated at 37°C in a humidified incubator with 5% CO_2. Primary HTFs that migrated out from the tissue were propagated in the same medium. All tissue culture reagents were obtained from Invitrogen Corp. (Carlsbad, CA, USA) unless otherwise stated. HTFs of less than passage 8 were used in this study.

For treatment of HTFs with MMC, a single application of 0.4 mg/ml MMC (Kyowa Hakko Kirin Co. Ltd., Shizuoko, Japan) for 1 min. was used. After treatment, cells were washed three times in PBS and maintained in DMEM for 72 hrs before analyses.

To induce a fibrotic response, cells were treated in the morning with either MMC for 1 min. or incubated with transfection media containing the siRNAs for 5 hrs before being stimulated in the evening with recombinant TGF-β_2 (PeproTech Inc., NJ, USA) for 72 hrs. The concentration of TGF-b2 used is based on the optimal concentration required to induce the maximal expression of collagen I mRNA in HTFs and is determined for every batch of TGF-β_2 procured. In this study, the concentration of TGF-β_2 used was between 2 and 4 ng/ml.

siRNA and transfection

To knockdown SPARC, a 21-base double-stranded siRNA for SPARC (si-SPARC: 5'-AACAAGACCUUCGACUCUUCC-3') was used. A non-silencing scrambled control (si-Scram: 5'-GCUCACAGCUCAAUCCUAAUC-3') was also used. The si-RNAs were synthesized and purified by Bioneer (Daedeok-gu, Korea). HTFs were transfected with 100 nM SPARC or scrambled si-RNA using Lipofectamine 2000 (Invitrogen Corp.) according to manufacturer's instructions.

RNA isolation and real-time PCR

Total RNA recovery, first-strand cDNA synthesis and quantitative real-time PCR (qPCR) was performed as described previously [19]. All PCR reactions were performed in triplicate. All mRNA levels were measured as C_T threshold levels and were normalized with the corresponding β-actin C_T values. Values were expressed as fold increase over the corresponding values for untreated control by the $2^{-\Delta\Delta C}T$ method. The presented data are representative of three independent experiments. The primers used are shown in Table S1.

Immunoblotting

Total cellular extracts were prepared by lysing cells in a solution containing 20 mM Tris-buffer, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 2 mM $MgCl_2$, 1 mM dithiothreitol and 1× Complete Protease Inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) followed by SDS-PAGE and immunoblotting as previously described [19]. Antibodies against SPARC, β-tubulin and GAPDH were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) while antibodies recognizing MMP-14 and α-SMA were obtained from Abcam (Cambridge, UK). The antibody against collagen I was from Novus Biologicals (Littleton, CO, USA) while the fibronectin antibody was from Epitomics Inc. (Burlingame, CA, USA). Horseradish peroxidase (HRP)–conjugated secondary antibodies were from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA,

USA). Densitometric quantitation was performed as reported previously [19] and potential errors in loading were corrected to levels of GAPDH, which was used as the housekeeping protein.

Real-time cell proliferation analysis

The xCelligence real-time cell analyser (Roche Diagnostics GmbH) was used to assess cell proliferation according to manufacturer's instructions. HTFs were trypsinized and counted with a haemocytometer. Treatment with MMC was performed at 0.4 mg/ml for 1 min. on trypsinized cells followed by three washes in PBS before being seeded onto the E-Plate 96 (Roche Diagnostics GmbH) wells at 6000 cells/well in normal culture medium, in quadruplicates. For experiments with MMP inhibitors, the cells were seeded in medium containing 0.15% DMSO (vehicle), 20 μM GM6001 (Calbiochem, San Diego, CA, USA) or 5 μM MMP-2/MMP-9 inhibitor I (MMP2/9i; Calbiochem) at 1000 cells/well in quadruplicates. The plated cells were allowed to equilibrate for at least 30 min. in the tissue culture incubator before electrode resistance was recorded. Cell growth was monitored continuously for up to 6 days.

Annexin V assay by flow cytometry

Apoptosis was assessed by flow cytometry using the Guava Nexin Reagent (Guava Technologies, Hayward, CA, USA). The nexin assay is based on the measurement of annexin V, which binds to phosphatidylserine that is translocated from the inner to outer surface of the cell membrane during apoptosis. HTFs were trypsinized at 72 hrs, and processed according to the manufacturer's instructions. Five thousand cells from each sample were analysed. Cell populations were quantified using the Guava EasyCyte Plus flow cytometry system (Guava Technologies) and the data were analysed using the Guava Nexin software (Guava Technologies).

TUNEL analysis

End-labelling of exposed 3'-OH ends of DNA fragments in HTFs was performed with the DeadEnd Fluorometric TUNEL System according to manufacturer's instructions (Promega, Madison, WI, USA). Nuclei were visualized by mounting the TUNEL-stained cells in DAPI-containing Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and photographed using the Zeiss Imager.Z1 microscope (Carl Zeiss Inc., Maple Grove, MN, USA).

In vitro scratch assay

HTFs were seeded into wells of six-well culture dishes and allowed to attach and grow to confluence. The cells were then scratched wounded by a pipette tip and washed with medium to remove loose or dead cells. For experiments with MMP inhibitors, the washed cells were replaced with medium containing 0.15% DMSO (vehicle), 20 μM GM6001 (Calbiochem, San Diego, CA, USA) or 5 μM MMP-2/MMP-9 inhibitor I (MMP2/9i; Calbiochem). The wound at the same spot, marked by a reference line drawn on the outside of the dish, was photographed at different time points for a period of 31 hrs with a phase-contrast Zeiss microscope equipped with a camera. Two different fields of each scratch wound were photographed. Each condition was performed in triplicate. The wound widths were analysed with Image J software. The data were expressed as averages of the percentage width of the wounds at the measured time point over the original width at time 0 hr.

Collagen gel cultures

Collagen gel contraction assays were performed as described previously [19]. All experiments were performed in triplicate. Recovery of HTFs from collagen gels and analyses with the rabbit antibody for MMP-14 (Abcam plc, Cambridge, UK) were also as described [19]. Three sets of independent experiments were performed.

Gelatin zymography

MMP-2 activity in the culture supernatant was determined as described previously [19].

Immunofluorescence

Human Tenon's fibroblasts (HTFs) were plated on cover slips and treated with MMC or transfected with si-RNAs for 72 hrs before immunostaining was performed. For immunofluorescent analysis, antibodies specific for SPARC, collagen I, fibronectin and α-SMA were obtained from the same manufacturers as mentioned for immunoblotting. Labelling by the SPARC antibody was detected using secondary antibodies conjugated to Alexa Fluor-488 (Invitrogen, Eugene, OR, USA) while co-labelling by the collagen I, fibronectin or α-SMA antibodies was detected with secondary antibodies conjugated to Alexa Fluor-594 (Invitrogen). Labelled cells were visualized using the Zeiss Imager.Z1 microscope (Carl Zeiss).

Statistical analysis

Data are expressed as mean ± S.D. where appropriate. The significance of differences among groups was determined by the two-tailed Student's *t*-test using the Microsoft Excel 5.0 software, with significance at $P \leq 0.05$.

Results

Silencing of SPARC in HTFs

To inhibit SPARC expression in HTFs, we used siRNA to mediate depletion of SPARC expression. HTFs transfected with SPARC siRNA (si-SPARC) showed efficient silencing of SPARC mRNA expression by 99.98% relative to that in HTFs transfected with the negative control scrambled si-RNA (si-Scram) 24 hrs after transfection. This level of inhibition was sustained for at least 1 week, as shown by quantitative real-time PCR (qPCR; Fig. 1A). The mRNA expression was corroborated by Western analysis, which confirmed that SPARC protein expression was dramatically reduced in HTFs transfected with si-SPARC for 7 days (Fig. 1B).

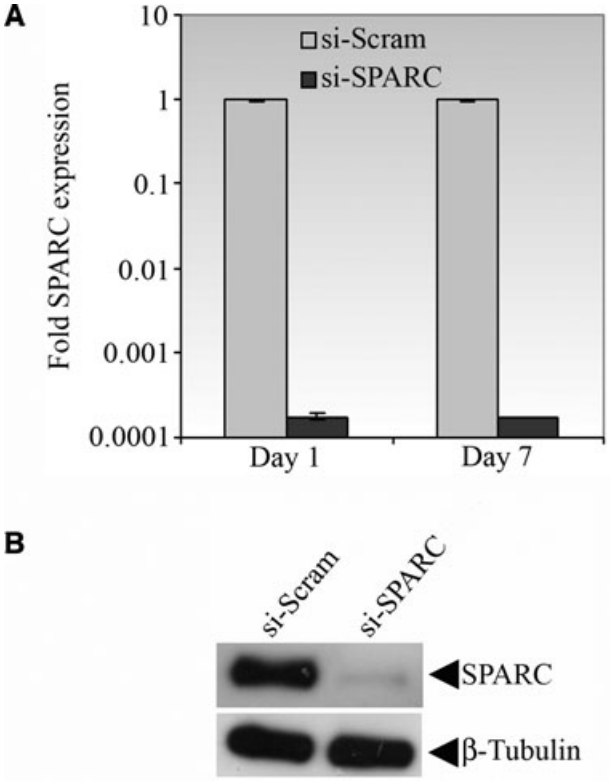

Fig. 1 Silencing of HTFs with si-SPARC was effective for at least 7 days. (**A**) HTFs were transfected with si-Scram (control) or si-SPARC and the mRNA level of SPARC was determined by quantitative real-time PCR after 1 or 7 days post-transfection. The β-actin transcript was used for normalization. Values are shown as fold expression relative to control for each time-point analysed. (**B**) HTFs were transfected as in (**A**) and the protein level of SPARC was determined by Western blotting with SPARC antibody after 7 days. Immunoblotting with β-tubulin was carried out to determine the loading levels in each sample.

SPARC-silenced HTF showed normal growth kinetics compared to growth inhibition by MMC

We investigated the effect of SPARC deficiency on HTF proliferation compared against treatment with MMC, which is well-established for its anti-proliferative activity. The real-time cell analyser SP instrument [20] was used to analyse HTF cell growth. Cell index values produced by the MMC-treated cells indicated the lack of proliferation while the cell index values of the untreated control cells increased progressively with time (Fig. 2A). Hence, a single application of MMC for 1 min. was able to suppress the growth of the HTFs effectively for up to 6 days. In comparison, the growth kinetics of si-SPARC-transfected HTFs were similar to that of the control si-Scram-transfected HTFs (Fig. 2B). Hence, silencing of SPARC in HTFs does not affect the growth properties of these cells.

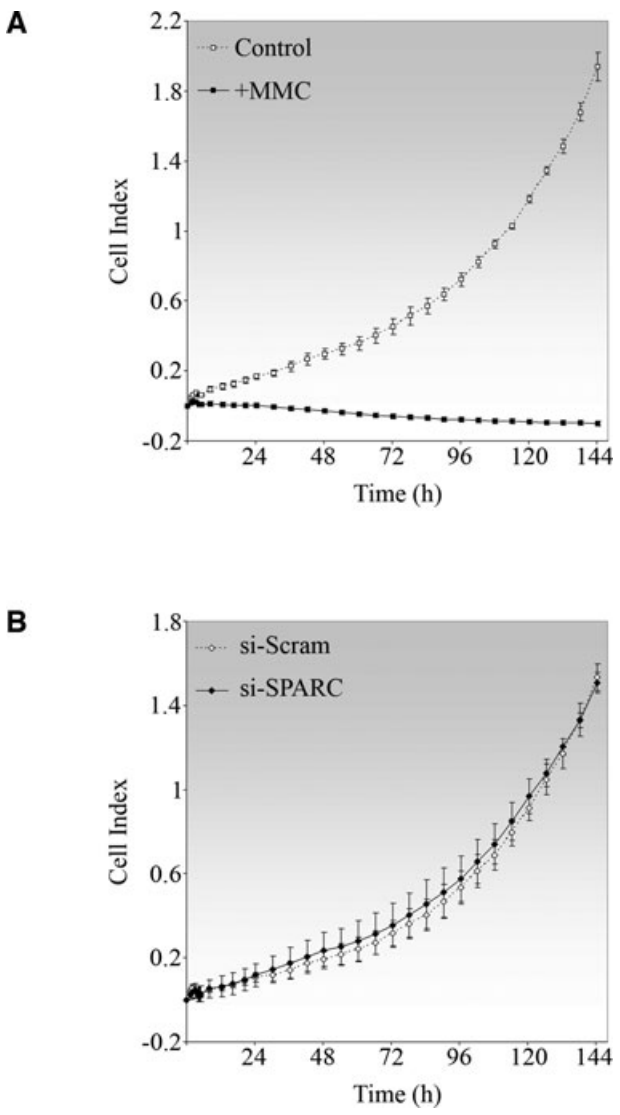

Fig. 2 Silencing of SPARC did not affect the cell proliferation capacity of HTFs. (**A**) HTFs were treated with 0.4 mg/ml MMC for 1 min. and washed before being plated on 96-well plates for real-time analysis of cell proliferation using the RTCA SP instrument. Untreated HTFs were similarly analysed to serve as controls. The cell index profiles of untreated cells (dotted line) and MMC-treated cells (solid line) reflected the logarithmic growth phase and response to treatment respectively for up to 6 days. The cell index values for MMC-treated cells did not demonstrate a logarithmic growth phase that was measured for the control cells, indicating that the MMC-treated cells did not proliferate. (**B**) HTFs were transfected with either si-Scram (control) or si-SPARC and plated the next day on 96-well plates for real-time analysis of cell proliferation as in (**A**). The cell index values for HTFs silenced for SPARC demonstrated a similar logarithmic growth phase as si-Scram–transfected control cells, indicating that knockdown of SPARC had no influence on the proliferative capacity of HTFs.

Silencing of SPARC reduced necrotic cell death compared to MMC

It has been proposed that MMC improves surgical outcome at least in part through the induction of apoptosis, which leads to early termination of the wound healing response [12]. To determine whether the induction of apoptosis or cell necrosis plays a role in the anti-fibrotic effect of SPARC knockdown, we analysed annexin V expression on the cell surface of si-SPARC-transfected HTFs using flow cytometry and compared that against MMC-treated cells. Surprisingly, MMC treatment resulted in 3.5-fold less early apoptotic cells compared to untreated HTFs at 72 hrs (Fig. 3A, upper panel, $P = 9.6\times10^{-6}$). However, MMC did cause a significant 12-fold increase in late apoptotic or necrotic cells compared to control (Fig. 3A, upper panel, $P = 6.1\times10^{-4}$). In comparison, SPARC knockdown did not result in a significant change in early apoptosis compared to si-Scram-transfected HTFs (Fig. 3A, lower panel, $P = 0.8$). Instead, SPARC silencing appeared to suppress necrotic cell death compared to control si-Scram-transfected HTFs by 1.6-fold (Fig. 3A, lower panel, $P = 0.03$). Analyses of the cells by TUNEL assay confirmed the flow cytometry results. As TUNEL preferentially labels cell apoptosis over necrosis, the number of TUNEL-positive cells upon MMC treatment was not observed to be greater than untreated HTFs (Fig. 3B). A dramatic difference between si-SPARC- and si-Scram-transfected HTFs was also not observed by TUNEL staining, in agreement with the flow cytometry results (Fig. 3B). Hence, the anti-fibrotic effect of SPARC depletion is not likely to be associated with the induction of apoptosis in HTFs.

SPARC knockdown delayed HTF cell migration

To determine whether silencing of SPARC impacts the migratory capacity of HTF, scratch wound assays were performed. As shown in Figure 4A, control (both untreated and si-Scram-transfected) HTFs migrated into the wound area and completely closed the wound within 31 hrs. On the other hand, SPARC-silenced HTFs maintained an open wound even at 31 hrs. Variations in cell proliferation were not factors for the differences between si-Scram- and si-SPARC-transfected HTFs because the cells exhibited similar growth kinetics (Fig. 2B) and HTFs closed the wound equally rapidly in the absence or presence of MMC within the same time frame (Fig. 4A and B). The reduction in migratory capacity of SPARC-silenced HTFs compared to si-Scram-transfected HTFs was significant at 23 and 31 hrs (Fig. 4B). Hence, the anti-fibrotic effect of SPARC silencing may be attributed in part to reduced cell migration.

SPARC-silenced and MMC-treated HTFs exhibited reduced fibroblast contractility to different extents and by distinct mechanisms

We next assessed the effects of SPARC silencing or MMC treatment in free-floating, mechanically unloaded collagen gel contraction by HTFs, a phenomenon that involves ECM remodelling [21]. The kinetics of collagen gel contraction were recorded over a 5-day period. MMC-treated HTFs were severely limited in the ability to contract the collagen gel compared to untreated control. Although lattice contraction by untreated HTFs has led to more than 90% reduction of the original lattice size by day 5, the MMC-treated HTFs never exceeded 70% (Fig. 5A, left panel, $P = 9.94\times10^{-7}$). HTFs silenced with respect to SPARC retained the capacity to exert collagen gel contraction (Fig. 5A, right panel). However, in agreement with our previous data with regard to SPARC-knockout mouse conjunctival fibroblasts [19], the contractile capacity of SPARC-silenced HTFs was reduced, as revealed by the significantly larger % gel size at day 5 compared to that of si-Scram-transfected HTFs (Fig. 5A, right panel, $P = 0.029$).

We showed previously that a reduction in MMP-2 activity may be partly responsible for the slower rate of collagen gel contraction in the SPARC knockout cells [19]. Accordingly, we analysed the conditioned media from the matrices on day 5 to determine if MMC treatment altered the MMP-2 activity level secreted by the cells. Gelatin zymography did not reveal an appreciable difference in MMP-2 activity between MMC-treated and control HTFs (Fig. 4B, left panel). In contrast, there was a striking reduction in MMP-2 activity in the si-SPARC-transfected cells compared to the si-Scram-transfected HTFs (Fig. 4B, right panel). Because the pro-MMP-2 activity appeared to be similar between the si-SPARC- and si-Scram-transfected HTFs, we proceeded to determine if the reduction in MMP-2 activity was due to an alteration in the expression of MMP-14, which along with pro-MMP-2 and TIMP-2, make up a trimolecular complex critical for MMP-2 activation [22]. Indeed, Western analysis of the cell lysates derived from the contracted matrices after 5 days showed that MMP-14 expression was reduced in the si-SPARC-transfected HTFs compared to the si-Scram-transfected HTFs (Fig. 4C, right panel). MMP-14 expression was not as prominently altered in the MMC-treated cells compared to control (Fig. 4C, left panel). This suggests that the inhibition of collagen contraction by MMC treatment is not predominantly due to reduced MMP-2 activity but that other signals are involved. Together, these results demonstrate that although SPARC silencing reduced the capacity of HTFs to contract collagen gels, MMC was far more effective in effecting this inhibition.

Silencing of SPARC exerted differential effects on the expression of pro-fibrotic genes in comparison to MMC treatment

We next examined whether silencing of SPARC or MMC treatment affect the expression of pro-fibrotic genes as well as their up-regulation by TGF-β, a cytokine viewed to play a master role during wound healing. The HTFs were analysed for the expression of collagen Iα1, fibronectin and α-SMA mRNAs in the absence or presence of TGF-β_2 by qPCR. Collagen I mRNA expression was

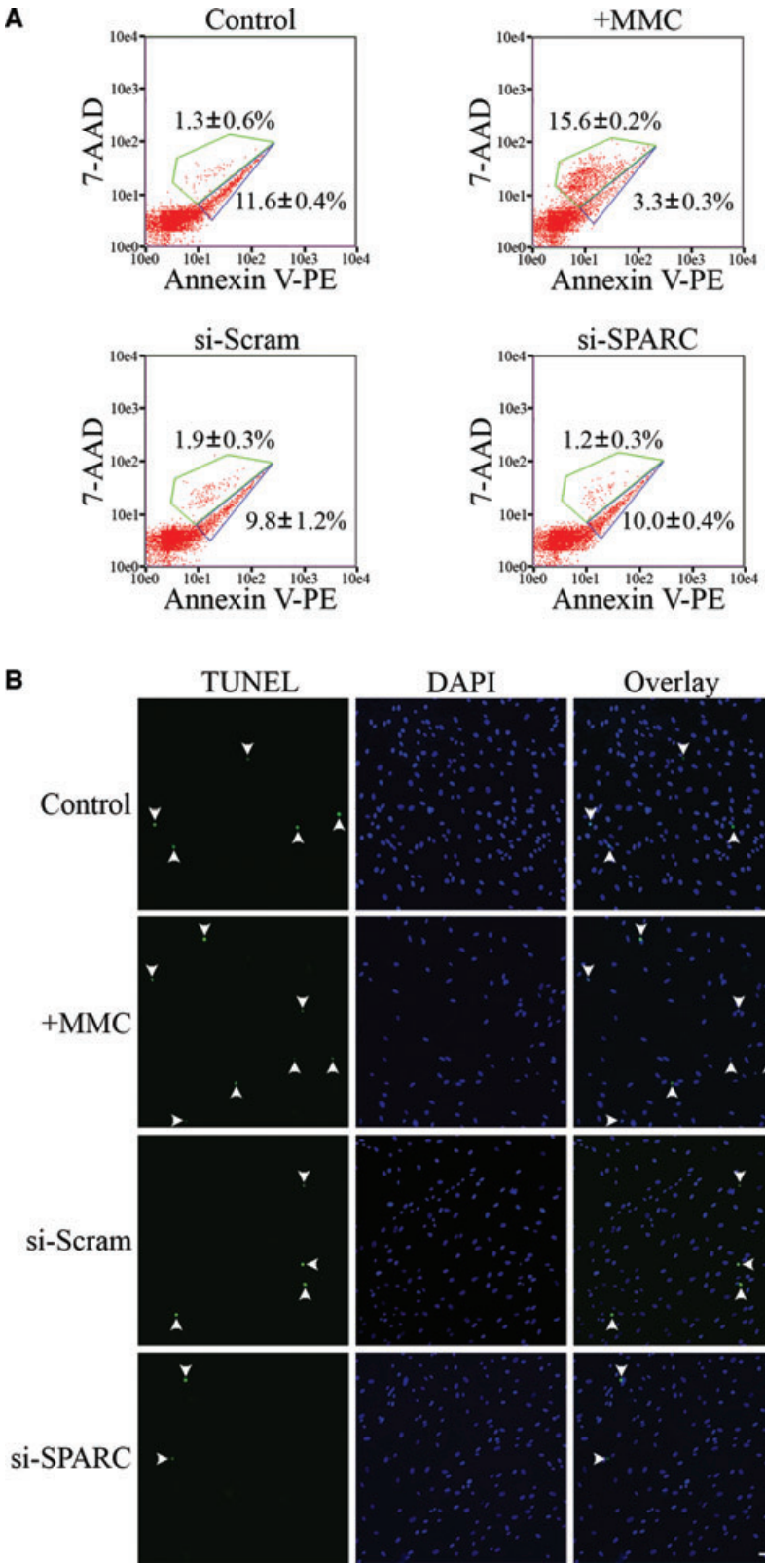

Fig. 3 Silencing of SPARC protected HTFs from necrosis. (**A**) HTFs, treated as indicated for 72 hrs, were harvested for both adherent and non-adherent cells and analysed for apoptosis and late apoptosis/necrosis. The representative scatterplots are shown. Apoptotic cells are enclosed within the blue perimeter and the mean percentage of apoptotic cells is indicated. Necrotic cells are shown within the green perimeter and the mean percentage of necrotic/late apoptotic is indicated. Data are presented as mean ± S.D. of the averages of three independent experiments, each performed in triplicate. (**B**) Cells were treated as in (**A**) and analysed by TUNEL staining (green). Nuclei were visualized by DAPI staining (blue). Cells positive for TUNEL staining are indicated by arrowheads; scale bar: 100 μm.

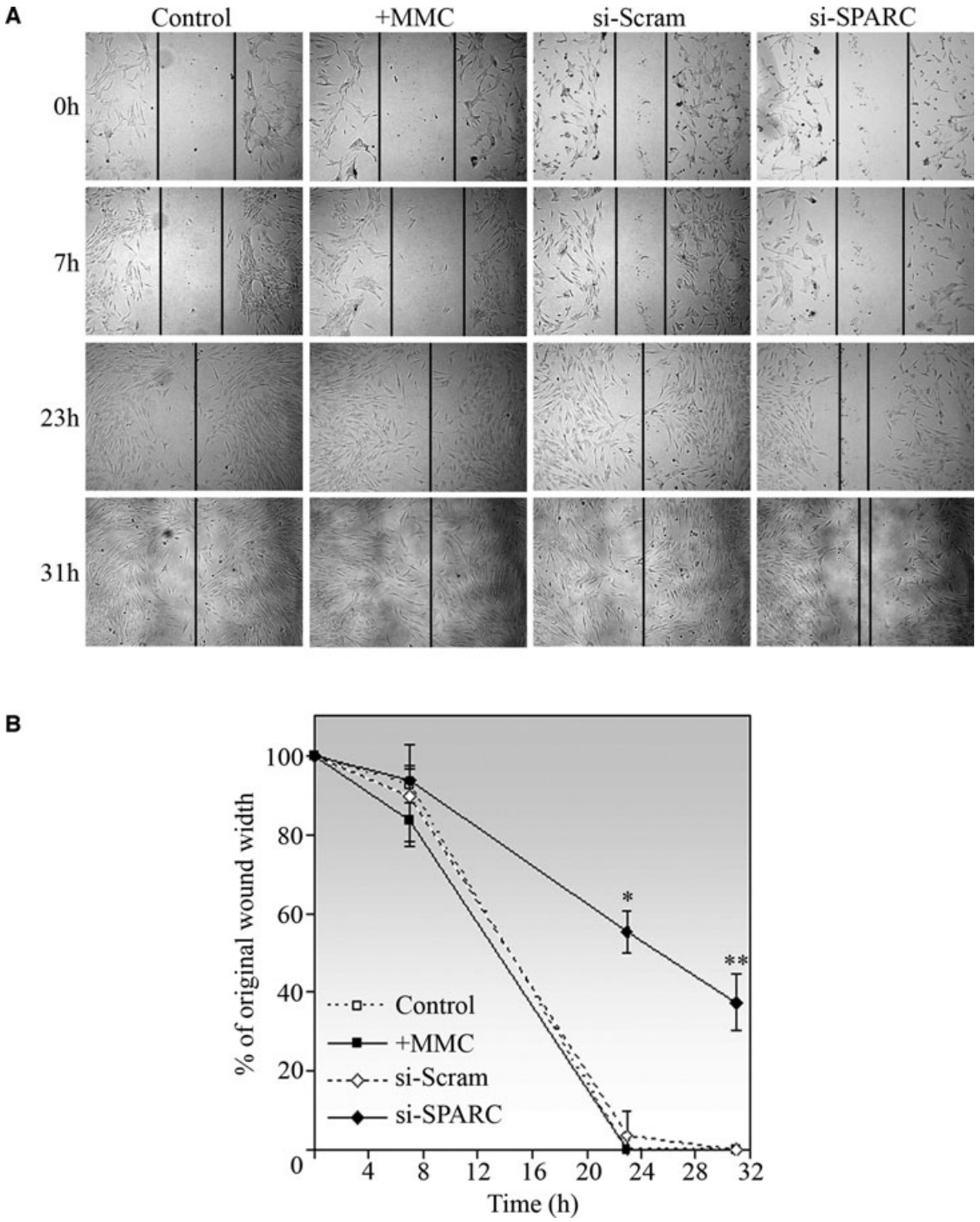

Fig. 4 Silencing of SPARC impaired HTF motility. (**A**) Plated HTFs were wounded with a pipette tip. After wounding, cell culture medium was replaced with fresh medium and wound closure was monitored by microscopy and photographed at the indicated times post-wounding. Triplicates were performed. A representative micrograph for each condition is shown. Vertical lines outline the wound edge. (**B**) Quantitative evaluation of HTF migration shown in (**A**). Values are expressed as percentages of the original wound widths. si-SPARC–transfected HTFs demonstrated significantly slower cell migration into the wound relative to si-Scram–transfected HTFs at 23 hrs (*$P = 0.0004$) and 31 hrs (**$P = 0.0009$) post-wounding. Data are presented as mean ± S.D. ($n = 3$).

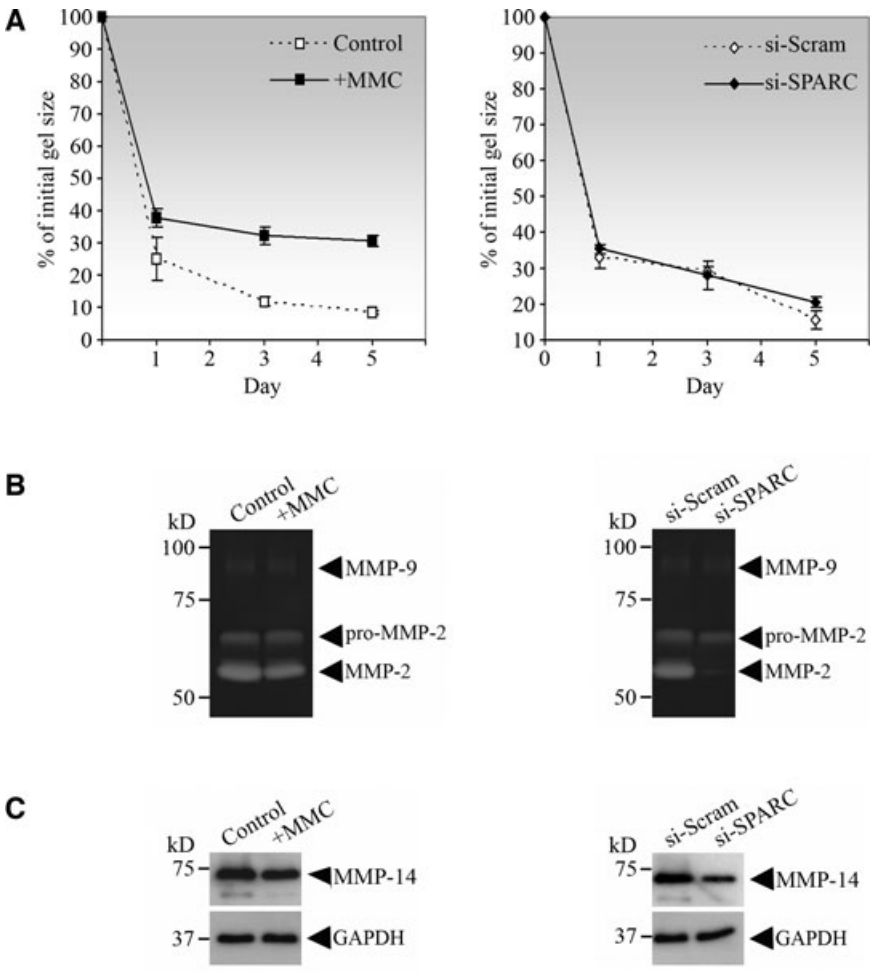

Fig. 5 Silencing of SPARC reduced the contractility of HTFs but to a lesser extent compared to MMC treatment. (**A**) Graphical representations of the contraction of free-floating collagen lattices seeded with control (untreated) or MMC-treated (left panel) and si-Scram- or si-SPARC–transfected HTFs (right panel) are shown. Data are presented as mean percentage of the initial gel size ± S.D. The data shown are from one experiment but is representative of three independent experiments, each performed in triplicate. (**B**) MMP-2 activity was reduced in medium conditioned by HTFs knocked down for SPARC seeded in collagen gels. Left panel: MMP-2 activity in medium conditioned by untreated or MMC-treated HTFs seeded in collagen gels for 5 days was analysed by gelatin zymography. Proteolytic activities corresponding to the molecular weights of MMP-9 (92 kD) and pro- and active MMP-2 (72 and 66 kD, respectively) are indicated by arrowheads. Right panel: MMP-2 activity in medium conditioned by si-Scram- or si-SPARC–transfected HTFs seeded in collagen gels for 5 days was similarly analysed. (**C**) MMP-14 expression was reduced in SPARC-silenced HTFs. Left panel: HTFs, control or treated with MMC, were seeded in collagen gels for 5 days before being lysed and equal amounts of protein subjected to immunoblotting with antibodies specific for MMP-14 and GAPDH (loading control). Right panel, similarly seeded si-Scram- or si-SPARC–transfected HTFs were subjected to the same immunoblot analysis for MMP-14 expression.

significantly reduced in both MMC-treated and SPARC-silenced HTFs relative to their respective controls (Table 1). MMC treatment appeared to be significantly more effective than SPARC silencing, although this is only a marginal 1.3-fold (Table 1). Incubation with TGF-β_2 resulted in marked up-regulation of collagen I mRNA, but to a significantly lesser extent in both SPARC-silenced and MMC-treated HTFs (Table 2). In fact, both SPARC-silenced and MMC-treated HTFs repressed collagen I gene expression induced by TGF-β_2 by the same magnitude, at 0.6-fold that of their respective TGF-treated controls (Table 2). In this capacity, there was no significant difference between SPARC-silenced and MMC-treated HTFs. These observations were verified at the protein level which showed that collagen I production was not significantly induced either in MMC-treated or in si-SPARC-transfected HTFs by TGF-β_2 (Fig. 6A and B).

In contrast to the effects on collagen I expression, neither silencing of SPARC nor MMC treatment was effective in significantly reducing fibronectin mRNA (Table 1) or protein (Fig. 6C and D) expressions. However, both SPARC-silenced and MMC-treated HTFs were able to maintain a significantly lower expression of fibronectin mRNA and protein in the presence of TGF-β_2 (Table 2, Fig. 6C and D). It also appeared that MMC treatment was significantly more effective, although marginal (1.2-fold), than SPARC silencing in maintaining a lower fibronectin mRNA expression relative to their respective controls in the presence of TGF-β_2 (Table 2). Hence, these data indicate that the steady-state level of fibronectin is not affected by either SPARC knockdown or MMC treatment, but that the stimulatory effect of TGF-β_2 can be inhibited.

Both SPARC knockdown and MMC treatment increased the mRNA expression of α-SMA, a marker for myofibroblasts (Table 1). The increase in α-SMA mRNA expression was significantly greater in SPARC-silenced HTFs compared to MMC-treated HTFs by 1.4-fold (Table 1). Surprisingly, TGF-β_2 induced less α-SMA mRNA expression in MMC-treated HTFs than control cells (Table 2). However, at the protein level, the silencing of

Table 1 Fold induction or suppression of mRNA expression by si-SPARC compared to MMC treatment as analysed by quantitative real-time PCR

mRNA	MMC/control	si-SPARC/si-Scram	*P* value
ECM/fibrotic genes			
Collagen Iα1	0.62 ± 0.04 (0.004)	0.81 ± 0.06 (0.03)	0.03
Fibronectin	1.28 ± 0.42 (0.4)	1.03 ± 0.16 (0.8)	0.4
α-SMA	1.57 ± 0.14 (0.02)	2.23 ± 0.08 (0.002)	0.007
MMPs			
MMP-1	2.56 ± 0.26 (0.01)	2.46 ± 0.28 (0.01)	0.7
MMP-2	1.61 ± 0.18 (0.03)	0.73 ± 0.07 (0.02)	0.004
MMP-3	3.06 ± 0.56 (0.02)	1.23 ± 0.16 (0.1)	0.03
MMP-9	2.01 ± 0.40 (0.048)	0.52 ± 0.11 (0.002)	0.02
MMP-14	0.95 ± 0.13 (0.6)	0.44 ± 0.07 (0.006)	0.009
Cytokines			
IL-8	1.81 ± 0.33 (0.049)	0.74 ± 0.15 (0.089)	0.01
MCP-1	1.72 ± 0.29 (0.05)	0.58 ± 0.03 (0.002)	0.02
TGF-β1	1.53 ± 0.16 (0.03)	1.01 ± 0.16 (0.9)	0.02
TGF-β2	1.21 ± 0.06 (0.02)	0.75 ± 0.02 (0.001)	0.005

Values are the means ± S.D. of the fold changes of MMC over control or si-SPARC over si-Scram from three independent sets of experiments, each set comprising of triplicates which were in turn measured in triplicates by qPCR. Values in parentheses denote the *P* values for the fold change of MMC over control or si-SPARC over si-Scram. *P* value in the last column indicates the significance between the mean MMC/Control and si-SPARC/si-Scram values from the three sets of data.

SPARC did not cause a significant increase in α-SMA expression compared to si-Scram-transfected control (Fig. 6E and F, right panels, $P = 0.35$) while MMC increased α-SMA expression relative to untreated HTFs by nearly twofold (Fig. 6E and F, left panels, $P = 0.002$). In the presence of TGF-β2, knockdown of SPARC could not prevent an induction of α-SMA protein expression while the already raised level of α-SMA protein expression in MMC-treated cells was not significantly raised further (Fig. 6E and F, left panel).

The expressions of collagen I, fibronectin and α-SMA in the HTFs transfected with si-SPARC or the negative control, si-Scram, were also analysed by immunofluorescent analysis. SPARC expression appeared to be localized in intracellular vesicular organelles in HTFs (Fig. 7). Collagen I expression was diminished in cells expressing low levels of SPARC (Fig. 7A, arrowheads). Alterations in fibronectin expression in cells expressing less SPARC were less obvious (Fig. 7B, arrowheads). In contrast, α-SMA expression in some HTFs expressing low levels of SPARC appeared slightly enhanced (Fig. 7C, arrowheads).

Table 2 Fold induction or suppression of mRNA expression by TGF-β2 after si-SPARC transfection or MMC treatment relative to their respective TGF-β2–induced controls

mRNA	MMC+TGF/ Control+TGF	si-SPARC+TGF/ si-Scram+TGF	*P* value
ECM/fibrotic genes			
Collagen Iα1	0.61 ± 0.04 (0.03)	0.62 ± 0.1 (0.04)	0.9
Fibronectin	0.70 ± 0.04 (0.0002)	0.85 ± 0.01 (0.02)	0.02
α-SMA	0.67 ± 0.09 (0.03)	1.66 ± 0.31 (0.2)	0.03

Values are the means ± S.D. of the fold changes of MMC+TGF over control+TGF or si-SPARC+TGF over si-Scram+TGF from three independent sets of experiments, each set comprising of triplicates which were in turn measured in triplicates by qPCR. Values in parentheses denote the *P* values for the fold change of MMC+TGF over control+TGF or si-SPARC+TGF over si-Scram+TGF. *P* value in the last column indicates the significance between the mean MMC+TGF/Control+TGF and si-SPARC+TGF/si-Scram+TGF values from the three sets of data.

Silencing of SPARC exerted differential effects on the expression and activity of matrix metalloproteinases (MMPs) in comparison to MMC treatment

MMPs are enzymes that modify the ECM during wound healing and tissue remodelling. These proteins control wound healing by regulating a myriad of biological activities including platelet aggregation, macrophage and neutrophil function, cell migration and proliferation, angiogenesis and collagen deposition. In this study, we examined the mRNA expression of MMP-1, MMP-2, MMP-3, MMP-9 and MMP-14 in HTFs following si-RNA transfection or MMC treatment. MMC treatment significantly increased the mRNA expressions of all the MMPs examined with the exception of MMP-14 (Table 1). In contrast, silencing of SPARC significantly reduced the mRNA expressions of MMP-2, MMP-9 and MMP-14 with no significant effect on MMP-3 expression (Table 1). Both SPARC silencing and MMC treatment increased MMP-1 expression by about the same extent (Table 1). We also studied the activities of MMP-2 in the culture supernatants of the si-RNA-transfected or MMC-treated HTFs by zymography. MMP-9 was not examined because its activity tended to be too low for accurate analysis. In agreement with the mRNA levels, si-SPARC-transfected HTFs expressed significantly less MMP-2 activity than its si-Scram-transfected counterpart (Fig. 8, right panels) while MMC-treated HTFs did not produce significantly different levels of MMP-2 activity from control (Fig. 8, left panels). To determine whether a reduction in MMP activity alters HTF cell migration, we performed scratch wound experiments using either a broad spectrum MMP inhibitor, GM6001, or a more potent MMP-2 and MMP-9 inhibitor, MMP-2/9i. As shown in Fig. S1, vehicle-treated HTFs migrated into the wound area

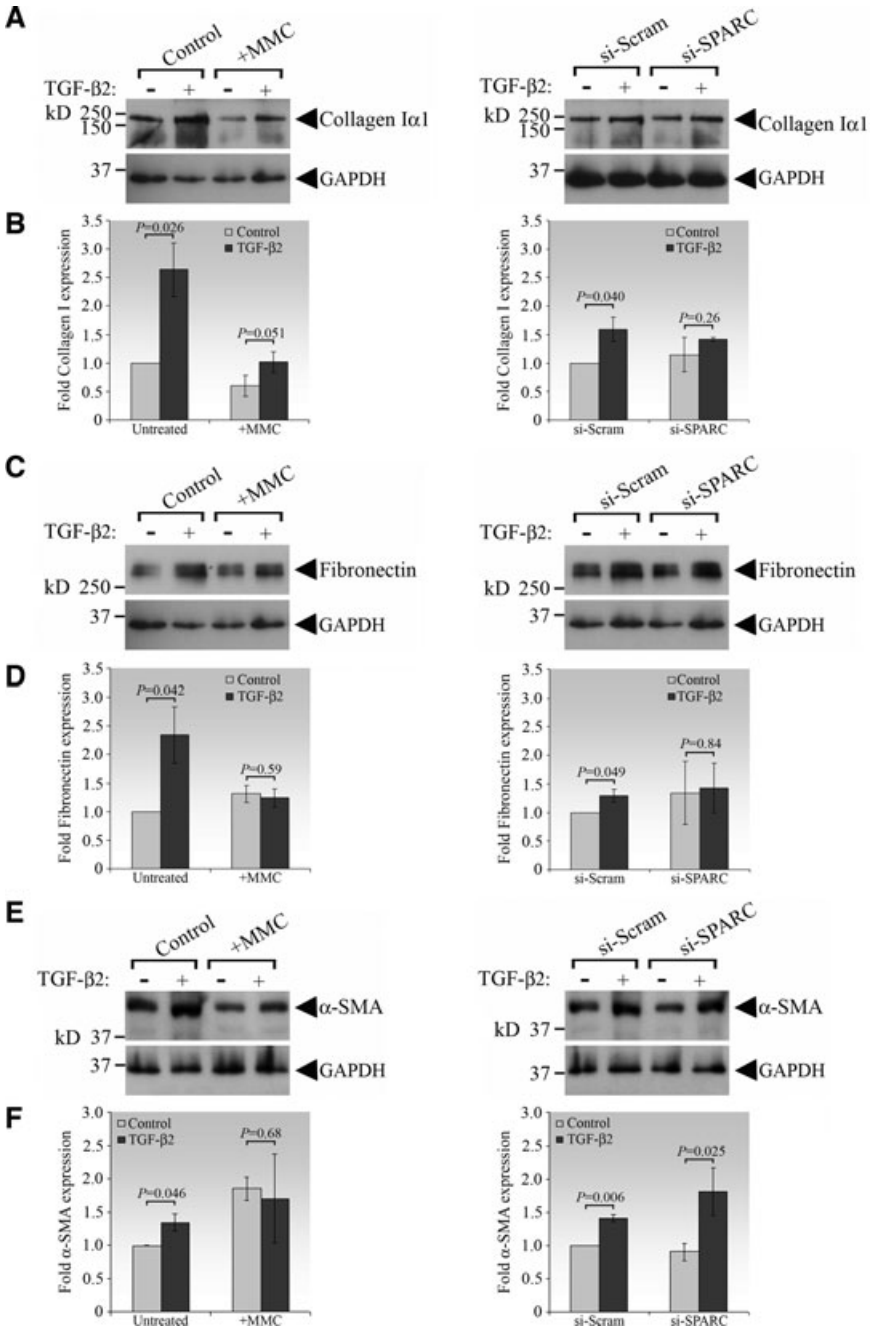

Fig. 6 Collagen I and fibronectin protein expressions were not induced in SPARC knockdown HTFs in response to TGF-β_2 but α-SMA expression remained inducible. (**A**) HTFs were treated with MMC (left panel) or transfected with siRNAs (right panel), with or without TGF-β_2, for 72 hrs before being analysed for collagen I protein abundance by immunoblotting for collagen Iα1 and GAPDH (loading control). The data shown are representative of three independent experiments. (**B**) Densitometric analysis of immunoblots from three independent experiments represented in (**A**). Data are presented as mean fold induction ± S.D. relative to their respective controls (untreated: left panel; si-Scram–transfected HTFs: right panel) from three independent experiments. The GAPDH level was used for normalization. The *P* value for each comparison is indicated above the bars. (**C**) HTFs were subjected to the indicated treatments as in (**A**) and analysed for fibronectin expression. The data shown are representative of three independent experiments. (**D**) Densitometric analysis of immunoblots from three independent experiments represented in (**C**). (**E**) HTFs were subjected to the indicated treatments as in (**A**) and analysed for α-SMA protein expression. The data shown are representative of three independent experiments. (**F**) Densitometric analysis of immunoblots from three independent experiments represented in (**E**).

and completely closed the wound within 23 hrs, as seen before with untreated HTF (Fig. 4). In comparison, MMP-2/9i-treated HTFs showed delayed migration with wound closure at 31 hrs while GM6001-treated HTFs maintained an open wound even at 31 hrs (Fig. S1A and B). The decrease in migratory capacity of HTFs treated with either GM6001 or MMP-2/9i was significant at 23 hrs (Fig. S1B). Variations in cell proliferation were not factors for the differences between the treated HTFs because treatment with either inhibitor did not significantly alter HTF growth kinetics (Fig. S1C). Our data therefore show that the migration of HTFs is mediated in part by MMP activity. Hence, the lower MMP expression and activity due to the silencing of SPARC is likely to contribute in part to the reduced migratory capacity of knockdown HTFs.

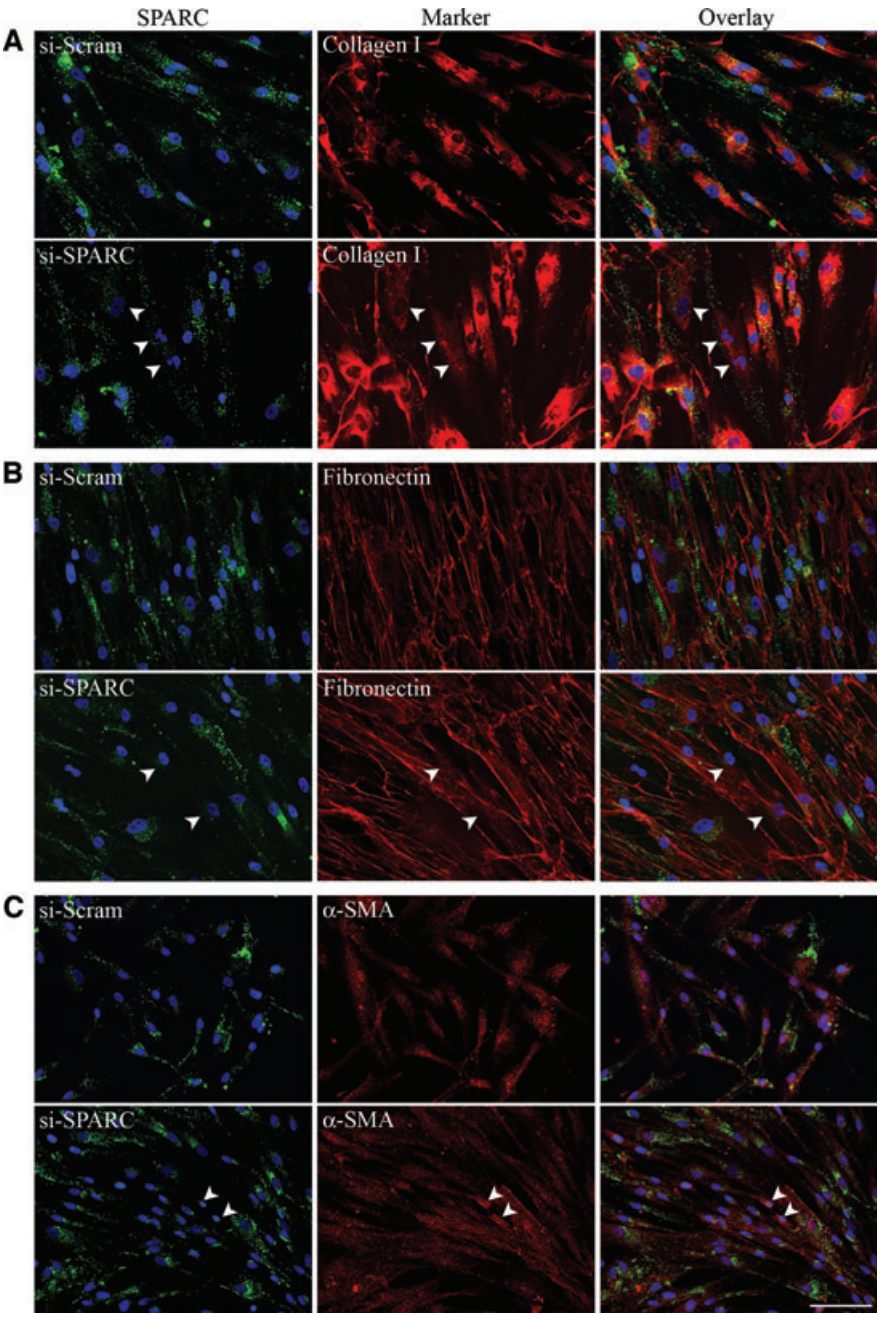

Fig. 7 Silencing of SPARC reduced collagen I in HTFs. (**A**) HTFs were transfected with either si-Scram or si-SPARC for 72 hrs and then simultaneously immunostained for SPARC (green) and collagen I (red). Nuclei were visualized by DAPI staining (blue). Cells expressing reduced levels of SPARC from transfection with si-SPARC also showed reduced collagen I expression (arrowheads). Overlapping staining is shown in the overlay. (**B**) HTFs transfected as in (**A**) were visualized for SPARC (green) and fibronectin (red) expressions simultaneously. Cells expressing reduced levels of SPARC from transfection with si-SPARC did not show obvious changes in fibronectin expression (arrowheads). (**C**) HTFs transfected as in (**A**) were visualized for SPARC (green) and α-SMA (red) expressions simultaneously. Cells expressing reduced levels of SPARC from transfection with si-SPARC showed slightly increased α-SMA staining (arrowheads). Scale bar: 100 μm.

Silencing of SPARC exerted differential effects on the expression of cytokine genes in comparison to MMC treatment

The wound healing process is frequently accompanied by the recruitment of inflammatory cells to the wound site. Inflammatory cells are typically attracted to wound sites by the secretion of chemokines such as interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1). We evaluated the effect of SPARC deficiency or MMC treatment on IL-8 and MCP-1 mRNA expressions in HTFs by qPCR. It has previously been reported that MMC upregulates the expressions of IL-8 and MCP-1 in corneal fibroblasts [23]. In agreement, conjunctival fibroblasts also responded to MMC treatment with significant increases in IL-8 and MCP-1 mRNA expressions (Table 1). In contrast, SPARC knockdown

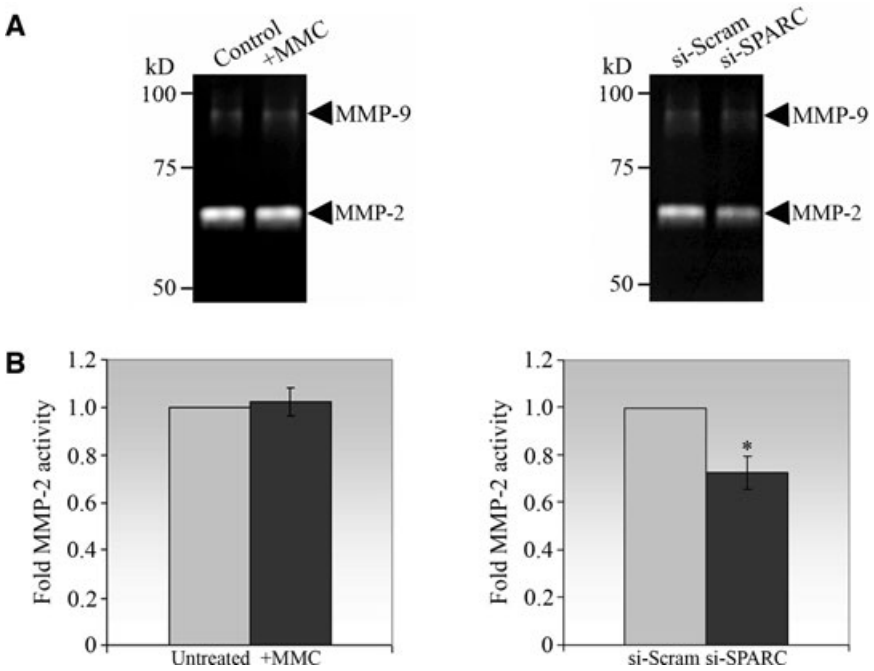

Fig. 8 Silencing of SPARC reduced MMP-2 activity. (**A**) MMP-2 activity was reduced in medium conditioned by HTFs knocked down for SPARC. Five μg of protein in the respective culture medium was analysed by gelatin zymography. Left panel: MMP-2 activity in medium conditioned by control (untreated) or MMC-treated HTFs cultured for 3 days was analysed. Proteolytic activities corresponding to MMP-9 and active MMP-2 are indicated by arrowheads. Right panel: MMP-2 activity in medium conditioned by si-Scram- or si-SPARC–transfected HTFs for 3 days was similarly analysed. The presented data are representative of three independent experiments. (**B**) Densitometric analysis of MMP-2 activity from three independent experiments represented in (**A**). Values are expressed as mean fold MMP-2 activity ± S.D. relative to that in control (untreated) HTFs (left panel; $P = 0.54$) or si-Scram–transfected HTFs (right panel; $^*P = 0.019$). Data are calculated based on the fold difference from three independent experiments.

resulted in a significant reduction in MCP-1 mRNA expression whereas IL-8 expression was unaffected (Table 1). Furthermore, because MMC is known to cause an increase in pro-fibrotic TGF-β production in HTFs within 7 days [14], we also examined the effect of either treatment on the expression of this cytokine. As shown in Table 1, MMC treatment resulted in significant increases in both TGF-β1 and TGF-β_2 mRNA expressions 72 hrs after treatment. SPARC knockdown, on the other hand, caused a striking and significant reduction in TGF-β_2 mRNA expression. Hence, our data revealed that SPARC knockdown may suppress the fibrotic response by down-regulating the expression of the inflammatory chemokine MCP-1 as well as the pro-fibrotic cytokine TGF-β_2.

Discussion

Many prior reports have indicated a strong association between elevated SPARC expression and tissue fibrosis. Conversely, the SPARC-null mouse exhibited diminished bleomycin-induced pulmonary fibrosis [24] as well as delayed scarring in a mouse model of GFS [19]. Hence, the targeting of SPARC expression is a potential therapeutic strategy for reducing conjunctival scarring. We examined in this study the mechanisms by which SPARC down-regulation modulate the key wound healing responses in HTFs. Silencing of SPARC did not affect HTF proliferation or apoptosis. However, SPARC down-regulation resulted in delayed cell migration, reduced collagen contractility and lower expressions of pro-fibrotic and pro-inflammatory genes.

Cellular hyperproliferation activated by wounding is generally considered an important cause of fibrosis as this process produces granulation tissue that fills in the wound after injury. For this reason, anti-proliferatives such as MMC are used to inhibit scarring and they are effective in delaying fibrosis in both clinical applications as well as in experimental animal models [25, 26]. However, a significant number of surgeries continue to fail even with their use [15]. Moreover, these drugs, being non-specific and un-targeted, tend to cause excessive and general cellular toxicity. Indeed, we showed that a single application of MMC caused significant necrotic cell death *in vitro*. Furthermore, the toxic effect of a single application of MMC seemed to have such a longevity and irreversibility that long-term tissue damage is a major concern [26]. Clinically, conjunctival thinning due to cell death at the site of MMC application is the most likely cause for many of the devastating and sight-threatening side effects observed, including blebitis, keratitis, bleb leakage, chronic hypotony and endophthalmitis [11, 16, 17, 27, 28]. Thus, targeting cell proliferation may not be the safest approach to inhibiting fibrosis in GFS.

Although SPARC knockdown did not inhibit cell proliferation and even seemed to protect the HTFs from necrotic cell death *in vitro*, SPARC deficiency was evidently effective in increasing surgical success in a mouse model of GFS, due in part to the reduced production and deposition of the scar protein, collagen, at the subconjunctival wound site *in vivo* [19]. Our results with the HTFs corroborated this notion. More importantly, stimulation by the pro-fibrotic cytokine TGF-β resulted in a comparatively lower level of collagen I expression when SPARC expression was suppressed. Also relevant to this is the finding that SPARC down-regulation

resulted in a decrease in MCP-1 expression in HTFs. MCP-1 is a multifunctional chemokine shown to be up-regulated in a variety of fibrotic diseases including idiopathic pulmonary fibrosis, systemic sclerosis and bleomycin-induced murine scleroderma [29–32]. Early on, it was thought that MCP-1 functioned mainly as a pro-inflammatory chemokine but there is now increasing evidence to suggest that MCP-1 may also directly up-regulate the expression of collagen I [33]. Recently, a study in MCP-1 null mice suggested that this chemokine may be involved in collagen fibre formation *in vivo* [34]. Hence, MCP-1 may have an ECM modulatory role in addition to its function as a pro-inflammatory mediator. It is thus tempting to speculate that SPARC regulates collagen production not only by modulating its stability during its production [35, 36], but also possibly *via* a secondary mediator such as MCP-1.

Cell migration is an important facet of wound healing as fibroblasts migrate to repair the wound at the surgical site. Depletion or down-regulation of SPARC has previously been shown to inhibit cell migration in various cell types [37, 38]. In agreement, we observed a delay in wound closure by SPARC-silenced HTFs *in vitro*. SPARC is thought to mediate cell migration by allowing cells to de-adhere from their normal attachments with their surrounding matrix as well as other neighbouring cells. Furthermore, SPARC is known to regulate MMP activity [39, 40], which is shown in this study and by others to modulate cell migration [41]. The reduced expression and activity levels of key MMPs due to SPARC knockdown in HTFs may thus partially account for the inhibition of cell migration in the knockdown cells.

The free-floating collagen lattice contraction model has been well-characterized for the study of cell-mediated ECM reorganization, wound contraction and tissue remodelling during wound healing. The mechanisms for fibroblast-mediated collagen gel contraction are not well understood, but are thought to be related to traction forces exerted by cells cultured on the underlying matrix resulting in a reorganization of the collagen network and subsequent phenotypic changes in both cell adhesion and signalling including the up-regulation of MMP-1 expression and secretion [42–45]. *In vivo*, a reorganized collagen matrix may encourage the production of a mature scar of high tensile strength because compacted collagen bundles may facilitate cross-linking by lysyl oxidase [46]. MMC treatment somehow incapacitated the ability of HTFs to remodel the collagen matrix and there was no obvious increase in MMP-2 activity, although the same treatment in two-dimensional HTF cultures increased the mRNA expression of MMPs. At this moment, we can only surmise that this phenomenon is related to the non-proliferative nature of the MMC-treated cells, as was also observed previously with cells that have lost both the *in vitro* potential to proliferate as well as contract collagen matrices [47]. It does suggest although, that there is a severe interruption in the dialogue between MMC-treated cells and their three-dimensional matrix environment, the specifics of which remains to be investigated. The less dramatic reduction in collagen lattice contraction exerted by SPARC-silenced HTFs is, on the other hand, possibly due in part to a reduction in MMP-2 activity, as was observed before for SPARC knockout mouse conjunctival fibroblasts [19]. We were inclined to believe that MMP-2 reduction plays a role in this function due to a previous finding which showed that inhibition of MMP activity significantly reduced HTF-mediated collagen lattice contraction in similar free-floating matrices [48]. Hence, our data suggest that, although both MMC and SPARC knockdown inhibit the reorganization of collagen matrices, there is a clear difference in the mechanisms by which they do so.

A scan of the current literature suggests that fibrosis commonly develops when inflammatory reactions are persistent [49]. As a matter of fact, most clinicians believe that a chronic inflammatory response is a poor prognostic sign in GFS. Indeed, treatment with anti-inflammatory medication has proven to be effective in attenuating the post-operative development of subconjunctival fibrosis in patients [50]. The evidence to support the importance of the inflammatory phase of wound healing to the extent of scar formation is extensive [51]. Of particular relevance is the importance of MCP-1, which was found in a recent study of 61 glaucoma patients to be specifically elevated in the tears of those who required surgical revision within 6 months of the original filtration operation due to subconjunctival fibrosis [52]. We found in this study that silencing of SPARC in HTFs resulted in reduced expression of MCP-1, which may potentially be further inactivated by the increased expression of MMP-1 known to cleave and inactivate MCP-1 [53]. Thus, the decrease in MCP-1 expression and possibly activity by SPARC down-regulation may have an impact in dampening the inflammatory response and the associated fibrosis. The anti-inflammatory property of SPARC knockdown may potentially also be enhanced by the lower expression level of MMP-9, which has the ability to cleave and activate IL-8 [54]. Thus, SPARC knockdown has the potential to confer on HTFs anti-inflammatory properties while MMC treatment is likely to be pro-inflammatory with the up-regulation of MCP-1 and IL-8 expressions.

Previous reports have shown that MMC-treated, growth-arrested fibroblasts are capable of performing wound-healing functions. MMC-treated HTFs maintained migratory ability and continued to express TGF-β and bFGF [14]. Furthermore, MMC-treated HTFs have been found to increase interferon-β production, suggesting a potential for HTFs to participate in aggressive wound healing reaction by mediating a chronic inflammatory phase *via* the inhibition of T cell apoptosis [10]. In agreement, we confirm in this study that MMC-treated HTFs continued to express TGF-β_1, TGF-β_2, as well as increased levels of α-SMA, MMPs and the pro-inflammatory cytokines, IL-8 and MCP-1, while cell migration remained unperturbed. These accumulating observations explain why some surgeries continue to fail despite the use of MMC. The current accepted use of MMC to attenuate the post-operative scarring response is evidently therapeutically suboptimal. Our reported findings that SPARC knockdown in HTFs modulates key cellular processes activated during the subconjunctival wound healing response without causing cellular toxicity, unlike with MMC, supports the targeting of SPARC expression as an improved anti-fibrotic strategy over MMC for treating post-operative scarring following GFS.

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Conflict of interest

The authors declare that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Inhibition of MMP activity reduced HTF motility.

Table S1 Primers used with real-time PCR.

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