

Research article

Impaired wound healing in mice deficient in a matricellular protein SPARC (osteonectin, BM-40)

Amitabha Basu^{1,3}, Lorraine H Kligman², Stefan J Samulewicz¹ and Chin C Howe^{*1}

Address: ¹The Wistar Institute, 3601 Spruce Street, Philadelphia, PA, 19104, USA, ²Department of Dermatology, University of Pennsylvania, Philadelphia, PA, 19104, USA and ³Germantown High School, The School District of Philadelphia, Germantown Avenue and High Street, Philadelphia, PA, 19144, USA

E-mail: Amitabha Basu - amitbasu72@hotmail.com; Lorraine H Kligman - LoriFBCR@aol.com; Stefan J Samulewicz - Ssamulewicz@wistar.upenn.edu; Chin C Howe* - CHowe@wistar.upenn.edu

*Corresponding author

Published: 7 August 2001

Received: 3 May 2001

BMC Cell Biology 2001, 2:15

Accepted: 7 August 2001

This article is available from: <http://www.biomedcentral.com/1471-2121/2/15>

© 2001 Basu et al; licensee BioMed Central Ltd. Verbatim copying and redistribution of this article are permitted in any medium for any non-commercial purpose, provided this notice is preserved along with the article's original URL. For commercial use, contact info@biomedcentral.com

Abstract

Background: SPARC is a matricellular protein involved in cell-matrix interactions. From expression patterns at the wound site and in vitro studies, SPARC has been implicated in the control of wound healing. Here we examined the function of SPARC in cutaneous wound healing using SPARC-null mice and dermal fibroblasts derived from them.

Results: In large (25 mm) wounds, SPARC-null mice showed a significant delay in healing as compared to wild-type mice (31 days versus 24 days). Granulation tissue formation and extracellular matrix protein production were delayed in small 6 mm SPARC-null wounds initially but were resolved by day 6. In in vitro wound-healing assays, while wild-type primary dermal fibroblasts showed essentially complete wound closure at 11 hours, wound closure of SPARC-null cells was incomplete even at 31 hours. Addition of purified SPARC restored the normal time course of wound closure. Treatment of SPARC-null cells with mitomycin C to analyze cell migration without cell proliferation showed that wound repair remained incomplete after 31 hours. Cell proliferation as measured by ³H-thymidine incorporation and collagen gel contraction by SPARC-null cells were not compromised.

Conclusions: A significant delay in healing large excisional wounds and setback in granulation tissue formation and extracellular matrix protein production in small wounds establish that SPARC is required for granulation tissue formation during normal repair of skin wounds in mice. A defect in wound closure in vitro indicates that SPARC regulates cell migration. We conclude that SPARC plays a role in wound repair by promoting fibroblast migration and thus granulation tissue formation.

Background

Wound healing involves reepithelialization, granulation tissue formation and contraction (reviewed in refs.

[1,2]). Reepithelialization and granulation tissue formation in turn involve proliferation and migration of cells from the wound edge to fill the wound site. In higher ver-

tebrate adult animals, reepithelialization of a wound begins within hours after injury while granulation tissue begins to form approximately 4 days after injury. Besides fibroblasts, granulation tissue consists of macrophages, blood vessels, and extracellular matrix (ECM) produced by fibroblasts. Once the wound is filled with granulation tissue and covered with a neoepidermis, a portion of the wound fibroblasts transforms into myofibroblasts, which contract the wound to reduce and strengthen the defect. Myofibroblasts are rich in F-actin bundles, which establish cell-cell and cell-matrix linkages and thus generate the force for wound contraction.

SPARC (secreted protein acidic and rich in cysteine) is an extracellular glycoprotein expressed in elevated levels in actively proliferating cells and organs such as developing embryos and adult tissues associated with remodeling (bone and gut), wound healing, angiogenesis (reviewed in refs. [3,4]), and tumorigenesis [5,6]. SPARC belongs to a class of recently denoted matricellular proteins that are involved in cell-ECM interactions. It binds to ECM proteins and interacts with cells. SPARC has been postulated to modulate cell shape [3,4] and cell adhesions [4,7,8], to regulate cell proliferation [3,4] and migration [9,10], and to influence matrix contraction [11]. In addition, SPARC is known to bind to certain growth factors [12] and to modulate expression of ECM components [13]. Many of these functions are requisite to wound repair.

To further investigate the role of SPARC in wound healing, we used cells from our recently generated SPARC-null mice [14]. As we reported previously SPARC plays a role in regulating expression of growth factors, growth factor receptors, and ECM proteins and in modulating cell proliferation. In the absence of SPARC, expression of transforming growth factor- β 1 and type I collagen is down-regulated in kidney mesangial cells [15]. The expression of cell surface receptor for insulin-like growth factor I in SPARC-null embryonic fibroblasts is also diminished [16]. Studies with SPARC-null cells have confirmed that SPARC functions in cell proliferation. Mesangial cells, adult skin fibroblasts and smooth muscle cells from SPARC-null mice proliferate faster than their respective wild-type counterparts in media containing fetal bovine serum (FBS) [17]. Although embryonic fibroblasts from the SPARC-null mice grow at the same rate as the wild-type cells when they are cultured in FBS, the SPARC-null embryonic fibroblasts exhibit down-regulated proliferation as compared to the wild-type cells when the fibroblasts are cultured in serum-free medium containing insulin as the sole mitogenic factor [16]. Thus, it appears that the role of SPARC in cell proliferation is cell type and environment dependent.

Although SPARC is expressed in fibroblasts and macrophages at wound sites [18] and functions in many processes important to wound repair, the role of SPARC in wound healing in a whole animal has not yet been studied. Previously, studies of phenotypes in this laboratory have shown that SPARC-null mice develop osteopenia around 2.5 months of age [19] in bones where SPARC is normally the most abundant non-collagenous glycoprotein [3]. The second phenotype thus far uncovered is in the lens where SPARC is normally produced by lens epithelial cells [20,21] and is a component of the lens capsule [20,22]. SPARC-null mice show progressive cataract formation beginning approximately 1.5 months after birth [14,21]. Electron microscopy of the SPARC-null lens revealed abnormality at the lens cell-ECM (capsule) interface with an intrusion of cellular processes into the basement membrane of the lens capsule, whereas wild-type lens exhibited a precise border at the cell-matrix interface [23]. In the present study, we show for the first time that SPARC plays a role in wound repair in vivo and in vitro. Wound healing in SPARC-null mice is retarded due to delayed granulation tissue formation. Repair of a wound in vitro by dermal fibroblasts is impaired due to defect in cell migration.

Results

Delayed wound healing in SPARC-deficient mice

Two independent experiments with 25 mm oblong full-thickness excision wounds (6 mice of each type per experiment), including the striated muscle layer (panniculus carnosus), on the dorsal skin of wild-type and SPARC-null mice indicated that the absence of SPARC impeded healing of skin wounds. One day after surgery a clear thin film of dried exudate covered the wounds in the wild-type and SPARC-null mice. Build-up of dehydrated wound crusts or scabs began at day 3 and became subsequently more extensive. In the SPARC-null mice scabs were thicker and extravasation of blood, visible in the gaps within the scabs, was more frequent and extensive than that in the control mice. In control wild-type mice, loss of wound scabs began at day 17 and was complete in all mice by day 24 (Fig. 1A, lower panel) with wounds well-healed and epithelial covering restored (Fig. 1A, upper panel). In contrast, the scabs in SPARC-null mice started to come off partially around day 14, resulting in a scab with a gaping red wound field, which was evident even at 22 days (Fig. 1A, upper panel). Healing of SPARC-null wounds remained incomplete on day 24, and complete healing in all mice was observed between 25 and 31 days (Fig. 1A, lower panel). Similar results were seen with 30-mm circular excision wounds (not shown).

Although the larger 25 mm oblong, full thickness excisions allowed us to observe visible differences in wound

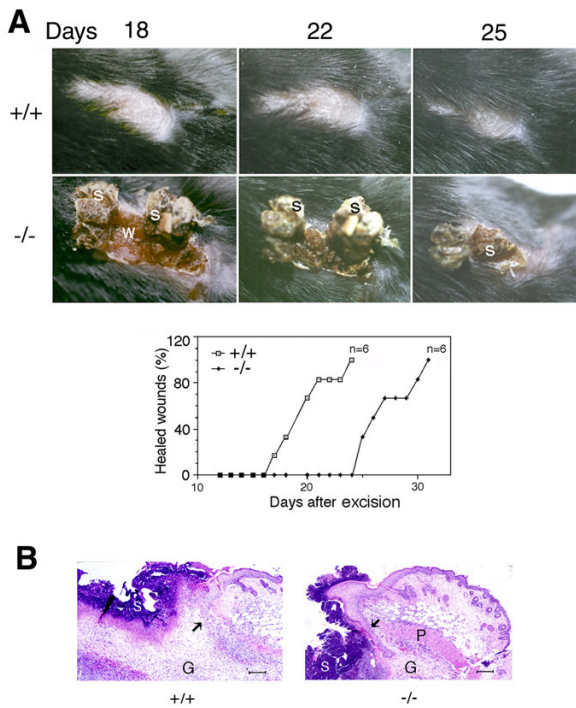


Figure 1
 Healing of 25 mm oblong skin wounds (A) and histology of 6 mm circular skin wounds (B) in wild-type (+/+) and SPARC-null (-/-) mice. In (A) upper panel: an example of the progress of repair processes at the indicated times (days) is shown. S: scab; W: open wound field. In (A) lower panel: percent of wild-type and SPARC-null mice (6 in each group) with healed wounds, defined as loss of the wound scabs and complete covering of the wounds with epidermis, is plotted versus days after the excision. Panel (B) illustrates histologic appearance of 6-mm wounds in a wild-type (+/+) mouse and an SPARC-null (-/-) mouse 4 days after surgery. Granulation (G) tissue formation is more extensive in the wild-type mouse than in the SPARC-null mouse. Arrows indicate reepithelialized wound edge. Reepithelialization appears to be as efficient in mutant mice as in control mice. S: scab; P: panniculus carnosus. Hematoxylin and eosin staining. Scale bar, 210 μ m.

healing progression, the wound sites were too fragile to be isolated for histological examination. We therefore made 6 mm full thickness circular lesions and examined the gross and microscopic appearance of skin wounds at various time points, with four wild-type and four SPARC-null mice at each time point. Examination of the gross appearance of wounds showed no apparent difference in healing progression between wild-type and SPARC-null mice, with build up of scab at day 1 and loss of scab by day 10. However, we detected histological differences between wild-type and SPARC-null mice. Histology of 6 mm wound tissues showed that at day 2 after

wounding the epidermis at wound edges had grown thicker in both wild-type and SPARC-null mice, indicating that proliferation of keratinocytes had begun. However, no granulation tissue formation was visible at this stage of healing in either wild-type or SPARC-null mice (results not shown). At day 4 reepithelialization and granulation tissue growth had begun as shown in the 6 mm biopsies in figure 1B. The epidermis had begun to migrate centrally to cover the wound edge and reepithelialization at this early stage of wound healing in a small biopsy appeared to be as efficient in SPARC-null mice as in the wild-type mice except for the more extensive granulation tissue formation in the wild-type than in the SPARC-null mice. In wild-type mice, the granulation tissue had formed at the edge and base of the wound. In SPARC-null mice, the granulation tissue covered the edge of the wound but did not extend to the base of the wound (Fig. 1B). At day 6, reepithelialization and granulation tissue formation appeared as elaborate in SPARC-null mice as in wild-type mice with the neopidermis and granulation tissue filling two third of the wound beds (not shown). By day 10, the wound areas in both control and mutant mice were smaller, reflecting some degree of wound contraction, and in addition, the wounds were now closed, being completely covered by epidermal cells and underlying granulation tissue (results not shown).

Northern analysis of 6 mm circular wound tissues isolated during a 6 day healing period showed similar patterns of cytokeratin 14 expression in SPARC-null mice and wild-type mice (Fig. 2). Cytokeratin 14 expression is associated with proliferative epidermal keratinocytes at wound sites [24]. Such expression in control unwounded skin (0 day) is, as expected, very low but increased appreciably by day 2 after wounding with peak expression at day 4. Interestingly, expression of cytokeratin 14 was up-regulated in the normal epidermis and neopidermis of SPARC-null mice relative to those of wild-type mice (Fig. 2), although histologically no differences in reepithelialization between the SPARC-null and wild-type wounds were apparent (Fig. 1B). Analysis of α 1(I) collagen and fibronectin transcripts, which are synthesized by fibroblasts of dermal granulation tissue [25–27], revealed that expression of both transcripts was delayed in the wounds of SPARC-null mice relative to those of wild-type mice (Fig. 2). In wild-type mice, both transcripts were expressed in the unwounded skin (0 day) used as controls. At day 2, the levels of the two transcripts decreased appreciably below the levels of the control skin followed by an increase in expression between day 2 and day 4. In the case of SPARC-null mice, expression of the two transcripts remained below the control unwounded skin at day 2 and day 4 and did not begin to increase until between day 4 and day 6 (Fig. 2). Thus, we conclude that SPARC deficiency compromises granulation tissue for-

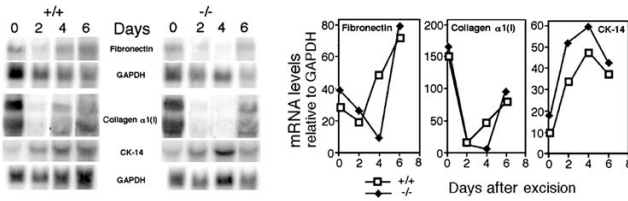


Figure 2
 Expression of fibronectin, α1(I) collagen and cytokeratin 14 (ck-14) transcripts (left panel) and levels of mRNA expression relative to that of GAPDH set at 100 (right panel) in wounds of wild-type (+/+) and SPARC-null (-/-) mice. Five 6 mm diameter wounds were made on the back of each mouse. Biopsies were collected from 3 wild-type and 3 SPARC-null mice 2, 4, and 6 days after the surgery. Skin from the backs of 2 unwounded wild-type and mutant mice (0 day) was collected and used as controls.

mation that involves fibroblast ingrowth and ECM protein deposition.

Impaired wound repair by cultured SPARC-null fibroblasts
 Granulation tissue formation after cutaneous wounding involves both cell proliferation and migration [1]. However, these two processes are difficult to analyze in vivo. To investigate these two processes, a small scrape wound was made across a confluent monolayer of primary dermal fibroblasts and the process of wound closure was monitored (Fig. 3A). In wild-type cells, at 11 hours after wounding, repair of the wound was essentially complete with the denuded area covered with fibroblasts (Fig. 3A, upper panel). In comparison, repair of the wounded SPARC-null fibroblast monolayer was retarded (Fig. 3A, lower panel). The denuded area remained unpopulated through 11 to 15 hours, even though cells at the edge of the wound showed forward movement. The first evidence of cell ingrowth did not appear until around 24 hours and wound repair was not complete until some time between 31 and 48 hours after wounding. Since the doubling time for these dermal fibroblasts was about 20 hours (our unpublished observation), the healing in wild-type cells at 11 hours implied that wild-type fibroblasts had extensively migrated from the edge of the wound into the wound site prior to the onset of cell proliferation. However SPARC-null cells did not show any migration prior to the onset of cell proliferation and ingrowth was retarded even after the onset of cell proliferation. This defect in cell motility was rescued to the level of wild-type cells by the addition of purified SPARC (Fig. 3B). The denuded area was repopulated by the SPARC-null cells by 11 hours in the presence of added SPARC (Fig. 3B, lower panel) as compared to 48 hours in its ab-

sence (Fig. 3A, lower panel). The repair of wild-type fibroblasts was not affected by the addition of SPARC and was complete by 11 hours in the presence of exogenous SPARC (Fig. 3B, upper panel) or in its absence (Fig. 3A, upper panel). The results indicate that SPARC regulates wound healing directly through cell migration. Although the long period of impeded ingrowth of SPARC-null cells after wounding already suggested defect in cell migration rather than cell proliferation, we determined cell migration in the absence of cell proliferation. Mitomycin C blocks mitosis and thus allows analysis of cell migration

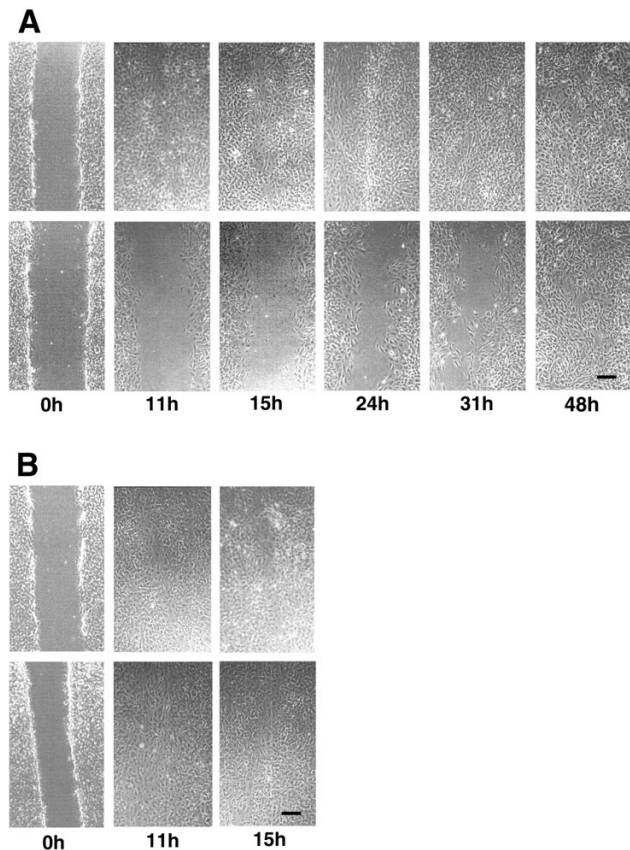


Figure 3
 Phase-contrast photomicrographs showing the process of wound repair by wild-type (upper panel) and SPARC-null (lower panel) fibroblasts cultured in the absence of SPARC (A) and in the presence of SPARC (B). In (A), wild-type (upper panel) and SPARC-null (lower panel) fibroblasts were grown to confluence in DMEM containing 10% FBS and then wounded (example shown at 0 hour). Wound closure was monitored at various time points. Wound healing is delayed in SPARC deficient fibroblasts. In (B), wounded monolayers of wild-type (upper panel) and SPARC-null (lower panel) fibroblasts were cultured in DMEM containing 10% FBS and SPARC protein purified from human platelets. Exogenous SPARC restores wound healing ability of SPARC-null cells to the level of wild-type cells. Scale bar, 62.5 μm.

in the absence of cell proliferation. Treatment with mitomycin C did not affect the time course of wound closure of either the wild-type or SPARC-null cells (Fig. 4). The wound in a monolayer of wild-type fibroblasts cultured in the absence or presence of mitomycin C healed by 15 hours after wounding (Fig. 4A). The wound in a monolayer of SPARC-null fibroblasts, whether cultured in the presence of mitomycin C or in its absence, remained unrepaired at 15 hours, began to show slight closure at 24 hours and healed by 48 hours (Fig. 4B). These results confirm that cell migration is a key step affecting the rate of wound healing and that this process is impaired in SPARC-null cells. To further test our conclusion, we investigated whether cell proliferation was affected by the lack of SPARC. We found that SPARC-null fibroblasts incorporated ^3H -thymidine as efficiently as control wild-type fibroblasts over the 25 hour period studied (Fig. 5). These results together show that indeed cell motility, rather than cell proliferation, is the major factor in the retarded wound repair process in SPARC-null cells.

Collagen-gel contraction by SPARC-null fibroblasts is not compromised

An important event during wound healing is the contraction of newly formed granulation tissue by fibroblasts to bring together the edges of the wound [1]. It has been reported [28] that fibroblasts incorporated in a collagen gel induce progressive contraction of the gel, resulting in the formation of a dense collagen disc of greatly reduced diameter. This phenomenon has been considered as an *in vitro* equivalent of the connective-tissue contraction that occurs during wound healing. We used this procedure to examine the contractile ability of SPARC-null fibroblasts. Gel diameter was measured at various time points following incorporation of fibroblasts in the collagen gel, and the area calculated and expressed as a percentage of the initial area. Primary dermal fibroblasts from SPARC-null mice contracted type I collagen gels as efficiently as primary dermal fibroblasts from wild-type mice, contracting to 30% of the initial area at 20 hours and to 15% by 40 hours after incubation of the fibroblasts in collagen gels (Fig. 6).

Discussion

In this study, we have shown for the first time that repair of excision wounds in SPARC-null mice is impaired. Histological examination and study of the expression patterns of ECM components, fibronectin and type I collagen, at the wound site suggested that retarded granulation tissue formation caused impaired wound repair. *In vitro* studies with dermal fibroblasts isolated from SPARC-null mice further showed that the ability of the cells to restore wounded areas was hindered and that a defect in cell migration, rather than cell proliferation, was the major cause of the delay in repopulating the de-

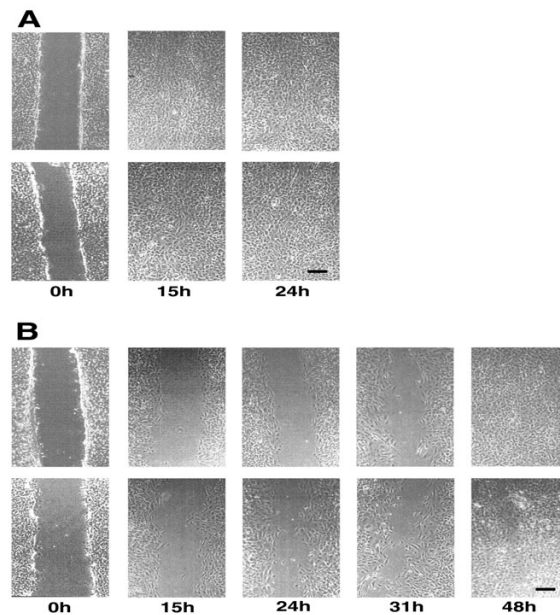


Figure 4

Wound repair by wild-type (A) and SPARC-null fibroblasts (B) cultured in the absence (upper panel) or presence (lower panel) of mitomycin C. Wounded monolayers of wild-type (A) and SPARC-null fibroblasts (B) were cultured in the absence (upper panel) or presence (lower panel) of mitomycin C and monitored for wound closure. Wound repair is not affected by the presence of mitomycin C, an inhibitor of cell mitosis. Scale bar, 62.5 μm .

nuded area. The delayed cell migration and restoration of the wound site were growth factor independent as these defects occurred in the presence of FBS. Furthermore these defects were reversed by the addition of purified SPARC, showing that the defect in cell migration and wound healing was attributable to the absence of SPARC. The mechanism by which SPARC influences cell migration is not known. One possibility is that the lack of SPARC causes alteration in ECM with respect to structure and/or function and thus contributes to a decrease in cell migration. Although SPARC has been postulated to be involved in wound healing due to its elevated expression in fibroblasts and macrophages at wound sites [18], our results are the first to demonstrate that SPARC indeed plays a role in wound repair and that it does so by promoting granulation tissue formation through modulation of fibroblast migration.

In large wounds (25 mm), visual examination of wounds showed that loss of wound scabs and restoration of epidermal covering were delayed in SPARC-null mice due to premature, partial loss of scabs and neopidermis, resulting in repeated setbacks in the reepithelialization process. However, similar setbacks in reepithelializa-

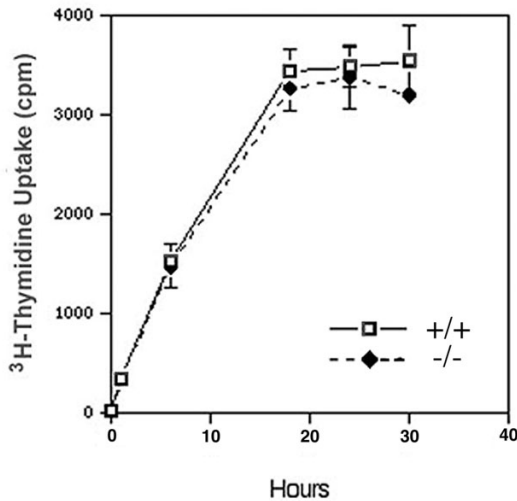


Figure 5

Incorporation of ^3H -thymidine by wild-type (+/+) and SPARC-null (-/-) dermal fibroblasts. Cells were plated and cultured overnight in DMEM containing 10% FBS. Cells were then washed with DMEM without FBS and incubated with ^3H -thymidine in DMEM containing 10% FBS. Data were derived from two independent experiments each performed in triplicate. Bars show mean \pm SD.

tion were not observed in small wounds (6 mm). Furthermore, histological examination of new epidermal cells in the wound bed and Northern analysis of expression of a wound keratinocyte marker, cytokeratin 14, in the biopsied tissues revealed that reepithelialization in SPARC-null mice was probably not compromised. It seems possible that reepithelialization occurs normally in SPARC-null mice. However, since SPARC is a matrix-cellular protein involved in cell-matrix interaction [3,4,23], cell-matrix interaction-related defects may exert a significant influence in large wounds of SPARC-null mice causing premature loss of a part of the scabs and of the neopidermis, a pathophysiological process requiring repeated healing. In small wounds, the pathophysiological process caused by the cell-matrix interaction-related defects may be too small to affect reepithelialization significantly with the result that restoration of epidermal covering progresses as normally as in the wild-type mice. Perhaps this size-dependent wound closure may have contributed to the enhanced wound healing in SPARC-null mice cited recently as an unpublished result [29].

Although SPARC is required for migration of dermal fibroblasts into the wound site, it is apparently not essen-

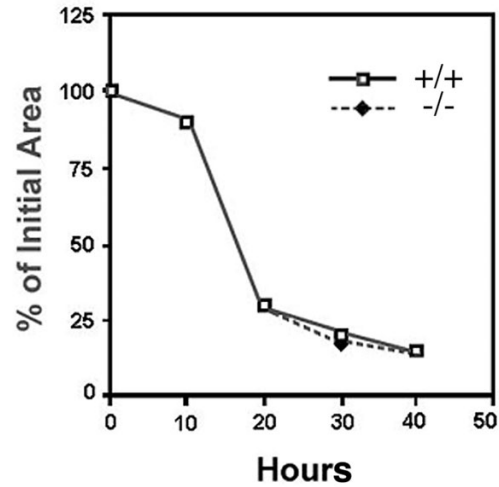


Figure 6

Contraction of collagen gels by wild-type (+/+) and SPARC-null (-/-) fibroblasts. Cells were incorporated in collagen gels and incubated in DMEM plus 2% FBS. Data represent the average of three independent experiments, each performed in triplicate.

tial to the motility of keratinocytes since reepithelialization appears normal in SPARC-null mice. Thus, the role of SPARC in cell migration may be cell type-specific, which has been shown to be the case with *in vitro* studies [7,9,10]. Cell motility is controlled by a cycle of cell attachment and detachment. It is generally accepted that the driving force for cell movement is provided by the dynamic reorganization of the actin cytoskeleton, directing protrusion (lamellipodia and filopodia) at the front of the cell and retraction at the rear [30,31]. Although we have not examined the ultrastructure of the dermal fibroblasts at the edge of wound sites, we showed previously by electron microscopy that in the lens of SPARC-null mice numerous cellular processes extended into the ECM of the lens capsule [23]. We further showed that, in contrast to the abnormal filopodial projections at the lens cell-ECM interface, cell-cell contacts between the neighboring lens epithelial cells and between the lens epithelial and fiber cells were normal [23]. The results indicate that disassembly of cell-cell contacts between lens cells is not taking place, despite the forward movement of the lens cells into the ECM of the capsule. In this study, the lack of ingrowth despite the forward movement of cells at the edge of wounded monolayers suggests that a similar failure of SPARC-null dermal fibroblasts to break away from its neighboring cells may account for the impaired cell motility observed during wound healing *in vitro*. Previously,

SPARC has been shown to regulate cell-cell contact through tyrosine phosphorylation of adherens junction proteins [32]. Electron microscopy of the SPARC-null dermal fibroblasts at wound sites should not only confirm the role of SPARC in cell motility but also reveal how SPARC mediates cell-ECM and cell-cell interactions to promote cell motility.

We showed in the present study that there are no overt differences in cell proliferation and type I collagen gel contraction between control and SPARC-null dermal fibroblasts. Wound contraction depends on migration of fibroblasts into the wound site where they lay down their own collagen-rich matrix [2]. Northern analysis of wound biopsies showed that production of type I collagen and fibronectin by the null mice, at the beginning of wound healing, was lower than that by the wild-type mice. Subsequently, the wound in the null mice was fully invaded by fibroblasts and expression of type I collagen and fibronectin by the SPARC-null mice was as abundant as that by the wild-type mice. Wound contraction takes place when a wound site is filled with fibroblasts and ECM [2]. Thus, it is likely that consistent with the *in vitro* study wound contraction in the SPARC-null mice is not compromised.

Conclusions

The delay in granulation tissue formation and healing of excision wounds in SPARC-null mice, and the defect in wound closure in *in vitro* assays indicate that SPARC plays a role in wound healing through its effect on cell migration. Cell migration is crucial not only to wound healing but also to a wide variety of normal or abnormal biological phenomena such as embryogenesis, leukocyte immigration into areas of insult, and tumor cell invasion. SPARC-null mice and their cells should provide a useful system to investigate how a matricellular protein regulates pleiotropic functions through cell migration and how its absence alters biological processes.

Materials and Methods

Animals

SPARC-null mice and control wild-type mice used in this study were generated and maintained on a mixed genetic background (F2) of 129SvEv × C57BL/6j as described previously [14].

Wound healing assays

Two- to 3-month-old male mice were anesthetized intraperitoneally with avertin (0.5 g/kg body weight). The hair on the back was cut and full-thickness 25 mm oblong or 30 mm circular wounds, including panniculus carnosus, were made. Wounds were neither dressed nor sutured. Mice were caged individually and progression of wound repair was monitored daily.

Histology

Five full-thickness 6 mm circular wounds were made on the back of an anesthetized mouse by a procedure similar to that described above. The 6 mm diameter wounds provided stable wound beds appropriate for collection of wound tissues for histological analysis. Mice were sacrificed by CO₂ asphyxia 2, 4, 6, and 10 days after wounding. An area 7 to 8 mm in diameter, which included the uninjured epithelial margins, was removed, bisected in the midtransversal plane, fixed in 10% formalin (Buffered Formalde-Fresh, Fisher Scientific, Fair Lawn, NJ) and embedded in paraffin. Sections (6 μm) were cut from both faces of bisected tissues and stained with hematoxylin and eosin.

RNA analysis

Circular, full-thickness dorsal cutaneous biopsies of 6 mm diameter were made on the backs of anesthetized mice and wound sites, including the uninjured epithelial margins, were excised 2, 4, and 6 days post wounding as described above. Control, unwounded dorsal skin (0 day) was also isolated. The harvested tissues were frozen immediately in liquid nitrogen. Total RNA was isolated using guanidine thiocyanate extraction and 15 μg of the total RNA was analyzed by Northern hybridization as described previously [16]. cDNA probes used in hybridization were human fibronectin [33], α1(I) collagen [34], or cytokeratin 14 cDNA (Hung Tseng, unpublished results). The same Northern hybridization filters were reprobated with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The amounts of transcripts were quantified by PhosphoImager (Molecular Dynamics) and normalized to GAPDH transcript. Two independent experiments gave similar hybridization patterns.

Isolation of primary dermal fibroblasts

Primary dermal fibroblasts were prepared from 3 day old newborn mice. Mice were euthanized by cervical dislocation and sterilized with 70% ethanol. Skin was removed, washed with phosphate buffered saline (PBS), and incubated overnight in 0.25% trypsin in PBS at 4°C. The dermis was separated from the epidermis and subjected to further incubation overnight in 200 U/ml of crude collagenase (Sigma, St. Louis, MO) in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies Inc., Gaithersburg, MD) at 37°C. Small cell aggregates were separated from large tissue fragments by gravitation sedimentation for 10 minutes at room temperature. The supernatant was carefully removed, and cells were collected by centrifugation and plated in DMEM containing 10% FBS (Hyclone, Logan, UT) for 48 hours. The cells were then re-plated twice to deplete from the culture the residual keratinocytes that do not survive re-plating in 10% FBS-DMEM. The fibroblasts were checked for their spindle shaped morphology (passage one) and used for

future experiments. Twenty fibroblast preparations were made from 10 wild-type or SPARC-null mice. In all experiments, wild-type and SPARC-null cells at identical passage numbers (<5) were used.

In vitro wound-healing and cell migration assays

Both wild-type and SPARC-null primary dermal fibroblasts were seeded in 6-well plates (5×10^5 cells/well), and grown to confluence overnight in DMEM containing 10% FBS. Monolayers were wounded using a 1 ml plastic micropipette tip and incubated at 37°C in DMEM containing 10% FBS or in DMEM containing 10% FBS and SPARC protein purified from human platelets (48 µg/ml; Calbiochem, La Jolla, CA). At various time points, cells were rinsed with PBS and visualized for migration of cells into denuded space by a phase-contrast microscope. The assays were performed in triplicate in two separate experiments. To investigate cell motility, monolayers of dermal fibroblasts were prepared and wounded as described above. The wounded monolayers were incubated at 37°C in DMEM containing 10% FBS with or without mitomycin C (10 µg/ml, Sigma, St Louis, MO) to block mitosis. Migration of cells into denuded areas was monitored for up to 48 hours.

Incorporation of ^3H -thymidine

Wild-type and SPARC-null dermal fibroblasts were plated in 96-well plates (1×10^4 cells/well) and cultured overnight in DMEM containing 10% FBS. The cells were then washed three times with DMEM without FBS and incubated with ^3H -thymidine (1 µCi/ml; NEN, Boston, MA) in DMEM containing 10% FBS for time periods varying between 0 and 30 hours. Due to the problem of cell death (>80%) in serum-free medium, cell cycle synchronization of the dermal fibroblasts in serum-free medium was not performed prior to ^3H -thymidine assays. DNA-bound ^3H -thymidine was immobilized on glass fiber filter paper (Packard, Meriden, CT) with the aid of an automated cell harvester (Tomtec, Orange, CT) and the tritium incorporation was determined by scintillation counting. Experiments were performed in triplicate with cells derived from two different dermal fibroblast preparations.

Collagen gel contraction assay

A standard procedure [11] for collagen disc contraction was followed with minor modifications. Briefly, 6-well tissue culture plates were made non-adhesive with a coating of 1% agarose (Sea-Kem LE, FMC BioProducts, Rockland, MD). One volume of a solution of 3 mg/ml bovine type I collagen (Vitrogen™, Celtrix Corp., Palo Alto, CA) was combined with 1/6 volume of 7X DMEM and sufficient 1X DMEM added to yield a final collagen concentration of 0.75 mg/ml. A suspension of mouse dermal fibroblasts at 2×10^6 cell/ml in DMEM was added to 9

volumes of the type 1 collagen solution, and FBS added to provide a concentration of 2% FBS in the final solution. The resulting cell suspension was dispensed into agarose-coated wells (2 ml/well) and gelled for 2 hours at 37°C. DMEM (2 ml) with 2% FBS was subsequently added to the wells and the gels were gently detached from the walls to float the collagen disks. The collagen/cell mixtures were incubated in humidified air at 37°C and contraction was monitored for 72 hours. The assay was performed three times, each in triplicate. The area of gels was calculated from the average diameter of the experiments and expressed as a percentage of the initial area.

Abbreviations

DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FBS, fetal bovine serum; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate buffered saline; SPARC, secreted protein acidic and rich in cysteine, also known as osteonectin and BM-40; SD, standard deviation.

Acknowledgments

We thank Dr. S. Amini for cDNA probes for fibronectin and type I collagen, and Dr. H. Tseng for cytokeratin 14 cDNA. This work was supported by NSF grant EEC-9814404.

References

1. Clark RAF: **Wound repair: overview and general considerations.** In: *The Molecular and Cellular Biology of Wound Repair* (Edited by Clark RAF) New York, Plenum Press 1996:3-50
2. Martin P: **Wound healing – aiming for perfect skin regeneration.** *Science* 1997, **276**:75-81
3. Lane TF, Sage EH: **The biology of SPARC, a protein that modulates cell-matrix interactions.** *FASEB J* 1994, **8**:163-173
4. Sage EH: **Terms of attachment: SPARC and tumorigenesis.** *Nat Med* 1997, **3**:144-146
5. Rempel SA, Ge S, Gutierrez JA: **SPARC: a potential diagnostic marker of invasive meningiomas.** *Clin Cancer Res* 1999, **5**:237-241
6. Vial E, Castellazzi M: **Down-regulation of the extracellular matrix protein SPARC in vSrc- and vJun-transformed chick embryo fibroblasts contributes to tumor formation in vivo.** *Oncogene* 2000, **19**:1772-1782
7. Ledda MF, Adris S, Bravo AI, Kairiyama C, Bover L, Chernajovsky Y, Mordoh J, Podhajcer OL: **Suppression of SPARC expression by antisense RNA abrogates the tumorigenicity of human melanoma cells.** *Nat Med* 1997, **3**:171-176
8. Rosenblatt S, Bassuk JA, Alpers CE, Sage EH, Timpl R, Preissner KT: **Differential modulation of cell adhesion by interaction between adhesive and counter-adhesive proteins: characterization of the binding of vitronectin to osteonectin (BM40, SPARC).** *Biochem J* 1997, **324**:311-319
9. Hasselaar P, Sage EH: **SPARC antagonizes the effect of basic fibroblast growth factor on the migration of bovine aortic endothelial cells.** *J Cell Biochem* 1992, **49**:272-283
10. Huynh M-H, Sage EH, Ringuette M: **A calcium-binding motif in SPARC/osteonectin inhibits chondrosarcoma cell migration during *Xenopus laevis* gastrulation: evidence of counter-adhesive activity in vivo.** *Dev Growth Differ* 1999, **41**:407-418
11. Iruela-Arispe ML, Vernon RB, Wu H, Jaenisch R, Sage EH: **Type I collagen-deficient Mov-13 mice do not retain SPARC in the extracellular matrix: implications for fibroblast function.** *Dev Dyn* 1996, **207**:171-183
12. Raines EW, Lane TF, Arispe-Iruela ML, Ross R, Sage EH: **The extracellular glycoprotein SPARC interacts with platelet-derived growth factor (PDGF)-AB and -BB and inhibits the binding of PDGF to its receptors.** *Proc Natl Acad Sci USA* 1992, **89**:1281-1285

13. Kamihagi K, Katayama M, Ouchi R, Kato I: **Osteonectin/SPARC regulates cellular secretion rates of fibronectin and laminin extracellular matrix proteins.** *Biochem Biophys Res Commun* 1994, **200**:423-428
14. Norose K, Clark JI, Syed NA, Basu A, Heber-Katz E, Sage EH, Howe CC: **SPARC deficiency leads to early onset cataractogenesis.** *Invest Ophthalmol Vis Sci* 1998, **39**:2674-2680
15. Francki A, Bradshaw AD, Bassuk JA, Howe CC, Couser WG, Sage EH: **SPARC regulates the expression of collagen type I and transforming growth factor- β 1 in mesangial cells.** *J Biol Chem* 1999, **274**:32145-32152
16. Basu A, Rodeck U, Prendergast GC, Howe CC: **Loss of insulin-like growth factor I receptor-dependent expression of p107 and cyclin A in cells that lack the extracellular matrix protein secreted protein acidic and rich in cysteine.** *Cell Growth Differ* 1999, **10**:721-728
17. Bradshaw AD, Francki A, Motamed K, Howe C, Sage EH: **Primary mesenchymal cells isolated from SPARC-null mice exhibit altered morphology and rates of proliferation.** *Mol Biol Cell* 1999, **10**:1569-1579
18. Reed MJ, Puolakkainen P, Lane TF, Dickerson D, Bornstein P, Sage EH: **Differential expression of SPARC and thrombospondin I in wound repair: immunolocalization and in situ hybridization.** *J Histochem Cytochem* 1993, **41**:1467-1477
19. Delany AM, Amling M, Priemel M, Howe C, Baron R, Canalis E: **Osteopenia and decreased bone formation in osteonectin-deficient mice.** *J Clin Invest* 2000, **105**:915-923
20. Sawhney RS: **Identification of SPARC in the anterior lens capsule and its expression by lens epithelial cells.** *Exp Eye Res* 1995, **61**:645-648
21. Bassuk JA, Birkebak T, Rothmier JD, Clark JM, Bradshaw A, Muchowski PJ, Howe CC, Clark JI, Sage EH: **Disruption of the SPARC locus in mice alters the differentiation of lenticular epithelial cells and leads to cataract formation.** *Exp Eye Res* 1999, **68**:321-331
22. Dziadek M, Paulsson M, Aumailley M, Timpl R: **Purification and tissue distribution of a small protein (BM-40) extracted from a basement membrane tumor.** *Eur J Biochem* 1986, **161**:455-464
23. Norose K, Lo W-K, Clark JI, Sage EH, Howe CC: **Lenses of SPARC-null mice exhibit an abnormal cell surface-basement membrane interface.** *Exp Eye Res* 2000, **71**:295-307
24. Watanabe S, Osumi M, Ohnishi T, Ichikawa E, Takahashi H: **Changes in cyokeratin expression in epidermal keratinocytes during wound healing.** *Histochem Cell Biol* 1995, **103**:425-433
25. French-Constant C, Van De Water L, Dvorak HF, Hynes RO: **Reappearance of an embryonic pattern of fibronectin splicing during wound healing in the adult rat.** *J Cell Biol* 1989, **109**:903-914
26. Scharffetter K, Kulozik M, Stolz W, Lankat-Bttgereit B, Hatamochi A, Sohnchen R, Krieg T: **Localization of collagen α 1(I) gene expression during wound healing by in situ hybridization.** *J Invest Dermatol* 1989, **93**:405-412
27. Gailit J, Clark RAF: **Wound repair in the context of extracellular matrix.** *Curr Opin Cell Biol* 1994, **6**:717-725
28. Bell E, Ivarsson B, Merrill C: **Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro.** *Proc Natl Acad Sci USA* 1979, **76**:1274-1278
29. Bradshaw AD, Sage EH: **SPARC, a matricellular protein that functions in cellular differentiation and tissue response to injury.** *J Clin Invest* 2000, **107**:1049-1054
30. Lauffenburger DA, Horwitz AF: **Cell migration: a physically integrated molecular process.** *Cell* 1996, **84**:359-369
31. Michison TJ, Cramer LP: **Actin-based cell motility.** *Cell* 1996, **84**:371-379
32. Young BA, Wang P, Goldblum SE: **The counteradhesive protein SPARC regulates an endothelial paracellular pathway through protein tyrosine phosphorylation.** *Biochem Biophys Res Commun* 1998, **251**:320-327
33. Kornblihtt AR, Vibe-Pedersen K, Baralle FE: **Isolation and characterization of cDNA clones for human and bovine fibronectins.** *Proc Natl Acad Sci USA* 1983, **80**:3218-3222
34. Chu ML, Myers JC, Bernard MP, Ding JF, Ramirez F: **Cloning and characterization of five overlapping cDNAs specific for the human pro α 1(I) collagen chain.** *Nucleic Acids Res* 1982, **10**:5925-5934

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMedcentral will be the most significant development for disseminating the results of biomedical research in our lifetime."

Paul Nurse, Director-General, Imperial Cancer Research Fund

Publish with **BMC** and your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours - you keep the copyright



Submit your manuscript here:

<http://www.biomedcentral.com/manuscript/>

editorial@biomedcentral.com