SPARC Represses E-Cadherin and Induces Mesenchymal Transition during Melanoma Development

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Abstract

During progression of melanoma, loss of the cell-cell adhesion molecule E-cadherin contributes to uncontrolled growth and invasive behavior of transformed melanocytes. Secreted protein acidic and rich in cysteine (SPARC) is a nonstructural matricellular protein that regulates cell-matrix interactions leading to alterations in cell adhesion and proliferation. Overexpression of SPARC has been associated with progression of various cancers, including melanoma; however, its role in primary tumor development is not well defined. We show that normal human melanocytes overexpressing SPARC adopt a fibroblast-like morphology, concomitant with loss of E-cadherin and P-cadherin expression, and increased expression of mesenchymal markers. Concurrent with these changes, SPARC expression stimulates melanocyte motility and melanoma cell invasion. Expression of SPARC results in transcriptional down-regulation of E-cadherin that correlates with induction of Snail, a repressor of E-cadherin. Conversely, SPARC depletion leads to up-regulation of E-cadherin and reduces Snail levels, and SPARC-null cells exhibit a marked change in their mesenchymal phenotype. Finally, analysis of SPARC, Snail, and E-cadherin levels in melanocytes and malignant melanoma cell lines further supports the functional relationship among these proteins during melanoma progression. Our findings provide evidence for the role of SPARC in early transformation of melanocytes and identify a novel mechanism, whereby tumor-derived SPARC promotes tumorigenesis by mediating Snail induction and E-cadherin suppression. (Cancer Res 2006; 66(15): 7516-23)

Introduction

Cutaneous melanoma is one of the most common lethal cancers among young adults. Its incidence has increased faster than any other neoplastic disease, thus making this malignancy a significant clinical problem. Melanoma has a high potential for invasion and metastatic spread. If undetected at early stages, the prognosis of melanoma is poor, and there are no effective treatment options available for patients with advanced and metastatic forms of the disease (1, 2).

Melanoma arises from neural crest-derived melanocytes that are located in the basal layer of the epidermis. Melanocytes are cells specialized in the synthesis of melanin pigments (3). The transition from a normal melanocyte to a metastatic melanoma occurs through a complex multistage process that has been well described based on clinical and histopathologic features (4). However, the genetic and molecular changes associated with the development and progression of melanoma have not been fully understood thus far.

In normal skin, melanocytes adhere to surrounding basal keratinocytes mainly through expression of E-cadherin (5, 6). These cell-cell contacts play an essential role in regulating melanocyte proliferation and differentiation. E-cadherin is a member of a family of functionally related transmembrane glycoproteins that mediate cell-cell adhesion in a calcium-dependent fashion. The extracellular domain of E-cadherin is involved in homophilic interaction with adjacent cells, whereas the cytoplasmic domain is linked noncovalently to the actin cytoskeleton via catenins (7). In addition to its functional importance in normal skin homeostasis and maintenance of epithelial tissue integrity, E-cadherin-mediated cell-cell adhesion plays a critical role in development of invasive tumors, including melanoma. Loss of E-cadherin has been observed in most melanoma cells (6, 8, 9). This event liberates melanoma cells from keratinocyte-mediated growth and phenotypic control. Reintroduction of E-cadherin results in restored control by keratinocytes, inhibits tumorigenicity, and suppresses the invasiveness of melanoma cells, supporting a role for E-cadherin as an invasion suppressor gene (10).

Control of E-cadherin transcription seems to be the main mechanism responsible for the down-regulation of this protein in melanoma. The zinc-finger factor Snail that belongs to the Snail superfamily of transcriptional repressors (11, 12) has been implicated in such repression in melanoma cells. Analysis of Snail mRNA expression profiles in a collection of melanoma cell lines showed an inverse relationship with E-cadherin levels, and suppression of Snail expression induced reexpression of E-cadherin in melanoma cells (8, 9). Snail exerts its effect through binding to specific E-boxes in the proximal E-cadherin promoter (13). In addition to repress E-cadherin expression, Snail promotes changes in cytoskeletal organization, increased expression of mesenchymal markers, and acquisition of migratory and invasive properties. This process referred to as epithelial to mesenchymal transition (EMT) occurs in

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normal embryonic development, such as gastrulation and neural crest migration, and during invasive phase of carcinoma. By contributing to EMT, Snail has been proposed to act as inducer of the invasive and metastatic phenotype (13, 14). Up to now, the implication of Snail family factors in acquisition of mesenchymal and transformed properties of neural crest-derived tumors (from which melanocytes are derived) remains largely unknown.

Secreted protein acidic and rich in cysteine (SPARC; also known as osteonectin or BM-40) belongs to the matricellular family of secreted proteins, which also includes thrombospondins 1 and 2, osteopontin, and tenascins C and X (15, 16). SPARC is a non-structural component of extracellular matrices that modulates cell-matrix interactions, particularly during tissue development, remodeling, and repair. SPARC interacts with several extracellular matrix components and functions as a de-adhesive molecule, as a cell cycle inhibitor, and as a modulator of cytokine and growth factor activities. SPARC is spatially and temporally regulated during development and expressed at high levels in remodeling tissues (15, 17, 18). Many types of cancers are characterized by deregulated expression of SPARC (19). Up-regulation of SPARC is associated with metastatic potential of melanomas and gliomas as well as invasive phenotype of breast, prostate, and colorectal carcinomas. In contrast, the levels of SPARC were inversely correlated with the development of ovarian carcinoma, neuroblastoma, and pancreatic adenocarcinoma (19). These observations suggest that tumorigenic effect of SPARC is cell type specific and may be dependent of the tumor cell surrounding environment.

SPARC seems to function as a melanoma tumor-promoting gene. Its production has been associated with aggressive stages of melanoma and is correlated with poor prognosis (20–22). SPARC is strongly expressed by advanced primary and metastatic melanomas but is weakly expressed and less frequent in nevus cells, and its expression is absent in normal melanocytes (20, 21). A recent report highlighted a critical role for SPARC produced by melanoma cells in antitumor immune responses (23). More importantly, inhibition of SPARC expression by antisense RNA diminished the adhesive and invasive capacities of human melanoma cells in xenotransplanted nude mice (24). Although this study defined a relevant role of SPARC in the biology of melanocytic tumors, the molecular events responsible for this activity are still unclear and remain to be defined.

Here, we aimed to investigate the molecular mechanisms by which SPARC affects melanocyte transformation, and we show that SPARC contributes to melanoma progression by repressing expression of E-cadherin likely through up-regulation of Snail. Our study reveals a previously unrecognized role for SPARC in the regulation of E-cadherin and suggests that alteration of cell microenvironment induced by SPARC can influence cellular contacts, leading to a migratory and invasive behavior. This demonstration that SPARC functionally contributes to E-cadherin repression and mesenchymal phenotype further suggests that

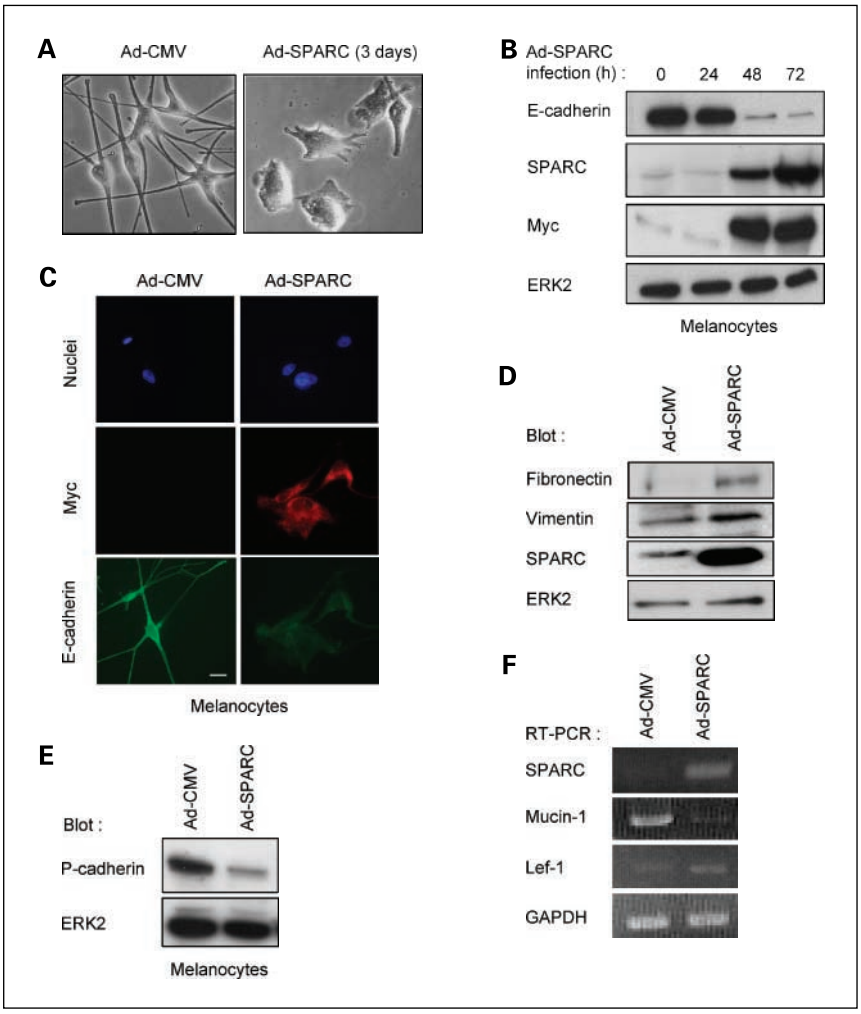

Figure 1. Adenovirus-mediated SPARC overexpression in human primary melanocytes promotes mesenchymal conversion and decreases E-cadherin and P-cadherin levels. *A,* SPARC induces morphologic changes in normal melanocytes. Phase-contrast pictures of cells infected for 3 days with a control empty adenovirus (Ad-CMV, multiplicity of infection of 2; *left*) or with adenovirus encoding SPARC (Ad-SPARC, multiplicity of infection of 2; *right*). A decrease in length of the dendrites and an increase in body cell size were observed in SPARC-transduced cells compared with vector-transduced cells. *B,* SPARC induces the loss of E-cadherin expression in melanocytes. Cells were infected with adenovirus encoding SPARC (multiplicity of infection of 2) for the indicated times. Whole-cell lysates were prepared, and expression of E-cadherin, SPARC-Myc transgene, and extracellular signal-regulated kinase 2 (*ERK2*) were visualized by Western blotting. *C,* immunofluorescent staining of vector- and SPARC-transduced melanocytes. Fluorescence signals specific to E-cadherin antibody were visualized as green, and fluorescence signals specific to Myc tag were visualized as red. Bar, 15 μm (*D-F*) SPARC decreases expression of epithelial markers and increases expression of mesenchymal markers in melanocytes. Three days after adenovirus infection, expression levels of fibronectin, vimentin, P-cadherin, SPARC, and extracellular signal-regulated kinase 2 were assessed by Western blotting (*D* and *E*). Expression levels of Mucin-1, lef-1, and SPARC were determined by semiquantitative reverse transcription-PCR (*RT-PCR*). GAPDH was used to normalize the reverse transcription-PCR reaction (*F*). From representative experiments that were repeated two or more times with identical results.

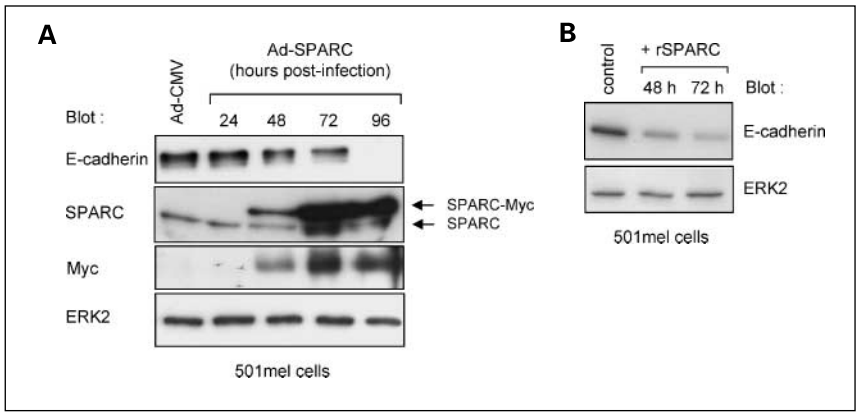

Figure 2. SPARC represses E-cadherin in E-cadherin-positive melanoma cells. *A,* 501mel cells were infected with Ad-CMV (multiplicity of infection of 2) for 4 days or with Ad-SPARC (multiplicity of infection of 2) for the indicated times. Whole-cell lysates were prepared, and expression of E-cadherin, SPARC, SPARC-Myc transgene, and extracellular signal-regulated kinase 2 were visualized by Western blotting. Representative of three independent experiments. *B,* 501mel cells were incubated with recombinant SPARC (*rSPARC*; 20 μg/mL) for the indicated times. Equal amount of proteins were analyzed by Western blotting.

SPARC may represent a candidate therapeutic target in the treatment of melanoma malignancy.

Materials and Methods

Cells and reagents. Epidermal melanocytes were isolated from foreskins and cultured as described (25). Human melanoma cell lines WM35, SBcl2, WM793, and WM9 were generously provided by Dr. M. Herlyn and maintained as described (9). Human melanoma cell lines M6, M113, and M67 were gifts from Dr. F. Jotereau. Human 501mel, MeWo, and A375 melanoma cells were cultured in DMEM supplemented with 7% fetal bovine serum as described previously (26). Primers and culture reagents were purchased from Invitrogen (Carlsbad, CA). All other reagents were obtained from Sigma (St. Louis, MO) unless stated otherwise. Purification of recombinant SPARC over Ni^{2+}-agarose was carried out as described (27).

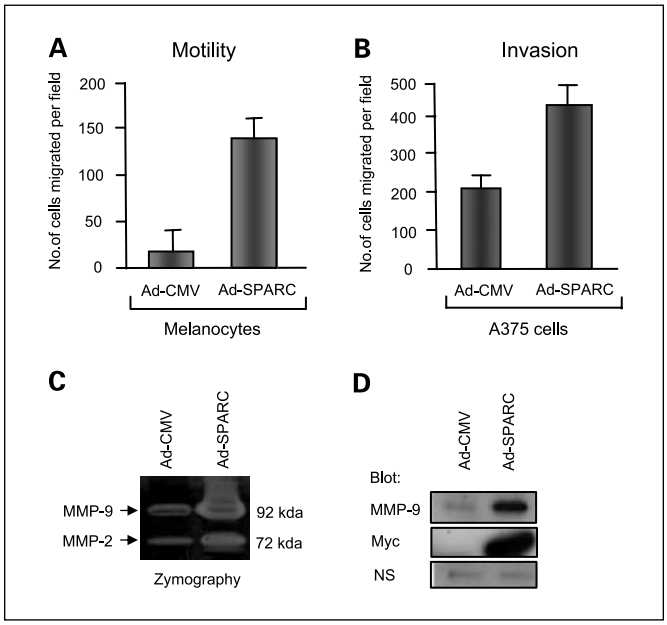

Figure 3. SPARC influences migratory and invasive potential of melanocytic cells. *A,* effects of SPARC expression on chemotaxis motility of primary melanocytes. Cells were infected with Ad-CMV (multiplicity of infection of 2) or with Ad-SPARC (multiplicity of infection of 2) for 4 days, and cell migration was assessed using Boyden chamber assays as described in Materials and Methods. *Columns,* means of triplicates from two independent experiments; *bars,* SD. *B,* effects of SPARC expression on melanoma cell invasion. A375 cells were infected with Ad-CMV (multiplicity of infection of 2) or with Ad-SPARC (multiplicity of infection of 2) for 4 days and then seeded onto a Matrigel invasion chamber. After 6 hours, the number of invading cells were determined by counting the stained cells in the lower surface of the membrane. *Columns,* means of triplicates from two independent experiments; *bars,* SD. *C,* serum-free conditioned media from A375 cells infected by Ad-CMV or Ad-SPARC were analyzed by collagen zymography. *D,* Western blot analysis to examine the expression of MMP-9 and SPARC-Myc transgene in A375 cells after 4 days of adenoviral infection.

Expression of SPARC using adenoviral gene transduction. The generation of the recombinant adenovirus expressing SPARC or SPARCΔEC was carried out as described (29). Viruses express SPARC (or SPARCΔEC) with a Myc-tag at its COOH terminus. Adenovirus vector carrying an empty expression cassette of pcDNA3 vector was used as control (Ad-CMV).

Immunofluorescence microscopy. Monolayers prepared for fluorescent staining were grown on glass coverslips. Cells were fixed with 3% paraformaldehyde and incubated successively with 5 μg/mL of E-cadherin-specific antibody (clone HECD-1, Zymed Laboratories, Inc., San Francisco, CA), the secondary FITC-labeled antibody (Molecular Probes, Carlsbad, CA), and with the Texas Red–coupled anti-Myc antibody (clone 9E10, Santa Cruz Biotechnology, Santa Cruz, CA). Stained cells were visualized with a Zeiss Axiophot fluorescence microscope.

Semiquantitative reverse transcription-PCR assays. Total RNA was isolated using the TRIzol reagent (Invitrogen), and first-strand cDNA was generated using the reverse transcriptase system from Promega (Madison, WI). PCRs were done with Taq DNA polymerase (Qiagen, Hilden, Germany), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification was used as an internal control. The optimal number of cycles for each set of primers was determined to keep signal amplification in the log phase of the reaction. Information on primers is given in Supplementary Table S1.

E-cadherin promoter analysis. Transfection of 501mel cells was carried out by using the FuGENE 6 reagent (Roche Applied Sciences, Crenjacherstrasse, Switzerland). Cells were transfected in 24-well plates with 200 ng of the wild-type (−178wt) or mutant (mE-pal) luciferase reporter constructs and 50 ng of pCMV-β-galactosidase as a control for transfection efficiency. Where indicated, cotransfections were carried out in the presence of 300 ng of empty pcDNA3, pcDNA3-SPARC, or pcDNA3-Snail vectors. Cells were harvested 72 hours after transfection and assayed for luciferase activity using the Luciferase assay system (Promega). All experimental values were determined from triplicate wells. Each experiment was repeated at least thrice.

Small interfering RNA–mediated down-regulation of SPARC. Stealth small interfering RNAs (siRNA) were designed by Invitrogen. The sense SPARC-siRNA sequence used was 5′-GCGGGUGAAGAAGAUCCAUGAGAAU-3′. As nonspecific control, a sequence targeting luciferase gene (Luc-siRNA) or enhanced green fluorescent protein gene (GFP-siRNA) was used. In rescue experiments, the sense SPARC-siRNA used was 5′-AAGUACAUCGCCCUGGAUGAGTT-3′. siRNA duplex was transfected into cells with Oligofectamine (Invitrogen). Transfections were done in six-well plates

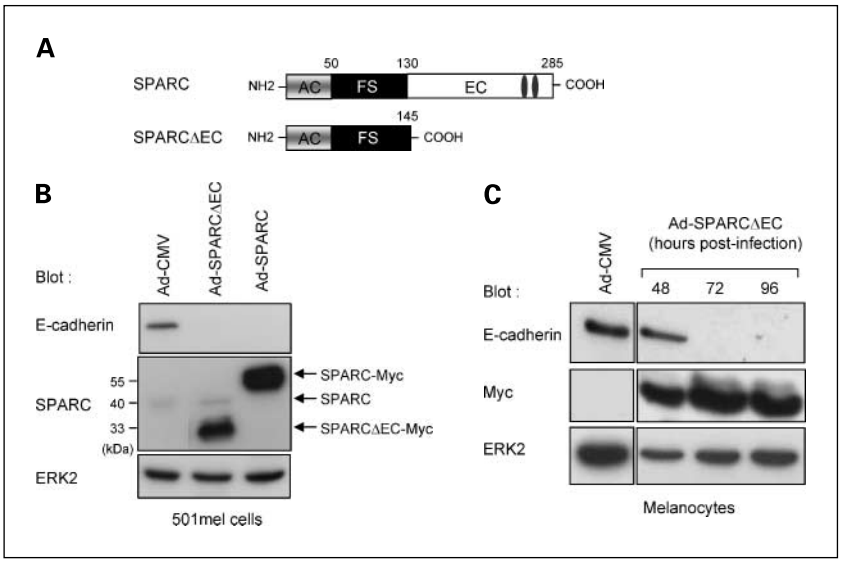

Figure 4. SPARC triggers E-cadherin down-regulation independently of its COOH-terminal domain. *A,* functional domains of the mature SPARC protein and schematic of the COOH-terminal truncation mutant (SPARCΔEC). The NH2-terminal acidic (*AC*) and follistatin (*FS*)–like domains are illustrated as gray and black boxes, respectively. The EC domain is shown as an empty box. Bars within the EC domain represent EF-hand motifs. *B,* SPARCΔEC promotes E-cadherin down-regulation in melanoma cells. 501mel cells were infected with Ad-CMV (multiplicity of infection of 2), Ad-SPARC (multiplicity of infection of 2), or with Ad-SPARCΔEC (multiplicity of infection of 2) for 4 days. Expression levels of E-cadherin, SPARC, SPARCΔEC, and extracellular signal-related kinase 2 were analyzed by Western blotting. Infection with adenovirus driving expression of full-length SPARC sequence was used as control. *C,* time-dependent loss of E-cadherin expression induced by SPARCΔEC in primary melanocytes. Cells were infected with Ad-CMV (multiplicity of infection of 2) for 4 days or with Ad-SPARCΔEC (multiplicity of infection of 2) for the indicated times. Expression of E-cadherin, SPARCΔEC-Myc transgene, and extracellular signal-regulated kinase 2 were visualized by Western blotting. Representative of four independent experiments. Infection with adenovirus driving expression of full-length SPARC sequence (Ad-SPARC) was used as a positive control.

at 50% to 60% confluency with a final concentration of 100 nmol/L of siRNA, and cells were harvested after 72 hours.

Cell lysis and Western blotting analysis. Cells were harvested in radioimmunoprecipitation assay buffer supplemented with 1% NP40. Whole-cell lysates were subjected to SDS-PAGE and Western blot analyses done using monoclonal antibodies to SPARC (AON-5031, Haematologic Technologies, Inc., Essex Junction, VT), Myc (clone 9E10, Santa Cruz Biotechnology), E-cadherin (clone 36, BD Biosciences, San Jose, CA), P-cadherin (clone 56, BD Biosciences), vimentin (clone V9, Sigma), Snail (clone 17EC3)[5], matrix metalloproteinase-9 (MMP-9; Research Diagnostics, Inc., Flanders, NJ), extracellular signal-regulated kinase 2 (clone D-2, Santa Cruz Biotechnology), or polyclonal antibodies to fibronectin (Sigma) or GAPDH (Santa Cruz Biotechnology). Immunoreactivity was detected with Amersham enhanced chemiluminescence system (Buckinghamshire, United Kingdom).

Zymography. Conditioned medium from A375 cells was collected, concentrated 5-fold using Centricon centrifugal filter unit (Millipore, Bedford, MA), and analyzed by collagen zymography as previously described (30).

Migration and invasion assays. Chemotaxis assays were monitored using modified Boyden chambers containing polycarbonate membranes (8.0-μm pores, Transwell, Corning, Inc., Corning, NY). Chambers were coated with 5 μm/mL fibronectin (BD Biosciences) and placed into 24-well chambers filled containing fibroblast-conditioned medium. Cells were loaded into the top side chamber and allowed to migrate for 22 hours at 37°C in 5% CO_2. Migratory cells on the lower membrane surface were fixed in 1% paraformaldehyde, stained with 0.1% crystal violet, and counted (five random 20× fields per well). Chemoinvasion was done using a Cell Invasion Assay kit (Chemicon International, Temecula, CA).

Results

Overexpression of SPARC promotes mesenchymal conversion and down-regulates E-cadherin and P-cadherin expression in melanocytes. The development of invasive tumors correlates with the loss of E-cadherin, and SPARC overexpression is associated with melanoma malignancies. Therefore, we investigated the possible relationship between E-cadherin and SPARC in normal melanocytes overexpressing SPARC using adenoviral delivery. Analysis of expression of the Myc-tagged SPARC transgene was depicted in Supplementary Fig. S1*A* and *B*.

Figure 1*A* shows cellular morphology of melanocytes after 3 days of infection. Compared with control infected cells, a significant conversion to a fibroblast-like phenotype was observed in cells infected by Ad-SPARC. Cells lost their melanocyte characteristics and became less dendritic, and the body cell size was increased. Western blotting analysis revealed that levels of E-cadherin protein were dramatically reduced 2 days after infection (Fig. 1*B*). This down-regulation was concomitant with appearance of SPARC transgene and occurrence of morphologic changes. Decrease of cell surface E-cadherin in melanocytes transduced with Ad-SPARC was further confirmed by immunofluorescence analysis (Fig. 1*C*). Consistent with a phenotypic transition, we also observed an increase in expression of vimentin, fibronectin, and Lef-1, which are markers of mesenchymal cells (Fig. 1*D* and *F*). Furthermore, we found a strong reduction of P-cadherin (or cadherin 3) expression, which has been recently associated with melanoma development (refs. 31, 32; Fig. 1*E*), and a decrease of Mucin-1 transcript level, an epithelial marker expressed in melanocyte (Fig. 1*F*). These data indicate that SPARC overexpression induces morphologic changes and influences expression of molecular markers that are associated with melanoma tumorigenesis. We also screened for changes in other markers associated with melanoma progression, such as N-cadherin, β_3 integrin, MelCAM, or CD44v6. We found that expression of these markers was not affected in response to SPARC in melanocytes (data not shown).

Increased SPARC expression in 501mel cells also promoted a time-dependent loss of E-cadherin expression, which is temporally

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correlated, with induction of SPARC-Myc transgene (Fig. 2*A*). Given that SPARC is a secreted protein, we also verified that treatment with recombinant SPARC induced a reduction of E-cadherin in this cell line (Fig. 2*B*), showing that SPARC induces E-cadherin down-regulation in E-cadherin-positive melanoma cells.

SPARC expression enhances melanocytes migration and melanoma cells invasion. The biological significance of SPARC expression on motility and invasion of melanocytic cells were evaluated using Boyden chamber assays. As shown in Fig. 3*A*, primary melanocytes exhibited almost no serum-stimulated chemotaxis motility, whereas motility was induced in melanocytes transduced by Ad-SPARC. However, these cells did not significantly invade through Matrigel (data not shown). In contrast, expression of SPARC in A375 melanoma cells led to an increase of the number of cells that had migrated through the Matrigel barrier (Fig. 3*B*). To explore whether the increased invasiveness potential of SPARC-transduced cells was associated with MMP induction and/or activation, collagen zymography on the conditioned medium from control- and SPARC-transduced cells was done. Figure 3*C* shows an increase in levels of both secreted 92- and 72-kDa gelatinases corresponding to latent MMP-9 and MMP-2, respectively. Western blot analysis indicated an increase in MMP-9 levels in cells expressing the SPARC-Myc transgene, whereas levels of MMP-2 remained unchanged (Fig. 3*D*; data not shown). These results show that overexpression of SPARC induces MMP activities and MMP-9 expression and can potentiate the invasive behavior of melanoma cells.

SPARC triggers E-cadherin down-regulation independently of its COOH-terminal extracellular Ca^{2+} binding domain. We next analyzed the role played by the COOH-terminal extracellular Ca^{2+} binding (EC) module of SPARC in mediating E-cadherin down-regulation. This bioactive domain, which is a hallmark of members of SPARC family, contains two EF-hand motifs and has been shown to mediate many of the effects of SPARC on cultured cells (15, 17). SPARC also includes a NH_2-terminal acidic domain followed by a follistatin-like module (Fig. 4*A*). We generated an adenoviral expression vector encoding SPARC deleted of the COOH-terminal EC domain and used it to infect 501mel cells and melanocytes. Expression of SPARCΔEC was confirmed using antibodies that recognize the Myc tag and the NH_2-terminal domain of SPARC. As for full-length SPARC sequence, expression of SPARCΔEC led to a decrease in E-cadherin expression as determined by Western blot analysis (Fig. 4*B* and *C*). These results suggest SPARC functions independently of its COOH-terminal EC module to mediate E-cadherin suppression.

Overexpression of SPARC induces Snail and represses the mouse E-cadherin promoter activity. To further investigate the mechanism by which SPARC induces E-cadherin loss, we analyzed E-cadherin mRNA levels by reverse transcription-PCR upon adenovirus-mediated SPARC expression in melanocytes. E-cadherin mRNA levels were present in control cells but nearly undetectable in cells overexpressing SPARC (Fig. 5*A*). Because previous studies have suggested a role of Snail in E-cadherin repression, we next investigated its potential involvement in mediating SPARC-triggered E-cadherin suppression. Expression of SPARC in primary melanocytes resulted in an increase of Snail transcript levels (Fig. 5*A*). By Western blotting, we confirmed that SPARC induced Snail expression in melanocytes (Fig. 5*B*). We also analyzed the effect of SPARC on E-cadherin promoter activity in 501mel cells. Luciferase reporter assays show that Snail resulted in a 50% decrease of the activity of E-cadherin promoter and that SPARC reduces promoter activity to levels similar to those induced by Snail (Fig. 5*C*). Note that no additive effect was observed when cells were transfected with the two vectors. We also showed that E2 box mutations in E-pal element of the mouse E-cadherin promoter abrogate SPARC-induced repression activity. As expected, mutation in the E-pal element abolished the effect of Snail on E-cadherin promoter inhibition. These data indicate that E-cadherin promoter repression induced by ectopic SPARC expression depends on E2 boxes integrity.

Taken together, our data show a reciprocal relationship between inhibition of E-cadherin expression and stimulation of Snail expression in cells overexpressing SPARC, and that SPARC mediates E-cadherin repression through Snail-binding sequences in the promoter.

SPARC contributes to E-cadherin repression in melanoma cells. The foregoing experiments showed a relationship between SPARC overexpression and E-cadherin loss; therefore, we tested whether suppression of SPARC in melanoma cells can up-regulate E-cadherin expression and reverses invasiveness. To this end, SPARC-specific siRNA (or unrelevant sequence siRNA for control) was

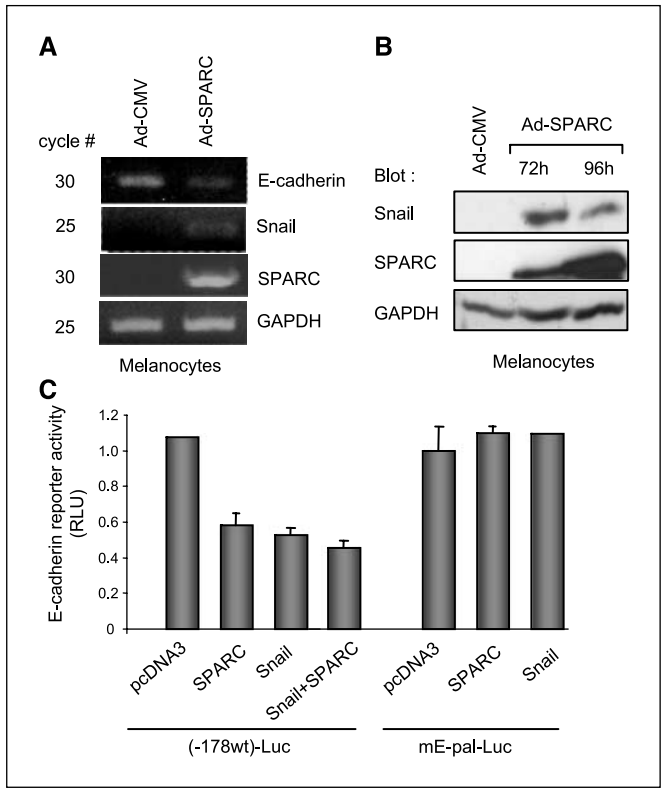

Figure 5. SPARC induces Snail and down-regulates expression of E-cadherin at the transcriptional level. *A,* expression of SPARC decreases E-cadherin mRNA levels and increases Snail mRNA levels in primary melanocytes. RNA was isolated from melanocytes infected with empty adenovirus (Ad-CMV) or SPARC-expressing adenovirus (Ad-SPARC) for 3 days, and levels of E-cadherin, Snail, and SPARC were determined by semiquantitative reverse transcription-PCR. GAPDH was used to normalize the reverse transcription-PCR reaction. The number of PCR cycle is indicated. *B,* SPARC induces Snail protein expression. Primary melanocytes were infected with Ad-CMV (multiplicity of infection of 2) for 4 days or with Ad-SPARC (multiplicity of infection of 2) for the indicated times. Expression of Snail, SPARC, and GAPDH were visualized by Western blotting. *C,* SPARC expression represses the activity of the E-cadherin promoter. 501mel cells were transfected with the wild-type (−178wt) or mutant (mE-pal) mouse E-cadherin promoter fused to the luciferase reporter gene together with empty vector, SPARC or Snail expression vector, or both. Luciferase activities were determined 2 days after transfection and normalized to β-galactosidase. The promoter activity is expressed relative to that detected in mock-transfected cells. *Columns,* mean of triplicates; *bars,* SD. Representative of three independent experiments.

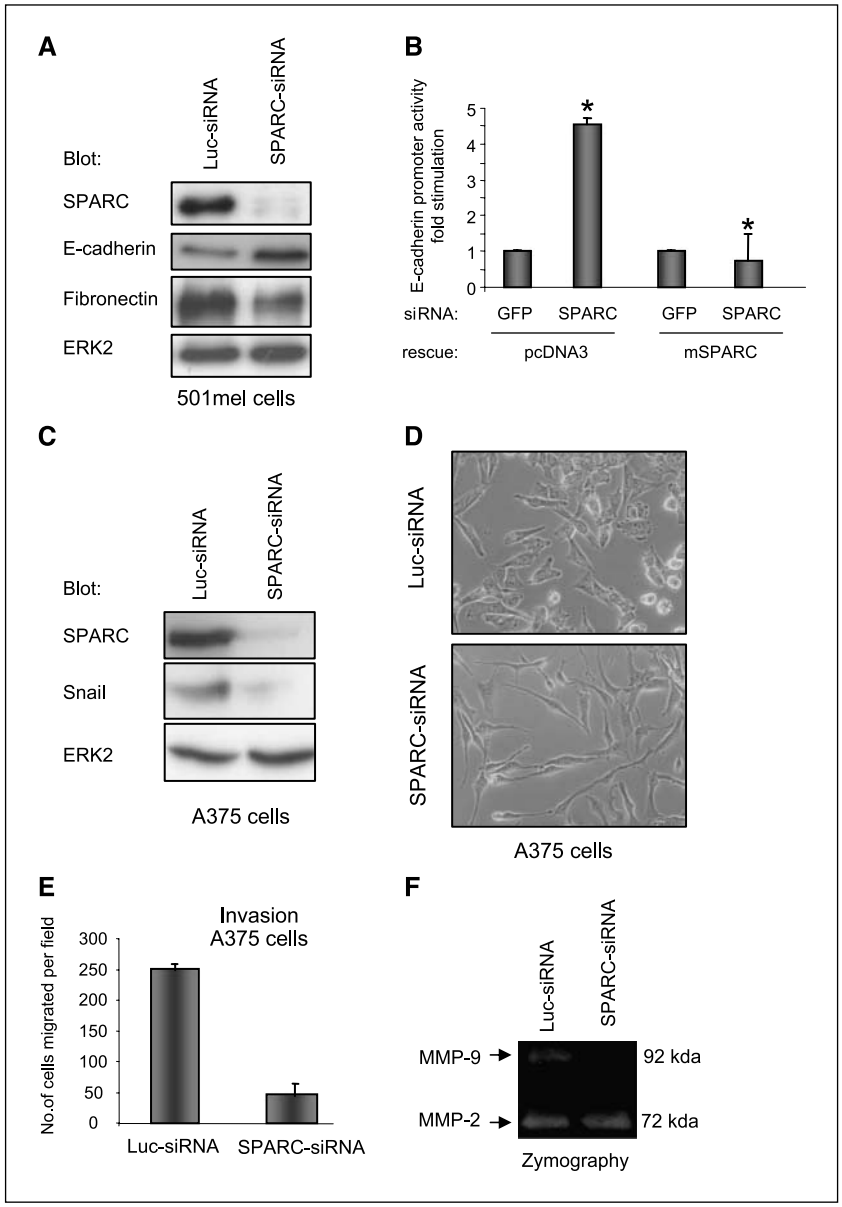

Figure 6. siRNA-mediated depletion of SPARC leads to reinduction of E-cadherin and down-regulation of Snail and decreases invasiveness of melanoma cells. *A,* silencing of SPARC leads to increased E-cadherin levels and decreased fibronectin levels. 501mel cells were transfected with SPARC-siRNA or Luc-siRNA. After 3 days of treatment, the expression levels of fibronectin, E-cadherin, SPARC, and extracellular signal-regulated kinase 2 were analyzed by Western blotting. extracellular signal-regulated kinase 2 was used as loading control. Representative of three independent experiments. *B,* rescue of SPARC silencing prevented E-cadherin promoter induction. 501mel cells were transfected with SPARC-siRNA or GFP-siRNA. Twenty-four hours later, cells were transfected with the wild-type mouse E-cadherin promoter luciferase reporter together with either pcDNA3-mSPARC encoding for the murine SPARC cDNA or empty pcDNA3 and cultured for additional 48 hours. Luciferase activities were assessed and normalized to β-galactosidase. *Columns,* mean fold induction of triplicates; *bars,* SD. Representative of three independent experiments. *, $P < 0.05$ (Student's *t* test). *C,* silencing of SPARC reduces Snail protein levels. Expression of Snail and SPARC in A375 cells transfected with SPARC-siRNA or Luc-siRNA were evaluated by Western blotting analysis. Extracellular signal-regulated kinase 2 was used as loading control (*D*) Morphology of A375 cells transfected with SPARC-siRNA or Luc-siRNA for 3 days using phase-contrast microscopy. Pictures were taken at 20-fold magnification. *E,* depletion of SPARC leads to decreased invasion capacity of A375 cells. Cells transfected with SPARC-siRNA or Luc-siRNA were seeded onto a Matrigel invasion chamber. *Columns,* average number of cells migrating through filters from five separate fields; *bars,* SD. *F,* serum-free conditioned media from A375 cells transfected with SPARC-siRNA or Luc-siRNA were analyzed by collagen zymography. Representative of three separate experiments.

transfected into 501mel cells, and whole-cell lysates were prepared at 24-hour intervals (0-96 hours). Maximal reduction in SPARC protein levels was achieved at the 72-hour time point and persisted after 6 days of treatment (Fig. 6*A*; data not shown). Interestingly, siRNA-mediated SPARC suppression resulted in a reinduction of E-cadherin expression and conversely a decrease in fibronectin levels. To verify that reinduction of E-cadherin was a consequence of specific inhibition of SPARC expression, the silencing of SPARC was rescued by expressing the murine SPARC mRNA. We used a siRNA that specifically targets the human SPARC mRNA and not the murine SPARC mRNA because its targets a 21-bp region in which the two homologues differ at three positions. This allowed us to show that SPARC siRNA-mediated induction of E-cadherin promoter activity could be prevent by cotransfection of the murine SPARC cDNA (Fig. 6*B*). These results confirm the specificity of the repression operated by SPARC on E-cadherin expression.

As expected, siRNA to SPARC led to a significant knockdown of SPARC expression in A375 cells (Fig. 6*C*). Depletion of SPARC dramatically lowered levels of Snail protein in A375 cells. Similar result was obtained in 501mel cells that is consistent with induction of E-cadherin expression (data not shown). Silencing of SPARC was also accompanied with significant morphologic changes (Fig. 6*D*), suggesting that SPARC contributes to the fibroblast-like morphotype of A375 cells. We also evaluated *in vitro* invasive properties of control and siRNA SPARC-depleted melanoma cells in Boyden chamber assays. Figure 6*E* shows that cells transfected with SPARC-siRNA had a dramatic diminished invasive ability when compared with control cells. This data corroborate previous observations made by Ledda et al. (24) using stable antisense RNA expression to target SPARC in IIB-MEL-LES cells. In addition, silencing of SPARC led to a strongly reduction in MMP-9 enzymatic activity (Fig. 6*F*). Taken together, these findings support the conclusion that SPARC functions as a mediator of Snail expression and E-cadherin repression in melanoma cells and indicate that expression of SPARC contributes to the mesenchymal and invasive phenotype of these cells.

Relationship among SPARC, Snail, and E-cadherin levels in melanoma lesions. Primary melanoma lesions progress to malignant tumors through a complex multi step process, including radial growth phase (RGP), invasive vertical growth phase, and metastasis. To gain further insights into the link among SPARC, Snail, and E-cadherin levels, we analyzed their expression in a series of human cell lines established from melanoma at various phases of development. Western blotting showed that SPARC increased early during primary melanoma development in the RGP and revealed that reduced E-cadherin levels were associated with high SPARC and Snail levels in most of melanoma cell lines tested (Fig. 7). These observations confirm at protein level the published findings that Snail transcript was expressed in melanoma cells (8, 9). Note that E-cadherin-negative SBcl2 cells showed SPARC expression but undetectable expression of Snail, suggesting that mechanism other than Snail induction may be involved in repression of E-cadherin in this cell line. Conversely, primary melanocytes express high amount of E-cadherin but show undetectable levels of SPARC and Snail. In conclusion, our observations provide independent support for the potential relationship among SPARC, Snail, and E-cadherin in melanocytic cells.

Discussion

The results presented in this article highlight a novel role for SPARC in controlling Snail and E-cadherin levels during oncogenic transformation of normal melanocytes and give important insights into the mechanism of action of SPARC in tumor progression.

Change in E-cadherin has emerged as a key factor in melanoma progression (8, 9, 33). It is well recognized that in addition to its crucial function in cell-cell adhesion, E-cadherin may act as a tumor suppressor negatively regulating several critical steps of invasion and metastasis (10, 34). In most melanoma cells, loss of expression has been attributed to transcriptional repression that could be achieved by Snail factors (8, 9). However, few studies have documented pathways that drive Snail expression and E-cadherin repression in these tumor cells (33, 35, 36). Our findings now identify a pathway involved in Snail regulation and support a role for SPARC in early primary melanoma development. We showed that SPARC induces up-regulation of Snail that occurs with E-cadherin transcriptional repression. Importantly, we observed that depletion of SPARC led to decreased Snail amount in A375 melanoma cells, implying that frequent aberrant production of SPARC in melanoma cells might contribute to Snail expression and subsequent phenotypic changes. However, in contrast to 501mel cells, decrease of Snail was not associated with reinduction of E-cadherin protein levels in A375 cells (results not shown). The lack of E-cadherin up-regulation is consistent with a recent report showing that other mechanisms, such as promoter hypermethylation, contribute to loss of E-cadherin in this cell line (37). Interestingly, this notion is in agreement with previous observations that Snail might act independently of E-cadherin repression to regulate epithelial phenotype and invasive behavior (38, 39). It will be interesting to determine whether the ability of SPARC to induce Snail and to repress E-cadherin is operant in epithelial cells and whether this mechanism is specific to melanocytic cells.

During melanoma progression, loss of E-cadherin often correlates with up-regulation of the mesenchymal cadherin N-cadherin (40). Although SPARC induces numerous events associated with mesenchymal transition, expression of SPARC was not sufficient to enhance N-cadherin or other markers associated with melanoma progression, such as β_3 integrin, MMP-2, MelCAM, or CD44v6. These findings indicate that SPARC-induced Snail expression is not sufficient to fully phenocopy alterations associated with mesenchymal transition and are consistent with the involvement of additional genetic or epigenetic events in this process. Nevertheless, invalidation of SPARC in melanoma cells is sufficient to down-regulate Snail and to revert melanoma invasiveness. This clearly stated that SPARC triggers signaling pathways that functionally contribute to the invasive phenotype of melanoma cells.

The mechanism and signaling pathways responsible for SPARC-induced Snail up-regulation and concomitant E-cadherin suppression remain to be determined. The modulation of cellular responses by SPARC is complex and thought to be cell context dependent. SPARC may down-regulate E-cadherin expression by several potential mechanisms. One can be indirect through interactions with other matrix components or through the ability of SPARC to regulate the activity of growth factors or of their cognate receptors. Alternatively, the effect of SPARC may be direct through yet unidentified cellular receptors binding and signaling. To clarify the mechanism by which SPARC influences E-cadherin in melanocytes, we deleted the highly conserved EC domain of SPARC and analyzed the role of SPARCΔEC mutant in mediating E-cadherin loss. We found that EC domain of SPARC is dispensable for this response, and that the NH_2-terminal acidic and follistatin-like modules contain determinants that are responsible for inhibiting E-cadherin expression. Interestingly, in contrast to its effect on E-cadherin expression, this mutant failed to inhibit cell spreading and attachment, suggesting that SPARC induces E-cadherin repression independently of its well-established counteradhesive property (results not shown). This observation allows us to distinguish this function from E-cadherin suppression, which is consistent with a recent study showing the role of EC domain in anti-spreading activity of SPARC in urothelial cells (41). Further work is clearly required to explain fully how SPARC functions to trigger signaling pathways that functionally regulate E-cadherin in melanocytes.

The mechanisms accounting for up-regulation of SPARC in malignant melanoma cells remain elusive. Because there is no cytogenetic evidence reported for abnormalities in the region of the long arm of chromosome 5 (5q31.3-q32) in which the *SPARC* gene is mapped, it is conceivable to postulate that SPARC up-regulation occurs secondary to oncogenic mutations of signaling pathways or aberrant cytokine or growth factors network present in the epidermal microenvironment. Data from gene profiling have

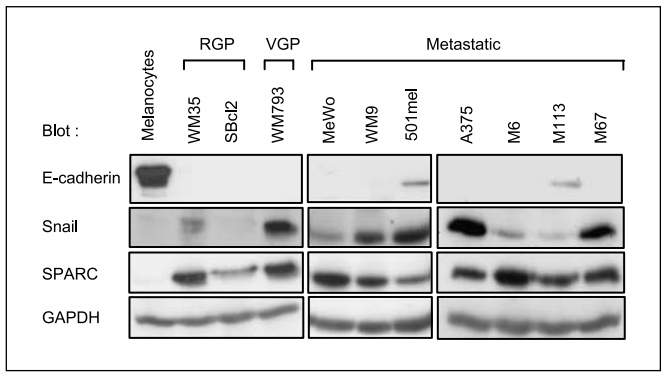

Figure 7. Relationship among SPARC, Snail, and E-cadherin levels in normal and transformed melanocytes. Expression of endogenous E-cadherin, Snail, and SPARC was analyzed by Western blotting in a series of human primary or metastatic melanoma cell lines. GAPDH was used as loading control. Representative of three independent analyses.

shown that SPARC and other melanoma-associated genes were modulated after enforced expression of the zinc finger PLZF protein or inhibition of melanoma inhibitory activity protein (42, 43). In addition, regulation of SPARC expression in RGP primary melanoma cells following β_3 integrin overexpression was documented (44). Another attractive possibility is to link the increase of SPARC with the production of transforming growth factor-β by melanoma cells (9) because this cytokine has been shown to induce SPARC in other cell systems (27, 45, 46).

The down-regulation of E-cadherin and SPARC overexpression have been separately reported to correlate with an invasive stage of tumor development. Here, we provide for the first time a mechanistic link between these two components during melanoma development. SPARC secreted by transformed melanocytes functions to mediate transcriptional down-regulation of E-cadherin likely through up-regulation of Snail that consequently facilitates mesenchymal transition. This demonstration of a relationship between SPARC function and E-cadherin expression provides an important insight for further understanding tumor progression and metastasis and also supports SPARC as a potential target for melanoma therapy.

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