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ORIGINAL ARTICLE

# SPARC/osteonectin is involved in metastatic process to the lung during melanoma progression

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**Abstract** The existence of a "metastasis gene signature" that predisposes primary breast cancer cells to metastasize to the lungs has been recently highlighted by gene expression profiling studies. The combination of genes responsible for this process includes genes encoding several metalloproteinases as well as the gene encoding SPARC (secreted protein acidic and rich in cysteine)/osteonectin. SPARC is involved in normal tissue remodeling as it regulates the deposition of extracellular matrix, but also plays a role in neoplastic transformation. Aberrant SPARC expression has been detected both in stromal cells associated with cancer and in cancer cells. The main aim of this study was to investigate whether or not SPARC might be involved in directing metastasis of other types of cancer to the lung. We constructed a tissue microarray containing lung metastases from a variety of primary tumors in different organs and used immunohistochemistry to assess SPARC expression. We found SPARC overexpressed mainly in lung metastases from melanoma. We then assessed the expression of SPARC mRNA and protein in metastatic melanoma from different anatomic sites and in their corresponding primary tumors, and found that it is overexpressed in lung metastases. Our data strongly support the hypothesis that SPARC is involved in directing melanoma metastases specifically to the lung, which underpins its potential as prognostic marker and novel target for specific therapy.

**Keywords** Melanoma progression · Lung metastases · SPARC expression

## Introduction

Metastasis is a key factor in melanoma progression and determines prognosis [1]. However, molecular mechanisms which determine the capacity of neoplastic cells in a primary tumor to invade surrounding tissues, migrate, and colonize different organ sites are still poorly understood [2]. The lung and bone are frequent sites of metastatic disease in many human cancers. Earlier gene expression profiling studies have identified a cluster of genes that selectively direct breast cancer cells to metastasize to the lungs. This includes the gene for SPARC (secreted protein acidic and rich in cysteine; also known as osteonectin), an acidic extracellular matrix glycoprotein [3].

SPARC is an intensively studied extracellular matrix protein in cancer and is involved in normal tissue remodeling as well as in neoplastic transformation, as it functions as a regulator of the deposition of extracellular matrix. It appears to play a fundamental role in the induction of a metastatic phenotype by modulating the adhesive capacity of neoplastic cells [4]. SPARC is differentially expressed in various cancer cells and their accompanying stroma in comparison with normal tissues, and its expression pattern is variable and highly dependent on the type of cancer [5–14].

In melanoma, gene expression profiling of primary non-metastatic cutaneous tumors and metastatic disease has allowed the identification of several extracellular matrix proteins that might play a determining role in metastatic potential and disease progression [15, 16]. In particular, expression of

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SPARC was found to be altered in a mouse model of melanoma characterized by high tumor aggressiveness [17].

Against this background, the main aim of this study was to further investigate the role of SPARC in the metastatic process. More specifically, we evaluated its propensity for directing the metastatic process of various tumor types to the lung, as this had been previously reported for breast cancer. In addition, we assessed whether in pulmonary metastases of melanoma SPARC are overexpressed. To this end, we selected a series of primary melanoma cases that had metastasized to different anatomical sites.

## Materials and methods

### Tissue samples

All patients had provided written informed consent for the use of samples, according to institutional regulations, and the study was approved by the ethics committee of the National Cancer Institute "G. Pascale."

All samples were from Caucasian patients and included formalin-fixed, paraffin-embedded (FFPE) tissue biopsies from primary melanomas, melanoma metastases, and lung metastases from primary tumors other than melanoma, as well as a series of cytology samples from melanoma metastases. All tissue sections, routinely stained as well as appropriate immunohistochemically stained sections, were reviewed by three pathologists using AJCC classification criteria. In addition, the medical records of patients providing samples were reviewed for clinical information. Clinicopathological parameters included patient age at initial diagnosis, characteristics of the primary lesion, lymph node involvement, tumor recurrence, and distant metastasis.

### Tissue microarray building

Tissue microarrays (TMA) were constructed based upon tissue samples of 43 lung metastases from different primary tumors and 73 melanoma metastases from different anatomical locations, selecting the morphologically most representative areas from each individual tissue sample. Tissue cores with a diameter of 0.6 mm were punched from each donor tissue block and inserted into a recipient paraffin block (3×2.5 cm), using a semi-automated tissue arrayer (Galileo TMA, Integrated System Engineering Srl, Milan, Italy). All tumor and control tissues (non-neoplastic skin and nevi) were independently reviewed by three experienced pathologists, any discrepancies being resolved by discussion of the case to reach a consensus diagnosis.

### SPARC immunostaining

SPARC expression was evaluated by immunohistochemical (IHC) staining of FFPE tissue sections and immunocytochemical (ICC) staining of cytology samples using a ThinPrep imager (Hologic, Inc., Marlborough, MA, USA). Paraffin sections were deparaffinized in xylene and rehydrated through a graded alcohol series. Antigen retrieval was performed by microwave pretreatment in 0.01 M citrate buffer for 10 min. After blocking of non-specific staining (5 % bovine serum albumin in PBS 1×), slides were incubated overnight with primary antibody against human SPARC (dilution 1:1200, cod.ab55251, Abcam, Cambridge, UK). Sections were incubated with mouse anti-rabbit or goat anti-mouse secondary IgG biotinylated secondary antibody for 30 min. Immunoreactivity was visualised by means of avidin-biotin-peroxidase complex kit (Novocastra, Newcastle, UK) with its chromogenic substrate. Finally, sections were weakly counterstained with hematoxylin and mounted, and the results were interpreted by light microscopy.

### Evaluation of SPARC immunostaining

SPARC stained tissue sections were evaluated by three pathologists. The percentage of cancer cells with cytoplasmic staining was determined by counting the number of positive cells as a fraction of the total number of cancer cells in ten non-overlapping fields at ×400 magnification. The median value of SPARC positive cells (15 %) was taken as cut-off level, to result in recording a case as negative/low when the percentage of positive cells was 0-15 % and high when this was ≥16 %.

### RNA extraction from cellular suspension and paraffin-embedded tissues

FFPE tissue sections were incubated at 37 °C in the presence of xylene for about 20 min. Total RNA was purified using the High Pure FFPE RNA Micro Kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. Total RNA was isolated from cytology samples using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. All samples were treated with RNase-free DNase (Qiagen GmbH, Hilden, Germany) to prevent amplification of genomic DNA. A total of 1 μg RNA was subjected to complementary DNA (cDNA) synthesis for 1 h at 37 °C using the Ready To Go You-Primer First-Strand Beads Kit (Amersham Biosciences Europe Gmbh, Freiburg, Germany, cod. 27-9264-01) in a reaction mixture containing 0.5 μg random hexamers (GeneAmp RNA PCR Random Hexamers Set N808-0127 Applied Biosystems, Foster City, CA, USA).

### Real-time reverse transcription polymerase chain reaction (RT-PCR)

Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed in a LightCycler® system (Roche) using TaqMan® analysis. In this system, all reactions were run in glass capillaries with the LightCycler® TaqMan® Master Mix (cod. 04735536001) 10 μl in a volume of 20 μl containing 2 μl of cDNA and 1 μl of specific TaqMan® Gene Expression Assays for human *SPARC* (RealTime Designer Assay cod. 04162498001), according to the manufacturer's directions. All reactions were performed in triplicate. The thermal cycling conditions included 20 s at 95 °C followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. The comparative $C_t$ method was employed to determine the human *SPARC* gene variation, using as reference gene TaqMan® Endogenous Controls Human *ACTB* (*β-actin*) Endogenous Control (RealTime Designer Assay cod.05532957001, Roche). We identified a calibrator cell line that represents the unitary amount of the target of interest, with samples expressing n-fold messenger RNA (mRNA) relative to this calibrator. Final amounts of target were determined using the formula: $\text{target amount} = 2\text{-}C_t$ , where $C_t=[C_t(SPARC)-C_t(ACTB)]_{sample}-[C_t(SPARC)-C_t(ACTB)]_{calibrator.}$

### Statistical analysis

The association between SPARC and other clinicopathological parameters was assessed using chi-squared and Student's *t* tests. The Pearson's chi-squared test was used to determine whether a relationship exists between the variables included in the study. The level of significance was defined as $p<0.05$. All statistical analyses were carried out using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA).

## Results

### SPARC expression in lung metastases from different primary tumors

The results of SPARC immunostaining of pulmonary metastases from different primary tumor sites are summarized in Table 1 and illustrated in Fig. 1. SPARC immunoreactivity was detected in 15 of 43 lung metastasis samples. Of note, all five melanoma metastases and 7 of 9 sarcoma metastases (78 %) were SPARC-positive. In contrast, 13 of 14 metastases from gastrointestinal tract tumors and 9 of 10 metastases from urogenital tract tumors were SPARC-negative, while only one lung metastasis from an adrenal gland tumor showed weak immunoreactivity. Moreover, SPARC mRNA expression was identified by RT-PCR in ten lung metastases (which were also analyzed by IHC) and in four selected cytology samples of metastatic lesions. Lung metastases of intestinal, endometrial, renal, and bladder cancers showed almost complete absence of SPARC expression. Lung metastases from breast cancer showed low SPARC expression, while metastases from melanoma, leiomyosarcoma, and chondrosarcoma showed high expression of SPARC (between 10- and 100-fold higher) (Fig. 2).

**Table 1** SPARC expression in pulmonary metastases. Frequencies are indicated in absolute values (percentage)

| IHC SPARC expression, *n* (%) (*n*=43) | | |
|---|---|---|
| Primary localization | (−) | (+) |
| Gastro-intestinal (*n*=14) | 13 (92.9) | 1 (7.1) |
| Soft tissue (*n*=9) | 2 (22.2) | 7 (77.8) |
| Melanoma (*n*=5) | 0 | 5 (100) |
| Breast (*n*=5) | 4 (80) | 1 (20) |
| Uro-genital | 9 (90) | 1 (10) |

### SPARC expression in metastatic melanoma samples

The results of SPARC immunostaining on the TMA of melanoma metastases to different sites are shown in Table 2. SPARC-immunoreactivity was detected in 41 of 73 (56 %) melanoma metastases. Seventeen of 24 lung metastases and 13 of 17 skin metastases were positive, while 12 of 18 lymph node metastases, 3 of 4 liver metastases, and 6 of 7 gastrointestinal metastases were negative. SPARC expression was also detected by ICC and RT-PCR on six selected cytology samples from metastatic lesions. Lymph node metastasis showed a complete absence of expression of SPARC by both ICC and RT-PCR, while lung metastases showed SPARC expression by ICC and high expression of SPARC (between 10- and 100-fold higher) (Fig. 3).

### SPARC expression in primary melanomas and corresponding metastases

Characteristics of the 32 patients of whom samples from primary melanoma and metastases were assessed are summarized in Table 3. Median age at time of surgery was 55.6 years; 13 of 32 patients (41 %) were female. Fourteen patients had stage IV disease, while stage I, II, and III each accounted for six patients. All patients were alive at last follow-up with a mean disease-free survival (DFS) of 26.8 months. Clark level was II in 2 patients, III in 8 patients, IV in 17 patients, and V in 5 patients, while mitotic rate was >1 in 28 patients (0 in four cases). Tumor-infiltrating lymphocytes (TILs) were absent in 23 patients, non-brisk in eight patients and brisk in a single patient. Ulceration was present in 15 patients and absent in 17 of the 32 cases.

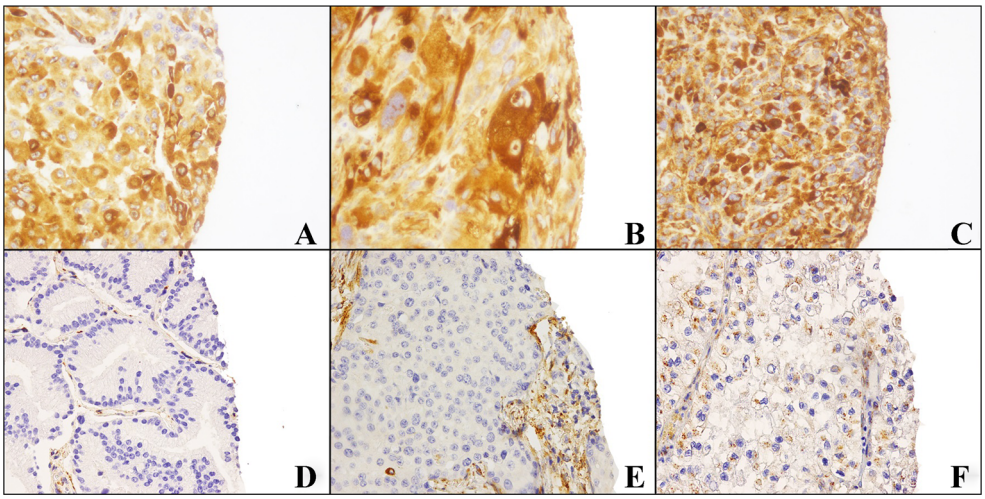


**Fig. 1** SPARC immunostaining (40×) of lung metastases: **a** Melanoma metastasis to the lung; **b** breast carcinoma metastasis to the lung; **c** lung metastasis of chondrosarcoma; **d** lung metastasis of gastrointestinal carcinoma; **e** lung metastasis urinary bladder cancer; **f** lung metastasis of renal cancer

Chi-squared analysis showed a significant positive relationship between SPARC expression in primary melanoma and corresponding metastases ($p<0.002$) (Table 4). Of note, in 19 of the 21 primary melanomas positive for SPARC (91 %), the corresponding metastases were also positive. SPARC was negative in 11 cases, eight of which negative also in the corresponding metastases (73 %) (Fig. 4).

## Discussion

Although metastasis is a key event in cancer, the molecular mechanisms which determine the capacity of neoplastic cells in a primary tumor to invade surrounding tissues, migrate, and colonize different organ sites are still poorly understood [18]. Current hypotheses suggest the existence of a "metastasis gene signature" in primary tumors that predispose it to metastasize to predefined sites [19–23]. An earlier gene expression array study identified a metastasis gene signature, which predisposed breast cancer to metastasize to the lung [3]. This signature encompassed the SPARC gene which, along with other extracellular matrix proteins, plays a crucial role in the induction of the metastatic phenotype by modulating the adhesive capacity of neoplastic cells [24].

In order to study whether SPARC might also direct the metastatic process to the lung in other human cancers, we study the expression of SPARC in lung metastases originating from different primary tumors. We detected SPARC overexpression in lung metastases of different types of sarcoma and melanoma. In lung metastases of gastrointestinal and urogenital cancer, SPARC was not expressed. In lung metastases of breast cancer, SPARC expression was absent or very low, in contrast with previously reported data [3]. This discrepancy might be due to the limited number of samples in our study and/or differences in the used methods. In sarcomas, high SPARC expression might be related to its mesenchymal derivation, as in normal tissues it is secreted by stromal cells that deposit the extracellular matrix.

We found high SPARC expression in all lung metastases from melanoma in our case series. We, therefore, studied SPARC expression in a series of metastatic melanoma cases

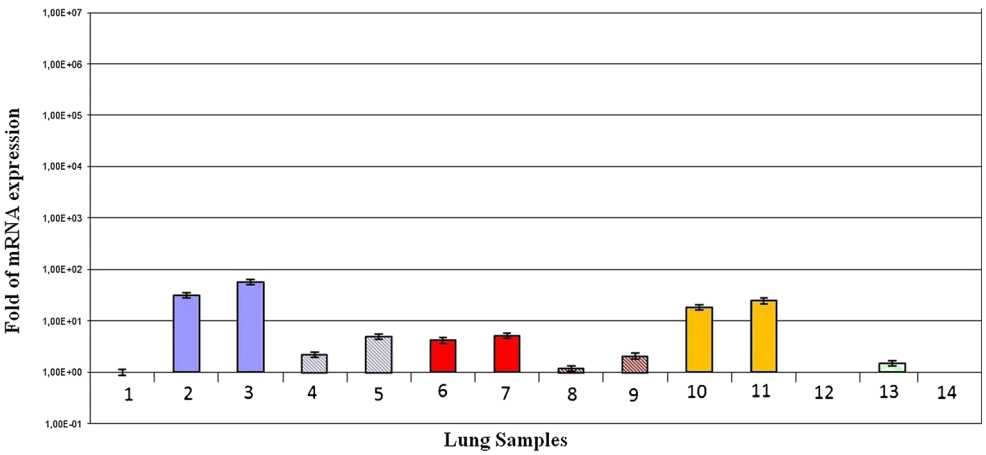


**Fig. 2** *SPARC* real-time expression in lung metastases: from intestinal cancer (*1*); from melanoma (*2, 3*) and cytology samples (*4, 5*); from metastatic breast cancer (*6, 7*) and cytology samples (*8, 9*); from lung metastases of leiomyosarcoma (*10*) and chondrosarcoma (*11*); and from metastases of endometrial (*12*), kidney (*13*), and bladder (*14*) cancer. All reactions were performed in triplicate, and data are expressed as mean of relative amount of mRNAs levels

**Table 2** SPARC expression in melanoma metastases. The frequencies are indicated in absolute values (percentage)

| SPARC expression, n (%) (*n*=73) | | |
|---|---|---|
| Site of metastasis | (−) | (+) |
| Lung (*n*=24) | 7 (29.2) | 17 (70.1) |
| Lymph node (*n*=18) | 12 (66.7) | 6 (33.3) |
| Skin (*n*=17) | 4 (23.5) | 13 (76.5) |
| Gastro-intestinal (*n*=7) | 6 (85.7) | 1 (14.3) |
| Liver (*n*=4) | 3 (75) | 1 (25) |
| Bone (*n*=1) | 0 | 1 (100) |
| Brain (*n*=1) | 0 | 1 (100) |
| Adrenal (*n*=1) | 0 | 1 (100) |

with high SPARC expression in lung metastases. Skin metastases were often positive but these represent local dissemination in an area close to the primary tumor. In primary melanomas and corresponding metastases to lymph nodes or to the gastrointestinal tract, SPARC expression was very low or even absent. Our immunohistochemical results are corroborated by gene expression studies, which showed a similar trend of SPARC expression.

In the induction of the metastatic phenotype of melanoma, changes in expression of cell adhesion molecules have been identified, which affect the ability of cells to adhere to each other and to the constituents of the extracellular matrix [16]. In the pathogenesis of melanoma, SPARC has been found to be involved in matrix remodeling during tumor progression, and it is expressed in both melanoma associated stromal cells as well as neoplastic cells [25]. Earlier immunohistochemical studies have shown moderate expression of SPARC in nevi and strong expression in primary and metastatic melanoma [26]. In addition, SPARC expression is strongly related to clinical outcome [27]. Mouse models of melanoma have shown that SPARC expression goes along with increased tumor aggressiveness [17]. This was recently confirmed by

**Table 3** Clinicopathological characteristics of melanoma patients and tumors and relation with SPARC expression. The frequencies are indicated in absolute values (percentage)

| SPARC expression, *n* (%) (*n*=32) | | | |
|---|---|---|---|
| | (−) | (+) | *p* value |
| Sex | | | |
| Female | 6 (46.2) | 7 (53.8) | 0.403 |
| Male | 6 (31.6) | 13 (68.4) | |
| Age | | | |
| ≤55 years | 5 (27.8) | 13 (72.2) | 0.277 |
| ≥56 years | 7 (50) | 7 (50) | |
| Ulceration | | | |
| No | 5 (29.4) | 12 (70.6) | 0.314 |
| Yes | 7 (46.7) | 8 (53.3) | |
| Stage | | | |
| I | 2 (33.3) | 4 (66.7) | 0.955 |
| II | 3 (50) | 3 (50) | |
| III | 2 (33.3) | 4 (66.7) | |
| IV | 5 (35.7) | 9 (64.3) | |
| TILs | | | |
| Absent | 8 (34.8) | 15 (65.2) | 0.797 |
| Non-brisk | 4 (50) | 4 (50) | |
| Brisk | 0 | 1 (100) | |
| Clark | | | |
| II | 2 (100) | 0 | 0.143 |
| III | 2 (25) | 6 (75) | |
| IV | 5 (29.4) | 12 (70.6) | |
| V | 3 (60) | 2 (40) | |
| Mitotic rate | | | |
| 0 | 2 (50) | 2 (50) | 0.783 |
| 1–5 | 6 (40) | 9 (60) | |
| >5 | 4 (30.8) | 9 (69.2) | |

TILs = tumor-infiltrating lymphocytes

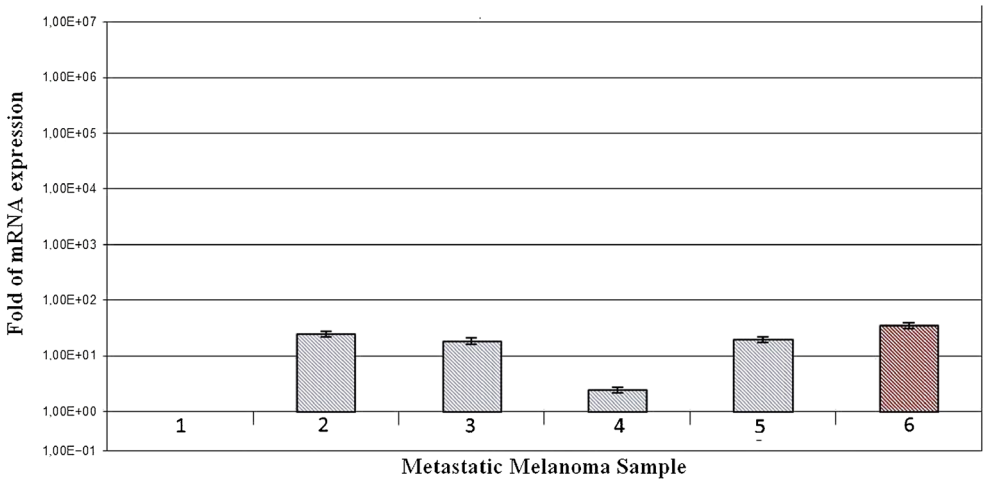


**Fig. 3** *SPARC* real-time expression in metastatic melanoma samples: from lymph node (*1*); from skin (*2–5*); and from lung (*6*). All reactions were performed in triplicate, and data are expressed as mean of relative amount of mRNAs levels

**Table 4** SPARC expression in primary melanoma and corresponding metastases. The frequencies are indicated in absolute values (percentage)

| | | Primary melanoma | | |
|---|---|---|---|---|
| | | (−) | (+) | *p* value |
| Metastasis | (−) | 8 (72.7) | 3 (27.3) | 0.0002 |
| | (+) | 2 (9.5) | 19 (90.5) | |

studies showing that SPARC promotes tumor invasion through an epithelial-mesenchymal transition (EMT) associated process, with activation of the AKT pathway and up-regulation of expression of SLUG [28].

## Conclusions

Considerable evidence supports the notion that extracellular matrix proteins of the tumor microenvironment play a fundamental role in the acquisition of invasive and migratory capacity, and are directly associated with the process of tumor progression. Our data, although on a limited number of samples, support the hypothesis that SPARC expression predisposes some primary tumors to preferentially metastasize to the lungs. This suggests that SPARC should be considered as a molecular target for new targeted therapies. SPARC expression has been reported to correlate with tumor response to nab-paclitaxel in head and neck squamous cell carcinoma (HNSCC) [29]. The use of specific small interfering RNA

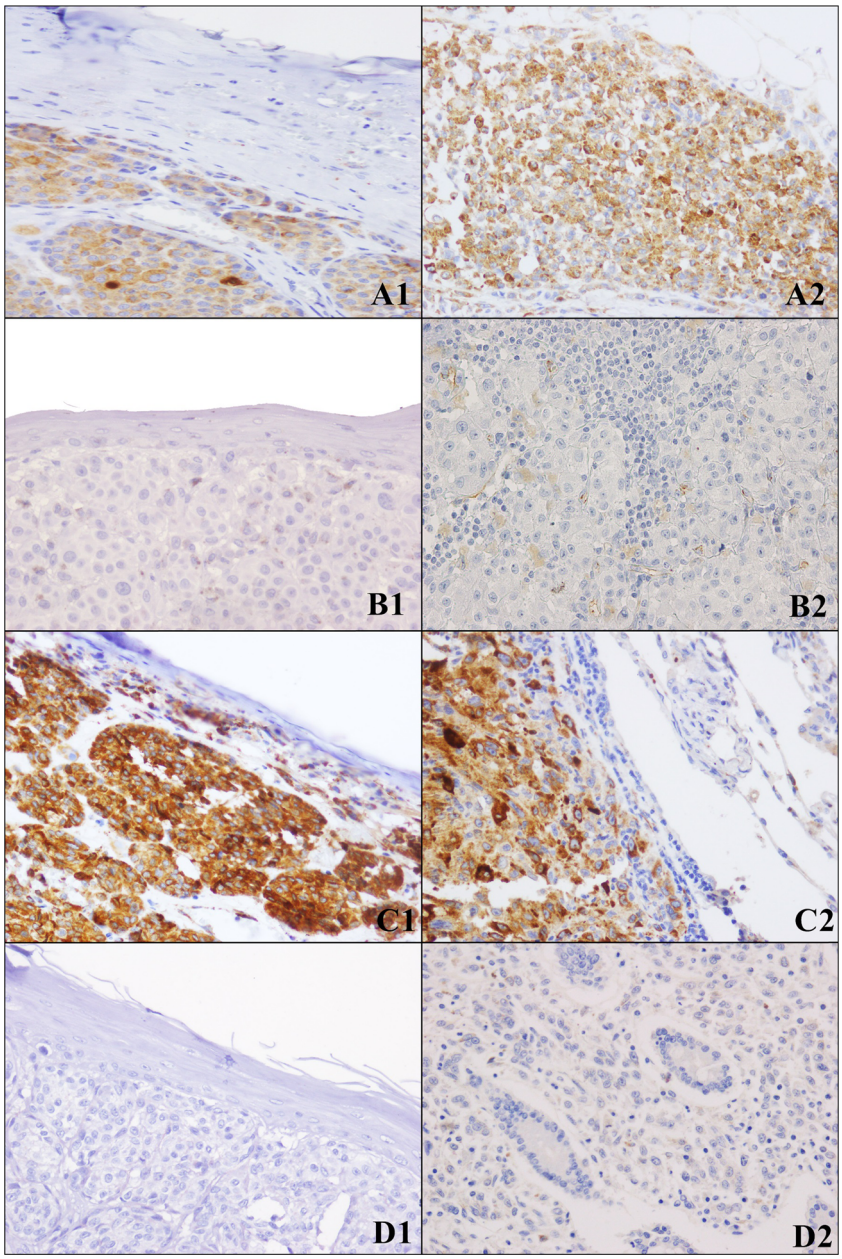


**Fig. 4** SPARC immunostaining (40×) in primary melanoma and corresponding metastases: SPARC immunoreactive primary melanoma (*a1*) and skin metastasis *a2*; SPARC negative primary melanoma (*b1*) and lymph node metastasis (*b2*); SPARC immunoreactive primary melanoma (*c1*) and lung metastasis (*c2*); SPARC negative primary melanoma (*d1*) and intestinal metastasis (*d2*)

(siRNA) to block gene expression, but also of specific antibodies targeting the function of its corresponding protein, represents approaches of which the potential has already been documented in experimental models. In melanoma cells silencing of SPARC, using an antisense expression vector, reduced their invasiveness and adhesive capacity [30]. Moreover, RNA interference (siRNA) silencing of SPARC arrested cell growth at the G1 phase in cell and animal models of melanoma [31]. Finally, small peptides have been synthesized capable of binding different domains of SPARC, which arrested angiogenesis and inhibited tumor growth in animal models [32, 33]. The tumor microenvironment represents a complex interaction between different processes (e.g., cell survival, apoptosis, angiogenesis, immunological activation or escape, invasion and metastasis process, etc.) [34]. Targeting of SPARC might be combined with other compounds active against melanoma, each targeting a different pathway. Targeting different pathways involved in the metastatic process might provide further opportunities to increase the efficacy of melanoma treatment, and we consider SPARC a strong candidate target for such an approach.

**Conflicts of interest** All authors report no conflict of interest with respect to any financial or personal relationships with other people or organizations that could inappropriately influence this work.

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