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SPARC Endogenous Level, rather than Fibroblast-Produced SPARC or Stroma Reorganization Induced by SPARC, Is Responsible for Melanoma Cell Growth

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SPARC (secreted protein acidic and rich in cysteine) is a matricellular protein whose overexpression in malignant or tumor-stromal cells is often associated with increased aggressiveness and bad prognosis in a wide range of human cancer types, particularly melanoma. We established the impact that changes in the level of SPARC produced by malignant cells and neighboring stromal cells have on melanoma growth. Melanoma cell growth in monolayer was only slightly affected by changes in SPARC levels. However, melanoma growth in spheroids was strongly inhibited upon SPARC hyperexpression and conversely enhanced when SPARC expression was downregulated. Interestingly, SPARC overexpression in neighboring fibroblasts had no effect on spheroid growth irrespective of SPARC levels expressed by the melanoma cells, themselves. Downregulation of SPARC expression in melanoma cells induced their rejection *in vivo* through a mechanism mediated exclusively by host polymorphonuclear cells. On the other hand, SPARC hyperexpression enhanced vascular density, collagen deposition, and fibroblast recruitment in the surrounding stroma without affecting melanoma growth. In agreement with the *in vitro* data, overexpression of SPARC in co-injected fibroblasts did not affect melanoma growth *in vivo*. All the data indicate that melanoma growth is not subject to regulation by exogenous SPARC, nor by stromal organization, but only by SPARC levels produced by the malignant cells themselves.

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INTRODUCTION

Cancer progression is the result of a dynamic interaction between malignant cells, neighboring stromal cells such as fibroblasts and endothelial cells, and the host's immune cells that infiltrate the tumor mass. It is increasingly clear that proteins secreted by the tumor cells or surrounding stromal cells that become structural or transient components of the extracellular matrix (ECM) might play a significant role in cell–ECM interactions, helping the tumor cell to invade the neighboring stroma and disseminate (Murphy-Ullrich, 2001). One of the transient components of the ECM is the matricellular protein SPARC (secreted protein acidic and rich in cysteine). SPARC is a secreted glycoprotein associated with cell–matrix interactions during tissue remodeling, morphogenesis, migration, and proliferation (Bradshaw *et al.*, 2001). SPARC is produced by different cell types, including endothelial cells, and was found to bind specific growth factors, such as vascular endothelial growth factor, platelet-derived growth factor, and basic fibroblast growth factor, or interact with their signaling pathways, suggesting that it might play a role in vasculogenesis (Yan and Sage, 1999). SPARC was also found to regulate matrix metalloproteinase expression and activity in normal and malignant cells, suggesting a role in enhanced tumor aggressiveness (Lane and Sage, 1994; Ledda *et al.*, 1997b; Yan and Sage, 1999).

Several studies including expression array analysis indicate that SPARC overexpression is associated with increased aggressiveness of various human cancers (Framson and Sage, 2004, and references herein). In several adenocarcinomas, SPARC is faintly or not at all expressed in the malignant cells; however, its expression in stromal fibroblasts of human lung and colon carcinoma correlates with poor prognosis (Koukourakis *et al.*, 2003; Porte *et al.*, 1995). It has been proposed that SPARC's capacity to induce cell disengagement from the ECM favors cell migration and dissemination (Ledda *et al.*, 1997a; Rempel *et al.*, 2001). Indeed, SPARC was found to enhance the migration and invasive capacity of melanoma

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Abbreviations: SPARC, secreted protein acidic and rich in cysteine; ECM, extracellular matrix; $TCID_{50}$, 50% tissue culture infectious dose; PMN, polymorphonuclear cells

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and glioblastoma cells (Golembieski *et al.*, 1999; Ledda *et al.*, 1997a; Rempel *et al.*, 2001, 1998). In addition, SPARC increased the migration of prostate cancer cells toward bone extracts, highlighting its potential role as a chemotactic factor in bone metastasis (Thomas *et al.*, 2000). More recently, SPARC was identified as one of the selected proteins that confer lung metastatic capacity to breast cancer cells (Minn *et al.*, 2005). Moreover, SPARC was found to modulate the capacity of PMNs to eliminate melanoma cells *in vitro* (Alvarez *et al.*, 2005).

Contrary to previous evidence, SPARC expression was associated with good prognosis in human neuroblastoma through the inhibition of angiogenesis (Yang *et al.*, 2004). Moreover, SPARC-null mice exhibited enhanced peritoneal dissemination of ovarian cancer cells (Said and Motamed, 2005). SPARC also inhibited the *in vitro* growth of some human pancreatic cancer cell lines (Sato *et al.*, 2003; Puolakkainen *et al.*, 2004). Furthermore, SPARC's effect on breast cancer growth also appears controversial because recent data demonstrated that its ectopic expression in MDA-MB231 cells inhibited cell capacity to metastasize to different organs, including bone and lungs (Koblinski *et al.*, 2005). In addition, reexpression of SPARC in colon cancer cells rendered the tumor more susceptible to proapoptotic chemotherapeutic molecules, suggesting that reduced levels of SPARC might promote apoptosis resistance (Tai *et al.*, 2005).

This controversial evidence might indicate that SPARC's effect in cancer could be strongly dependent on the tumor cell type. Because SPARC is produced not only by the malignant cells but also by neighboring fibroblasts and endothelial cells, it is unclear whether the protein produced by the different cell components of a tumor mass might have different roles in tumor growth. In fact, recent attempts to elucidate the role of host-derived SPARC using SPARC-null mice led to opposite results; one group claimed enhanced tumor growth (Brekken *et al.*, 2003), whereas others showed impaired tumor growth in SPARC-null mice (Sangaletti *et al.*, 2003). Despite this discrepancy both groups claimed that the major role of SPARC is associated with the assembly and function of the ECM.

In an attempt to understand the role of SPARC in melanoma, we modulated SPARC expression in malignant melanoma cells and in neighboring fibroblasts by overexpressing or downregulating SPARC levels through adenoviral gene transfer. The effects of SPARC were assessed *in vitro,* using both melanoma cell monolayers and spheroids made of melanoma cells alone or combined with stromal cells, and *in vivo* following xenograft transplantation of melanoma cells alone or combined with stromal cells. We observed that the modulation of SPARC levels only in melanoma cells, and not in the neighboring stromal cells, affected melanoma cell growth.

RESULTS

Positive and negative modulation of SPARC levels does not profoundly affect melanoma cell growth *in vitro*

The human SPARC sense and antisense full-length cDNAs were cloned into an E1/E3-deleted recombinant adenoviral vector (Ad-SP and Ad-SPas, respectively) containing a Rous sarcoma virus (RSV) promoter driving gene transcription. Using 5×10^8 50% tissue culture infectious doses ($TCID_{50}$)/ml as the viral dose, 60–80% of melanoma cells, depending on the cell line, were transduced as assessed with Ad-βgal (data not shown). Conditioned media obtained from A375N and IIB-Mel-J melanoma cells transduced with 5×10^8 $TCID_{50}$/ml Ad-SPas showed an important decrease in SPARC levels compared both to mock- and to Ad-βgal-transduced melanoma cells (Figure 1a). Unexpectedly, transduction of melanoma cells with Ad-βgal led to increased SPARC expression (Figure 1a). This increase was independent of the transgene because it was also observed with an adenoviral vector carrying CRE recombinase (data not shown). Time course analysis of SPARC expression showed that the maximal suppression of SPARC secretion was observed between days 4 and 5 (Figure 1b). Similarly, maximal SPARC secretion was observed at day 4 (Figure 1b). At day 15, SPARC levels returned to basal levels (Figure 1b). A day 4 loading control of total protein was also included (Figure 1c). Hyperexpression of SPARC in melanoma cells resulted only in a nonsignificant growth inhibitory effect in the two melanoma cell lines, whereas its downregulation had essentially no effect (Figure 1d and e).

Positive and negative modulation of SPARC levels inhibits and enhances, respectively, melanoma spheroid growth

To assess the role of SPARC produced by the different cell components of a tumor mass, we performed *in vitro* assays using spheroids made of melanoma cells alone or combined with fibroblasts. SPARC hyperexpression in A375N cells (A375N-SP) had a profound inhibitory effect on spheroid growth that was increasingly evident with time (Figure 2a). On the contrary, downregulation of SPARC expression in melanoma cells (A375N-SPas) induced a statistically significant increase in spheroid growth capacity compared to Ad-βgal-transduced cells (Figure 2a). Interestingly, cells transduced with Ad-βgal (A375N-βgal) also exhibited reduced growth capacity at the earliest time points, probably due to the increase in SPARC levels induced by Ad-βgal transduction (Figure 2a). Western blot analysis shows that increased SPARC production by spheroids made of A375N-SP lasted for at least 10 days compared to the other groups (Figure 2b). On the other hand, spheroids made of A375N-SPas cells showed 20–25% reduced secretion of SPARC throughout the experiment, which is probably an underestimation of the reduction in SPARC secreted levels (Figure 2b). A375N-βgal and A375N-SPas spheroids showed a closely packed inner core surrounded by an outer layer of more loosely associated cell aggregate and neat borders (Figure 2c and d). On the contrary, A375N-SP spheroids were essentially devoid of the external layer, which was completely disassembled after 4 days of incubation (Figure 2e). Very similar results were obtained with IIB-Mel-J cells (data not shown). As a whole, these data indicate that SPARC hyperexpression in melanoma cells inhibits spheroid growth, whereas downregulation of SPARC expression showed the opposite effect, enhancing spheroid growth capacity.

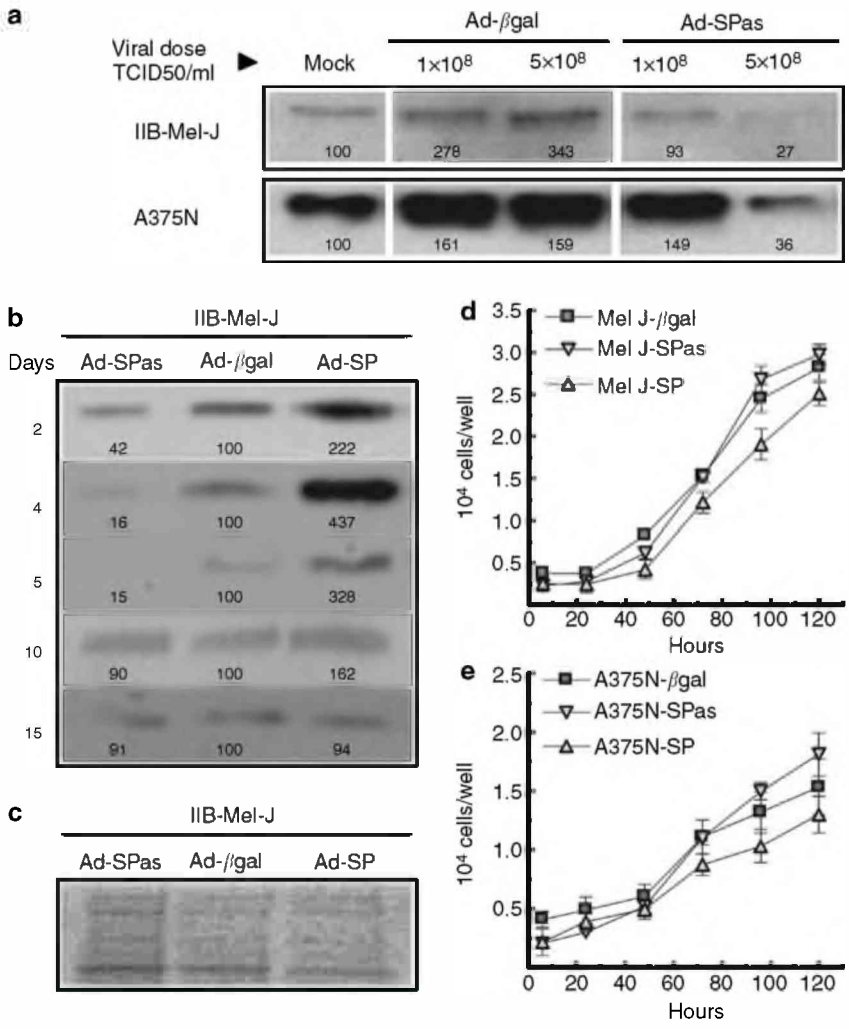

Figure 1. Modulation of SPARC levels in melanoma cells does not greatly impair their *in vitro* growth. (**a**) Western blot of conditioned media obtained from IIB-Mel-J and A375N melanoma cells following transduction with the different adenoviral vectors. Samples were collected at day 4 after cell transduction. (**b**) Time course of SPARC secretion by IIB-Mel-J cells transduced with the various adenoviral vectors. (**c**) Loading control of total protein at day 4. (**d**) *In vitro* growth of IIB-Mel-J cells transduced with the different adenoviral vectors. (**e**) *In vitro* growth of A375N cells transduced with the different adenoviral vectors. Data are expressed as means ± SD. An adenoviral $TCID_{50}$/ml of 5×10^8 was used in **b**, **c**, **d**, and **e**.

SPARC overexpression by neighboring fibroblasts does not alter the growing rate of heterotypic melanoma spheroids

We next evaluated whether melanoma spheroid growth might be affected by the presence of neighboring fibroblasts. Heterotypic spheroids made of A375N-SPas and WI-38 cells showed an enhanced growth, although not statistically significant ($P > 0.05$), whereas heterotypic spheroids made of A375N-SP and WI-38 cells were completely growth inhibited (Figure 3a).

Next, we assessed whether changes in the expression levels of SPARC in neighboring WI-38 cells might affect heterotypic spheroid growth. For this purpose, we transduced WI-38 cells, which express low levels of SPARC that are not detected in Western blots unless 10-fold concentrated medium is loaded (data not shown), with Ad-SP (WI38-SP). WI38-SP cells showed strong SPARC expression, similar to the levels produced by A375N-SP cells (Figure 3b). Figure 3 shows that spheroid growth was not affected by SPARC levels secreted by neighboring fibroblasts, because no difference was found in heterotypic spheroid growth regardless of whether WI-38 cells overexpressed SPARC (Figure 3c). Because the endogenous levels of SPARC produced by melanoma cells could be sufficient to attain its maximal effect, we prepared spheroids made of WI38-SP mixed with A375N-SPas cells instead of native A375N melanoma cells. Even in this case, when SPARC levels produced by A375N-SPas cells were only 10–15% of the amount produced by native A375N cells, spheroid growth was not affected by the levels of SPARC produced by neighboring fibroblasts (Figure 3d). It can be concluded as a whole that heterotypic spheroid growth was affected only by the amount of SPARC secreted by the melanoma cells themselves, whereas SPARC produced by neighboring fibroblasts had no effect on spheroid growth.

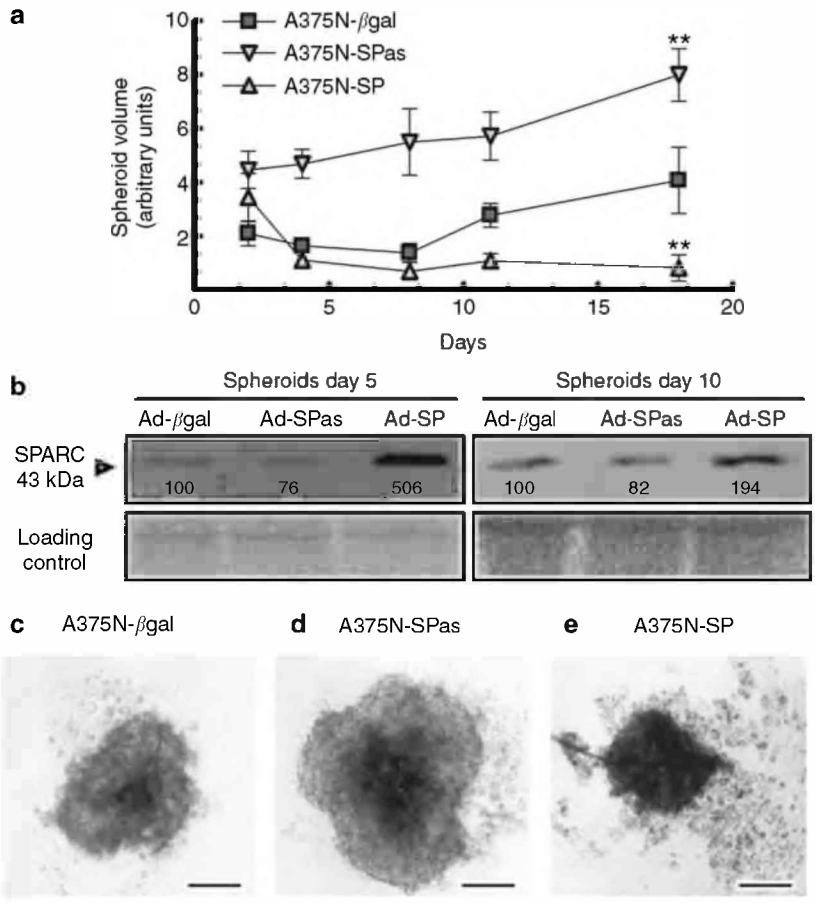

Figure 2. Modulation of SPARC levels in melanoma cells affects their growth as spheroids. (**a**) Growth rate of spheroids made of A375N cells transduced with 5×10^8 $TCID_{50}$/ml of the different vectors. Data are expressed as means ± SD of at least seven spheroids and three different experiments (**$P < 0.01$). (**b**) Conditioned media obtained from spheroids at the indicated time points were used to assess SPARC secretion. Loading control of total protein was included. (**c–e**) A375N spheroid morphology at day 4. Bar = 150 μm.

Host polymorphonuclear cells are responsible for the rejection of human melanoma cells expressing reduced SPARC levels

The next experiments aimed to establish whether changes in SPARC levels produced by melanoma cells or by neighboring stromal cells might affect the *in vivo* growth capacity of melanoma cells. Confirming previous studies (Alvarez *et al.*, 2005), the *in vivo* growth capacity of melanoma cells expressing reduced SPARC levels (Mel J-SPas) was strongly inhibited in nude mice compared to control Mel J-βgal or mock-transduced melanoma cells (Figure 4a). Interestingly, more than 50% of mice injected with Mel J-SPas survived after 100 days, compared to none in the other two groups (Figure 4b). Similar effects were observed with A375N cells (Alvarez *et al.*, 2005, and data not shown). Confirming previous results (Alvarez *et al.*, 2005), tumor growth inhibition was accompanied by a fourfold increase in the amount of intratumoral recruited polymorphonuclear cells (PMN; data not shown). Because previous studies suggested that host PMN might be responsible for the rejection of malignant cells, we depleted PMN by using specific neutralizing antibodies. Initial experiments showed that anti-Gr-1 antibody administration (RB6-8C5 clone) depleted host PMN for at least 15 days (data not shown). Administration of anti-PMN antibodies completely reverted the inhibition of melanoma growth induced by downregulation of SPARC expression, indicating that host PMN were responsible for melanoma rejection (Figure 4c). Interestingly, depletion of host PMN also accelerated the *in vivo* growth of non-genetically modified IIB-Mel-J cells, highlighting a general role of host PMN in controlling human melanoma xenograft growth (Figure 4c).

SPARC hyperexpression by melanoma cells promotes stroma reorganization without affecting their *in vivo* tumor growth

Our previous data demonstrated that melanoma spheroid growth was greatly inhibited following SPARC overexpression. To establish whether SPARC hyperexpression might also affect the *in vivo* growth of melanoma cells, we xenotransplanted nude mice with melanoma cells transduced *ex vivo* with Ad-SP. In contrast to the results obtained with spheroids, SPARC hyperexpression in A375N cells did not alter their *in vivo* growth at all compared to control cells transduced *ex vivo* with Ad-βgal (Figure 4d). Similar results were obtained with IIB-Mel-J cells (Figure 4e).

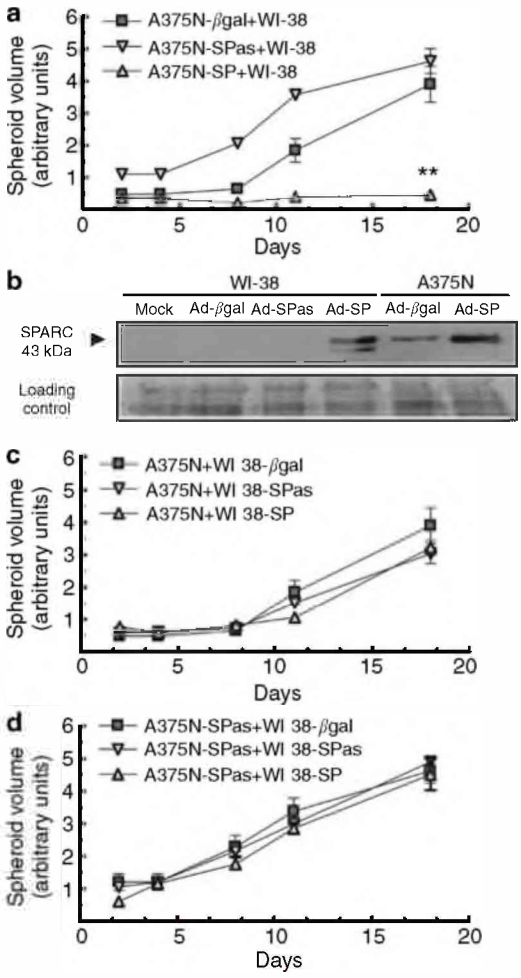

Figure 3. Modulation of SPARC levels in neighboring fibroblasts has no effect on heterotypic spheroid growth. (**a**) Growth rate of spheroids made of wild-type WI-38 fibroblasts mixed with A375N cells transduced with the various adenoviral vectors. (**b**) Western blot of the conditioned media of WI-38 cells transduced with the various vectors. A375N-βgal and A375N-SP conditioned media were included as a comparison. Loading control of total protein was also included. (**c**) Growth rate of spheroids made of A375N cells mixed with WI-38 fibroblasts transduced with the various adenoviral vectors. (**d**) Growth rate of spheroids made of A375N-SPas cells mixed with WI-38 fibroblasts transduced with the various adenoviral vectors. Data are expressed as means±SD of at least seven spheroids and three different experiments (**$P<0.001$). An adenoviral $TCID_{50}$/ml of 5×10^8 was used.

Interestingly, however, clear differences were observed when immunohistological studies were performed. Mel J-SP tumors hyperexpressed SPARC *in vivo* for at least the first 5 days compared to control cells transduced with Ad-βgal (Figure 5a and b). SPARC hyperexpression by melanoma cells was also accompanied by an increased amount of fibroblasts that were mainly located in a collagen-rich tumor area (Figure 5c). Indeed, Masson's trichrome staining revealed a twofold increase in collagen deposition in melanoma tumors overexpressing SPARC compared to control tumors (Figure 5f). This collagen was observed at the tumor interface between the malignant nests and the surrounding host tissue (compare Figure 5d and e). On the other hand, we observed no differences in the amount of the recruited inflammatory infiltrate following SPARC hyperexpression (data not shown). Immunohistochemical analysis using an anti-CD31 antibody revealed increased angiogenesis, as peritumoral microvessel density was significantly augmented in melanoma tumors hyperexpressing SPARC compared to the control (Figure 5g and h). This difference was clearly evidenced at day 5 after cell administration (Figure 5i). Interestingly, autopsies of the mice at 40 days revealed no differences, neither in the amount of vessels nor in fibroblast recruitment or collagen deposition, between melanomas obtained from cells that hyperexpressed SPARC and control melanomas (data not shown). Finally, no difference in the *in vivo* proliferation rate was observed as assessed with an anti-Ki-67 antibody (data not shown). Thus, all the evidence indicates that the remarkable changes in stroma composition observed in tumors transiently hyperexpressing SPARC did not affect melanoma growth.

Coadministration of fibroblasts overexpressing SPARC or not has no effect on melanoma outcome

In the previous studies it could be argued that human melanoma cells could have been unable to establish proper interactions with murine stroma. Therefore, we co-injected melanoma cells with human fibroblasts and followed their *in vivo* growth capacity in nude mice. We initially transduced IIB-Mel-J cells *ex vivo* with Ad-SP and co-injected them into nude mice with an equivalent amount of WI-38 cells. Coadministration of WI-38 cells had no effect on the *in vivo* growth capacity of Mel J-SP cells compared to control Mel J-βgal cells coadministered with WI-38 cells (Figure 6a). In concordance with the spheroid data, overexpression of SPARC in coadministered WI-38 cells also had no effect on the *in vivo* growth of unmodified IIB-Mel-J cells (Figure 6b). To establish whether coadministration of transformed fibroblasts might have a different effect, we co-injected IIB-Mel-J cells with WI-38 VA fibroblasts, a transformed variant stably expressing the SV40 large T antigen, which were transduced *ex vivo* with Ad-SP. Figure 6 shows no effect of SPARC overexpression in coadministered WI-38 VA fibroblasts on melanoma growth outcome (Figure 6c). This set of experiments indicates as a whole that SPARC overexpression in fibroblasts had no effect on melanoma growth.

DISCUSSION

Here, we show that the *in vitro* and *in vivo* growth capacity of human melanoma cells depends essentially on SPARC levels produced by the malignant cells themselves, whereas SPARC produced by stromal cells has no apparent role on melanoma growth. Indeed, manipulation of SPARC levels in neighboring human fibroblasts in heterotypic spheroids or *in vivo* did not affect melanoma cell growth. Moreover, changes in stroma organization induced by SPARC did not substantially affect melanoma growth.

Despite the contradictory results on tumor growth that we have extensively presented in the introduction, it was proposed that the main role of host-derived SPARC in cancer progression is in the appropriate organization of the ECM

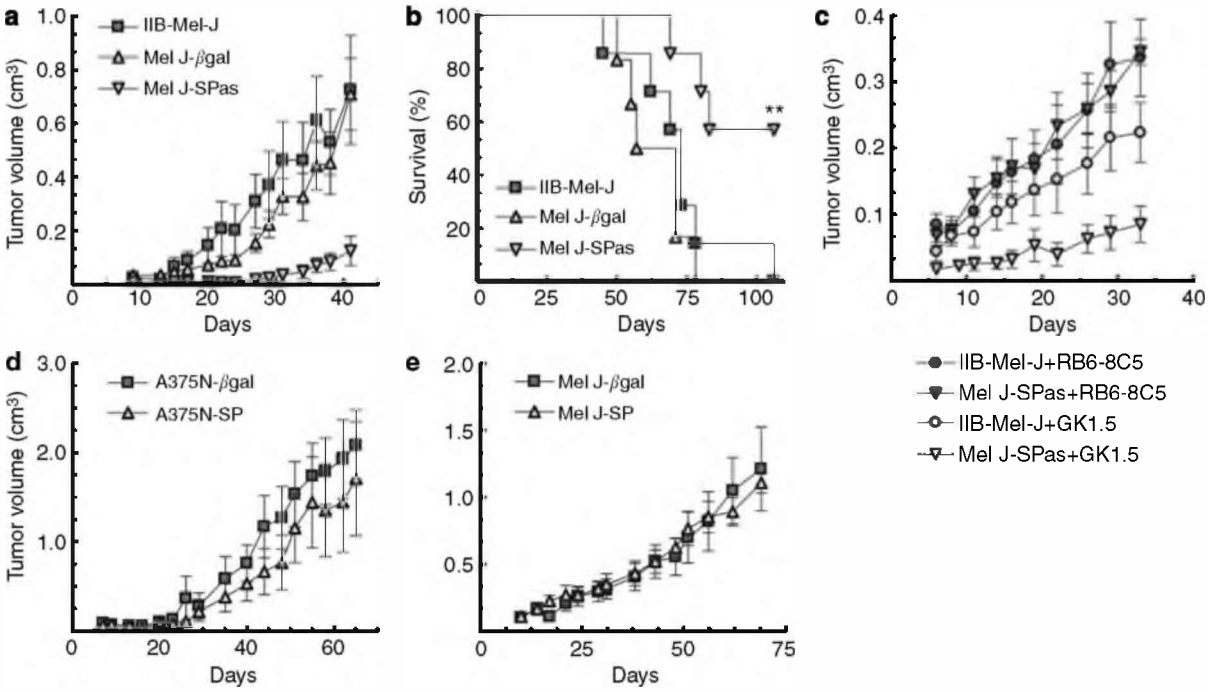

Figure 4. Downregulation but not upregulation of SPARC expression affects the *in vivo* melanoma growth capacity. (**a**) IIB-Mel-J cells transduced with the different vectors were xenotransplanted in nude mice as described. (**b**) Kaplan–Meier curve showing mouse survival. (**c**) IIB-Mel-J cells transduced or not with Ad-SPas and injected into nude mice in the presence of anti-PMN or anti-CD4 antibodies (RB6-8C5 and GK1.5 hybridoma clones, respectively). (**d**) *In vivo* growth of A375N cells transduced with Ad-SP. (**e**) *In vivo* growth of IIB-Mel-J cells transduced with Ad-SP. Tumor volume was expressed as the mean±SE of at least five mice and at least two independent experiments (*$P<0.05$ and **$P<0.01$). An adenoviral $TCID_{50}$/ml of 5×10^8 was used.

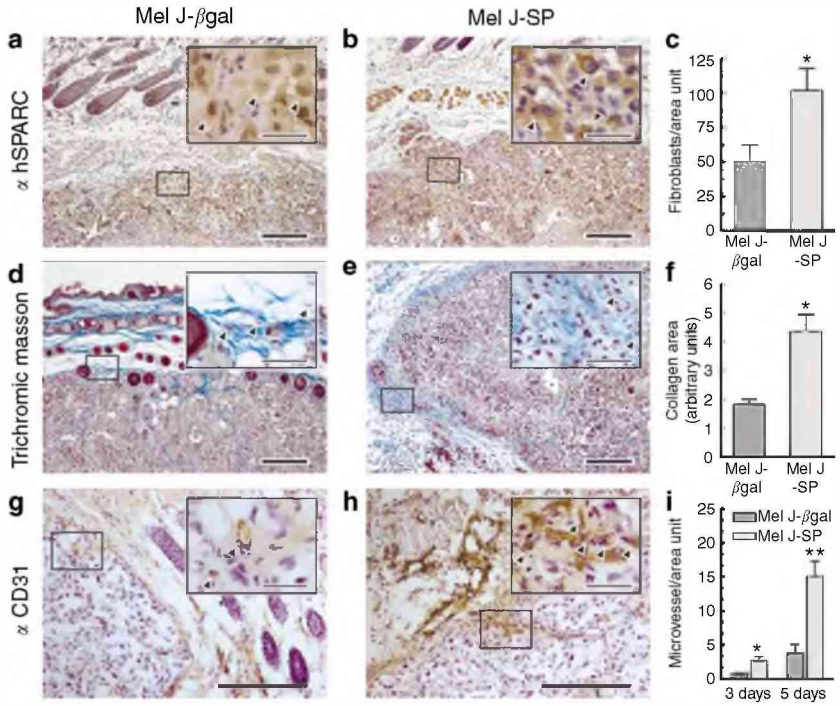

Figure 5. Increased collagen deposition, angiogenesis, and fibroblast recruitment in melanoma tumors hyperexpressing SPARC do not affect tumor growth. Immunohistochemical expression of SPARC in (**a**) control Mel J-βgal and (**b**) Mel J-SP cells. The square represents the area of magnification shown in the inset. The arrows show malignant cells (**b**) hyperexpressing or (**a**) not hyperexpressing SPARC. (**c**) Quantification of recruited fibroblasts. (**d** and **e**) Trichromic Masson staining in (**d**) control Mel J-βgal and (**e**) Mel J-SP tumors. (**f**) Quantification of collagen surrounding the tumor area. (**g** and **h**) Immunohistochemical staining of CD31-positive vessels in (**g**) control Mel J-βgal and (**h**) Mel J-SP tumors. The arrows in the insets show microvessel areas. (**i**) Quantification of microvessels. Data in **c**, **f**, and **i** are expressed as means±SD (*$P<0.05$ and **$P<0.01$). Bar = 200 μm; inset bar = 50 μm. Data were obtained from samples collected 5 days after injection.

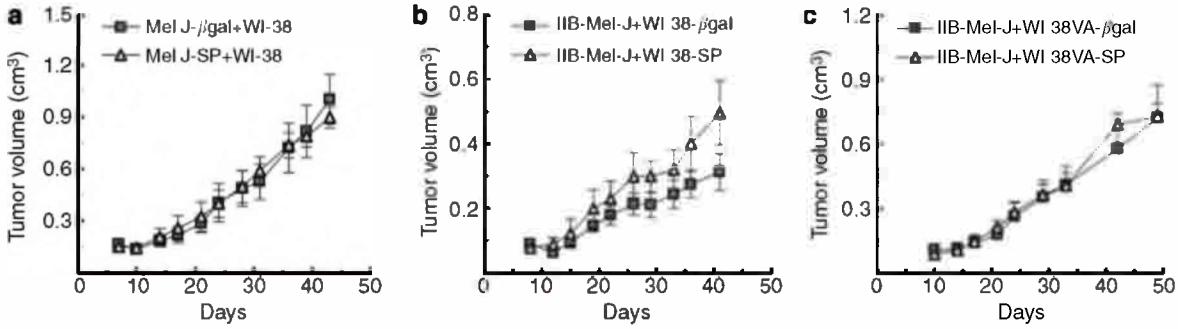

Figure 6. Coadministration of fibroblasts overexpressing SPARC or not has no effect on melanoma cell growth. (**a**) WI-38 fibroblasts mixed either with Mel J-βgal or with Mel J-SP were co-injected into nude mice. (**b**) IIB-Mel-J cells mixed either with WI38-βgal or with WI38-SP cells were co-injected into nude mice. (**c**) IIB-Mel-J cells mixed either with WI38 VA-βgal or with WI38 VA-SP cells were co-injected into nude mice. Tumor volume was expressed as the mean±SE of at least five mice and at least two independent experiments. An adenoviral $TCID_{50}$/ml of 5×10^8 was used.

(Brekken *et al.*, 2003). It was proposed that the stroma may act as a shield to protect tumors from the immune infiltrate; thus, a less-dense stroma such as the one observed in SPARC-null mice may allow recruitment of immune infiltrate and hence tumor rejection (Sangaletti *et al.*, 2003). Alternatively, the less-dense stroma in SPARC-null mice also showed a reduced blood vessel number, suggesting that a smaller blood supply might have also affected tumor growth (Sangaletti *et al.*, 2003). The discrepancy regarding tumor growth was suggested to be based on the different interactions that tumor cells establish with the surrounding stroma; certain tumor cells might favor proteolysis of stromal proteins leading to angiogenesis inhibition, whereas other cell types might induce the opposite, thus inhibiting or promoting tumor growth (Sangaletti *et al.*, 2003; Chlenski *et al.*, 2006).

On the basis of previous discrepancy, we attempted to establish the ECM role in melanoma growth by initially modulating SPARC levels in melanoma cells and monitoring tumor growth and ECM organization. We conclude that the increases in angiogenesis, collagen deposition, and fibroblast recruitment induced by SPARC hyperexpression in melanoma cells have no significant effect on melanoma outcome, clearly indicating that melanoma growth is essentially not affected by the composition or organization of the neighboring stroma. It is of note that stromal reorganization was observed at the initial stages of melanoma growth, when transient adenoviral-directed hyperexpression of SPARC was higher. In fact, necropsy of the mice at 40 days revealed no differences in ECM organization between control and SPARC-hyperexpressing melanomas. Whether sustained SPARC overexpression in melanoma cells and concomitant remodeling of ECM might result in increased melanoma growth *in vivo* remains to be tested. However, in initial studies, we observed that stable hyperexpression of SPARC in melanoma cells resulted in a nonsignificant inhibition of *in vivo* tumor growth, supporting the data obtained in the present studies (Viale *et al.*, manuscript in preparation). Moreover, the fact that tumor cell rejection occurs during the first 72 hours suggests that 10 days of SPARC hyperexpression must suffice for the protein to exert an effect on tumor growth. Because human melanoma cells might also be disadvantageous in the generation of proper interactions with rodent stromal cells, we modulated SPARC levels in coadministered human fibroblasts. We conclude that the presence or absence of neighbor human fibroblasts expressing or not expressing SPARC has no effect on melanoma growth, strengthening the fact that the changes in surrounding stroma induced by SPARC have no profound influence on melanoma outcome. The question as to whether the amount and biochemical characteristics of SPARC produced by neighboring fibroblasts might define its potential paracrine effect is currently under investigation.

It was of note that SPARC overexpression almost obliterated melanoma cell growth as spheroids, whereas SPARC downregulation increased spheroid growth. Because SPARC hyperexpression had little or no effect at all when cells were grown *in vitro* as monolayers or *in vivo*, it is tempting to speculate that SPARC might have affected the anchorage-independent growth capacity of melanoma cells. In addition, we observed a reduced clonogenic capacity of melanoma cells grown in semisolid agar following overexpression of SPARC (data not shown). No previous evidence was presented for the potential role of SPARC on anchorage-independent growth of tumor cells. Recent studies using cells obtained from SPARC-null mice indicated that SPARC might regulate the cell cycle through the concurrently induced expression of p107 and cyclin A (Basu *et al.*, 1999). Cyclin A has been shown to be a direct target of c-Jun and to be necessary for c-Jun-induced anchorage-independent growth of certain cancer cells (Katabami *et al.*, 2005). Interestingly, SPARC expression has been shown to be both upregulated and downregulated in response to c-Jun overexpression (Kraemer *et al.*, 1999; Vial *et al.*, 2000; Briggs *et al.*, 2002;). Overall these data suggest a potential link between SPARC, cyclin A, Jun, and anchorage-independent growth of tumor cells. It might be hypothesized that the timely regulation of SPARC levels might allow melanoma cells to grow in an anchorage-independent way favoring metastatic dissemination.

The role of SPARC in angiogenesis is a matter of controversy. Several studies have shown that SPARC might impair angiogenesis through the inhibition of the vascular endothelial growth factor mitogenic effect on endothelial cells, which in some cases inhibited tumor growth (Kupprion

et al., 1998; Said and Motamed, 2005; Chlenski *et al.*, 2006; Nozaki *et al.*, 2006), whereas others have shown that vascular endothelial growth factor might upregulate SPARC levels produced by endothelial cells (Kato *et al.*, 2001) and that SPARC by itself might promote angiogenesis (Brekken *et al.*, 2003). This data indicate that SPARC hyperexpression promotes angiogenesis in melanoma tumors as assessed by the increased number of microvessels stained with an anti-CD31 antibody. Recent studies demonstrated that SPARC cleavage by MMPs released fragments that stimulated or inhibited proliferation and migration of endothelial cells in mutually exclusive manners (Sage *et al.*, 2003). Moreover, SPARC has been shown to modulate MMP expression in melanoma cells (Ledda *et al.*, 1997a). Thus, the evidence indicates that the role of SPARC in angiogenesis is dependent on the tumor cell type and the availability of concurrent factors such as MMPs that might also influence the same process.

Here we show that depletion of host PMN by specific antibodies is sufficient to completely revert melanoma rejection, thereby providing definitive evidence on the central role of host PMN in this process. The role of host PMN in tumor promotion or rejection is still under discussion. Various studies have shown that PMN are responsible for rejection of tumor cells ectopically expressing cytokines such as G-CSF, IL-2, or Fas L (Alvarez *et al.*, 2005; Motomu Shimizu *et al.*, 2005; Stoppacciaro *et al.*, 1994). On the other hand, others have also shown that PMN were required for acquisition of a metastatic phenotype of benign murine fibrosarcoma cells (Tazawa *et al.*, 2003). Also, PMN depletion in nude mice by using the RB6-8C5 antibody inhibited growth of tumors induced by UV light (Pekarek *et al.*, 1995). It appears plausible, then, that PMN might play a different role in different tumor types or they might behave differently at different stages of tumor growth.

In conclusion, these studies show that SPARC levels produced only by the melanoma cells themselves affect tumor growth, whereas these malignant cells are essentially resistant to external SPARC influence as assessed both *in vitro* and *in vivo*. Although the precise role of the stroma in melanoma growth is still elusive, we speculate that it might become crucial only at certain stages of melanoma development.

MATERIALS AND METHODS

Cells and cell cultures

A375N human melanoma cells were grown in melanoma medium (DMEM/F12, 3.6 g/l HEPES, 1.5 g/l $NaHCO_3$, 5 mg/l insulin, 17.6 mg/l ascorbic acid, and 0.3 g/l galactose). IIB-Mel-J melanoma cells were grown in melanoma medium supplemented with 5 μg/l epidermal growth factor, 500 μg/l transferrin, and 0.92 g/l $NaHCO_3$. Human embryonic fibroblasts (WI-38 and WI-38 VA-13) were grown in DMEM supplemented with MEM nonessential amino acids. All complete media were supplemented with 10% v/v fetal bovine serum and antibiotics. Cell cultures were maintained at 37°C in a 5% CO_2 humidified incubator. Cell culture reagents were from Gibco (Invitrogen, Grand Island, NY).

Construction of adenoviral vectors and cell transduction

The adenoviral vectors carrying human SPARC sense (Ad-SP) and antisense (Ad-SPas) were prepared using the full-length human SPARC cDNA (Podhajcer *et al.*, 1996). A 1.7 kb *Sal*I fragment containing the cDNA was released from pBluescript SK+ and cloned into the *Sal*I site of the shuttle vector pADPSY-LTRSVpolyA. To obtain the recombinant adenoviruses, each of the shuttle vectors containing the SPARC cDNA in both orientations was cotransfected into human embryonic kidney 293 cells with the large *Cla*I fragment (2.6–100 mu) of Ad5 DNA lacking the E3 regions (Lieber *et al.*, 1996). Viral stock titers were determined by optic density and $TCID_{50}$ (Reed, 1938). An additional adenoviral vector, containing 527 bp of *Escherichia coli* β-galactosidase coding sequence, was prepared following the same protocol (Ad-βgal). All viral stocks contained <12.5 endotoxin units/ml as assessed using the E-Toxate kit (Sigma Co., St Louis, MO).

For cell transduction, cells were grown up to 80% confluence in monolayer and transduced with 5×10^8 $TCID_{50}$/ml of the different adenoviral vectors for 6 hours. At the end, the transduction medium was replaced with fresh complete medium; cells were incubated for an additional 20 hours, trypsinized, counted, and used. Transduction efficiency was assessed 36 hours after transduction with Ad-βgal followed by X-gal (Alam and Cook, 1990). Experiments were run only when transduction efficiency was better than 60 and 75% for IIB-Mel-J and A375N cells, respectively.

Production of monoclonal antibodies and Western blot analysis

Monoclonal antibodies recognizing human SPARC were obtained by intrasplenic immunization of BALB/c mice with 200 μg of human rSPARC in 50 μl of phosphate-buffered saline (PBS) (Wayne et al., 2006). Positive clones were mostly of the IgMκ and IgMλ isotypes (IsoStrip; Roche Molecular Biochemicals, Indianapolis, IN). Anti-SPARC (IgMκ) 7A5 monoclonal antibody was purified from an ascitic fluid by using Sephacryl S-400 (GE Healthcare, Uppsala, Sweden). To obtain conditioned media, nearly confluent cells were washed twice with PBS and kept in serum-free medium. After 24 hours, the medium was collected, supplemented with 1 mM phenylmethylsulfonyl fluoride, and quantified using a NanoOrange Protein Quantitation Kit (Molecular Probes, Eugene, OR). One microgram of protein was separated in SDS-PAGE under reducing conditions and transferred onto nitrocellulose membranes (Schleicher & Schuell Bioscience, London, UK). Loading controls were performed to validate protein quantification using Sypro Ruby protein gel stain (Sigma). After being blocked with skimmed milk in PBS, membranes were incubated with mAb-7A5 overnight at 4°C. After being washed with 0.05% Tween-20 in PBS, membranes were further incubated for 2 hours at room temperature with horseradish peroxidase-conjugated goat anti-mouse antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Bands were detected using the ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). SPARC levels were quantified by densitometer analysis using the ImageJ 1.33u software (National Institutes of Health, Bethesda, MD).

Proliferation assays

Cell proliferation was measured using the colorimetric MTT assay (Sigma). Briefly, 3×10^3 cells/well were seeded onto a 96-well plate in a final volume of 100 μl per well. At each time point, 10 μl of

5 mg/ml 3-(4,5-dimethylthiazol-3-yl)-2,5-diphenyl tetrazolium bromide was added to each cell culture plate. Two hours later, the formazan dye was solubilized and read at 550 nm optical density as described (Denizot and Lang, 1986).

Three-dimensional spheroids

Ninety-six-well tissue culture plates were coated with 75 μl of 1% agarose in PBS. Nearly confluent cells were washed twice with PBS, trypsinized, and seeded at 10^4 cells/well in 150 μl of melanoma medium for melanoma cells and DMEM for fibroblasts to obtain a single homotypic spheroid per well. Heterotypic spheroids were built using 5×10^3 each of A375N and WI-38 cells grown in a mix of melanoma medium and DMEM. Seventy-five microliters of supernatant was carefully removed from each well every 3 days and replaced with fresh medium. Spheroid size was measured every 2 days using an inverted microscope containing a $\times 10$ eyepiece ruler graduated to 0.01 mm. Spheroid volume was determined using the equation $V = (LW^2) \times 0.5$, where L is length and W is width of the spheroid. Spheroid conditioned media were obtained by pooling five spheroids followed by three washes with PBS and incubation in the absence of serum for 24 hours.

In vivo tumor growth

For assessment of the *in vivo* tumor growth, A375N and IIB-Mel-J human melanoma and WI-38 fibroblast cell lines were transduced *ex vivo* with Ad-SP, Ad-SPas, and Ad-βgal. Twenty hours later cells were trypsinized, counted, and resuspended in 100 μl of PBS. Six- to 8-week-old male athymic N:NIH(S)-nu mice were subcutaneously injected into the left flank with 5×10^6 IIB-Mel-J or 3×10^6 A375N melanoma cells or a mix of 5×10^6 IIB-Mel-J and 4×10^6 fibroblasts. Perpendicular diameters were used to determine tumor volume, as $V = d_s^2 \times d_l/2$, where d_s is the smaller diameter and d_l is the larger one. All *in vivo* experiments followed institutional guidelines approved by NIH authorities.

Depletion of PMCs

Conditioned medium containing anti-Gr-1 antibody was prepared from RB6-8C5 hybridoma clone (Dr. Coffman, DNAX Research Institute, Palo Alto, CA), whereas the anti-CD4 antibody was prepared from a GK1.5 hybridoma clone (TIB 207™, ATCC, Manassas, VA). For PMN depletion, mice were intraperitoneally injected every 2 days starting 2 days before tumor inoculation with the specific RB6-8C5 antibody. To quantify the effect of RB6-8C5 on PMN depletion, we induced PMN mobilization by injecting casein and then recovered PMN from the abdominal cavity (Luo and Martin, 2006). Briefly, animals were injected twice (18 and 3 hours before they were killed) intraperitoneally with 1 ml of 4% casein in PBS. The cellular suspension obtained from the peritoneal exudates was centrifuged 10 minutes at 1000 r.p.m. and the relative amount of PMN was assessed by differential counting using May Grunwald-Giemsa staining of the smear.

Histology and immunohistochemistry

Eight-micrometer-thick tumor sections were deparaffinized in xylene and rehydrated in graded ethanol. For antigen unmasking, sections were immersed in citrate buffer (pH = 6) and boiled twice in a microwave oven. Endogenous peroxidase activity was blocked by soaking the sections in 3% hydrogen peroxide in methanol for 15 minutes. Nonspecific binding sites were blocked by incubating the sections in normal goat serum (10% in PBS). Excess serum was then removed and the tissue sections were incubated overnight with anti-hSPARC rabbit polyclonal antibody (Ledda *et al.*, 1997b) at 1/200 dilution. After the slides were washed twice in PBS for 10 minutes, the sections were incubated with biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) at 1/400 dilution for 45 minutes, followed by a PBS wash. The sections were subsequently incubated with Vectastain ABC reagent (Vector Laboratories, Burlingame, CA) for 45 minutes. Color was developed by incubating the sections with liquid diaminobenzidine (substrate chromogen system, DakoCytomation, Carpinteria, CA). Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted.

For total collagen detection, deparaffinized sections were stained with Masson's trichrome according to standard protocols. Collagen content was quantitatively determined using the Image-Pro Plus software (Media Cybernetics, Silver Springs, MD). Briefly, five areas from serial sections of tumor explants obtained from at least four different animals were digitally selected. The collagen-rich area of each selected field was measured using the "area measurement" within the "count tool" belonging to the Image-Pro Plus software. Fibroblasts were counted by our pathologist (AIB) under a light microscope.

For CD31 detection, tumors were fixed in 4% paraformaldehyde for 1 hour, cryopreserved overnight in 30% sucrose, embedded in tissue OCT, and stored at -20°C. Cryostat sections of 9 μm were mounted on gelatin-coated slides and rehydrated in graded ethanol. For antigen retrieval, a 3 minute pepsin digest was performed. Slides were incubated overnight with anti-CD31 (PharMingen, San Diego, CA) at a 1/600 dilution. After being washed, the sections were incubated with 1/400 dilution of biotinylated goat anti-rat antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Product visualization was performed as described above. Slides were counterstained with hematoxylin. PBS was used instead of the first antibody as a control. After the immunostaining, the entire tumor section was scanned at low power ($\times 40$) to identify areas of neovascularization. Individual or small-caliber microvessels were then counted under high power ($\times 400$) to obtain a vessel count in a defined area. Data were obtained from three animals at each time point. We analyzed five areas of each sample.

Statistical analysis

Survival rates were calculated with the Kaplan–Meier method and their differences were evaluated by the log-rank test. Statistical difference between groups was determined by one-way analysis of variance follow by a Dunnett's multiple comparison test. A P-value of < 0.05 was considered statistically significant. Data analysis was performed with the Prism GraphPad (GraphPad Software, Inc., San Diego, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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