Enhanced Growth of Pancreatic Tumors in *SPARC-Null* Mice Is Associated With Decreased Deposition of Extracellular Matrix and Reduced Tumor Cell Apoptosis

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Abstract

SPARC, a matricellular glycoprotein, modulates cellular interaction with the extracellular matrix (ECM). Tumor growth and metastasis occur in the context of the ECM, the levels and deposition of which are controlled in part by SPARC. Tumor-derived SPARC is reported to stimulate or retard tumor progression depending on the tumor type, whereas the function of host-derived SPARC in tumorigenesis has not been explored fully. To evaluate the function of endogenous SPARC, we have examined the growth of pancreatic tumors in *SPARC-null* ($SP^{-/-}$) mice and their *wild-type* ($SP^{+/+}$) counterparts. Mouse pancreatic adenocarcinoma cells injected s.c. grew significantly faster in $SP^{-/-}$ mice than cells injected into $SP^{+/+}$ animals, with mean tumor weights at sacrifice of 0.415 $\pm$ 0.08 and 0.086 $\pm$ 0.03 g ($P < 0.01$), respectively. Lack of endogenous SPARC resulted in decreased collagen deposition and fiber formation, alterations in the distribution of tumor-infiltrating macrophages, and decreased tumor cell apoptosis. There was no difference in microvessel density of tumors from $SP^{-/-}$ or $SP^{+/+}$ mice. However, tumors grown in $SP^{-/-}$ had a lower percentage of blood vessels that expressed smooth muscle α-actin, a marker of pericytes. These data reflect the importance of ECM deposition in regulating tumor growth and demonstrate that host-derived SPARC is a critical factor in the response of host tissue to tumorigenesis. (Mol Cancer Res 2004;2(4):215–44)

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Introduction

The development, growth, and spread of cancer occur in the context of the extracellular matrix (ECM), which consists of growth factors, certain matricellular and structural proteins, and proteoglycans (1). The ECM provides a dynamic environment for cellular function and also acts to maintain homeostasis in tissues by influencing cell communication and adhesion. The progression toward malignant disease is dependent on alteration of the local microenvironment to favor proliferation of tumor cells, recruitment of vasculature, and metastatic spread. Thus, it is important to identify ECM proteins that affect cell-matrix interactions during transformation and the development of malignancy.

SPARC, also known as BM-40 and osteonectin, is a 32 kDa calcium binding glycoprotein secreted by many cells (2). As a matricellular protein, SPARC does not contribute structurally to ECM; rather, it binds to a variety of structural proteins (*e.g.*, collagens) and modulates the interaction of cells with the ECM. Two principal functions of SPARC *in vitro* are its modification of cell shape and inhibition of cell cycle progression (3). It is antiadhesive for cells from diverse sources and regulates the production of several ECM proteins. SPARC also influences the levels and activity of several growth factors, including platelet-derived growth factor, fibroblast growth factor-2, vascular endothelial growth factor (VEGF), transforming growth factor-β (TGFβ), and insulin-like growth factor-1 (4–6). In the adult, the expression of SPARC is limited largely to tissues that exhibit high rates of cellular proliferation and remodeling, such as healing cutaneous wounds and bowel anastomoses (7, 8). Targeted disruption of the *SPARC* gene in mice results in a complex phenotype characterized by early cataractogenesis, increased amounts of adipose tissue, and progressively severe osteopenia (9–12). The curled tails and lax skins of these mice are suggestive of altered collagen fibrillogenesis (13, 14). Furthermore, accelerated cutaneous wound healing, enhanced fibrovascular invasion of sponge implants, and reduced foreign body reaction have been reported in *SPARC-null* ($SP^{-/-}$) mice (13, 15, 16). The mechanism responsible for most of these findings in $SP^{-/-}$ mice devolves from compromised maturation and assembly of ECM.

The expression of SPARC is variable in different types of malignancies (17). Production of SPARC by melanoma and glioma cells is linked to an invasive phenotype *in vivo* (18, 19). In contrast, overexpression of SPARC by ovarian carcinoma cells led to increased tumor cell apoptosis, and the levels of

SPARC were inversely correlated with tumor progression *in vivo* (20). We have used $SP^{-/-}$ mice to evaluate the function of endogenous (host-derived) SPARC in tumor progression. We have recently reported (21) that implanted tumors grow more quickly in $SP^{-/-}$ mice due in part to a decrease in the physical restraint imposed by the ECM resulting from the lack of endogenous SPARC.

Pancreatic adenocarcinoma is an aggressive malignancy with a high metastatic rate, which results in high mortality (22, 23). The desmoplastic and invasive characteristics of pancreatic adenocarcinoma are likely influenced by the response of the host to tumor progression. Therefore, given the phenotype of $SP^{-/-}$ mice, we asked whether the lack of SPARC might influence the growth of s.c.-injected pancreatic adenocarcinoma cells.

SPARC is an ECM protein that influences the soil in which tumors develop. Using mice with a targeted deletion of the *SPARC* gene as well as *wild-type* ($SP^{+/+}$) counterparts, we have tested the contribution of host SPARC to the progression of syngeneic tumors. Here, we report that pancreatic tumor growth was enhanced in mice lacking endogenous SPARC. The increased tumor size in the $SP^{-/-}$ mice was not due to an increase in tumor angiogenesis but results from changes in the ECM that create a more permissive environment for tumor progression. One of these changes appears to affect the overall rate of apoptosis in these tumors, which is reduced significantly in $SP^{-/-}$ animals.

Results

Host-Derived SPARC Influences Tumor Growth

Given the phenotype of mice lacking different matricellular proteins (2, 24), we investigated the importance of host-derived SPARC on the growth of implanted pancreatic tumors. $SP^{-/-}$ mice and their age- and sex-matched $SP^{+/+}$ counterparts were injected s.c. with murine pancreatic adenocarcinoma cells, termed PAN02. At the time of sacrifice, the $SP^{-/-}$ mice had significantly larger tumors than the $SP^{+/+}$ mice (Fig. 1), with a mean tumor volume of 532 and 103 mm^3 ($P < 0.01$), respectively. The mean tumor weight was 0.415 g in $SP^{-/-}$ mice and 0.086 g in $SP^{+/+}$ animals ($P < 0.01$). A similar difference in tumor size between $SP^{-/-}$ and $SP^{+/+}$ mice was also seen in an independent experiment with five $SP^{+/+}$ and five $SP^{-/-}$ mice (data not shown). No distant metastases were detected in either genotype.

PAN02 cells were also injected s.c. into C57BL/6 mice ($n = 10$) purchased from The Jackson Laboratory (Bar Harbor, ME). The tumors in these mice grew in a similar fashion to tumors injected into $SP^{+/+}$ mice from our colony (data not shown).

SPARC Influences Matrix Deposition

We suspected that the lack of host-derived SPARC might influence the growth of the tumor cells *in vivo* by alteration of the deposition of ECM in and around the tumor mass. The gross morphology of tumors grown in both genotypes appeared similar (Fig. 2A). However, differences between the tumors were found after staining with Masson's trichrome and picrosirius red, both of which provide information on extracellular collagen. Collagen fibers (mainly collagens I and III), stained bright blue by Masson's trichrome, were more abundant in tumors from $SP^{+/+}$ mice in comparison with those from $SP^{-/-}$ mice. Representative sections (Fig. 2B) showed a reduction in collagen in tumors grown in $SP^{-/-}$ mice as well as a reduction in the birefringence of the collagen fibers stained with picrosirius red (Fig. 2C). This reduction indicates that the collagen fibers (yellow-green) within the tumors of the $SP^{-/-}$ mice were of a smaller diameter and had fewer cross-links than the more mature collagen fibers (red) in tumors grown in the corresponding $SP^{+/+}$ mice. These results are identical to the results we found with Lewis lung carcinoma (LLC) and EL4 tumors (21), except that the capsule was less defined around the pancreatic tumors, likely due to the invasive nature of the PAN02 cells. In summary, the collagen that is laid down in response to the tumor did not mature at the same rate in the absence of host-derived SPARC.

SPARC Is Expressed in PAN02 Cells and Tumors

Immunoblotting with an anti-SPARC antibody showed that SPARC was present in PAN02 cell lysates (Fig. 3A) and conditioned media (Fig. 3B) and that levels of secreted SPARC increased with time. Immunohistochemical analysis indicated that SPARC was present in tumors from both $SP^{+/+}$ and $SP^{-/-}$ animals (Fig. 3C). The reaction product was enhanced in tumors from $SP^{+/+}$ mice and was seen both intracellularly and in the ECM of the tumors (Fig. 3C). That the PAN02 tumors grown in $SP^{-/-}$ mice contained SPARC mRNA and protein (Fig. 3) indicated that the tumor cells themselves produced

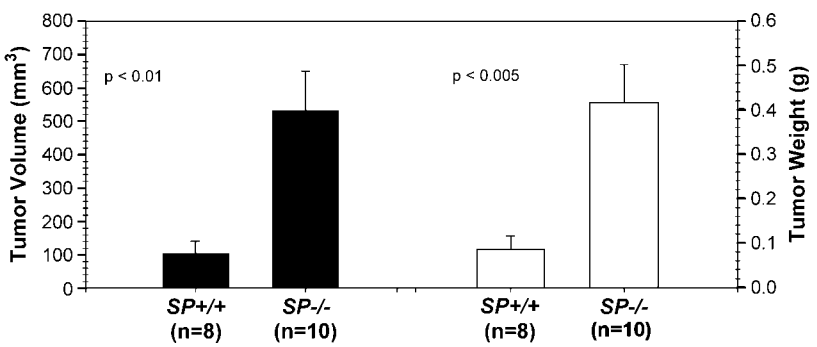

FIGURE 1. Growth of pancreatic tumors is enhanced in $SP^{-/-}$ mice. PAN02 cells were injected s.c. into $SP^{-/-}$ mice and into age- and sex-matched $SP^{+/+}$ counterparts. After 6 weeks, tumors were sampled, weighed, and measured. A comparison of the mean final volume (*filled bars*) and weight (*open bars*) of PAN02 tumors. The enhancement in growth of the tumors in $SP^{-/-}$ mice as compared with $SP^{+/+}$ mice was statistically significant (tumor volume $P < 0.01$, tumor weight $P < 0.05$, Student's t test).

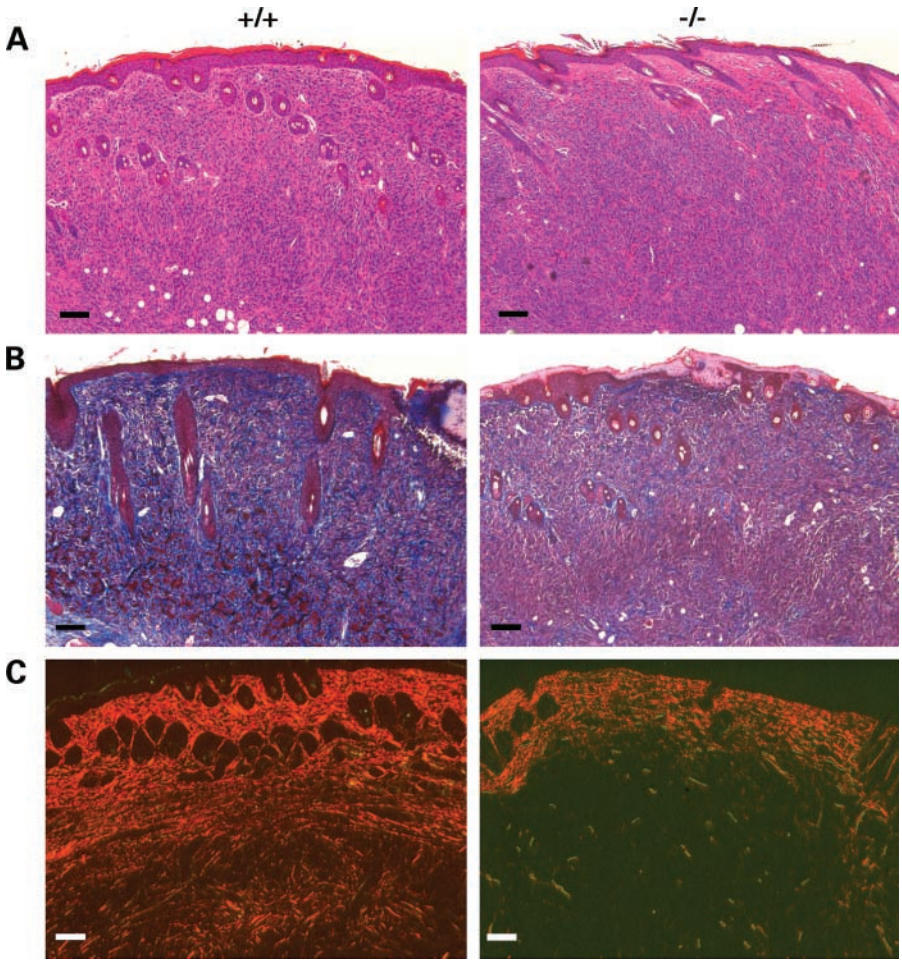

FIGURE 2. Tumor growth in $SP^{-/-}$ mice is associated with changes in collagen organization. PAN02 cells were injected s.c. **A–C.** Representative sections of PAN02 tumors grown in $SP^{+/+}$ (*+/+*) and $SP^{-/-}$ mice (*–/–*). **A.** H&E staining showed a similar morphology between tumors grown in $SP^{+/+}$ and $SP^{-/-}$ mice. **B.** Masson's trichrome staining of the tumors revealed an increased amount of collagen fibers (*blue*) in the tumors grown in $SP^{+/+}$ mice compared with those in $SP^{-/-}$ mice. **C.** Picrosirius red staining showed a significantly increased amount of mature collagen fibers in the tumors from $SP^{+/+}$ (*red* under polarized light) in comparison with $SP^{-/-}$ mice (*yellow-green* under polarized light). Bar, 50 μm.

SPARC *in vivo*. Reverse transcription-PCR (RT-PCR) analysis of mRNA from PAN02 cells and tumors grown in $SP^{+/+}$ and $SP^{-/-}$ mice confirmed that SPARC is produced by PAN02 cells *in vitro* and *in vivo* (data not shown).

Influence of Host SPARC on Protein Expression in PAN02 Tumors

There were no obvious differences in the distribution or level of laminin 1 or collagen IV in tumors from $SP^{+/+}$ or $SP^{-/-}$ mice as determined by immunostaining (data not shown; Fig. 4A). Additionally, mRNA corresponding to the laminin α1 chain was not detected in the tumors or in PAN02 cells by RT-PCR (data not shown). Collagen VI associates with collagen I and was shown to be elevated in the dermis of $SP^{-/-}$ mice over 20 weeks of age by Western blot analysis of acetic acid-extracted collagen (14). However, neither the level of collagen VI nor its distribution was substantially different in PAN02 tumors grown in $SP^{+/+}$ or $SP^{-/-}$ mice (Fig. 4B).

Angiogenesis in PAN02 Tumors From $SP^{+/+}$ and $SP^{-/-}$ Mice

We measured the density of blood vessels in the pancreatic tumors by immunohistochemical identification of endothelial cells with two antibodies, one specific for VEGFR2, and MECA32, a pan-endothelial cell marker. Counting the number of MECA32-immunoreactive vessels in high-power fields (hpf) yielded no significant differences in the number of vessels/unit area in tumors from $SP^{+/+}$ or $SP^{-/-}$ mice ($SP^{+/+}$ = 7.5 ± 2.2 capillaries/hpf, $SP^{-/-}$ = 8.2 ± 5.0 capillaries/hpf). In a similar fashion, VEGFR2-positive capillaries were counted, again with no significant differences ($SP^{+/+}$ = 6.3 ± 2.0 capillaries/hpf, $SP^{-/-}$ = 5.1 ± 2.5 capillaries/hpf). We also evaluated the number and percentage of blood vessels that showed colocalization between smooth muscle α-actin (SMA), a marker of pericytes, and MECA32 (Fig. 5). Table 1 shows that there was a decrease in the percentage of blood vessels displaying colocalization between MECA32 and SMA in tumors grown in $SP^{-/-}$ mice.

We examined the levels of VEGF and TGFβ-1 protein by ELISA of lysates from the PAN02 tumors and found no significant differences in the levels of either growth factor, irrespective of genotype: VEGF was present at 5.8 ± 3.1 and 4.5 ± 2.2 pg/100 μg PAN02 lysate from $SP^{+/+}$ and $SP^{-/-}$ mice, respectively. Corresponding values for TGFβ-1 were 8.2 ± 0.8 and 8.3 ± 0.8 pg/100 μg lysate from $SP^{+/+}$ and $SP^{-/-}$ mice, respectively, with 60% in the active form in each lysate.

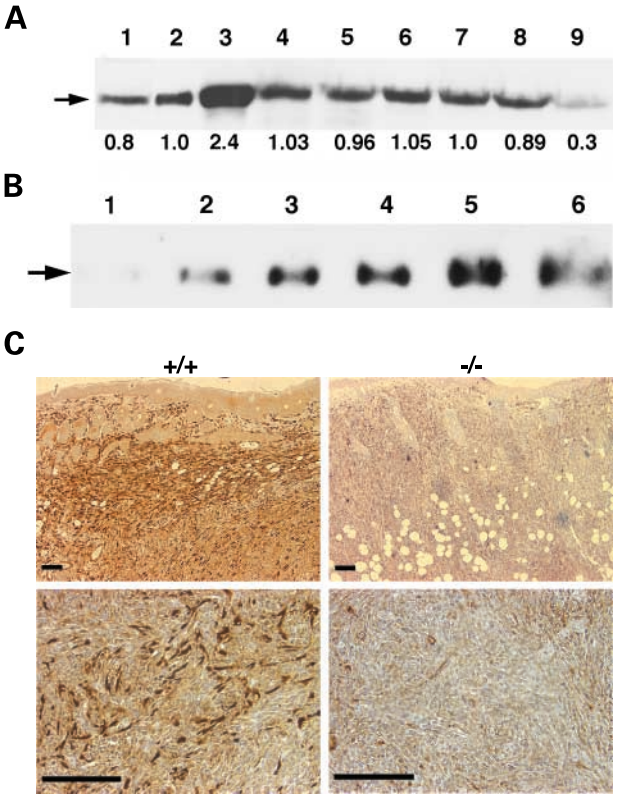

FIGURE 3. Expression of SPARC by PAN02 cells and tumors. SDS-PAGE and Western blot analysis of tumor lysates (**A**) and conditioned media from cultured PAN02 cells (**B**). **A.** *Lane 1,* PAN02 cell lysate; *lane 2,* rhuSPARC (500 ng); *lane 3,* bEnd.3 cell lysate; *lanes 4, 5,* and *6,* PAN02 tumor lysates from three $SP^{+/+}$ mice; *lanes 7, 8,* and *9,* PAN02 tumor lysates from three $SP^{-/-}$ mice. *Numbers under individual lanes,* results from scanning densitometry, with *lane 2* (500 ng rhuSPARC) set at 1.0. **B.** *Lanes 1 and 2,* conditioned media from PAN02 cells cultured for 24 h; *lanes 3* and *4,* conditioned media from PAN02 cells cultured for 48 h; *lanes 5* and *6,* conditioned media from PAN02 cells cultured for 72 h. Levels of SPARC were elevated at 48 and 72 h postculture. **C.** Immunohistochemical staining of the tumors with anti-SPARC IgG indicated its presence in tumors from both $SP^{+/+}$ (+/+) and $SP^{-/-}$ (−/−) mice. Note the enhanced signal in tumors from $SP^{+/+}$ animals. Bar, 50 μm.

Host SPARC Supports Macrophage Infiltration

We found no evidence of rejection or spontaneous regression of PAN02 tumors grown in either $SP^{+/+}$ or $SP^{-/-}$ mice. However, there were differences in the distribution of macrophages (Fig. 6). The antigen F4/80 expressed on mature murine macrophages was more apparent in tumors from $SP^{+/+}$ mice. F4/80-positive cells were found near the margin of the tumor in four of five tumors excised from $SP^{+/+}$ mice. In contrast, in tumors from $SP^{-/-}$ mice, F4/80-positive macrophages were distributed uniformly throughout the tumors, and the strong staining seen at the margins of $SP^{+/+}$ tumors was found in only one of five $SP^{-/-}$ tumors. Coincident staining was found with an antibody specific for Mac-3, another marker of mature macrophages (data not shown).

Proliferation and Apoptosis in PAN02 Tumors

We evaluated the proliferation of PAN02 cells *in vivo* with an antibody against phosphohistone H3 (pH3; Table 2). Quantification of the number of nuclei positive for pH3 in tumors grown in $SP^{+/+}$ and $SP^{-/-}$ mice revealed no significant differences, although the tumors were larger in $SP^{-/-}$ mice. Because tumor growth is dependent on the rates of proliferation as well as cell death, we also examined the extent of apoptosis in the PAN02 tumors by determination of the number of tumor cells that were positive for active caspase-3. As shown in Table 3, the number of caspase-3-positive cells was higher in tumors from $SP^{+/+}$ animals in comparison with those from $SP^{-/-}$ mice. Similarly, a higher number of cells positive for cleaved poly(ADP-ribose) polymerase (PARP; data not shown) was found in tumors from $SP^{+/+}$ animals in comparison with those from $SP^{-/-}$ mice. These results indicate that there were no substantial differences in the rate of cell proliferation in the PAN02 tumors grown in $SP^{+/+}$ and $SP^{-/-}$ mice. However, the rate of programmed cell death or apoptosis was higher in tumors from $SP^{+/+}$ mice.

Because SPARC is known to block proliferation of some cell types in mid-late G_1 (25), we evaluated whether SPARC could inhibit serum-induced proliferation of PAN02 cells *in vitro*. Using a [^{3}H]thymidine incorporation assay, we found that stimulation with 2% serum in the presence of 20 or 60 μg/ml purified recombinant human SPARC (rhuSPARC; Ref. 26) resulted in proliferation rates of PAN02 cells that were 86% and 85% of control, respectively (Table 4). Another proliferation assay, based on cell number determined by a nonradioactive method, produced similar results (data not shown). Thus, proliferation assays *in vitro* demonstrated that PAN02 cells were not appreciably inhibited by exogenous SPARC. However, the proliferation of other cell types, such as LLC cells and bovine aortic endothelial (BAE) cells, was inhibited by a similar range of concentrations of rhuSPARC (data not shown; Ref. 21). Consistent with previous experiments on other cells, exogenous SPARC alone was not associated with apoptosis in PAN02 cells *in vitro* (Fig. 7). However, SPARC appears to prime PAN02 cells to undergo apoptosis, as evidenced by the increased level of cleaved caspase-3 in PAN02 cells treated with staurosporine and SPARC, as opposed to staurosporine alone, for an extended period of time (30 h; Fig. 7).

Discussion

The major findings to emerge from this study are that the matricellular protein SPARC influences the response of host tissue to implanted pancreatic carcinoma cells. Lack of host endogenous SPARC results in (*a*) enhanced growth of implanted pancreatic tumors, (*b*) alteration in the deposition of ECM constituents within the tumor, (*c*) reduced rates of tumor cell apoptosis, (*d*) a decrease in the percentage of blood vessels that maintain pericyte support, and (*e*) altered distribution of macrophages within the tumor.

SPARC is expressed in several different types of malignancies that include cancer of the stomach (27), renal carcinoma (28), hepatocellular carcinoma (29), esophageal carcinoma (30), breast (31), and gliomas (32). Normal human pancreatic acinar and islet cells produce SPARC (17), and SPARC expression correlates with the desmoplastic response of host tissue to pancreatic carcinoma (33, 34). Recently, Sato *et al.* (35) identified SPARC as a frequent target for aberrant methylation in pancreatic adenocarcinoma. Interestingly, they

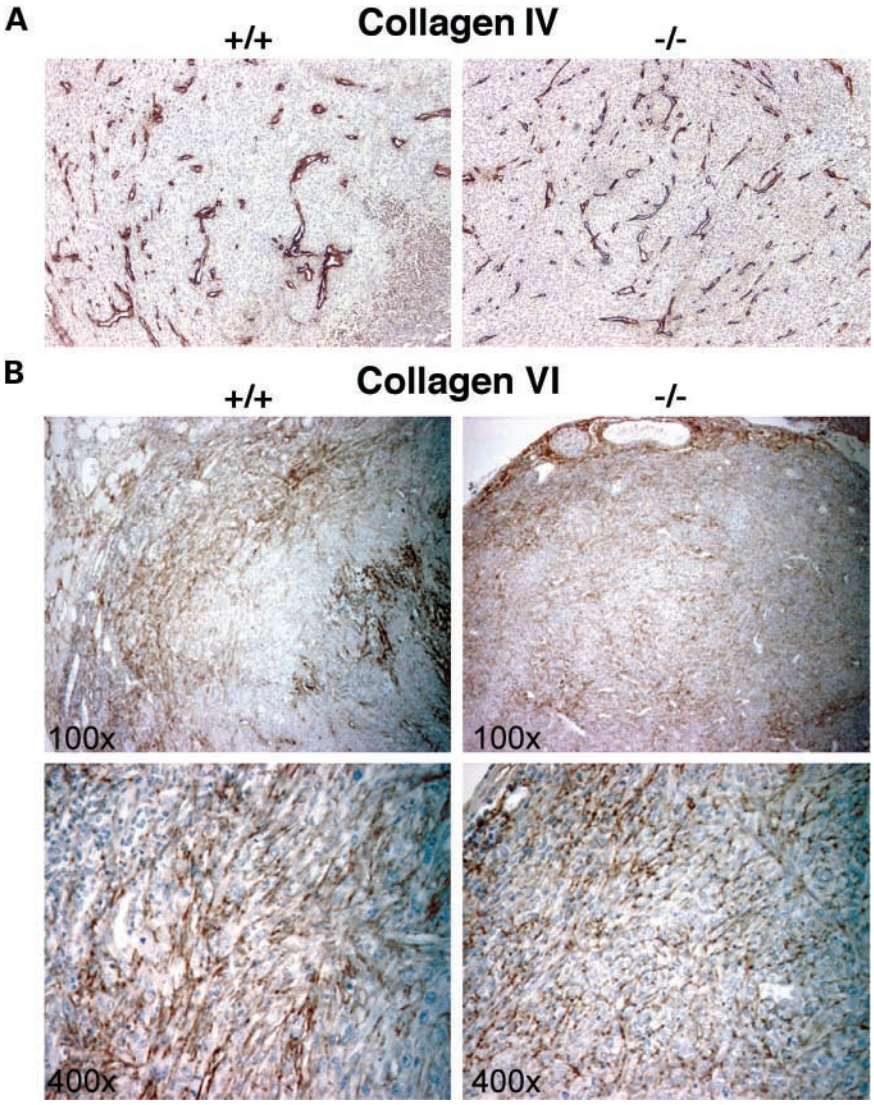

FIGURE 4. Immunolocalization of ECM proteins in PAN02 tumors. Immunostaining with (**A**) anti-type IV collagen IgG and (**B**) anti-type VI collagen IgG. Anti-type IV and type VI collagen IgG each revealed similar patterns in tumors from $SP^{+/+}$ (*+/+*) and $SP^{-/-}$ (*–/–*) mice. Magnification for **A** is 200×. Magnifications for **B** are as indicated.

found that SPARC expression was a common feature of normal pancreatic ductal epithelium, but SPARC was not transcribed in several pancreatic cancer cell lines due to promoter methylation. In fact, treatment with the methylation inhibitor 5Aza-dC restored SPARC mRNA expression in seven of eight pancreatic cancer cell lines. Furthermore, these authors demonstrated that exogenous SPARC (10 μg/ml) inhibited the growth of two pancreatic cancer cell lines (Panc1

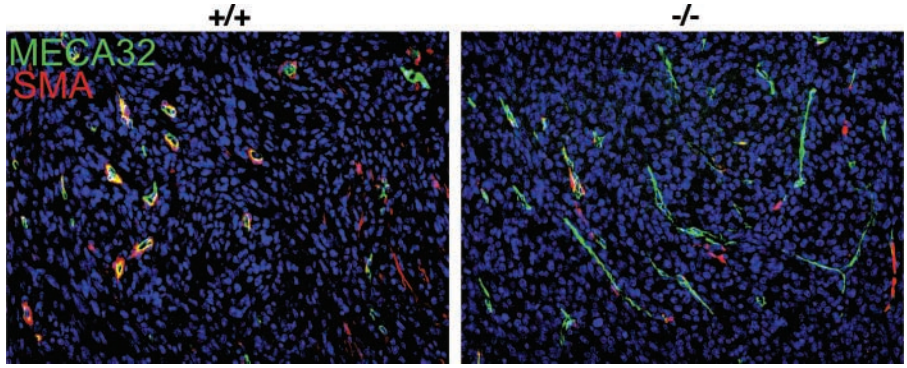

FIGURE 5. PAN02 tumors grown in $SP^{-/-}$ mice show a decreased percentage of pericyte-associated blood vessels. Paraffin-embedded sections of PAN02 tumors grown in $SP^{+/+}$ (*+/+*) or $SP^{-/-}$ (*–/–*) mice were stained for endothelial cells (MECA32; *green*) and pericytes (*SMA*; *red*). Representative sections (total magnification of 200×) are shown.

Table 1. Density of Mature Vessels in Tumors Grown in $SP^{+/+}$ and $SP^{-/-}$ Mice

	$SP^{+/+}$			$SP^{-/-}$		
	MECA	Mature	% Mature	MECA	Mature	% Mature
Mean	17.2	13.5	78*	20.1	13.2	65.7*
SD	4.98	4.8	19.04	6.25	5.24	16.1
n	17	17	17	23	23	23
Min	10	3	30	11	5	20
Max	28	20	100	37	23	95.8

Note: The number of MECA32 (MECA)-positive blood vessels and the number of blood vessels that were positive for MECA and SMA (Mature) were counted in PAN02 tumors grown s.c. in $SP^{+/+}$ and $SP^{-/-}$ mice in the number (n) of 200× fields indicated. Min, minimum; Max, maximum number counted/hpf.
*$P < 0.04$, Student's t test.

and AsPC1) *in vitro* (35). Although these results contrast with our results showing virtually no inhibition of cultured PAN02 cells by exogenous SPARC, they nevertheless support the concept that SPARC is an important regulator of tumor-stroma interaction that is known to influence the progression of pancreatic cancer.

Carcinoma of the pancreas is the fifth leading cause of death in Western industrialized countries (36, 37). More than 80% of pancreatic tumors are diagnosed only when the tumor has infiltrated into neighboring organs or when distant metastases are present, which results in poor prognosis and an overall 5-year survival rate of <5% (36, 38, 39). A variety of regulatory mechanisms are thought to contribute to the development and aggressive growth and spread of pancreatic carcinoma. Pancreatic carcinomas are characterized by a dense desmoplastic stromal component, in which only a subset of cells is neoplastic. Therefore, understanding the interaction of the tumor cells with stromal components is critical to developing improved therapeutic options for patients. $SP^{-/-}$ mice provide us with a unique opportunity to study the relationship between pancreatic tumor cells, ECM, and cells that reside in the ECM.

PAN02 cells are derived from a murine pancreatic adenocarcinoma and, after s.c. injection, cause tumors characterized by highly aggressive growth (40). We now report that the size of PAN02 tumors was significantly increased in $SP^{-/-}$ mice compared with $SP^{+/+}$ mice after s.c. injection. As reported previously for other tumors, SPARC was produced by both host cells and tumor cells (17, 20, 21). Previous studies have implicated tumor-derived SPARC as an important modulator of tumor progression and metastatic spread (41–43). Our findings with PAN02 and two other murine tumor cell lines, LLC and EL4, a mouse lymphoma (21), show an inverse correlation between the production of SPARC by tumor cells and the rate of tumor growth *in vivo*. It is unknown whether tumor-derived SPARC is functionally different from host-derived SPARC produced by stromal cells (*e.g.*, glycosylation of SPARC is heterogeneous). Host-derived SPARC might preferentially affect stromal cells in the tumor and would therefore have a greater influence on the response of the host to the tumor.

SPARC binds to several collagen types, including collagen I and III (4, 44, 45), the major structural proteins of the ECM produced by host cells in response to cutaneous wounds (46), implanted biomaterials (47), and s.c.-injected tumor cells (48). Recent studies have revealed that the skin from $SP^{-/-}$ mice contains less collagen than that from $SP^{+/+}$ counterparts (13). By electron microscopy, the mean diameter of collagen fibrils was reduced in $SP^{-/-}$ compared with $SP^{+/+}$ dermis (14, 16). These results are consistent with our studies (Fig. 2) showing a reduced amount of collagen in the tumors grown in $SP^{-/-}$ mice as well as a reduction in the size and cross-linking of collagen fibrils. The faulty deposition of collagen could lead to decreased mechanical resistance to tumor growth and transport

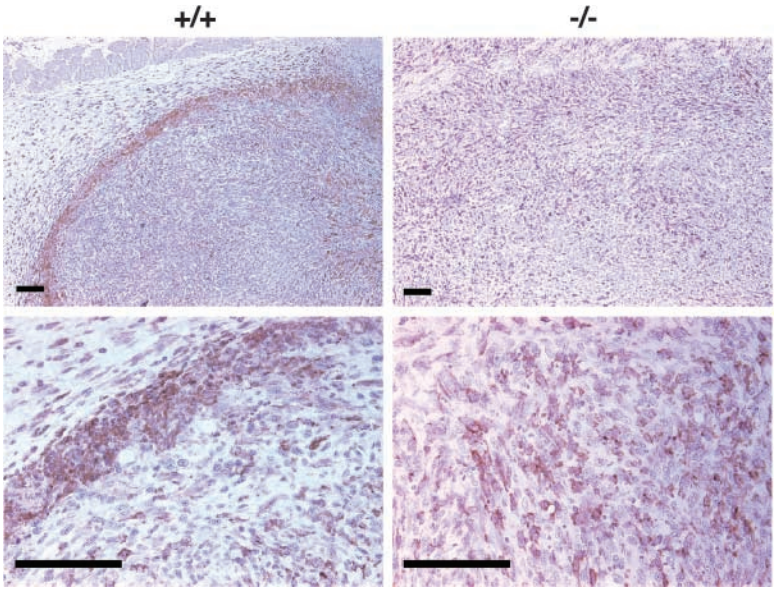

FIGURE 6. Macrophage infiltration into PAN02 tumors. Representative immunohistochemical staining with F4/80, a marker of mature macrophages, shows the extent of infiltration into the PAN02 tumors grown in $SP^{+/+}$ (+/+) or $SP^{-/-}$ mice (–/–). F4/80-positive cells were found near the margins of the tumor in four of five tumors from $SP^{+/+}$ animals. In tumors from $SP^{-/-}$ mice, the distribution of F4/80-positive macrophages was uniform, and concentrated staining at the margin was found in only one of five tumors. Bar, 50 μm.

Table 2. Cell Proliferation in Tumors Grown in $SP^{+/+}$ and $SP^{-/-}$ Mice

	$SP^{+/+}$	$SP^{-/-}$
Mean	2.5	2.9
SD	1.9	1.6
n	95	97

Note: The number of cells/hpf positive for pH3 in PAN02 tumors grown in $SP^{+/+}$ and $SP^{-/-}$ mice was counted by two independent observers in the number (*n*) of fields indicated.

Table 4. Proliferation of PAN02 Cells *in Vitro* in the Presence of SPARC

Treatment	rhuSPARC (μg/ml)	% of Control
No stimulation	0	11
2% serum	0	91
2% serum	20	86
2% serum	60	85

Note: PAN02 cells were serum starved for 48 h and stimulated with 2% serum in the presence of the indicated concentration of rhuSPARC. The level of DNA synthesis was assessed by [^{3}H]thymidine incorporation.

and might also affect the influx of host cells into the tumor mass. Taken together, these results suggest a function for SPARC in the regulation of the ECM in response to tumor growth.

The ECM is known to influence tumor growth directly. Solid stress inhibits the growth of tumor spheroids in culture (49–51). Our current results therefore indicate that the less restrictive ECM seen in $SP^{-/-}$ mice is permissive for enhanced tumor growth. This concept is supported by a recent mathematical study, which predicts that a more robust ECM results in slower growth of the tumor (52). Several reports have also demonstrated the importance of the tumor microenvironment, particularly the ECM, as a regulator of drug delivery, gene expression, and angiogenesis (53, 54). These studies show that an increase in the collagen content of the ECM enhances the mechanical stiffness and transport resistance of tumors (53). Increased collagen content and mechanical stiffness are properties that would also increase solid stress and therefore slow the growth of tumors. Clearly, regulation of collagen production by SPARC is a contributing mechanism to tumor mass in $SP^{-/-}$ animals.

Tumors are angiogenesis dependent for growth and progression to metastatic disease (55, 56). SPARC has been shown to influence angiogenesis (*e.g.*, it can be a proangiogenic factor after cleavage by proteases such as plasmin or MMP3; Ref. 57). Therefore, it was surprising that there was no significant difference in the microvessel density between tumors grown in $SP^{-/-}$ *versus* $SP^{+/+}$ mice. However, the overall decrease in the percentage of blood vessels associated with pericytes (SMA-positive cells) indicates that host-derived SPARC might be important in the maintenance of blood vessel stability in remodeling tissues (*e.g.*, *via* its control of ECM synthesis). That these differences occur in the presence of seemingly unchanged levels of VEGF and TGFβ underscores the importance of matricellular regulation of growth factor activity and cell-matrix interaction. These results are consistent with those of our earlier study (21), in which we found no difference in the microvessel density of LLCs grown in $SP^{-/-}$ and $SP^{+/+}$ mice but did in fact find a decrease in the overall area occupied by blood vessels in tumors grown in $SP^{-/-}$ mice. Our working hypothesis is that perfusion of the tumor in the $SP^{-/-}$ animals is more efficient than perfusion in tumors grown in $SP^{+/+}$ mice; therefore, microvessel density does not have to be greater to result in a net increase in tumor size.

Macrophages influence the response of host tissue to tumor growth and can directly affect the growth of tumors (58). Macrophages were evenly distributed in tumors from $SP^{-/-}$ mice, whereas they appeared to be preferentially localized to margins of tumors from $SP^{+/+}$ mice. Our results indicate that host-derived SPARC influences the infiltration of tumors by host macrophages, although it is unclear whether this effect is due to a direct interaction between SPARC and macrophages. A recent study by Sangaletti *et al.* (59) demonstrated that SPARC expression by leukocytes was critical for stroma production and collagen IV deposition. The study also suggests that SPARC influences leukocyte/macrophage migration *in vivo*, which is consistent with our previous observations (21) and the macrophage immunohistochemistry presented herein (Fig. 7).

Although SPARC inhibits the proliferation of many cell types in culture, exogenous SPARC did not influence the proliferation of PAN02 cells *in vitro*. We measured the number of pH3-positive cells by immunohistochemistry and were unable to show differences in the number of proliferating tumor cells *in vivo* between the genotypes. In contrast to cell proliferation, there was a significant difference in the number of cells undergoing apoptosis in the pancreatic tumors. The number of cells positive for active caspase-3 or cleaved PARP (data not shown) was higher in tumors from $SP^{+/+}$ animals in comparison with those from $SP^{-/-}$ mice. Thus, a reduced amount of programmed cell death could contribute to the enhanced growth of pancreatic tumors in $SP^{-/-}$ mice. Our results are consistent with the study from Yiu *et al.* (20), who reported an inverse correlation between levels of SPARC and degree of malignancy in human ovarian carcinoma. This result is comparable with our finding that tumor growth is slower in tissues expressing endogenous

Table 3. Active Caspase-3 Levels in Tumors Grown in $SP^{+/+}$ and $SP^{-/-}$ Mice

	$SP^{+/+}$	$SP^{-/-}$
Mean*	27.7	22.6
SD	15	8.6
n	49	66
Min	8	7
Max	76	43

Note: The number of tumor cells positive for cleaved caspase-3 was counted by three independent observers in PAN02 tumors grown in $SP^{+/+}$ or $SP^{-/-}$ mice in the number (*n*) of 200 × fields indicated. Min, minimum; Max, maximum number/hpf.
*$P < 0.025$, Student's *t* test.

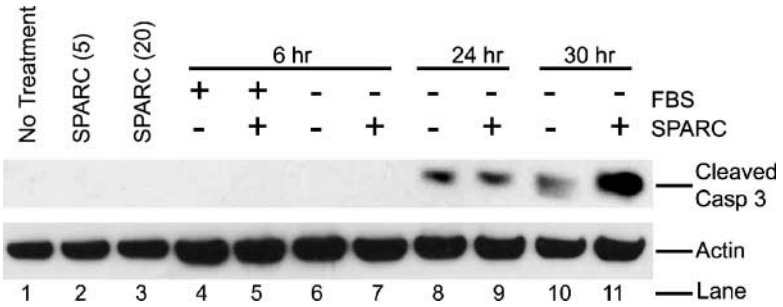

FIGURE 7. SPARC primes PAN02 cells to undergo programmed cell death. PAN02 cells grown *in vitro* were treated with growth media only (*lane 1*, *No Treatment*) or rhuSPARC at 5 μg/ml (*lane 2*) or 20 μg/ml (*lane 3*) for 48 h or with staurosporine (200 nM; *lanes 4, 5, 6, 7, 8, 9, 10,* and *11*) for the indicated time period in the presence or absence of fetal bovine serum (*FBS*) and/or SPARC (20 μg/ml). Cell lysate (20 μg) was resolved by SDS-PAGE and analyzed by Western blotting for the presence of cleaved caspase-3 as an indication of the induction of programmed cell death by staurosporine or SPARC. Staurosporine induced caspase-3 cleavage by 24 h, and cleavage of caspase-3 was enhanced at 30 h in the presence of SPARC. β-actin (*Actin*) levels were also evaluated by Western blotting to normalize for loading of protein among the lanes.

SPARC. A therapeutic approach using SPARC has been suggested previously for neuroblastoma (60). Accordingly, SPARC might also be an effective candidate for the treatment of pancreatic cancer.

In summary, the growth of pancreatic tumors was enhanced in $SP^{-/-}$ mice. How the lack of host-derived SPARC results in increased tumor growth is both multifactorial and presently unresolved but certainly reflects a compromised ECM. In tumors grown in $SP^{-/-}$ mice, the decreased deposition/maturation of collagenous ECM was associated with reduced levels of tumor cell apoptosis and a lower percentage of blood vessels associated with pericytes. These changes emphasize the importance of host-derived SPARC in the appropriate organization of the ECM in response to tumors.

Materials and Methods

Cell Culture

A murine pancreatic adenocarcinoma cell line (PAN02) was purchased from the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Frederick, MD). BAE cells were isolated as described previously (61), and bEnd.3 cells (transformed endothelial cell line from mouse brain) were provided by the late Dr. Werner Risau (Bad Nauheim, Germany). All cell lines were grown in DMEM supplemented with L-glutamine (2 mM), penicillin G (100 units/ml), streptomycin sulfate (100 μg/ml), and 5–10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY). The PAN02 cell line was tested (Impact III PCR profiles; MU Research Animal Diagnostic Laboratory, Columbia, MO) and was found to be pathogen free. Staurosporine was purchased from Sigma-Aldrich, Inc. (St. Louis, MO).

Tumor Growth in Vivo

C57BL/6 × 129SvJ $SP^{-/-}$ mice were generated as described previously (13). The mice were backcrossed against wild-type C57BL/6 at least four generations. The mice were housed in a pathogen-free facility, and experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the Hope Heart Institute and Fred Hutchinson Cancer Research Center (Seattle, WA). For s.c. tumor growth, PAN02 cells were removed from tissue culture flasks with trypsin. The cells were washed twice with HBSS, counted, and resuspended at 2.5×10^7 cells/ml. Cells (200 μl in HBSS) were injected s.c. (26 gauge needle) into the right flank of $SP^{+/+}$ and $SP^{-/-}$ mice (3–7 months old) or C57BL/6 mice (n = 10; The Jackson Laboratory). After tumor cell injection, the mice were monitored thrice a week for weight, signs of discomfort or morbidity, and tumor size. At the time of sacrifice, the mice were weighed and the tumors were excised, weighed, measured, and evaluated as described (21). A blood sample was drawn from each animal and frozen. The peritoneal cavity (including liver and spleen), inguinal and axillar regions, kidneys, thoracic cavity (including lungs and heart), and brain were screened for metastases by visual inspection under a dissecting microscope.

Histology and Immunohistochemical Analysis

Formalin- and methyl Carnoy's-fixed tissues embedded in paraffin were sectioned by the Histopathology Laboratory at the University of Washington (Seattle, WA). Formalin-fixed sections were stained with H&E, Masson's trichrome, and picrosirius red according to standard protocols (14, 21). Methyl Carnoy's-fixed sections were evaluated by immunohistochemistry as described (21). Some of the sections were subsequently treated with AutoZyme (10 μl enzyme concentrate/ml buffer for 6 min at room temperature; BioMeda Corp., Foster City, CA). Antibodies requiring AutoZyme treatment were affinity-purified goat anti-mouse SPARC IgG (R&D Systems, Inc., Minneapolis, MN), rat anti-F4/80 (Serotec, Raleigh, NC), and rabbit anti-VEGFR2 (62). The antibodies that did not require antigen retrieval were rabbit anti-collagen type IV (BD Biosciences, San Diego, CA), rabbit anti-pH3 (Upstate Biotechnology, Inc., Lake Placid, NY), rabbit anti-active caspase-3 (R&D Systems), rabbit anti-cleaved PARP (Chemicon International Inc., Temecula, CA), rabbit anti-collagen type VI (Biodesign International, Saco, ME), rabbit anti-SMA (Lab Vision, Fremont, CA), and rat anti-mouse endothelial cell MECA32 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). Sections were examined on a Leica DMR microscope, and images were captured digitally with a RT-Spot camera (Diagnostic Instruments, Sterling Heights, MI).

The numbers of MECA32-positive and VEGFR2-positive capillaries/hpf were counted in five tumors from both $SP^{+/+}$ and $SP^{-/-}$ mice (10 hpf/tumor). Similarly, to assess the rate of cell proliferation and apoptosis, we counted the number of cells/hpf labeled with pH3 or active caspase-3 antibodies.

Western Blotting and ELISA

Pieces of frozen tumor were homogenized in a modified lysis buffer (63), and protein concentration was determined by a bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL). Lysate (25 μg) from each tumor was loaded onto duplicate 12% SDS polyacrylamide gels, one of which was stained with Coomassie brilliant blue R and the other was used for electrophoretic transfer to a polyvinylidene fluoride membrane. The membrane was blocked with Aquablock (East Coast Biologics, Inc., Berwick, ME) for 1 h at room temperature. The membrane was probed overnight with an anti-SPARC antibody. Proteins secreted by PAN02 cells *in vitro* were probed with 303, a mouse anti-SPARC monoclonal antibody that binds to mouse SPARC (64). The blots were developed as described (21). For ELISA, 100 μg of tumor lysate were assayed for VEGF (R&D Systems) or 20 μg for transforming growth factor-β1 (Promega, Madison, WI) according to the manufacturers' instructions.

In Vitro Proliferation Assay

Quiescent PAN02 and BAE cells were stimulated with serum in the presence or absence of rhuSPARC, and incorporation of [^{3}H]thymidine (Perkin-Elmer, Torrance, CA) into DNA was measured as described (25).

RT-PCR

RNA was isolated from tumor tissues and cell lines with Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. PCR was performed as described (65) with the following primer sequences (sense/antisense): SPARC (5′-GTCCCACACTGAGCTGGC-3′)/(5′-AAGCACAGAGTCTGGGTGAGTG-3′); laminin α1 (5′-GATGCCATTGGCCTAGAGATTG-3′)/(5′-GGATGGGAATGGGAGCTGA-3′); murine hevin (5′-TGGTTCTTGCACGAACTTCC-3′)/(5′-GAGAAGTTCAATGGGATGGTCTC-3′); and rpS6 (5′-AAGCTCCGCACCTTCTATGAGA-3′)/(5′-TGACTGGACTCAGACTTAGAAGTAGAAGC-3′).

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Molecular Cancer Research

Enhanced Growth of Pancreatic Tumors in *SPARC-Null* Mice Is Associated With Decreased Deposition of Extracellular Matrix and Reduced Tumor Cell Apoptosis[1]

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