STC1 Expression By Cancer-Associated Fibroblasts Drives Metastasis of Colorectal Cancer

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Abstract

Platelet-derived growth factor (PDGF) receptor signaling is a major functional determinant of cancer-associated fibroblasts (CAF). Elevated expression of PDGF receptors on stromal CAFs is associated with metastasis and poor prognosis, but mechanism(s) that underlie these connections are not understood. Here, we report the identification of the secreted glycoprotein stanniocalcin-1 (STC1) as a mediator of metastasis by PDGF receptor function in the setting of colorectal cancer. PDGF-stimulated fibroblasts increased migration and invasion of cocultured colorectal cancer cells in an STC1-dependent manner. Analyses of human colorectal cancers revealed significant associations between stromal PDGF receptor and STC1 expression. In an orthotopic mouse model of colorectal cancer, tumors formed in the presence of STC1-deficient fibroblasts displayed reduced intravasation of tumor cells along with fewer and smaller distant metastases formed. Our results reveal a mechanistic basis for understanding the contribution of PDGF-activated CAFs to cancer metastasis. *Cancer Res; 73(4); 1287–97. ©2012 AACR.*

Introduction

Studies on metastasis have traditionally focused on properties of the malignant cells. However, recent studies in tumor biology have shown that the tumor microenvironment exerts major influence on tumor behavior, including the metastatic process (1–3). Several inflammatory cell types of the tumor microenvironment have been shown to affect the metastatic capacity of the malignant cells (4). Also, vascular characteristics, determined by properties of endothelial cells and pericytes, contribute to the formation of a more or less metastasis-permissive microenvironment (5). Moreover, factors derived from cancer-associated fibroblasts (CAF) have been shown to promote metastasis (6).

CAFs constitute a functionally important component of tumor stroma in many types of cancer (7, 8). Clinical data indicate that carcinomas with desmoplastic stroma, consisting of fibroblast cells and extracellular matrix, are associated with a poorer prognosis, which is consistent with the idea that CAF-derived factors stimulate invasion and metastases. Clinical associations between activated CAFs and metastasis have also been shown through prognostic fibroblast-derived gene signatures, including the core serum response signature derived from serum-stimulated fibroblasts and stroma-derived breast and lung cancer gene signatures (9–11).

CAF-derived secreted proteins include growth factors, cytokines, chemokines, extracellular matrix proteins, proteases, protease inhibitors, and lipid products, which may be involved in proinvasive effect of CAFs (7, 8). Production of these factors is generally induced by growth factors that activate and recruit CAFs. Growth factors linked to CAF activation include members of the TGF-β and hedgehog families, and chemokines including CXCL14 and CCL3 (12–15).

Members of the PDGF family have also been identified as key regulators of CAFs (16, 17). Paracrine activation of PDGF receptors on fibroblasts acts as a potent signal for tumor stroma recruitment (18–20). Antitumor effects have been shown following targeting of stromal PDGF receptors

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(21, 22). PDGF-activated fibroblasts have also been shown, in animal tumor models, to exert a negative influence on tumor drug uptake, presumably through effects on interstitial fluid pressure (23–27).

Recent analyses of primary tumors have established that stromal PDGF receptor expression is associated with worse prognosis in breast and prostate cancer (28, 29). Similar findings have been made in studies of smaller series of colorectal cancer (21). These clinical findings indicate that PDGF receptor–activated fibroblasts are involved in cross-talk with malignant cells that promotes metastasis. However, there have been few experimental analyses of the potential proinvasive effects of PDGF-activated fibroblasts, and the identity of potential PDGF-induced prometastatic factors remains unknown.

The aim of this study was to analyze the effects of PDGF-activated fibroblasts on migration and invasion of cancer cells and to identify novel proinvasive factors induced by PDGF activation of fibroblasts.

Collectively, the studies show promigratory and -invasive effects of PDGF-stimulated fibroblasts. Importantly, stanniocalcin 1 (STC1), a poorly characterized, secreted, and hypoxia-regulated protein, was identified as a fibroblast-derived mediator of the PDGF-dependent stimulatory *in vitro* effects. Fibroblast-derived STC1 was also shown in animal studies to contribute to prometastatic effects of fibroblasts.

Materials and Methods

Culture cells and identification of PDGF β-receptor expression

Colorectal tumor cell lines LIM1215, SW620, HT29 and HCT116, immortalized fibroblast BJhTERT and wild-type, and *STC1*−/− mouse embryonic fibroblasts (MEF) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco Life Technologies) containing 1% heat-inactivated fetal calf serum (FCS), 2 mmol/L L-glutamine, penicillin (100 U/mL), and streptomycin (100 ng/mL) at 37°C in a 5% CO_2 humidified atmosphere. Cells were stimulated with PDGFBB (Peprotech) for 5 minutes at 37°C, and the expression of PDGF β-receptor and its activation were analyzed by Western blotting using a specific PDGF β-receptor antibody (Cell Signaling) and an antibody against phospho-tyrosine (phospho-Y99, sc-7020, Santa Cruz).

LIM1215, SW620, HT29, and BJhTERT cell lines were obtained from commercial provider (Cell Bank Australia and American Type Culture Collection). HCT116 Cell lines were purchased from the Cancer Cell Line Repository of the Parc de Recerca Biomèdica de Barcelona (Barcelona, Spain). *STC1* (+/+) and (−/−) MEFs were provided by Roger R. Reddel. These cells were derived from previously described wild-type and knockout *STC1* C57BL/6 mice (30). MEFs were used at low passages and without experimentally induced or spontaneous immortalization.

Coculture assays with tumor colon cells and fibroblasts for migration and invasion assays

Colon cancer cells were cocultured with PDGF-stimulated or nonstimulated fibroblasts on 8.0 μm pore Transwells (Costar, Corning Incorporated) without coating for migration assays, or coated with Matrigel matrix (BD Biosciences) for invasion assays. Before coculture, epithelial cells were labeled with a molecular probe (Cell Tracker green CMFDA C2925, Invitrogen), according to the manufacturer's instructions, to distinguish them from fibroblasts. After 48 hours, epithelial cells that had reached the lower surface of the filter were recovered by trypsin treatment and counted by fluorescence with WALLAC plate reader (excitation at 485 nm and emission at 535 nm) by interpolation using a standard curve. For migration and invasion studies with physical separation, stimulated or nonstimulated fibroblasts were seeded in the bottom compartment of the Transwell and the labeled colon cells in the top one.

RNA Isolation, cDNA synthesis, qRT-PCR analyses and gene expression microarray

Analyses were conducted largely using standard procedures. Details are given in Supplementary Information.

siRNA STC1 downregulation in BJhTERT fibroblasts

BJhTERT cells were seeded under standard conditions and transfected with either control or STC1 siRNA (Dharmacon RNA Technologies) using DharmaFect transfection reagent (Dharmacon RNA Technologies) according to the manufacturer's instructions. The cells were then cocultured with colon cells for invasion experiments as described above.

Colorectal cancer patients and tissue microarray construction

The tissue microarray was derived from a population-based cohort of 320 patients with colorectal cancer. Details about patients and technical aspects related to the generation of the tissue microarray is provided in Supplementary Information.

Orthotopic cell microinjection in nude mice and histopathologic analysis of primary and disseminated cells

The animal experiments were conducted in accordance with National guidelines and approved by the Stockholm North Ethical Committee on Animal Experiments. The animal experiments and tissue analyses largely followed previously published procedures. For details, see Supplementary Information.

PCNA, caspase-3, vimentin, Ki67, and PDGF β-receptor immunohistochemistry and analyses of mitotic rate

Immunohistochemistry analyses, and analyses of mitotic rate, followed established and previously published procedures. For details, see Supplementary Information.

Analyses of *STC1* expression in human colorectal cancer

Sense and antisense RNA probes were generated using the PCR primer sequences detailed in Supplementary Information Methods. Labeled RNA probes were generated by *in vitro* transcription from PCR products in the presence of digoxigenin–UTP. For *in situ* hybridization, tissues were digested by

Proteinase K (Roche) and incubated with digoxigenin-labeled RNA probe. High-stringency washing was conducted after hybridzation, followed by detection of the bound probe by chromogenic anti-digoxigenin antibody immunohistochemistry. Blocking of various endogenous enzymes was conducted throughout the entire process.

Establishment of primary colon cancer CAFs

Fresh tissue from primary tumors of patients operated for colorectal cancer at Linköping University Hospital (Linköping, Sweden) was used for the propagation of primary CAFs. Primary colon cancer CAFs were propagated from minced tumor tissue pieces in RPMI-1640 (Fisher Scientific), supplemented with 20% FCS (Invitrogen), 10 mmol/L HEPES (Invitrogen), 30 μg/mL gentamicin (Invitrogen), and 2.5 μg/mL Fungizone (Invitrogen). Subcultivation and experiments were carried out in DMEM (# 21969, Gibco) with 1% or 10% FCS and penicillin/streptomycin antibiotics in standard conditions. CAFs were used at low passages (between 6 and 8) and without experimentally induced or spontaneous immortalization.

Statistical analysis

All *t* tests were conducted following evaluation of equality of variance or not with Levene test. Two-tailed *P* values $\leq$ 0.05 were considered statistically significant. Comparison of *STC1* and PDGF β-receptor expression, or Ki67, was done with χ^2 test.

Results

PDGF activation of fibroblasts increases their ability of paracrine stimulation of colon cancer cell migration and invasion

The colon cancer cell lines, LIM1215, SW620, HT29, and HCT116 were initially characterized with regard to expression of PDGF β-receptors and production of their PDGF ligands. As shown in Fig. 1A, none of the cell lines expressed PDGF β-receptors as determined by immunoblotting. Furthermore, none of the cultures displayed an increase in tyrosine phosphorylation following addition of exogenous PDGF. In the case of LIM1215 cells, absence of detectable receptors was also confirmed under coculture conditions (Supplementary Fig. S1A).

To measure the expression of PDGF ligands in colon cancer cells, BJhTERT fibroblasts were incubated with conditioned medium from the different colon cancer cell lines, and effects on PDGF receptor phosphorylation were analyzed. This analysis showed that the colon cancer cell lines produced different levels of PDGF ligands, with HCT116 and SW620 cells showing the highest levels of PDGF production and LIM1215 with the lowest levels (Supplementary Fig. S1B). Basal levels of migration among the different epithelial cell lines were characterized before coculture experiments. These analyses identified HCT116 as the most migratory cells and the LIM1215 cells as the least migratory cells (Supplementary Fig. S1C).

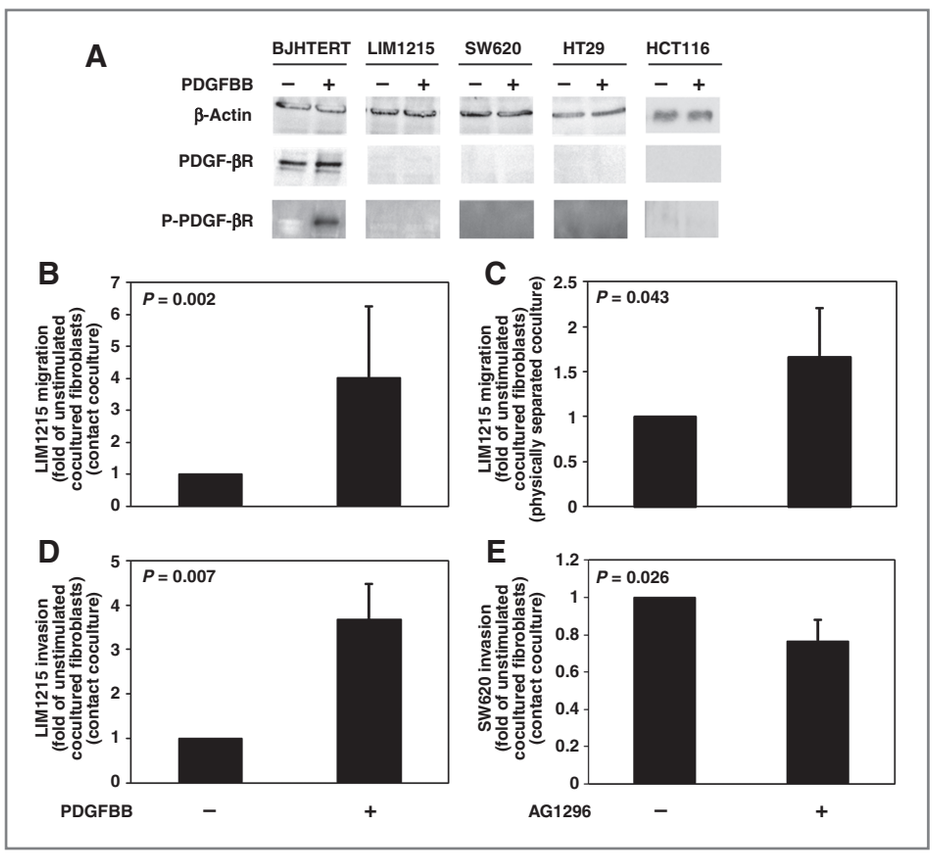

Figure 1. PDGF-dependent stimulation by fibroblasts of colon cancer cell migration and invasion. A, PDGF receptor expression in colon cancer cells and fibroblasts was characterized by PDGF β-receptor and phosphotyrosine immunoblotting. Effects of cocultured fibroblasts on migration (B and C) or invasion (D) of indicated colon cancer cells were determined in the absence or presence of PDGF-BB (B–D), or in the absence or presence of the PDGFR tyrosine kinase inhibitor AG1296 (E). Coculture experiments were carried with the two cell types together in the top chamber (B, D, and E) or with the fibroblasts in the bottom chamber (C). Results in B–E are derived from two to four independent experiments, each carried out in duplicate.

Activation of the fibroblasts with PDGF induced a significant increase in the migration of cocultured LIM1215 cells in formats where the 2 cell types were either cultured together in the upper chamber of a Transwell culture system (Fig. 1B) or when fibroblasts were separated from the cancer cells (Fig. 1C). Furthermore, a PDGF dose dependency of the promigratory effects of stimulated fibroblasts were also shown (Supplementary Fig. S1D). A potent PDGF-dependent effect was also observed on LIM1215 invasion in coculture experiments. This was shown when the 2 cell types were cultured together in the top chamber of the Transwell culture system (Fig. 1D).

As shown above, SW620 and HCT116 cells produced high levels of PDGF (Supplementary Fig. S1B). Addition of exogenous PDGF ligand in cocultured fibroblasts did not show a significant increase in the migration of HCT116 cells (Supplementary Fig. S1E). Invasion experiments were carried out, which analyzed invasion of SW620 cells when cultured with fibroblasts in the absence or presence of the AG1296 PDGF receptor tyrosine kinase inhibitor (31). As shown in Fig. 1E, SW620 cell invasion was significantly reduced upon PDGF receptor inhibition.

Together, these experiments thus show PDGF-dependent paracrine promigratory and proinvasive effects of fibroblasts on colon cancer cells.

STC1 contributes to the PDGF-induced paracrine stimulatory effects of fibroblasts on migration and invasion of colon cancer cells

To identify mediators involved in PDGF-dependent paracrine signaling between fibroblasts and colon cancer cells, the gene expression profile of nonstimulated and PDGF-stimulated BJhTERT fibroblasts was analyzed by microarray analysis (GEO Series accession number: GSE40720). *STC1* was among the most upregulated genes encoding secreted factors (see GEO dataset). This poorly characterized, secreted hypoxia-induced protein was recently linked to ovarian cancer (32). Furthermore, earlier studies have also linked this protein to progression of colorectal cancer (33, 34), although negative effect on prosurvival signaling pathways has also been observed (35).

Quantitative real-time PCR (qRT-PCR) analyses of *STC1* expression confirmed a 15-fold *STC1*-induction upon PDGF stimulation of fibroblasts (Fig. 2A). On the basis of these findings, STC1 was selected for further analyses.

Previously isolated and characterized MEFs from *STC1* knockout mice (30, 35) were used to analyze the contribution of STC1 to the PDGF-induced paracrine effects. These cells were observed to have similar levels of expression, and ligand-induced phosphorylation, of PDGF β-receptor as wild-type MEFs (Fig. 2B). Previous studies have reported that proliferation rate of *STC1*−/− MEFs is increased as compared with wild-type MEFs (35).

Analyses using the wild-type MEFs showed an increase of invasion in LIM1215 colon cells upon coculture with PDGF-activated wild-type MEFs (Supplementary Fig. S2A). Furthermore, the wt MEFs enhanced the invasion of the PDGF-producing SW620 cells in a manner that was not affected by PDGF but was reduced by PDGF receptor inhibitors (Supplementary Fig. S2B). Together, these results support the findings of Fig. 1, showing PDGF-dependent proinvasive effects of fibroblasts on colon cancer cells.

Importantly, experiments analyzing LM1215 cell invasion, in the presence of PDGF stimulation, revealed significantly lower LIM1215 migration in the *STC1*−/− MEF cocultures, as compared with that observed in wild-type MEF cocultures (Fig. 2C). Experiments using SW620 and HCT116 cells also showed that loss of STC1 significantly reduced the invasion-stimulatory effects of PDGF-stimulated fibroblasts (Fig. 2D and E). Similar results were observed when the promigratory effects of fibroblasts on HT29 migration were analyzed (Supplementary Fig. S2C). In some cases, the experiments with the MEF pair were also carried out in the absence of PDGF stimulation. Also, in this setting, the *STC1*−/− fibroblasts displayed a lower invasion-stimulatory effect than the wild-type fibroblasts, but this difference was consistently smaller than that observed in the presence of PDGF (Fig. 2C).

To obtain independent evidence for a role of STC1 in the paracrine proinvasive effects of fibroblasts, siRNA experiments were carried out using the BJhTERT fibroblasts. As shown in Fig. 2F, downregulation of STC1 dramatically reduced the proinvasive effect of BJhTERT cells in cocultures with LIM1215 cells.

Together, these experiments thus identify STC1 as a previously unrecognized important mediator of PDGF-dependent paracrine effects of fibroblasts on cancer cell migration and invasion.

PDGF β-receptor and STC1 are coexpressed in tumor stroma of colorectal cancer

PDGF β-receptor and Ki67 were analyzed by immunohistochemistry, STC1 expression was analyzed by *in situ* hybridization, and expression in tumor stroma and the malignant cells were scored separately in a tissue microarray of a population-based collection of human primary colorectal cancers.

In agreement with previous studies, PDGF β-receptor expression was predominantly observed in the tumor stroma, whereas variable expression of *STC1* was found both in tumor stroma and in malignant cells. When results from PDGF β-receptor and *STC1* analyses were combined, a significant positive association between stromal PDGF β-receptor and stromal *STC1* expression was detected ($P = 0.032$; Fig. 3A and B). Analyses of stroma abundance, based on α-smooth-muscle-actin (ASMA) immunohistochemistry analyses, showed that the *STC1* expression was not correlated to stroma abundance (Supplementary Fig. S3A).

To substantiate these findings, *STC1* expression in primary cultures of CAFs isolated from colon cancers was also analyzed. Induction of *STC1* was also observed in these cells following PDGF stimulation (Fig. 3C and D). Tissue analyses in 105 patients with TMA available from both primary tumor and normal mucosa revealed that the stromal expression of *STC1* was significantly higher in the primary tumors than in the corresponding normal mucosa ($P < 0.001$). In addition, a significant positive correlation was observed between stromal *STC1* expression and colon cancer cell proliferation, as determined by Ki67 analysis (Fig. 3E).

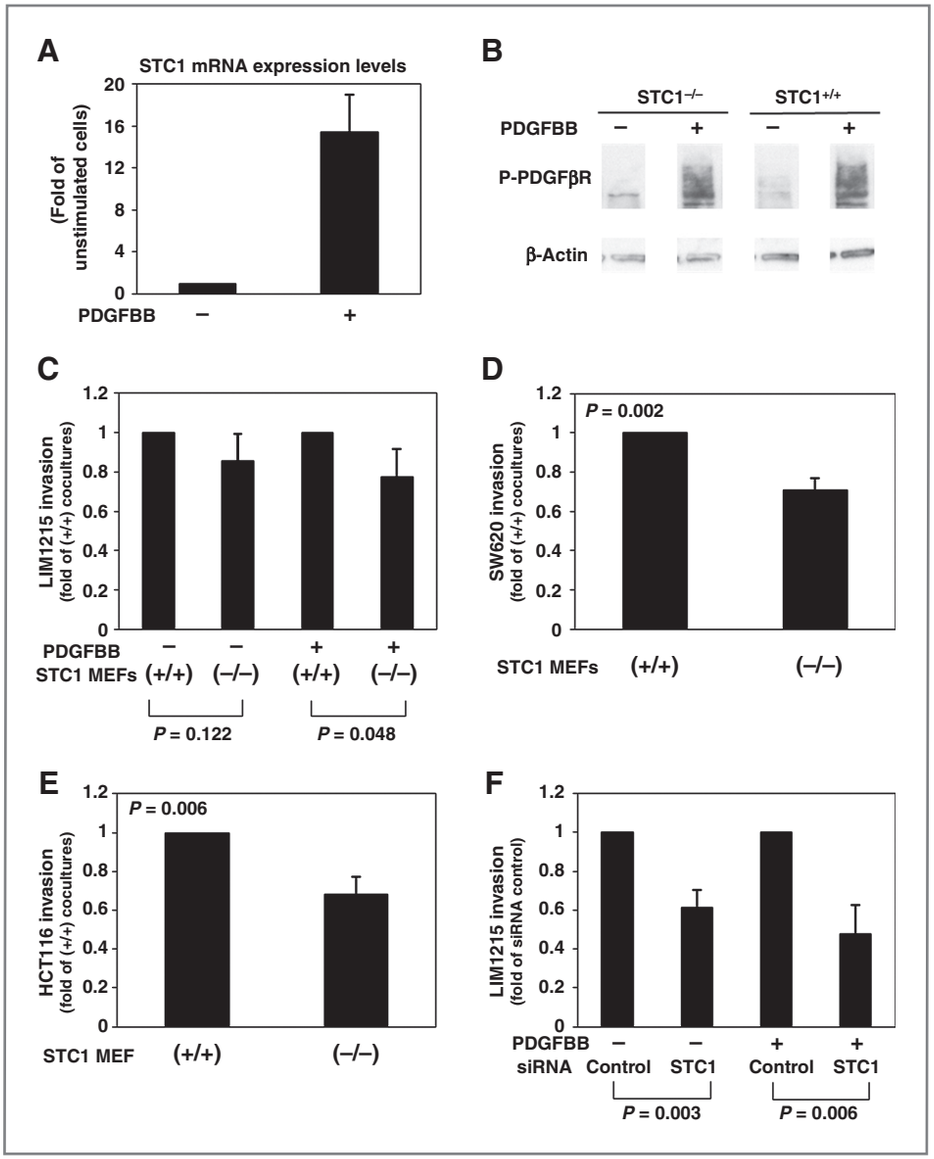

Figure 2. STC1 contributes to the PDGF-induced fibroblast-derived paracrine effects on colon cancer cells migration and invasion. A, STC1 mRNA expression in unstimulated and PDGF-stimulated fibroblasts was analyzed by qRT-PCR. B, PDGF β-receptor expression and ligand-induced tyrosine phosphorylation in *STC1*+/+ and *STC1*−/− fibroblasts were analyzed by PDGF β-receptor and phospho-tyrosine immunoblotting. Effects of *STC1*−/− and *STC1*+/+ fibroblasts (C–E), and control siRNA or STC1 siRNA-transfected fibroblasts (F) on invasion of indicated colon cancer cells were determined. C–F, all coculture experiments were carried out with the 2 cell types together in the top chamber. Results in C–F are derived from 2 to 4 independent experiments, each carried out in duplicate.

Together, these analyses thus show an upregulation of STC1 in tumor stroma, as compared with normal mucosa, and also suggest that the upregulation of STC1 in tumor stroma is related to stromal PDGF receptor signaling and cancer cell proliferation. No conclusive evidence was obtained concerning associations between STC1 expression, as presently analyzed, and survival (Supplementary Fig. S3B and S3D; see Discussion).

STC1−/− fibroblasts display reduced ability to support tumor growth in an orthotopic colon cancer model

A previously described orthotopic colon cancer model was used to evaluate the importance of fibroblast-derived STC1 for metastasis (36). In previous studies, HCT116 cells have been shown to recapitulate critical features of colon cancer growth including invasion into the muscle layer, infiltration into lymphatic and blood vessels, and formation of distant metastases in, for example, the liver and the lung (36).

HCT116 cells were coinjected with wild-type or *STC1*−/− MEFs in a total of 24 animals. The take rate in both groups was similar with 9 and 8 animals forming tumors in the wild-type and *STC1*−/− MEF groups, respectively.

The tumors in animals coinjected with HCT116 cells and either wild-type or *STC1*−/− MEFs expanded from their initial location in the submucosa throughout all of the intestinal wall layers, ulcerating the mucosa and reaching the serosal surface. The local tumors gave rise to poorly differentiated adenocarcinomas in both groups. They were highly cellular, including atypical cells with pleomorphic nuclei and multinucleated cells. Tumors also formed diffuse fronts that invaded the local normal colon (Supplementary Fig. S4A and S4B).

Tumor volume was measured at necropsy. Tumors derived from coinjection of HCT116 cells and *STC1*−/− MEFs were significantly smaller than the tumors of the control group (Fig. 4A). The presence of fibroblast-like cells was observed in all

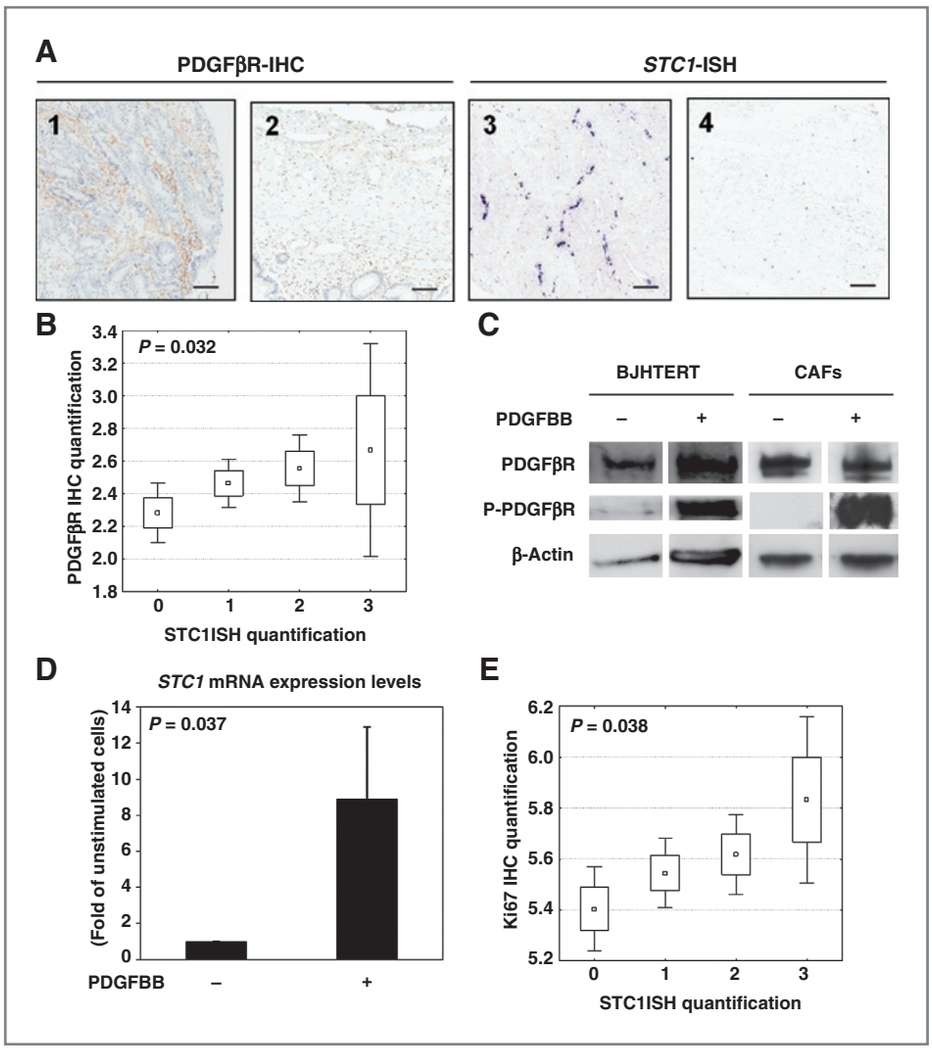

Figure 3. Stromal STC1 is associated with expression of stromal PDGF β-receptor and cancer cell proliferation in human colorectal cancer, and STC1 expression in colorectal CAFs is induced by PDGF stimulation. PDGF β-receptor (A1 and A2) and *STC1* (A3 and A4) expression were analyzed by immunohistochemistry and *in situ* hybridization, respectively, on a tissue microarray of a population-based series of human colorectal cancers. Expression in malignant cells and tumor stroma was scored separately. Microphotographs show representative examples of tumors with positive (A1 and A3) or negative (A2 and A3) PDGF β-receptor expression and *STC1* expression in tumor stroma (scale bar, 100 μm). B, box plot showing the association between PDGF β- receptor and *STC1* immunohistochemistry quantification. C, PDGF receptor expression in primary human CAFs was characterized by PDGF β-receptor and phospho-tyrosine immunoblotting. D, expression of *STC1* in primary cultures of CAFs was analyzed by qRT-PCR in cells cultured in the absence or presence of PDGFBB. E, box plot showing the association between Ki67 and *STC1*.

tumors. Staining with PDGF β-receptor antibodies and cytokeratin did not reveal major differences between the tumor groups with regard to stromal–epithelial ratio (Fig. 4B and Supplementary Fig. S4C). Necrotic areas were also detected in most tumors but did not differ between the 2 experimental groups (Fig. 4B and Supplementary Fig. S4C).

Proliferation and apoptosis were evaluated in primary tumors by mitotic figures counting and caspase-3 immunohistochemistry, respectively. Tumors derived from coinjection with *STC1*−/− MEFs displayed reduced proliferation and also increased apoptosis (Fig. 4C–F). PCNA immunohistochemistry also confirmed the reduced proliferation in tumors derived from coinjection with *STC1*−/− MEFs (Supplementary Fig. S4D and S4E).

Vascular density was evaluated in primary tumors following immunohistochemical analyses using Isolectin B4 antibodies. No differences were observed between groups (Supplementary Fig. S4F and S4G).

Together, these analyses showed that the depletion of STC1 from fibroblasts decreased their ability to support orthotopic colon cancer growth through mechanisms involving reduced proliferation and increased apoptosis.

Tumors derived from coinjection with *STC1*−/− fibroblasts display reduced ability to form distant metastasis

The lymphatic and hematogenous cancer cell dissemination was analyzed by macroscopic and microscopic examination of lymph nodes, peritoneum, diaphragm, pancreas, liver, and lungs. Interestingly, there was a difference in the number of affected organs such that the tumors derived from the *STC1*−/− group showed a significantly lower number of affected organs per mouse (Fig. 5A). Characterization of the distant metastatic lesions also revealed that the foci in the *STC1*−/− MEF group were significantly smaller than those in the control group (Fig. 5B).

The potential relationship between dissemination and size of primary tumor was also analyzed. Tumors were divided into 2 groups based on size (50% cut off), regardless of experimental group. This analysis did not reveal significant differences between the 9 "small primary tumor group" and the 8 "large

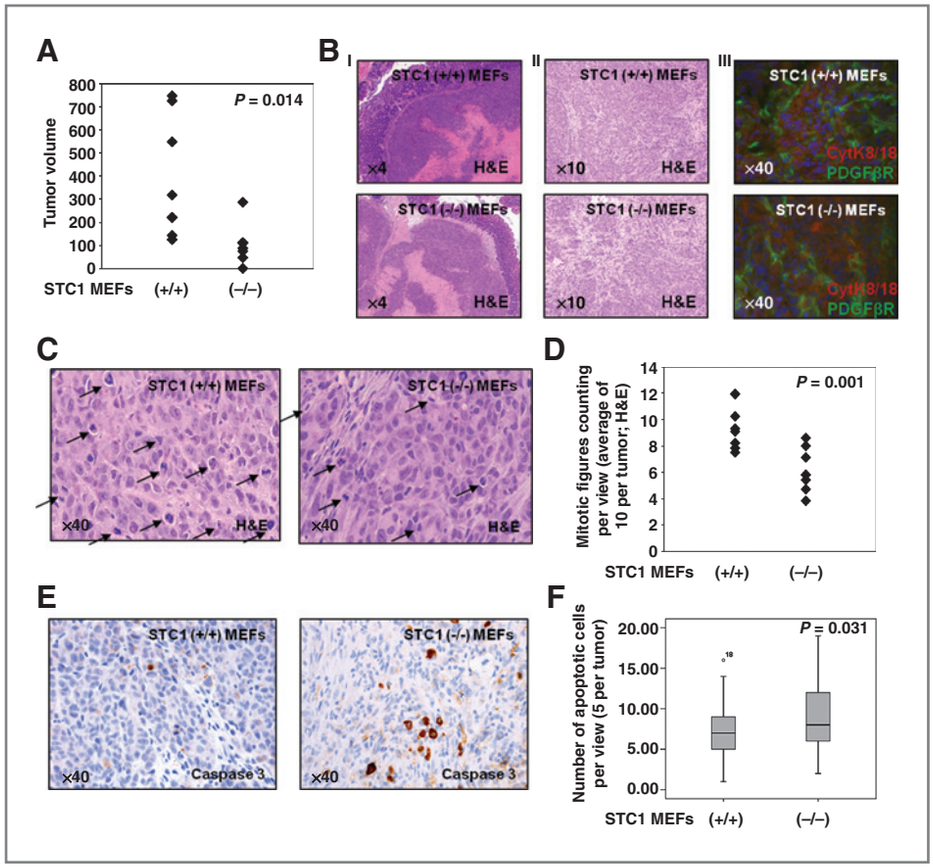

Figure 4. *STC1*−/− cells display a reduced ability to support growth of primary orthotopic colon cancer. Primary tumor size was determined after sacrifice conducted 45 days after coinjection of HCT116 cells together with *STC1*−/− or *STC1*+/+ MEFs ($n = 24$; A). B, I, hematoxylin and eosin-stained sections from primary tumors were analyzed with regard to overall histology and to determine areas of necrosis. The stromal–epithelial ratio in tumors generated from coinjection of HCT116 together with +/+ or −/− MEFs was determined by hematoxylin and eosin staining (B, II) and by PDGF β-receptor and cytokeratin immunofluorescence (B, III). Quantification of mitotic figures (C and D) and apoptosis (E and F) were conducted following staining of tumor sections with hematoxylin and eosin and antibody against caspase-3, respectively. Quantifications in F are based on the analyses of 5 sections from each of 8 and 9 tumors, respectively, of each of the 2 types.

primary tumor group" with regard to number of organs affected per mouse, or average lesion size (Supplementary Fig. S5A and S5B). Calculations of "metastatic index", relating the number of affected organs to the size of the primary tumors, also indicated a reduced metastatic potency of tumors from the *STC1*−/− MEF group (Supplementary Fig. S5C).

Metastases were divided into groups of lesions formed through the peritoneal route (peritoneum and diaphragm), the lymphatic route (lymph nodes and pancreas), and the hematogenous route (liver and lungs). As shown in the Supplementary Fig. S6, this analysis indicated that the metastasis formation via all 3 routes, based on the size of foci, was reduced in the *STC1*−/− MEF group (Supplementary Fig. S6A–S6I).

This analysis thus showed that the STC1 status of coinjected fibroblasts determined metastatic behavior of orthotopically implanted colon cancer cells.

STC1 status of fibroblasts affects lymph vessel density and vascular invasion in primary orthotopic tumors

The possibility that STC1 status affected vascular infiltration was evaluated. Interestingly, the number and size of intravasated tumor emboli was significantly reduced in the

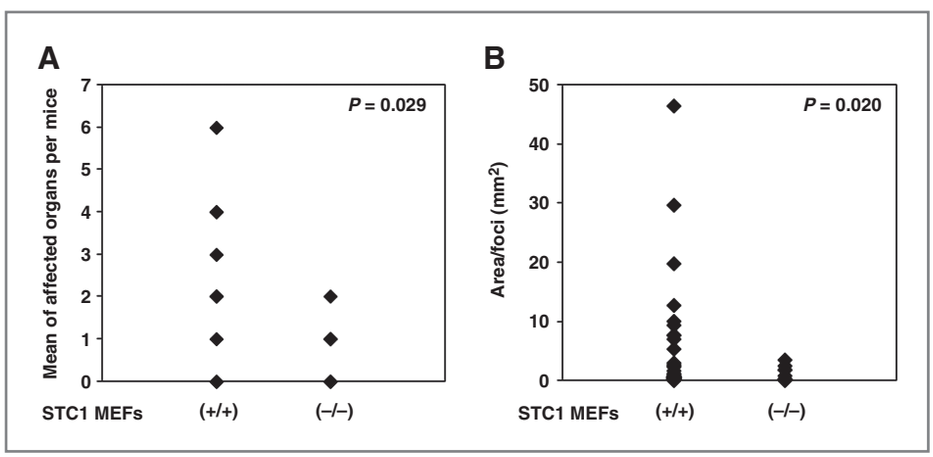

Figure 5. *STC1*−/− cells display a reduced ability to promote metastasis to lymph nodes and distant organs from orthotopic colon cancer. A, the number of organs affected by metastasis was determined by macroscopic and microscopic analyses of regional and distant lymph nodes, peritoneum and diaphragm, and liver and lungs from 8 and 9 animals of each group. B, average area of each focus (57 and 18 foci from the *STC1*+/+ and *STC1*−/− groups, respectively) was determined after analyses of the same organs in 8 and 9 animals of the *STC1*−/− and *STC1*+/+ groups, respectively.

STC1−/− MEF group (Fig. 6A and B). Neither of these parameters differed significantly when tumors were divided according to size (Supplementary Fig. S7A and S7B).

Decreased lymphatic vessel density, evaluated by LYVE-1 immunohistochemistry, was also considered as a factor contributing to the reduced metastasis in the *STC1*−/− MEF group. A significant decrease of lymph vessel density was observed in primary tumors with *STC1*−/− MEFs (Supplementary Fig. S7C and S7D). However, a significant difference in lymphatic vessel density was also observed when tumors were grouped on the basis of size (Supplementary Fig. S7E), making it difficult to evaluate whether the phenotype was secondary to the effect of STC1 on tumor size, or reflected a more direct relationship between STC1 and lymphangiogenesis.

These findings thus suggest that STC1 status of fibroblasts affects metastasis by regulating the intravasating ability of colon cancer cells.

Characterization of tumors with regard to EMT, cancer stem cell content, and macrophage infiltration

The presence of cell displaying an EMT phenotype, the abundance of cancer stem cells, and the amount of macrophages was evaluated as additional possible factors involved in the reduction of metastases from tumors with *STC1*−/− MEFs.

CD44 was used as stem cell marker (Supplementary Fig. S7F–S7H). Tumors with *STC1*−/− or +/+ MEFs did not show changes in the CD44 immunostainning. F4–80 Immunohistochemistry was analyzed to determine the amount of

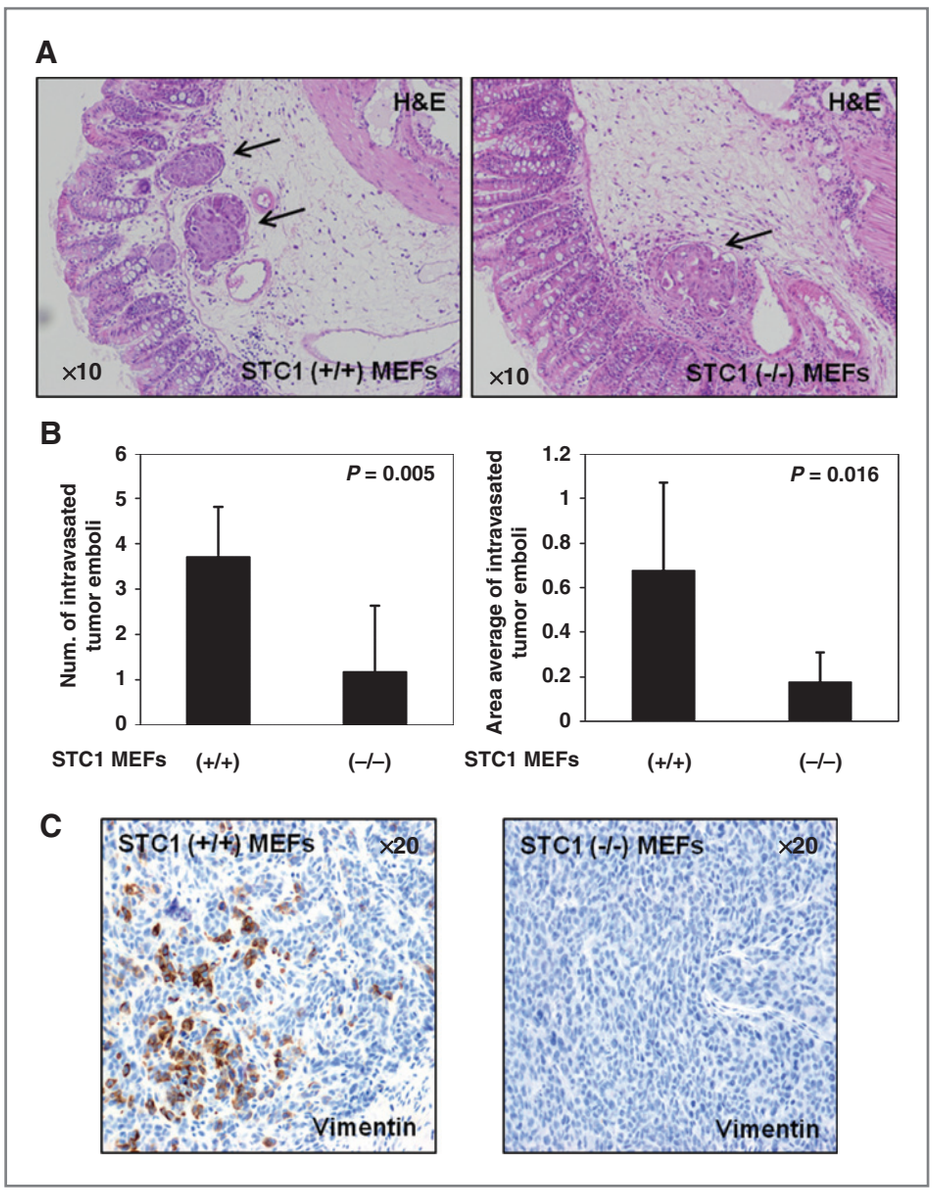

Figure 6. Primary tumors with *STC1*−/− fibroblasts display reduced intravasation and decreased EMT. A, tumor cell intravasation was determined by microscopic analyses of H&E-stained tumor sections. B, quantification of data was conducted after analysis of 10 sections from each tumor from the two tumor types. C, presence of cells under EMT was evaluated by vimentin immunostaining. Two animals from each group were excluded in the analysis of intravasation because of absence of tumor-free submucosa.

macrophages in tumors (Supplementary Fig. S7I–S7K). A reduction of the macrophage number was observed in the *STC1*−/− MEF group. However, the analyses of size-divided tumor groups also revealed differences macrophage density, and the differences between the experimental groups could thus be secondary to the generally smaller size *STC1*−/− tumors, rather than a direct consequence of the fibroblast genotype.

Vimentin immunostaining, using an antibody specific for human vimentin, was used as an EMT cell marker. EMT was detected in 4 of 9 tumors in the *STC1*+/+ group. In contrast, no sample in the *STC1*−/− MEF group showed vimentin expression ($P = 0.053$; χ^2 test; Fig. 6C).

Together, these analyses suggest that STC1 directly contributes to EMT, which could be involved in the effects on metastasis to lymph nodes and distant organs.

Discussion

In summary, this study shows that PDGF receptor stimulation of fibroblasts significantly increases migration and invasion of cocultured colorectal cancer cells in an STC1-dependent manner. The *in vivo* significance of these findings is documented by the observations that tumors with *STC1*−/− fibroblasts formed fewer and smaller distant metastases in a manner involving reduced intravasation of tumor cells and a reduction in cells displaying an EMT phenotype. Furthermore, clinical relevance is indicated by the observation that PDGF receptor and *STC1* expression is significantly correlated in the stroma of human colorectal cancers.

A series of other studies based on tumor tissue analyses have provided findings, which support a role for STC1 in cancer progression and metastasis (32–34, 37). Increased levels of circulating STC1 mRNA have also been associated with worse prognosis in lung cancer (38).

The findings imply stromal STC1 as a prometastatic factor. Preliminary analyses of the prognostic significance of stromal *STC1* expression, based on the *in situ* analyses, showed no prognostic effect of stromal *STC1* in a multivariate analyses including tumor stage (Supplementary Fig. S3). Surprisingly, univariate analyses indicated an association between high stromal *STC1* and better prognosis (Supplementary Fig. S3). These inconclusive analyses should be extended. Such studies should particularly explore STC1 expression in the invasive front of colorectal cancer. It should also be noted that the present analyses relied on *in situ* expression analyses of *STC1*, which might not reflect protein expression. This approach was selected because validation experiments, using various antibodies and cells with known STC1 expression status, failed to confirm the specificity of the available antibodies. Future studies should also address the possibility that the prognostic significance of stromal STC1 expression might be restricted to certain molecular subsets of colorectal cancer.

This study, dominated by experimental findings, presents some findings that indicate clinical relevance, including the demonstration of a significant association between PDGF β-receptor expression and *STC1* in a large clinical cohort. Importantly, PDGF receptor-dependent upregulation of *STC1* expression was also shown in a primary culture of colorectal CAFs. The cell culture experiments suggest that the proinvasive effects are not restricted to a particular cell line. Moreover, the orthotopic animal model used in this study has been well characterized and is recognized to model human colorectal cancer well (36).

The results from the animal experiments strongly support a stimulatory effect of fibroblast-derived STC1 on metastasis to lymph nodes and distant organs. An important topic for future studies will be to further describe the *in vivo* mechanism(s) that are most important for the prometastatic effects of STC1. On the basis of the coculture experiments, it is likely that a promigratory and proinvasive effect of STC1 contributes to the prometastatic *in vivo* results. Furthermore, the analyses of tumor tissue suggest that STC1 also enhances the ability of cancer cells to intravasate. The potential involvement of epithelial–mesenchymal transition in the STC1-dependent differences in metastasis should be further explored on the basis of the preliminary findings of the present study (Fig. 6C). In the present study, no major alterations in cell type composition of tumors from the 2 groups were observed. However, this issue should be revisited by expanded analyses of various subsets of additional tumor stromal cells, such as fibroblasts, pericytes, and neutrophils. These future analyses should also consider recent studies, which have implicated STC1, in non-cancer models, as a regulator of inflammation, macrophage mobility, and vascular permeability (39–42). Interestingly, earlier studies have also shown that hypoxia, a prometastatic stimuli, also leads to the upregulation of STC1 (43).

Tumors with wild-type fibroblasts were significantly larger than the tumors with *STC1*−/− fibroblasts, and it could be suggested that the differences in metastasis burden between the 2 groups are secondary to differences in tumor size. However, it should be noted that when animals were divided according to the size of primary tumor, no significant differences in metastasis number or size was detected. The lack of correlation between the size of primary tumor and metastasis is also in agreement with clinical findings showing that the size of the primary tumor is not a consistent risk factor for metastasis (44).

According to preliminary results (data not shown), fibroblast-derived STC1 did not affect cancer cell proliferation *in vitro*. However, a potential indirect effect of STC1 on cancer cell proliferation should not be excluded since Ki67 staining of human samples showed an association with STC1 expression levels (Fig. 3E). Also, the results from the study of experimental tumors support an effect on proliferation, as a decrease in PCNA immunostaining and a reduction in the number of mitotic figures, was observed in tumors derived from HCT116 cells coinjected with *STC1* (−/−) MEFs (Fig. 4D and Supplementary Fig. S4D and S4E). This effect of STC1 on *in vivo* proliferation should be further explored and could be secondary to the effect of interactions with other cells in the tumor microenvironment.

The accumulating evidence suggesting an important role of STC1 in cancer progression prompts further studies on the molecular mechanism(s) of action of this still poorly characterized protein. Identification of its molecular receptor and delineation of the intracellular pathways mediating

the promigratory and -invasive effects are highly warranted. Analyses of *STC1*−/− fibroblasts have shown that these cells display resistance to apoptosis and higher levels of activation of MEK and ERK1/2 than wild-type MEFs (35). To what extent these pathways are also important in the paracrine effects described in the present study merits further investigations. A better characterization of the signaling involved in the PDGF-induced activation of STC1 is also motivated. Preliminary studies with PI3K and MEK inhibitors showed that monotreatment with either of these agents were not sufficient to significantly reduce the PDGF-induced *STC1* expression (data not shown).

The findings of the present study, in general terms, show the importance of CAFs in modulating metastatic potential of cancer cells. Obvious and important implications of these findings are, first, that inhibition of fibroblast–epithelial interactions represents a strategy for interference with metastasis and, second, that careful analyses of fibroblast characteristics should be emphasized in ongoing efforts to identify markers for progression in colorectal and other cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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