STC1 expression by cancer-associated fibroblasts drives metastasis of colorectal cancer

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ABSTRACT

PDGF receptor signaling is a major functional determinant of cancer-associated fibroblasts (CAFs). Elevated expression of PDGF receptors on stromal CAFs are associated with metastasis and poor prognosis, but mechanism(s) that underlie this these connections are not understood. Here 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 co-cultured 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.

PRÉCIS

Findings reveal a mechanistic basis for understanding how cancer-associated fibroblasts activated in the tumor microenvironment by PDGF can act to promote cancer metastasis, with implications for arresting this deadly process.
INTRODUCTION

Studies on metastasis have traditionally focused on properties of the malignant cells. However, recent studies in tumor biology have demonstrated 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 (CAFs) 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 demonstrated 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, ECM proteins, proteases, protease inhibitors and lipid products, which may be involved in pro-invasive 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-beta 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). Anti-tumor effects have been demonstrated following targeting of stromal PDGF receptors (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 pro-invasive effects of PDGF-activated fibroblasts, and the identity of potential PDGF-induced pro-metastatic 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 pro-invasive factors induced by PDGF-activation of fibroblasts.

Collectively the studies demonstrate pro-migratory 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 pro-metastatic effects of fibroblasts.
MATERIAL 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-/- MEFs were cultured in Dulbecco’s modified Eagle medium (DMEM; Gibco Life Technologies) containing 1% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 ng/ml) at 37°C in a 5% CO2 humidified atmosphere. Cells were stimulated with PDGFBB (Peprotech) for 5 min 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 ATCC). HCT116 Cell line were purchased in the Cancer Cell Line Repository of the Parc de Recerca Biomèdica de Barcelona. STC1 (+/-) and (-/-) MEFs were provided by Roger R. Reddel. These cells were derived from previously described wild type and knock out STC1 C57BL/6 mice (30). MEFs were used at low passages and without experimentally induced or spontaneous immortalization.

Co-culture assays with tumor colon cells and fibroblasts for migration and invasion assays

Colon cancer cells were co-cultured with PDGF-stimulated or non-stimulated 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. Prior to co-culture, 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 h, 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 485nm; emission at 535nm) by interpolation using a standard curve. For migration and invasion studies with physical separation, stimulated or non-stimulated fibroblasts were seeded in the lower compartment of the Transwell and the labeled colon cells in the upper one.

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

Analyses were performed largely using standard procedures. Details are given in Supplemental Information.

siRNA STC1 down-regulation 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 co-cultured with colon cells for invasion experiments as described above.

Colorectal cancer patients and tissue microarray construction

The tissue micro array was derived from a population-based cohort of 320 patients with CRC. Details about patients and technical aspects related to the generation of the Tissue microarray is provided in Supplemental Information.

Orthotopic cell microinjection in nude mice and histopathological 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 Supplemental Information.

**PCNA, Caspase-3, Vimentin, Ki-67 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 Supplemental Information.

**Analyses of STC1 expression in human colorectal cancer**

Sense and antisense RNA probes were generated using the PCR primer sequences detailed in SI 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 performed after hybridization, followed by detection of the bound probe by chromogenic anti-digoxigenin antibody immunohistochemistry. Blocking of various endogenous enzymes was performed 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 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 mM HEPES (Invitrogen), 30 μg/mL gentamicin (Invitrogen), and 2.5 μg/mL Fungizone (Invitrogen). Subcultivation
and experiments were performed in DMEM (# 21969, Gibco) media 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-test were performed following evaluation of equality of variance or not with Levene’s test. Two-tailed P-values ≤0.05 were considered statistically different. Comparison of STC1 and PDGF β-receptor expression, or KI67, was done with chi-square 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 Figure 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 co-culture conditions (Supplemental Figure 1A).

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 demonstrated 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 (Supplemental Figure 1B). Basal levels of migration among the different epithelial cell lines were characterized prior to co-culture experiments. These analyses identified HCT116 as the most migratory cells and the LIM1215 cells as the least migratory cells (Supplemental Figure 1C).

Activation of the fibroblasts with PDGF induced a significant increase in the migration of co-cultured LIM1215 cells in formats where the two cell types were either cultured together in the upper chamber of a trans-well culture system (Figure 1B) or when fibroblasts were separated from the cancer cells (Figure 1C). Furthermore, a PDGF-dose-dependency of the pro-migratory effects of stimulated fibroblasts were also
demonstrated (Supplemental Figure 1D). A potent PDGF-dependent effect was also observed on LIM1215 invasion in co-culture experiments. This was demonstrated when the two cell types were cultured together in the upper chamber of the trans-well culture system (Figure 1D).

As shown above, SW620 and HCT116 cells produced high levels of PDGF (Supplemental Figure 1B). Addition of exogenous PDGF ligand in co-cultured fibroblasts did not show a significant increase in the migration of HCT116 cells (Supplemental Figure 1E). Invasion experiments were performed 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 Figure 1E, SW620 cell invasion was significantly reduced upon PDGF receptor inhibition.

Together these experiments thus demonstrate PDGF-dependent paracrine pro-migratory and pro-invasive 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 non-stimulated and PDGF-stimulated BJhTERT fibroblasts was analyzed by microarray analysis (GEO Series accession number: GSE40720). Stanniocalcin 1 (STC1) was among the most up-regulated genes encoding secreted factors (see GEO data set). 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 pro-survival signaling pathways has also been observed (35).
qRT-PCR analyses of STC1 expression confirmed a 15-fold STC1- induction upon PDGF stimulation of fibroblasts (Figure 2A). Based on these findings, STC1 was selected for further analyses.

Previously isolated and characterized mouse embryo fibroblasts (MEFs) from STC1 knock-out 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 (Figure 2B). Previous studies have reported that proliferation rate of STC/- MEFs is increased as compared to wild-type MEFs (35).

Analyses using the wild-type MEFs demonstrated an increase of invasion in LIM1215 colon cells upon co-culture with PDGF-activated wild type MEFs (Supplemental Figure 2A). Furthermore, the wt MEFs enhanced the invasion of the PDGF-producing SW620 cells, in a manner which was not affected by PDGF but was reduced by PDGF receptor inhibitors (Supplemental Figure 2B). Together, these results support the findings of Fig. 1, showing PDGF-dependent pro-invasive 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 STC-/- MEF co-cultures, as compared to that observed in wild-type MEF co-cultures (Figure 2C). Experiments using SW620 and HCT116 cells also demonstrated that loss of STC1 significantly reduced the invasion-stimulatory effects of PDGF-stimulated fibroblasts (Figure 2D and E). Similar results were observed when the pro-migratory effects of fibroblasts on HT29 migration were analyzed (Supplemental Figure 2C). In some cases the experiments with the MEF pair were also performed 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 (Figure 2C).

To obtain independent evidence for a role of STC1 in the paracrine pro-invasive effects of fibroblasts, siRNA experiments were performed using the BJhTERT fibroblasts. As shown in Figure 2F, down-regulation of STC1 dramatically reduced the pro-invasive effect of BJhTERT cells in co-cultures with LIM1215 cells.

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

**PDGF β-receptor and STC1 are co-expressed in tumor stroma of colorectal cancer**

PDGF β-receptor and Ki67 were analyzed by immunohistochemistry and 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 ($p = 0.032$; Figure 3A and B).

Analyses of stroma abundance, based on ASMA IHC analyses, demonstrated that the STC1 expression was not correlated to stroma abundance (Supplemental Figure 3A).

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 (Figure 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 (Figure 3E).

Together these analyses thus demonstrate an up-regulation of STC1 in tumor stroma, as compared to normal mucosa, and also suggest that the up-regulation 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 (Supplemental Figure 3B-3D) (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 employed 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, e.g., the liver and the lung (36).

HCT116 cells were co-injected 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 co-injected 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 (Supplemental Figure 4A and B).

Tumor volume was measured at necropsy. Tumors derived from co-injection of HCT116 cells and STC1-/- MEFs were significantly smaller than the tumors of the control group (Figure 4A). The presence of fibroblast-like cells was observed in all tumors. Staining with PDGF β-receptor antibodies and cytokeratin did not reveal major differences between the tumor groups with regard to stromal-epithelial ratio (Figure 4B, Supplemental Figure 4C). Necrotic areas were also detected in most tumors but did not differ between the two experimental groups (Figure 4B and Supplemental Figure 4C).

Proliferation and apoptosis were evaluated in primary tumors by mitotic figures counting and caspase-3 immunohistochemistry, respectively. Tumors derived from co-injection with STC1-/- MEFs displayed reduced proliferation and also increased apoptosis (Figure 4C-F). PCNA immunohistochemistry also confirmed the reduced proliferation in tumors derived from co-injection with STC1-/- MEFs (Supplemental Figure 4D and E).

Vascular density was evaluated in primary tumors following immunohistochemical analyses using Isolectin B4 antibodies. No differences were observed between groups (Supplemental Figure 4F and G).

Together these analyses demonstrated that 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 co-injection 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 tumors derived from the STC1-/- group showed a significantly lower number of affected organs per mouse (Figure 5A). Characterization of the distant metastatic lesions also revealed that the foci in the STC-/- MEF group were significantly smaller than those in the control group (Figure 5B).

The potential relationship between dissemination and size of primary tumor was also analyzed. Tumors were divided into two 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 primary tumor-group” with regard to number of organs affected per mouse, or average lesion size (Supplemental Figure 5A and B). 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 STC -/- MEF group (Supplemental Figure 5C).

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 Supplemental Figure 6, this analysis indicated that metastasis formation via all three routes, based on the size of foci, was reduced in the STC1-/- MEF group (Supplemental Figure 6A-6F).

This analysis thus demonstrated that the STC1 status of co-injected 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 STC1-/- MEF group (Figure 6A and B). Neither of these parameters differed significantly when tumors were divided according to size (Supplemental Figure 7A and B).

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 (Supplemental. Figure 7C and D). However, a significant difference in lymphatic vessel density was also observed when tumors were grouped based on size (Supplemental Figure 7E), making it difficult to evaluate if 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 (Supplemental Figure 7F-H). Tumors with STC1 -/- or +/- MEFs did not show changes in the CD44 immunostainning. F4-80 IHC was analyzed to determine the amount of macrophages in tumors (Supplemental Figure 7I-K). A reduction of the macrophage number was observed in the STC1 -/- MEF group. However, the analyses of size-divided tumor groups also revealed differences in macrophage density, and the differences between the experimental groups could thus be considered secondary to the generally smaller size STC-/- 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 out of 9 tumors in the STC1 +/+ group. In contrast, no sample in the STC1 -/- MEF group showed vimentin expression ($p = 0.053$; $\chi^2$ test) (Figure 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 demonstrates that PDGF receptor stimulation of fibroblasts significantly increases migration and invasion of co-cultured colorectal cancer cells in a 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 has also been associated with worse prognosis in lung cancer (38).

The findings imply stromal STC1 as a pro-metastatic factor. Preliminary analyses of the prognostic significance of stromal STC1 expression, based on the in situ analyses, demonstrated no prognostic effect of stromal STC1 in a multivariate analyses including tumor stage (Supplemental Figure 3). Surprisingly, univariate analyses indicated an association between high stromal STC1 and better prognosis (Supplemental Figure 3). 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 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 which 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 up-regulation of STC1 expression was also demonstrated in a primary culture of colorectal CAFs. The cell culture experiments suggest that the pro-invasive 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 pro-metastatic effects of STC1. Based on the co-culture experiments, it is likely that a pro-migratory and pro-invasive effect of STC1 contributes to the pro-metastatic 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 based on the preliminary findings of the present study (Figure 6 C). In the present study, no major alterations in cell type composition of tumors from the two groups were observed. However, this issue should be revisited by expanded analyses of various subsets of additional 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 demonstrated that hypoxia, a pro-metastatic stimuli, also leads to up-regulation of STC1 (43).

Tumors with wild-type fibroblasts were significantly larger than the tumors with STC-/- fibroblasts, and it could be suggested that the differences in metastasis burden between the two groups are secondary to differences in tumor size. However, it should be noted that when animals were divided according to size of primary tumor, no significant differences in metastasis number or size was detected. The lack of correlation between size of primary tumor and metastasis is also in agreement with clinical findings showing that 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 Ki-67 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, since a decrease in PCNA immunostaining, as well as a reduction in the number of mitotic figures, was observed in tumors derived from HCT116 cells co-injected with STC1 (-/-) MEFs (Fig. 4D and Supplemental Figure 4D-E). This effect of STC1 on in vivo proliferation should be further explored and could be secondary to 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 pro-migratory and -invasive effects are highly warranted. Analyses of STC-/- fibroblasts have demonstrated 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 demonstrated that mono-treatment 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, demonstrate the importance of CAFs in modulating metastatic potential of cancer cells. Obvious and important implications of these findings are, firstly, that inhibition of fibroblast-epithelial interactions represents a strategy for interference with metastasis and, secondly, that careful analyses of fibroblast characteristics should be emphasized in ongoing efforts to identify markers for progression in colorectal and other cancers.
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FIGURE LEGENDS

Figure 1: PDGF-dependent stimulation by fibroblasts of colon cancer cell migration and invasion. PDGF receptor expression in colon cancer cells and fibroblasts were characterized by PDGF β-receptor and phospho-tyrosine immunoblotting (A). Effects of co-cultured fibroblasts on migration (B-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). Co-culture experiments were performed with the two cell types together in the upper chamber (B, D and E) or with the fibroblasts in the lower chamber (C). Results in B-E are derived from 2-4 independent experiments, each performed in duplicate.

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

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: 100μM). Box plot showing the association between PDGF β- receptor and STC1 IHC quantification (B). PDGF receptor expression in primary human CAFs was characterized by PDGF β-receptor and phospho-tyrosine immunoblotting (C). Expression of STC1 in primary cultures of CAFs was analyzed by qRT-PCR in cells cultured in the absence or presence of PDGF-BB (D). Box plot showing the association between Ki67 and STC1 (E).

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

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

**Figure 6:** Primary tumors with STC1-/- fibroblasts display reduced intravasation and decreased EMT. Tumor cell intravasation was determined by microscopic analyses of H&E-stained tumor sections (A). Quantification of data was performed after analysis of 10 sections from each tumor from the two tumor types (B). Presence of cells under EMT was evaluated by vimentin immunostaining (C). Two animals from each group was excluded in the analysis of intravasation because of absence of tumor-free submucosa.
**Figure 3**

A) **PDGFbr-IHC**

- 1
- 2

B) **STC1-ISH**

- 3
- 4

B) **Box Plot**

- PDGF br-IHC quantification
- STC1-ISH quantification

- $p = 0.032$

C) **Western Blot**

- BJH/TERT
  - PDGFBB
  - PDGFβR
  - P-PDGFβR
  - β-actin

- CAFs
  - PDGFBB
  - PDGFβR
  - P-PDGFβR
  - β-actin

D) **STC1 mRNA expression levels**

- $p = 0.037$

- (Fold of un-stimulated cells)

- PDGFBB
  - -
  - +

E) **K167 IHC quantification**

- $p = 0.038$

- STC1-ISH quantification
Figure 5

A

Mean of affected organs per mice

STC1 MEFs

(++)

(+)  

(-+)

P = 0.029

B

Area/foci (mm²)

STC1 MEFs

(++)

(+)  

(-+)

P = 0.020
Figure 6

A

H&E

10x

STC1 (+/+ ) MEFs

H&E

10x

STC1 (-/-) MEFs

B

Num. of intravasated tumor emboli

p = 0.005

STC1 MEFs

(+/+)

(−/−)

Area average of intravasated tumor emboli

p = 0.016

STC1 MEFs

(+/+)

(−/−)

C

STC1 (+/+ ) MEFs

20x

Vimentin

STC1 (-/-) MEFs

20x

Vimentin
## STC1 expression by cancer-associated fibroblasts drives metastasis of colorectal cancer

Cristina Peña, María Virtudes Céspedes, Maja Bradic Lindh, et al.

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