PROTEOME PROFILING OF CANCER-ASSOCIATED FIBROBLASTS IDENTIFIES NOVEL PRO-INFLAMMATORY SIGNATURES AND PROGNOSTIC MARKERS FOR COLORECTAL CANCER

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Translational relevance

Our knowledge of cancer-associated stroma is rather limited in comparison with cancer epithelial cells. Stroma constitutes the cancer microenvironment that nurtures cancer cells and facilitates invasion and metastasis. Moreover, stromal myofibroblasts have been associated to recurrence and survival. Novel biomarkers are required for the isolation and characterization of colorectal cancer (CRC)-associated fibroblasts (CAFs) in order to improve overall survival and recurrence prediction in CRC. We here describe a strategy based on an in-depth proteome profiling of CAFs obtained from a mouse model of human sporadic CRC. The use of this model facilitates the isolation of pure populations of CAFs, avoiding other cell contaminants. The validity of this strategy is supported by the identification of multiple stromal biomarkers previously described in humans. In addition, we report a collection of novel stromal biomarkers as well as comprehensive pro-inflammatory and desmoplastic CAFs signatures for CRC. The value and relevance of these novel stromal biomarkers for colon cancer prognosis and survival were validated in human samples.
ABSTRACT

Purpose: Cancer-associated fibroblasts (CAFs) are essential components of the stroma that play a critical role in cancer progression. This study aimed to identify novel CAFs markers that might contribute to the invasion and the prognosis of colorectal cancer.

Experimental design: The azoxymethane/dextran sodium sulfate mouse model of sporadic colon cancer represents an adequate source for the isolation of CAFs and normal fibroblasts (NFs). By using the explants technique we purified CAFs and NFs from colon tissues. Whole cell extracts and supernatants were subjected to in-depth quantitative proteomic analysis by tandem mass spectrometry. Further validations of up-regulated proteins in CAFs were carried out by chemokine microarray analysis and immunohistochemistry of mouse and human tissues.

Results: Using a fold-change ≥1.4, we found 132 and 125 differentially-expressed proteins in whole cell extracts and supernatants, respectively. We found CAFs-associated pro-inflammatory and desmoplastic signatures. The pro-inflammatory signature was composed of several cytokines. Among them, CCL2 and CCL8 caused an increase in migration and invasion of colorectal cancer KM12 cells. The desmoplastic signature was composed of 30 secreted proteins. In mouse and human samples, expression of LTBP2, CDH11, OLFML3 and, particularly, FSTL1 was significantly increased in the tumoral stroma, without significant expression in the cancer epithelial cells. The combination of CALU and CDH11 stromal expression showed a significant association to disease-free survival and poor prognosis.
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**Conclusion.** We have identified LTBP2, CDH11, OLFML3 and FSTL1 as selective biomarkers of cancer stroma and CALU and CDH11 as candidate stromal biomarkers of prognostic significance in colon cancer.
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INTRODUCTION

The tumor stroma comprises most of the cancer mass and is mainly composed of fibroblasts and endothelial cells, although also contains infiltrating immune cells and pericytes (1). Stroma nurtures cancer cells and facilitates tumor development and invasion. Clinical and experimental data support the hypothesis that tumor stroma promotes invasion and cancer metastasis (2). Within stroma, fibroblasts are key components for cancer progression. Stromal fibroblasts are called activated fibroblasts, myofibroblasts or cancer-associated fibroblasts (CAFs) and they acquire a particular phenotype similar to fibroblasts present in skin wounds. Carcinoma progression is associated with an increase in the production of fibrosis, known as desmoplasia, similar to that present in wound healing (3). CAFs respond to profibrotic and promigratory factors, such as TGFβ, PDGF, HGF or FGF2, promoting cancer progression. They are characterized by increased expression of myofibroblastic markers like α-smooth muscle actin (α-SMA), FSP1 or prolyl-4-hydroxilase (4, 5). Cancer fibroblasts proliferate more than their normal counterparts and secrete more extracellular matrix (ECM) constituents and ECM-degrading proteases (6). In addition, CAFs can mediate inflammation and angiogenesis, as reported in skin cancer (7). Despite the significant number of markers and secreted proteins already associated to activated fibroblasts, the study of their contribution to tumor growth and invasion would benefit of additional markers for cell selection, prognosis and invasion prediction (8).

Regarding human colon adenocarcinomas, CAFs synthesize ECM components such as fibronectin, tenasin, collagens types I, III, IV, V and XII and proteoglycans (biglycan, fibromodulin, perlecan and versican) (9, 10). They also contribute to the formation of basement membranes by secreting collagen type IV and laminin. For a more comprehensive molecular analysis, we propose the use of sensitive proteomic
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techniques combined with a mouse model of colon cancer, cell isolation and mass spectrometry. We have chosen the well-established murine model of colitis-associated cancer (CAC) based on the use of azoxymethane (AOM) and dextrane sodium sulphate (DSS). Although the model does not progress to metastasis (11), it mimics quite well many steps in cancer progression of sporadic colorectal cancer. In fact, this model has been very useful for the elucidation of the role of TNF-α, IL-6, NFκB and other molecules in the initiation and progression of inflammation-associated cancer (see (12) for a review).

Fibroblast characterization has been largely complicated by the necessity to isolate primary fibroblasts from the colonic tissue. In other publications, the characterization of CAFs was carried out by co-culturing normal fibroblasts and colon cancer cell lines (10), cell sorting (7) or cell immortalization (13). We propose the use of direct intestinal explants in culture for fibroblast isolation and proteomic characterization (14). This strategy strongly reduces sample complexity and heterogeneity. The use of inbred mouse model reduces biologic variation due to genetic and environmental heterogeneity. Since primary fibroblasts duplicate only a few times before entering senescence, we discarded metabolic labeling for quantification. Therefore, iTRAQ (isobaric tag for relative and absolute quantification) was preferred because of its reproducibility and reliability (15).

Here, we compared the protein component of the whole cell extracts and conditioned medium of primary CAFs and normal fibroblasts (NFs) isolated from AOM/DSS-induced sporadic colorectal cancer mice or controls, respectively. We identified 132 and 125 proteins deregulated in whole cell extracts and conditioned medium, respectively, of CAFs versus NFs. *In silico* studies demonstrated a predominant association of up-regulated proteins to deposition of extracellular matrix,
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wound healing and inflammation. Pro-inflammatory and desmoplastic signatures, specific for colon cancer, were defined for CAFs-associated proteins. A number of proteins were identified as promising tumor-associated stromal prognostic markers. Among these proteins, high expression of CALU and CDH11 was associated to poor survival in human cancer samples.
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MATERIAL AND METHODS

AOM/DSS-induced colon cancer model
All mouse studies were performed under the approval of the Animal Ethics Committee of the Spanish Research Council (CSIC, Madrid, Spain). Animals were housed under pathogen-free conditions and were given autoclaved food and water *ad libitum*. For the AOM/DSS model, FVB/N mice (4-6 weeks-old) were weighed and given a single intraperitoneal injection of azoxymethane (AOM; 10 mg/kg) or vehicle (PBS). Five days later, animals received either 2.5% DSS or normal drinking water. Chronic colitis-derived colon cancer was induced after three cycles of DSS treatment, which consisted of 5 days with 2.5% DSS followed by 16 days with normal water. At the end of the protocol, animals were sacrificed and distal colons were longitudinally cut, rinsed twice with ice-cold PBS and cut in small pieces. Intestine pieces were either cultured to isolate fibroblasts or fixed in 10% buffered formalin overnight for immunohistochemistry analysis.

Cell line culture
KM12C and KM12SM human colon cancer cells were obtained from I. Fidler’s laboratory (MD Anderson Cancer Center, USA). A large batch of working aliquots of KM12 cells was stored in liquid nitrogen. For each experiment, cells were thawed and kept in culture for a maximum of 10 passages in DMEM (Gibco-Life Technologies) containing 10% fetal calf serum and antibiotics at 37°C. Cells were tested for mycoplasma but not authenticated, as Dr Fidler’s laboratory is where these lines were originally isolated. CT26 murine colon cancer cell line was obtained directly from the ATCC (which authenticated the cell line by short tandem repeat profiling), and
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passaged for fewer than 6 months after receipt or resuscitation according to provider instructions.

**Fibroblast isolation and culture**

CAFs and NFs cultures were established from colonic tissues from AOM/DSS-treated mice (n=16) and vehicle-treated (n=16) by the explant technique (14, 16). Tissues were cut into 2-3 mm fragments and planted in Fibroblast Growth Medium-2 (Lonza) containing 2% fetal calf serum, 1% penicillin/streptomycin/amphotericin B (Invitrogen) at 37°C in a 5% CO₂-humidified atmosphere. NFs and CAFs grew around the explants and were cultured approximately for 3 weeks. Then, tissue fragments were removed and cells were trypsinized and seeded at a 70-90% confluence and cultured in complete medium. When cells reached 95-100% confluence (approximately 1.7-2.0x10⁴ cells/cm²) were washed with PBS, incubated with serum-free medium for 1 h, washed again and incubated for 24 h in serum-free medium. Then, cells and conditioned medium were collected, pooled in two different batches for NFs and CAFs and mixed for performing the different experiments. We recovered 7.0 and 13.5 x 10⁶ cells from NFs and CAFs cultures, respectively.

**Sample preparation and iTRAQ labeling**

For a detailed description of sample preparation see Supplementary Methods. iTRAQ labeling was carried out using iTRAQ Reagent 4-Plex kit (AB SCIEX), following the manufacturer’s protocol with minor modifications. Briefly, 100 µg of protein from whole cell lysate or 50 µg of protein from conditioned medium from NFs and CAFs were digested with 5 µg trypsin (Promega) overnight at 37°C. Peptide mixtures were labeled with iTRAQ reagents, 114, 116 for NFs and 115, 117 for CAFs as illustrated in
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Supplementary Fig. S1. To remove interfering substances, SCX chromatography was carried out using a Resource S column (GE healthcare). To adjust the pH between 2.5 and 3.3 the sample mixture was diluted with 15 vol of 10mM KH₂PO₄ pH 3.0 containing 25% acetonitrile (ACN). Peptides were eluted in 1ml of elution buffer (25% ACN, 350mM KCl, 10mM KH₂PO₄, pH 3.0). Then, peptides were desalted with Sep-Pak C18 cartridges (Waters), dried and reconstituted in OFFGel solution (Agilent Technologies). All chemicals were obtained from Sigma-Aldrich.

Peptide Fractionation and Mass Spectrometry Analysis

Peptides were recovered in 12 fractions by isoelectric focusing using a low resolution strip (pH 3-10) in a 3100 OFF-GEL fractionator (Agilent Technologies). Five µl of 25% trifluoroacetic acid (TFA) were added to each fraction and desalting was performed with Zip-Tip. Peptide mixtures were vacuum-dried and reconstituted with 6 µl 0.1% formic acid. Then, peptides were trapped onto a precolumn C18-A1 ASY-Column (2 cm, ID100 µm, 5µm) (Thermo Scientific) and run with a linear gradient of 2–35% ACN in 0.1% aqueous solution of formic acid. The gradient was performed over 180 min using an Easy-nLC (Proxeon) at a flow-rate of 300 nL/min onto a Biosphere C18 column (75 µm, 16 cm, 3 µm) (NanoSeparations). Then, peptides were scanned and fragmented with a linear ion trap-Orbitrap Velos (Thermo Scientific). The Orbitrap Velos was operated in data-dependent mode to automatically switch between MS and MS/MS. Survey full-scan MS spectra were acquired from m/z 400 to 1600 after accumulation to a target value of 10⁶ in the Orbitrap at a resolution of 60,000 at m/z 400. For internal mass calibration, we used the 445.120025 ion for lock mass. Charge state screening was enabled and precursors with charge state unknown or 1 were excluded. After the survey scan, the 10 most intense precursor ions were selected for
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CID-HCD MS/MS fragmentation. Peptide identification was performed in both CID and HCD spectra and quantification of iTRAQ reporter ions was performed in HCD. HCD was carried out with excess of collision energy for effectively maximizing abundance of the reporter ions. For CID fragmentation the target value was set to 10,000 and normalized collision energy to 35%. For HCD, target value was set to 50,000 and collision energy was set to 55%. Dynamic exclusion was applied during 30s. All MS data were analyzed and quantified with Proteome Discoverer (version 1.3.0.339) (Thermo) using standardized workflows (see supplementary Methods for a more detailed description).

Mouse cytokine array

Conditioned medium from CAFs and NFs were collected after 24h in serum-free medium and incubated with Mouse Cytokine Antibody Array C6 for semi-quantitative analysis of 97 mouse cytokines (RayBiotech) according to manufacturer’s instructions. Then, membranes were scanned and analyzed using Redfin, a 2D-gel image analysis software (Ludesi). Relative semi-quantitative cytokine intensities were normalized in comparison to control spots on the same membrane. Expression ratios were calculated comparing the signal intensities for each spot of the different cytokines. The limit of detection, sensitivity and the dynamic range of the measured cytokines were above 1 pg/mL, where most of cytokines can be detected.

Cell proliferation, adhesion, migration and invasion assays

KM12C and KM12SM human colon cancer cells (17) were cultured in Dulbecco’s modified Eagle medium (Life Technologies) containing 10% fetal calf serum and antibiotics at 37ºC in a 5% CO2-humidified atmosphere. Invasion, migration, adhesion
and cell proliferation assays were carried out following established procedures (18), with the addition of the indicated cytokines: CCL2 (10 ng/ml), CCL8 (50 ng/mL) and IL-9 (2 ng/ml). See Supplementary Methods for a detailed description.

**Immunohistochemistry analysis**

Intestine pieces from AOM/DSS treated or control mice were fixed in 10% buffered formalin overnight to perform hematoxylin/eosin and immunohistochemical staining. For the prognostic studies in humans, a total of 80 patients diagnosed and treated of colorectal adenocarcinoma between 2001 and 2006 in Fundación Jiménez Diaz (Madrid) and followed in the long term were used for the study. Human samples were prepared as described in (18). All human biopsies were obtained with the patient’s consent and the approval of the Ethical Committee of Hospital Fundación Jiménez Diaz according to Spanish official regulations. We reviewed the clinical records of the patients to determine tumor stage at the time of diagnosis and outcome (18). Each sample was deparaffinized for antigen retrieval using the PT Link Module (Dako) at high pH for 20 min, rehydrated and then incubated with the primary antibody against α-SMA, Snail1, S100A4, CALU, CDH11, LTBP2, FSTL1 or OLFML3 (*Supplementary Table S1*). The reaction was revealed using DAB as chromogen and hematoxylin for counterstaining, and observed in an Olympus microscope. ImageJ was used to quantify the DAB staining of immunohistochemistry images.

**Bioinformatics**

Ingenuity Pathway Analysis (IPA) (Ingenuity systems, Inc) was used to analyze the predicted biological functions of the proteins deregulated in CAFs and to determine protein interactions and network analysis. Bio-GPS software was used to determine the
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top-5 tissues with highest expression of each altered protein compartment in order to
determine the desmoplastic signature (10). FatiGo was used to get further insight in
deregulated functions using a cut-off <0.1 for the adjusted $p$ value (19).
RESULTS

Isolation and characterization of fibroblasts from AOM/DSS colon cancer tumors

After AOM/DSS treatment, development of adenocarcinomas in the distal colon of mice was visible by visual inspection and histological staining (Fig. 1A). Mouse adenocarcinomas were equivalent to human colorectal adenocarcinomas, corresponding to a well-differentiated infiltrating enteroid adenocarcinoma, with presence of large numbers of flat and polypoid malignant tumors. The mucosa from control mice was normal, without dysplastic changes (Fig. 1A). By immunohistochemical analysis, AOM/DSS tumors showed high expression level of α-SMA, S100A4 and Snail, considered markers of activated fibroblasts in desmoplastic tumors (Fig. 1B). To establish CAFs and NFs cultures, distal colons from 16 AOM/DSS-treated and vehicle-treated mice were cut in small fragments and placed in culture. Fibroblasts migrated outside the explants and expanded (Fig. 1C). Cells at 95-100% confluence (approximately 2x10^4 cells/cm^2) were collected, divided in two fractions and mixed for iTRAQ experiments. By western blot, AOM/DSS-isolated fibroblasts confirmed the higher expression of markers corresponding to activated fibroblast like α-SMA, S100A4 and Snail1 (Fig. 1D). Expression was equivalent to the immunohistochemical staining in the original tumor (Fig. 1B). As expected, purified fibroblasts expressed vimentin, a mesenchymal marker, but not the epithelial marker Epcam.

Protein identification, iTRAQ quantification, gene ontology and functional networks alterations

Briefly, 100 µg (cell lysates) and 50 µg (conditioned media) of proteins from CAFs and NFs cultures were trypsin-digested. Peptides were labeled with iTRAQ for relative quantification and fractionated with OFFGEL, pH 3-10. To avoid biases in peptide
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labeling that could affect the final quantification, we performed two replicate analyses using the 114 and 116 iTRAQ tags for NFs peptides and 115 and 117 for CAFs peptides (Supplementary Fig. S1). In total, 3,846 and 734 peptides corresponding to 1,353 and 295 proteins were identified in the whole cellular extract and conditioned medium, respectively. For quantification, peptide ratios were calculated by comparing the intensity of the reporter ions in the MS/MS spectra and normalized either using the ratios corresponding to seven different house-keeping proteins in the whole cell extract or the median in the secretome (Supplementary Fig. S2). A total of 1,102 and 250 proteins were quantified in the whole cell extract and conditioned medium, respectively (Supplementary Tables S2 and S3). We used several criteria for protein selection, 1) a fold-change \( \geq 1.4 \) relative to control samples, 2) proteins present in both replicates, 3) expression ratios following a similar trend in both replicates and 4) proteins identified with a single unique peptide or variability higher than 50% were manually inspected to verify that the peptide only corresponded to a single protein. We selected 1.4 fold-change as a compromise value to include all relevant proteins that showed systematic change and to compensate the compression of iTRAQ ratios that leads to underestimation of fold changes (20). Using a fold-change \( \geq 1.4 \), we found 132 proteins deregulated in complete cell lysates, 109 up-regulated and 23 down-regulated (Supplementary Table S4), and 125 proteins in the secretome, with 72 up-regulated and 53 down-regulated (Supplementary Table S5).

Gene ontology analysis of whole cellular extracts results from CAFs showed a clear up-regulation of ECM constituents (collagen \( \alpha1 \) types I, V, III and XII, collagen \( \alpha2 \) types I and V, fibulin 2), proteins for matrix assembly (decorin, prolyl 4-hydroxylase \( \alpha2 \) and leprecan 1), proteins related to TGF\( \beta \) signaling, such as LTBP2 (Latent transforming growth factor beta binding protein 2), cadherin 11, Fibrillin 1 and IGF2R
Molecular signatures of colon cancer-associated fibroblasts (insulin-like growth factor 2 receptor) (Supplementary Table S6). In the secretome, 26 out of 125 proteins were related to ECM. In addition, we identified differentially-expressed chemokines such as CCL11, CCL8, CXCL5 or CCL2, insulin growth factor-related proteins such as IGFBP7, or IGF1, cell adhesion and wound response (Supplementary Table S7). Collectively, these data suggest that activated fibroblasts participate in extracellular matrix remodeling, wound healing, epithelial differentiation and are important regulators of inflammation.

Altered biological functions, networks and pathways were analyzed using IPA (Supplementary Table S8) and FatiGO (Supplementary Table S9). Using IPA in whole cellular extract, “Connective Tissue disorders”, related with ECM remodeling, was the top altered function (n= 21 proteins and p values ranging from 1.59E-11 to 1.34E-02). In secretome, we found “Cancer” as the most represented biological function altered (n= 97 proteins and p values ranging from 2.82E-14 to 1.35E-04), and “Organismal Injuries and Abnormalities” as the top biological function altered (n= 54 proteins and p values ranging from 2.55E-14 to 1.54E-04). Regarding altered network function “Dermatological Diseases and Conditions, Cellular Assembly and Organization, Cellular Function and Maintenance” with 31 proteins emerging as identified in cell extracts (Fig. 2A) and “Cell morphology, Cellular Assembly and Organization, Cellular Function and Maintenance” was significantly altered in the secretome with 28 proteins identified (Fig. 2B).

With FatiGo we found that oxidative phosphorylation and molecular functions related to redox processes were altered in whole cellular extract. Transport of proteins and their cellular localization emerged as the top altered biological processes. In the secretome, the extracellular matrix and focal adhesion were the most altered processes. In addition, we observed a focus towards modulations of molecular functions through
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receptor binding, whereas the most altered biological processes were the response to wounding, external stimulus and inflammatory response. Collectively, these protein networks are indicating the association of CAFs proteins with desmoplastic and pro-inflammatory signatures, respectively.

Validation and fibroblast specificity of selected biomarkers

After previous network analyses and data-mining using GeneCards database, we selected top up-regulated proteins that were not previously associated to colon cancer and pertained to major networks (Fig. 2, Supplementary Tables S8, S9). So, eleven differentially-expressed proteins (5 from whole cellular extract and 6 from secretome) were validated using Western Blot. We selected a relatively large number of proteins from both locations to make a more comprehensive analysis, as they would represent different family proteins and functionalities. Western blot results were consistent with the iTRAQ quantification data (Fig. 3A, B). Molecules like CRABP1, LTBP2, FDPS and CDH11 were up-regulated in whole cellular extracts (Fig. 3A), whereas OLFML3, CALU, AEBP1, LMOD1, SPON2 and FSTL1 were up-regulated in CAFs supernatants (Fig. 3B). In order to confirm that purified fibroblasts were equivalent to those present in the tumor, we validated a subset of these markers in the original tissues of the AOM/DSS model by quantitative RT-PCR and western blot. Protein and mRNA expression values were similar between purified fibroblasts and original tissues (Supplementary Fig. S3).

To confirm fibroblast specificity of stromal biomarkers, we compared the presence of these proteins in CAFs respect to CT26, a colon adenocarcinoma murine cell line, by western blot. LTBP2, FDPS, CDH11 and OLFML3 expression was specific or clearly up-regulated in fibroblasts respect to the epithelial CT26 cells (Fig. 3C).
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When we tested the supernatants, SPON2 and FSTL1 were also specific of fibroblasts. Finally, we investigated the capacity of CDH11 for cell sorting of fibroblasts. PDGFRα was used as a positive control (7). CDH11 antibodies were able to isolate fibroblast cell lines, like mouse NIH-3T3 or human BJ-hTERT, but did not work with epithelial cell lines like CT26 or RKO (Fig. 3D). These results suggested that all these six proteins were specific colon cancer stromal biomarkers.

Cytokines and growth factors deregulated in the secretome of activated fibroblasts are pro-inflammatory and pro-tumorigenic on colon cancer cells

CAFs secrete chemokines that control critical steps of the adhesion-invasion-metastasis cascade and affect recruitment of inflammatory cells and enhancement of angiogenesis (21, 22). By mass spectrometry, we observed a significant increase in the expression of cytokines like CCL11 (Eotaxin-1), CCL8 (MCP-2), CCL2 (MCP-1), SAA3 and CXCL5 (ENA-78), whereas CSF1 was down-regulated in CAFs (Supplementary Table S7). To investigate other additional changes in cytokines, we used a mouse chemokine microarray for CAFs/NFs supernatants (Fig. 4A). CCL11, CCL8, SAA3 and CXCL5 antibodies were not present in the arrays. Microarray semi-quantitative data confirmed the increase in CCL2 expression, although not as relevant as in iTRAQ, and showed an increase in other pro-inflammatory cytokines like IL-6, CXCL2, CCL20 and osteopontin (OPN) (Fig. 4B). Overall, our chemokine profile was similar to the one described for cancer skin fibroblasts that showed cancer-promoting activity, NF-κB dependent (7).

To be activated by cytokines, cancer cells should express the corresponding receptors. In order to detect CCL2 and CCL8 receptors (CCR1, 2, 3 and 5) as well as the receptor for IL-9 (IL9R), we performed RT-PCR assays with KM12C and SM cells.
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These assays showed that both cell lines express IL9R and CCR1 and CCR3, whereas CCR2 and CCR5 were detected only in KM12C and KM12SM, respectively (Fig. 4C). Then, we investigated the effect of CCL2, CCL8 and IL-9 on cell proliferation, adhesion, migration and invasion of colon cancer cell lines (Fig. 4D). A >2-fold increase was observed at the adhesion, migration and invasion level in KM12 cells, after CCL2 and CCL8 addition, but no effect for IL-9. In contrast, IL-9 induced a significant increase in the proliferation of KM12 cell lines. Collectively, these data suggest that CCL2 and CCL8 play an important role in tumorigenesis and the acquisition of invasive capacity of cancer cells.

Desmoplastic signature in AOM/DSS colon fibroblasts and prognostic value

Since activated fibroblasts are characterized by the acquisition of a myofibroblast phenotype consisting of different smooth-muscle-like proteins, like α-SMA, one aim of this work was to define a desmoplastic signature for colon cancer. We searched for desmoplastic markers on the differentially-expressed proteins using the Bio-GPS database. In the secretome, we found 30 secreted proteins (24% of the total) preferentially expressed in myofibroblasts (Table 1), including seven collagens, collagen metabolism-related proteins (LOX, PCOLCE), fibronectin, proteoglycans (perlecan and byglican), cytokines (CCL11, CCL8 and CCL2) and calcium metabolism proteins (calumenin (CALU)), insulin growth factor binding proteins (IGFBP6, 7) and FSTL1. The cell lysate showed a similar profile.

Then, we studied the value of the proteins in this desmoplastic signature as prognostic biomarkers in human patients. Given the large number of candidates, we carried out a meta-analysis using databases from the “cBio Cancer Genomics Portal” (23), which contains mRNA expression data sets from 274 samples of colon cancer.
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Using Kaplan-Meier for overall survival analysis, we found some combinations of proteins with prognostic value from whole cellular extract (Supplementary Fig S4A) and the secretome (Supplementary Fig S4B). The most significant combination included RPN2, IVL, RP1 and CALU (Supplementary Fig S4C), which displayed a highly significant association to overall survival (log-rank test \( p \) value = 0.000095). In contrast, the association to survival in ovarian or lung adenocarcinoma did not deliver significant \( p \) values (data not shown).

Verification of stromal markers and prognostic association in human cancer samples

Immunohistochemical staining was performed to validate the expression of the five selected candidate stromal biomarkers CALU, CDH11, FSTL1, OLFML3 and LTBP2 in AOM/DSS-induced tumor samples. SPON2 was not tested due to the lack of suitable antibodies. The selected markers were strongly expressed in the stroma of AOM/DSS-induced murine adenocarcinoma with little or no expression in normal colon mucosa (Fig 5A). The expression of these markers was more intense in the leading edge of tumors, which could be associated to desmoplastic invasion front.

Finally, to investigate the relevance of these markers in human colon cancer samples we used a tissue microarray. Clinical information together with the stromal expression of the selected CAF markers is summarized in Supplementary Table S10. We observed an increased expression for CALU, CDH11, FSTL1, OLFML3 and LTBP2 in the tumoral respect to normal stroma (Fig. 5B). All of them showed stronger expression in the leading edge of the tumors, where the tumor infiltrates the surrounding areas. Tumoral epithelial cells showed an increased expression in >70% of the tumors for CALU, CDH11 and OLFML3, but not significant expression was detected for
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FSTL1 (Fig. 5B). In contrast, in the stroma of normal tissue the staining of these markers was mainly observed to be negative or weak in contrast to tumoral stroma where the markers were highly over-expressed (Fig. 5B).

Then, we determined if the expression of these proteins in the stroma was associated to survival in human cancer using the tissue microarray data for patients followed on the long term (more than five years). The series was retrospectively selected (Supplementary Table S10). High stromal expression of CALU correlated significantly with lymph node involvement at the moment of diagnosis ($p=0.034$) (Fig. 5C) and with poor prognosis ($p = 0.010$) (Fig. 5D). Also stromal expression of CDH11 correlated with disease-free survival ($p=0.051$, Fig. 5E), but no with prognosis ($p=0.105$). In addition, the combination of CALU with CDH11 expression displayed a significant association to disease-free survival (log-rank test $p$ value = 0.015) and overall survival (log-rank test $p$ value = 0.009) in human cancer samples (Fig. 5D). The rest of biomarkers did not reach significant correlation with metastasis or survival (Supplementary Fig. S5A-K).
DISCUSSION

The use of the AOM/DSS murine model of sporadic colon cancer for the isolation of purified and homogeneous populations of CAFs, together with in-depth proteomic analysis, allowed the identification of an elevated number of proteins deregulated after fibroblast activation in cancer. The identification of many of the currently known stromal biomarkers confirmed the validity of our experimental approach. As an example, we found an increase in collagens Type III and Type XII, which are mainly implicated in desmoplasia and colorectal cancer metastasis (10). At least six identified proteins: LTBP2, CDH11, FDPS, OLFML3, SPON2 and FSTL1 were novel candidate stromal biomarkers, as no expression was observed in the murine adenocarcinoma cell line CT26. In addition, CDH11 was useful to isolate colon cancer fibroblasts. The relevance of these biomarkers for CRC prognosis was confirmed by studies with human tissue microarrays. The expression of CDH11, FSTL1, OLFML3, and LTBP2 together with CALU, was increased in the tumoral stroma of patients with CRC. Moreover, we observed an association of CALU and CDH11 with poor survival and prognosis in human cancer patients. Together, these data support the value of the AOM/DSS murine model for the discovery of stromal biomarkers applicable in human patients.

Despite this, the AOM model presents some limitations. The most relevant is its inability to develop metastasis. Recently, an APC mutant mouse model of sporadic colon cancer was used to analyze the proximal fluid proteome of whole tumor tissues, without isolation of pure cell populations (24). Looking at the candidate protein biomarkers identified in that model, we have observed several coincidences in ECM fibroblast-related proteins: biglycan, cingulin, collagens, decorin or lamin A/C, confirming the relative similarity of both models.
CAFs exhibited specific proinflammatory and desmoplastic protein signatures for colon cancer. Several reports support the role of the desmoplastic microenvironment in tumor promotion (2). For instance, Tsujino et al (25) reported a strong association between presence of abundant stromal myofibroblasts, liver metastasis and shorter disease-free survival. In addition, it has been reported that tumor-derived ECM is stiffer than normal ECM. Moreover, increased matrix stiffness and ECM remodeling was observed in pre-malignant tissue. This increase was shown to contribute to malignant transformation in the breast (26). In colorectal cancer, previous studies showed a correlation between the presence of myofibroblasts (25), vimentin (27) or fibroblast activation protein (FAP) expression (28) with prognosis and recurrence.

Here, we identified several new prognostic markers associated to fibroblasts. Calumenin is a calcium-binding protein that regulates the activity of coagulation factors (29) and was reported as overexpressed in colon cancer (30, 31). Although calumenin expression is not restricted to the stroma, we have found a good association with stromal expression, lymph node invasion and lower survival, alone or in combination with other proteins like CDH11. Cadherin-11 is a mesenchymal cadherin, up-regulated by TGFβ, which has been identified as a novel target for pulmonary fibrosis and associated with invasion in squamous cell carcinoma (32, 33). The combination of CALU with CDH11 improved significantly the disease-free survival prognostic value.

We found several other TGFβ-related deregulated proteins as stromal biomarkers. OLFML3 belongs to the olfactomedin domain-containing proteins. They are known BMP antagonists (34) and showed proangiogenic activity in cancer (35). FSTL1 expression was the most specific for human stromal compartment. FSTL1 is a TGFβ-inducible gene, SPARC-related, that enhances inflammatory cytokine/chemokine expression (36) and seems to be implicated in angiogenesis and revascularization (37).
Another TGFβ signaling-related protein was LTBP2, which co-localizes with fibronectin and collagen type 1 for ECM regulation and remodeling (38, 39). LTBP2 is overexpressed in pancreatic cancer, being mainly located in the cancer stroma (40). TGFβ activates CAFs by changing the secretion profile of chemokines to assist CRC cells in the progression of the disease. An inflammatory microenvironment can promote malignant progression and participate in the recruitment and retention of infiltrating leukocytes to inhibit the resolution of inflammation (41, 42).

Evidence was provided of a significant increase in pro-inflammatory chemokines (CCL2, CCL11, CCL8, CCL20, CXCL5, IL-9) in the conditioned media of mouse CAFs either by mass spectrometry or chemokine microarrays. In addition to function as leukocyte chemoattractants and pro-angiogenic properties, human CCL2, CCL8 and IL-9 promoted tumorigenesis in two human colorectal cancer cell lines. CCL8, also called MCP2, a pluripotent chemokine, attracts monocytes (43) and lymphocytes (44). From our results, CCL2 and CCL8 regulated the proliferation, adhesion, migration and invasion of two colorectal cancer cell lines, whereas IL-9 was only efficient in tumor growth proliferation. Since CCL2 and CCL8 share, at least, 3 receptors (CCR2, CCR3 and CCR5) (45), this could explain the similar effect induced by both chemokines in colorectal cancer. In fact, several solid cancers, including CRC, showed expression of functional chemokine receptors on tumor cells, able to promote proliferation and metastasis (46). Then, CCL8 and IL-9 are new colon cancer-associated chemokines that deserve further investigation. Stromal chemokines might work either in paracrine or autocrine ways. CAFs and myofibroblasts could differentiate from resident tissue fibroblasts under various paracrine stimuli (47). In this regard, resident monocytes or other immune cells might secrete chemokines that activate the pro-inflammatory signature in CAFs by paracrine stimulation. Also, carcinoma cells...
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may produce chemokines that affect CAFs. In turn, CAFs secrete cytokines that can modulate tumor cells or other fibroblasts in different ways.

Our data provide a comprehensive view of the many molecules secreted by CAFs that favor the progression and invasion of tumors, from angiogenesis to desmoplasia and pro-fibrotic mechanisms. A panel of new stromal markers in colon cancer was identified. Among them, the combination of CDH11 with CALU gave a significant prognostic value. In addition, verification of the results with human samples confirmed the value of the model and the strategy for identification of novel candidate prognostic biomarkers with clinical potential. In summary, this study defines a new set of stromal biomarkers with good prognostic value that might constitute an alternative to current markers like α-SMA or the presence of myofibroblasts in colon cancer.
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ACKNOWLEDGEMENTS

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REFERENCES

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Table 1. Myofibroblastic signature associated to proteins\(^1\) in the CAFs secretome

<table>
<thead>
<tr>
<th>Accession Number</th>
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<th>Std Dev</th>
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1. Differentially-expressed proteins were identified as preferentially expressed in smooth muscle if they were among the top-5 tissues with highest expression using the Bio-GPS database.
Legend to Figures

Figure 1. Isolation and culture of fibroblast from colorectal tissue from AOM/DSS treated mice. A, Mice treated with AOM/DSS showed engrossed intestine with polypoid tumors, whereas mice treated with vehicle displayed a normal colon (left). Hematoxylin/eosin staining of tumoral and normal colon (right). B, Immunohistochemical analysis of cancer and normal tissue using antibodies against S100A4, Snail1 and α-SMA. C, Fibroblast isolation from colonic tissues by the explants technique between days 1 and 25. Tissues were cut into 2-3 mm fragments and placed in culture medium for 3 weeks to allow fibroblast migration. Top: Control Explants. Bottom: AOM/DSS explants. Explants are indicated with white arrows. D, Western blot analysis of isolated fibroblasts using antibodies against S100A4, Snail1 and α-SMA. Vimentin was used as positive fibroblast marker and EpCAM as negative control. B, D, Ratio AOM-DSS/Control was calculated for Snail1, α-SMA and S100A4 using ImageJ or QuantityOne 1-D Analysis Software, respectively.

Figure 2. Network functions affected in CAFs. Protein networks were identified by IPA using the 132 and 125 differentially-expressed proteins identified in whole cellular extract and secretome, respectively. A, “Dermatological Diseases and Conditions, Cellular Assembly and Organization, Cellular Function and Maintenance” network was identified in whole cellular extracts with a score of 47. The network consisted of 26 up-regulated and 5 down-regulated proteins from 34 direct-interacting proteins. B, “Cell Morphology, Hematological System Development and Function, Inflammatory Response” was significantly altered in the secretome with a score of 44. The network consisted of 27 up-regulated and 1 down-regulated proteins from 35 direct-interacting proteins.
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**Figure 3. Western Blot validation of iTRAQ data and biomarker expression on stromal cells.** Protein samples from A, whole cellular extract or B, concentrated conditioned media of CAFs and NFs were separated on SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with the indicated antibodies. Tubulin was used as a control. Protein abundance was quantified by densitometry and CAFs/NFs ratios were compared with the iTRAQ ratios. C, western blot analysis of the expression of selected stromal biomarkers in the murine adenocarcinoma cell line CT26 and CAFs using complete cell extracts and concentrated supernatants, respectively. D, Flow cytometry analysis for testing CDH11 and PDGFRα specificity (grey areas) on fibroblasts and cancer cell lines. An irrelevant antibody was used as a control (white areas).

**Figure 4. Chemokine expression in conditioned medium from CAFs and NFs.**

**Functional studies with selected chemokines.** A, A representative image of a chemokine microarray after screening of conditioned medium from CAFs and NFs. White arrows indicate the most deregulated cytokines in CAFs and NFs. B, Most abundant chemokines and cytokines differentially-expressed in CAFs versus NFs. Bar graphs were calculated in arbitrary units with the mean value of the duplicates present in the microarrays. C, RT-PCR analysis of the indicated cytokine receptors in KM12C and KM12SM cell lines. D, Proliferation, adhesion, migration and invasion of colon cancer KM12 cells in presence or absence of the indicated chemokines. Proliferation was determined by MTT assays after 24 h of culture. Cell adhesion to Matrigel was performed after 2 min of incubation with the indicated chemokines and 4 min of adhesion. Migration speed was calculated as the distance covered by the cells in 24h. Cell invasion was done across Matrigel of the KM12 cell lines treated as indicated.
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Experiments were performed three times. Error bars: Standard Deviation (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$).

**Figure 5. Validation of stromal biomarkers and prognostic value of CALU and CDH11 in patients with colorectal cancer.** A, Immunohistochemical staining of AOM/DSS cancer and normal mouse tissues using antibodies against CALU, CDH11, FSTL1, OLFML3 and LTBP2. B, immunohistochemical analysis of CALU, CDH11, FSTL1, OLFML3 and LTBP2 expression in human tissue microarrays. White arrows indicate the leading edge of the tumor. Pictures were taken at x200 magnification. C, Percentage of patients with invasion of regional lymph nodes at time of diagnosis in function of CALU stromal expression; $p$ values were calculated with Chi-square test. D, Kaplan–Meier analyses of overall and disease-free survival of patients according to the stromal expression of CALU or CDH11 and their combination, respectively. For statistical analysis of significance we employed the log-rank test.
Figure 1
Figure 2

Dermatological Diseases and Conditions, Cellular Assembly and Organization, Cellular Function and Maintenance

Cell morphology, Cellular Assembly and Organization, Cellular Function and Maintenance
Figure 3
Figure 4

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

A

Galectin-1  NPs  CAFs

Eotaxin  IL-6  CCL-20

CCL-5  CCL-2  CXCL-2

Galectin-1  CAFs  Eotaxin  IL-6  CCL-20

CCL-5  CCL-2  CXCL-2

B

Arbitrary units

C

KM12C  KM12SM

CCR1  CCR2  CCR3  CCR5  IL9R  GAPDH

D

KM12C  KM12SM

PROLIFERATION

Absorbance (560 nm)

KM12C  KM12SM

ADHESION

Adhered cells/mm²

KM12C  KM12SM

MIGRATION

Cell migration speed (µm/h)

KM12C  KM12SM

INVASION

Invasive cells
Figure 5
Clinical Cancer Research

PROTEOME PROFILING OF CANCER-ASSOCIATED FIBROBLASTS IDENTIFIES NOVEL PRO-INFLAMMATORY SIGNATURES AND PROGNOSTIC MARKERS FOR COLORECTAL CANCER

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