Proteome Profiling of Cancer-Associated Fibroblasts Identifies Novel Proinflammatory Signatures and Prognostic Markers for Colorectal Cancer

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Abstract

Purpose: Cancer-associated fibroblasts (CAF) 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. By using the explants technique, we purified CAFs and normal fibroblasts from colon tissues. Whole-cell extracts and supernatants were subjected to in-depth quantitative proteomic analysis by tandem mass spectrometry. Further validations of upregulated proteins in CAFs were carried out by chemokine microarray and immunohistochemical analyses of mouse and human tissues.

Results: Using a fold-change of 1.4 or more, we found 132 and 125 differentially expressed proteins in whole-cell extracts and supernatants, respectively. We found CAFs-associated proinflammatory and desmoplastic signatures. The proinflammatory 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, OLFLM3, 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 with disease-free survival and poor prognosis.

Conclusion: We have identified LTBP2, CDH11, OLFLM3, 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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Translational Relevance

Our knowledge of cancer-associated stroma is rather limited in comparison with that of cancer epithelial cells. Stroma constitutes the cancer microenvironment that nurtures cancer cells and facilitates invasion and metastasis. Moreover, stromal myofibroblasts have been associated with recurrence and survival. Novel biomarkers are required for the isolation and characterization of colorectal cancer–associated fibroblasts (CAF) to improve overall survival and recurrence prediction in colorectal cancer. We, here, describe a strategy based on an in-depth proteome profiling of CAFs obtained from a mouse model of human sporadic colorectal cancer. 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 proinflammatory and desmoplastic CAFs signatures for colorectal cancer. The value and relevance of these novel stromal biomarkers for colon cancer prognosis and survival were validated in human samples.

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 with 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).

With regard to human colon adenocarcinomas, CAFs synthesize ECM components such as fibronectin, tenascin, collagens types I, III, IV, V, and XII, and proteoglycans (biglycan, fibromodulin, perlecan, and versican; refs. 9, 10). In addition, they 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 techniques combined with a mouse model of colon cancer, cell isolation, and mass spectrometry (MS). We have selected the well-established murine model of colitis-associated cancer (CAC) based on the use of azoxymethane (AOM) and dextran sodium sulfate (DSS). Although the model does not progress to metastasis (11), it mimics quite well tumor progression of sporadic colorectal cancer. In fact, this model was used very useful for the elucidation of the role of TNF-α, interleukin (IL)-6, NF-κB, and other molecules in the initiation and progression of inflammation–associated cancer (see ref. 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 coculturing 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 an inbred mouse model reduces biologic variation due to genetic and environmental heterogeneity. Because 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 (NF) 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 normal fibroblasts. In silico studies demonstrated a predominant association of upregulated proteins to deposition of ECM, wound healing, and inflammation. Proinflammatory 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.

Materials 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-week-old) were weighed and given a single intraperitoneal injection of azoxymethane (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 into small pieces. Pieces of intestine were either cultured to isolate fibroblasts or fixed in 10% buffered formalin overnight for immunohistochemical analysis.

Cell line culture

KM12C and KM12SM human colon cancer cells were obtained from the laboratory of I. Fidler (MD Anderson Cancer Center, Houston, TX). 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 Dulbecco’s modified Eagle medium (DMEM; Gibco-Life Technologies) containing 10% fetal calf serum (FCS) and antibiotics at 37°C. Cells were tested for Mycoplasma but not authenticated, because...
these lines were originally isolated in Dr Fidler’s laboratory. CT26 murine colon cancer cell line was obtained directly from the American Type Culture Collection (which authenticated the cell line by short-tandem repeat profiling), and passaged for fewer than 6 months after receipt or resuscitation according to the manufacturer’s instructions.

**Fibroblast isolation and culture**

CAFs and normal fibroblasts 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 to 3 mm fragments and planted in fibroblast growth medium-2 (Lonza) containing 2% FCS and 1% penicillin/streptomycin/amphotericin B (Invitrogen) at 37°C in a 5% CO2-humidified atmosphere. Normal fibroblasts and CAFs grew around the explants and were cultured for approximately 3 weeks. Then, tissue fragments were removed, and cells were trypsinized and seeded at 70% to 90% confluence and cultured in complete medium. When cells reached 95% to 100% confluence (~1.7–2.0 × 10⁶ cells/cm²), they were washed with PBS, incubated with serum-free medium for 1 hour, washed again, and incubated for 24 hours in serum-free medium. Then, cells and conditioned medium were collected, pooled in two different batches for normal fibroblasts and CAFs, and mixed for performing the different experiments. We recovered 7.0 and 13.5 × 10⁶ cells from normal fibroblasts 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), according to the manufacturer’s instructions but with minor modifications. Briefly, 100 µg of protein from whole-cell lysate or 50 µg of protein from conditioned medium from normal fibroblasts and CAFs were digested with 5 µg trypsin (Promega) overnight at 37°C. Peptide mixtures were labeled with iTRAQ reagents: 114 and 116 for normal fibroblasts and 115 and 117 for CAFs as illustrated in Supplementary Fig. S1. To remove interfering substances, strong cation exchange (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 volume of 10 mmol/L KH₂PO₄ pH 3.0 containing 25% acetonitrile (ACN). Peptides were eluted in 1 mL of elution buffer (25% ACN, 350 mmol/L KCl, 10 mmol/L KH₂PO₄, pH 3.0). Then, peptides were desalted with Sep-Pak C18 cartridges (Waters), dried, and reconstituted in OFF-GEL solution (Agilent Technologies). All chemicals were obtained from Sigma-Aldrich.

**Peptide fractionation and MS 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 microliters 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% to 35% ACN in 0.1% aqueous solution of formic acid. The gradient was performed over 180 minutes using an Easy-nLC (Proxeon) at a flow-rate of 300 nL/min onto a Biosphere C18 column (75 µm, 16 cm, and 3 µm; NanoSeparations). Then, peptides were scanned and fragmented with a linear ion trap-Orbitrap Velos (Thermo Scientific). The Orbitrap Velos was operated in a data-dependent mode to automatically switch between MS and tandem mass spectrometry (MS–MS). Survey full-scan MS spectra were acquired from m/z 400 to 1,600 after accumulation to a target value of 10⁵ 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 collision-induced dissociation—higher energy collision dissociation (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 30 seconds. All MS data were analyzed and quantified with Proteome Discoverer (version 1.3.0.339; Thermo Scientific) using standardized workflows (see Supplementary Materials and Methods for a more detailed description).

**Mouse cytokine array**

Conditioned medium from CAFs and normal fibroblasts were collected after 24 hours in serum-free medium and incubated with Mouse Cytokine Antibody Array C6 for semiquantitative analysis of 97 mouse cytokines (RayBiotech) according to the manufacturer’s instructions. Then, membranes were scanned and analyzed using Redfin, a two-dimensional gel image analysis software (Ludesi). Relative semiquantitative cytokine intensities were normalized in comparison with 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 more than 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 DMEM (Life Technologies) containing 10% FCS and antibiotics at 37°C in a 5% CO₂-humidified atmosphere. Results were measured at 24 hours as described in Supplementary Materials and Methods.
atmosphere. Invasion, migration, adhesion, and cell proliferation assays were carried out by 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 Materials and Methods for a detailed description.

**Immunohistochemical analysis**

Pieces of intestine from AOM/DSS–treated or control mice were fixed in 10% buffered formalin overnight to perform hematoxylin and eosin (H&E) 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 Díaz (Madrid, Spain) and followed in the long term, were included for the study. Human samples were prepared as described previously (18). All human biopsies were obtained with the patient’s consent and approval of the Ethical Committee of Hospital Fundación Jiménez Díaz in accordance with the official Spanish 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 minutes, 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 3,3′-diaminobenzidine (DAB) as the chromogen and hematoxylin for counterstaining, and observed in an Olympus microscope. Imagel software was used to quantify the DAB staining of immunohistochemical images.

**Bioinformatics**

Ingenuity Pathway Analysis (IPA; Ingenuity systems, Inc.) was used to analyze the predicted biologic functions of the proteins deregulated in CAFs and to determine protein interactions as well as for network analysis. BioGPS software was used to determine the top-5 tissues with highest expression of each altered protein compartment to determine the desmoplastic signature (10). FatiGo was used to get further insight in deregulated functions using a cutoff value of less than 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 histologic staining (Fig. 1A). Mouse adenocarcinomas were equivalent to human colorectal adenocarcinomas, corresponding to a well-differentiated infiltrating endorectum adenocarcinoma, with the presence of large numbers of flat and polypoid malignant tumors. The mucosa from control mice was normal, without dysplastic changes (Fig. 1A). On 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 normal fibroblasts in cultures, distal colons from 16 AOM/DSS–treated and vehicle-treated mice were cut into small fragments and placed in culture. Fibroblasts migrated outside the explants and expanded (Fig. 1C). Cells at 95% to 100% confluence (~2 × 10⁴ cells/cm²) were collected, divided into two fractions, and mixed for iTRAQ experiments. On Western blot analysis, it was confirmed that AOM/DSS–isolated fibroblasts had higher expression of markers corresponding to activated fibroblasts such as α-SMA, S100A4, and Snail1 (Fig. 1D). The 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 (cell lysates) and 50 μg (conditioned media) of proteins from cultures of CAFs and normal fibroblasts were trypsin-digested. Peptides were labeled with iTRAQ for relative quantification and fractionated with OFFGEL, pH 3–10. To avoid biases in peptide labeling that could affect the final quantification, we performed two replicate analyses using the 114 and 116 iTRAQ tags for normal fibroblasts 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-cell 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 housekeeping proteins in the whole-cell extract or the median in the secretome (Supplementary Table 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, including: (i) a fold-change of 1.4 or more relative to control samples; (ii) proteins present in both replicates; (iii) expression ratios following a similar trend in both replicates; and (iv) 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 fold-change of 1.4 as a minimum value to include all relevant proteins that showed systematic change and to compensate for the compression of iTRAQ ratios, which leads to underestimation of fold-changes (20). Using a fold-change of 1.4 or more, we found 132 proteins deregulated in complete cell lysates, 109 upregulated, and 23 downregulated (Supplementary Table S4), as well as 125 proteins in the secretome, with 72 upregulated and 53 downregulated (Supplementary Table S5).

Gene Ontology analysis of whole-cell extracts results from CAFs showed a clear upregulation of ECM proteins, including: CCL2 (10 ng/mL), CCL8 (50 ng/mL), and IL-9 (2 ng/mL).
constituents (collagen α1 types I, V, III, and XII, collagen α2 types I and V, and fibulin 2), proteins for matrix assembly (decorin, prolyl 4-hydroxylase α2, and leprecan 1), proteins related to TGF-β signaling, such as LTBP2 (latent TGF-β-binding protein 2), cadherin 11, fibrillin 1, and insulin-like growth factor (IGF) 2 receptor (IGF2R; Supplementary Table S6). In the secretome, 26 of 125 proteins were related to ECM. In addition, we identified differentially expressed chemokines, such as CCL11, CCL8, CXCL5, or CCL2, IGF-related proteins such as IGFBP7, or IGF1, cell adhesion, and wound response (Supplementary Table S7). Collectively, these data suggest that activated fibroblasts participate in ECM remodeling, wound healing, and epithelial differentiation, and are important regulators of inflammation.

Altered biologic functions, networks, and pathways were analyzed using IPA (Supplementary Table S8) and FatiGO (Supplementary Table S9). Using IPA in whole-cell 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 the secretome, we found “Cancer” to be the most represented biologic 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 biologic function altered (n = 54 proteins and P values ranging from 2.55E−14 to 1.54E−04). With regard to altered network function “Dermatological Diseases and Conditions, Cellular Assembly and Organization, Cellular Function and Maintenance” with 31 proteins emerged 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-cell extract. Transport of proteins and their cellular localization emerged as the top altered biologic processes. In the secretome, the ECM and focal adhesion were the most altered processes. In addition, we observed a focus toward modulation of molecular functions through receptor binding, whereas the most altered
Figure 2. Network functions affected in CAFs. Protein networks were identified by IPA using the 132 and 125 differentially expressed proteins identified in whole-cell extract and secretome, respectively. A, "Dermatological Diseases and Conditions, Cellular Assembly and Organization, Cellular Function and Maintenance" network was identified in whole-cell extracts with a score of 47. The network consisted of 26 upregulated and five downregulated 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 upregulated and one downregulated proteins from 35 direct-interacting proteins.
biologic processes were the response to wounding, external stimulus, and inflammatory response. Collectively, these protein networks indicate the association of CAF proteins with desmoplastic and proinflammatory signatures, respectively.

**Validation and fibroblast specificity of selected biomarkers**

After previous network analyses and data-mining using the GeneCards database, we selected top upregulated proteins that were not previously associated to colon cancer and pertained to major networks (Fig. 2 and Supplementary Tables S8 and S9). Therefore, 11 differentially expressed proteins (five from whole-cell extract and six from secretome) were validated using Western blot analysis. 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. Results of Western blot analysis were consistent with the iTRAQ quantification data (Fig. 3A and B). Molecules such as CRABP1, LTBP2, FDPS, and CDH11 were upregulated in whole-cell extracts (Fig. 3A), whereas OLFM3, CALU1, AEBP1, LMOD1, SPON2, and FSTL1 were upregulated in CAFs supernatants (Fig. 3B). 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 real-time PCR (RT-PCR) and Western blot analysis. Protein and mRNA expression values were similar between purified fibroblasts and original tissues (Supplementary Fig. S4A). Therefore, 11 differentially expressed proteins (five from whole-cell extract and six from secretome) were validated using Western blot analysis (Supplementary Fig. S3).

To confirm fibroblast specificity of stromal biomarkers, we compared the presence of these proteins in CAFs with regard to CT26—a colon adenocarcinoma murine cell line—by Western blot analysis. Expression of LTBP2, FDPS, CDH11, and OLFM3 was specific or clearly upregulated in fibroblasts with regard to the epithelial CT26 cells (Fig. 3C). When we tested the supernatants, SPON2 and FSTL1 were also specific for fibroblasts. Finally, we investigated the capacity of CDH11 for cell sorting of fibroblasts. PDGF receptor α (PDGFRα) was used as a positive control (7). CDH11 antibodies were able to isolate fibroblast cell lines, such as mouse NIH-3T3 or human BJ-hTERT, but did not work with epithelial cell lines such as CT26 or RKO (Fig. 3D). These results suggested that all these six proteins were stromal biomarkers specific for colon cancer.

**Cytokines and growth factors deregulated in the secretome of activated fibroblasts are proinflammatory and protumorigenic 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 MS, we observed a significant increase in the expression of cytokines such as CCL11 (Eotaxin-1), CCL8 (MCP-2), CCL2 (MCP-1), SAA3, and CXCL5 (ENA-78), whereas CSF1 was downregulated in CAFs (Supplementary Table S7). To investigate other additional changes in cytokines, we used a mouse chemokine microarray for CAF/NF 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 proinflammatory cytokines such as 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 NF-κB–dependent cancer-promoting activity (7).

To be activated by cytokines, cancer cells should express the corresponding receptors. To detect CCL2 and CCL8 receptors (CCR1, 2, 3, and 5) as well as the receptor for IL-9 (IL-9R), we performed RT-PCR assays with KM12C and SM cells. These assays showed that both cell lines express IL-9R as well as 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 more than 2-fold increase was observed at the adhesion, migration, and invasion level in KM12 cells, after addition of CCL2 and CCL8, but there was 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**

Because activated fibroblasts are characterized by the acquisition of a myofibroblast phenotype consisting of different smooth–muscle-like proteins, such as 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 and PCOLCE), fibronectin, proteoglycans (perlecan and hyaluronic), cytokines (CCL11, CCL8, and CCL2), and calcium metabolism proteins (calumenin (CALU1), IGF-binding proteins (IGFBP6 and -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 datasets from 274 samples of colon cancer. Using Kaplan–Meier for overall survival analysis, we found some combinations of proteins with prognostic value from whole-cell extract (Supplementary Fig. S4A) and the secretome (Supplementary Fig. S4B). The most significant combination
Figure 3. Western blotting validation of iTRAQ data and biomarker expression on stromal cells. Protein samples from whole-cell extract (A) or concentrated conditioned media (B) of CAFs and normal fibroblasts 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 CAF/NF 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 cytometric analysis for testing CDH11 and PDGFRα specificity (gray areas) on fibroblasts and cancer cell lines. An irrelevant antibody was used as a control (white areas).
Survival (stromal expression of CDH11 correlated with disease-free survival (log-rank test \( P = 0.000095 \)). In contrast, the association with 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 because of 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 with the 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 CAI 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 more than 70% of the tumors for CALU, CDH11, and OLFML3, but no significant expression was detected for 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 overexpressed (Fig. 5B).

Then, we determined if the expression of these proteins in the stroma was associated with survival in human cancer using the tissue microarray data for patients followed on the long term (>5 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). In addition, stromal expression of CDH11 correlated with disease-free survival (\( P = 0.051 \); Fig. 5E), but not with prognosis (\( P = 0.105 \)). In addition, the combination of CALU with CDH11 expression displayed a significant association with disease-free survival (log-rank test \( P = 0.013 \) and overall survival (log-rank test \( P = 0.009 \)) in human cancer samples (Fig. 5D). The rest of the 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 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 colorectal cancer 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 colorectal cancer. Moreover, we observed an association of CALU and CDH11 with poor survival and prognosis in human patients with cancer. 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 azoxymethane model presents some limitations. The most relevant is its inability to develop metastasis. Recently, an adenomatous polyposis coli (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, Tsujiino and...
colleagues (25) reported a strong association between the 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 premalignant 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 expression of fibroblast activation protein (FAP; ref. 28) with prognosis and recurrence.

Here, we identified several new prognostic markers associated with fibroblasts. Calumenin is a calcium-binding protein that regulates the activity of coagulation factors (29), and was reported as being 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 such as CDH11. Cadherin-11 is a mesenchymal cadherin, upregulated by TGF-β, 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 significantly improved the prognostic value of disease-free survival.

We found several other TGF-β–related deregulated proteins as stromal biomarkers. OLFML3 belongs to the olfactomedin domain-containing proteins. They are known BMP antagonists (34), and also showed proangiogenic activity in cancer (35). FSTL1 expression was the most specific for the human stromal compartment. FSTL1 is a TGF-β–inducible gene, SPARC-related, that enhances inflammatory cytokine/chemokine expression (36) and seems to be implicated in angiogenesis and revascularization (37). Another TGF-β signaling-related protein was LTBP2, which colocalizes with fibronectin and collagen type I for ECM regulation and remodeling (38, 39). LTBP2

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**Table 1. Myofibroblastic signature associated with proteins in the CAF secretome**

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*Differentially expressed proteins were identified as preferentially expressed in smooth muscle if they were among the top five tissues with highest expression using the Bio-GPS database.*
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 ×200 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 χ² 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 used the log-rank test.
is overexpressed in pancreatic cancer, being mainly located in the cancer stroma (40). TGF-β activates CAFs by changing the secretion profile of chemokines to assist colorectal cancer 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 proinflammatory chemokines (CCL2, CCL11, CCL8, CCL20, CCL5, and IL-9) in the conditioned media of mouse CAFs, either by MS or chemokine microarrays. In addition to function as leucocyte chemotractants and proangiogenic 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. Because CCL2 and CCL8 share at least three receptors (CCR2, CCR3, and CCR5; ref. 45), this could explain the similar effect induced by both chemokines in colorectal cancer. In fact, several solid cancers, including colorectal cancer, showed expression of functional chemokine receptors on tumor cells, able to promote proliferation and metastasis (46). Therefore, CCL8 and IL-9 are new colon cancer–associated chemokines that deserve further investigation. Stromal chemokines might work through either paracrine or autocrine pathways. 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 proinflammatory signature in CAFs by paracrine stimulation. In addition, carcinoma cells 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 profibrotic 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 such as α-SMA or the presence of myofibroblasts in colon cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M.J. Fernandez-Aceñero, A. García de Herreros, I. Casal Development of methodology: S. Torres, M. Mendes, M.J. Fernandez-Aceñero, C. Petía Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Torres, R.A. Bartolomé, R. Barderas, M.J. Fernandez-Aceñero, A. Peláez-García, M. Lopez-Lucendo, R. Villar-Vázquez, A. García de Herreros, F. Bonilla Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): S. Torres, R.A. Bartolomé, M. Mendes, R. Barderas, M.J. Fernandez-Aceñero, R. Villar-Vázquez, I. Casal Writing, review, and/or revision of the manuscript: S. Torres, M. Mendes, M.J. Fernandez-Aceñero, F. Bonilla, I. Casal Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Torres, C. Petía, M. Lopez-Lucendo, R. Villar-Vázquez Study supervision: I. Casal

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