

Functional Heterogeneity of Cancer-Associated Fibroblasts from Human Colon Tumors Shows Specific Prognostic Gene Expression Signature

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Abstract

Purpose: Cancer-associated fibroblasts (CAF) actively participate in reciprocal communication with tumor cells and with other cell types in the microenvironment, contributing to a tumor-permissive neighborhood and promoting tumor progression. The aim of this study is the characterization of how CAFs from primary human colon tumors promote migration of colon cancer cells.

Experimental design: Primary CAF cultures from 15 primary human colon tumors were established. Their enrichment in CAFs was evaluated by the expression of various epithelial and myofibroblast specific markers. Coculture assays of primary CAFs with different colon tumor cells were performed to evaluate promigratory CAF-derived effects on cancer cells. Gene expression profiles were developed to further investigate CAF characteristics.

Results: Coculture assays showed significant differences in fibroblast-derived paracrine promigratory effects on cancer cells. Moreover, the association between CAFs' promigratory effects on cancer cells and classic fibroblast activation or stemness markers was observed. CAF gene expression profiles were analyzed by microarray to identify deregulated genes in different promigratory CAFs. The gene expression signature, derived from the most protumorigenic CAFs, was identified. Interestingly, this "CAF signature" showed a remarkable prognostic value for the clinical outcome of patients with colon cancer. Moreover, this prognostic value was validated in an independent series of 142 patients with colon cancer, by quantitative real-time PCR (qRT-PCR), with a set of four genes included in the "CAF signature."

Conclusions: In summary, these studies show for the first time the heterogeneity of primary CAFs' effect on colon cancer cell migration. A CAF gene expression signature able to classify patients with colon cancer into high- and low-risk groups was identified. *Clin Cancer Res*; 19(21); 5914–26. ©2013 AACR.

Introduction

Tumor epithelial cells within a tumor coexist with a complex microenvironment. In this microenvironment, extracellular matrix, growth factors, cytokines, and a variety of nonepithelial cell types, including vascular space-related cells (endothelial cells, pericytes, and smooth-muscle cells), inflammatory response cells (lymphocytes, macrophages,

and mast cells), and fibroblasts, come together (1). In this complex scenario, a variety of interactions take place between its various components and determine a series of events, such as tumor cell proliferation, metastatic potential, and location of metastases (2, 3).

Fibroblasts are one of the most active cell types of the stroma. They form the basic cell component of the connective tissue that contributes to structural integrity, as well as the maintenance of the extracellular matrix (4). In addition, fibroblasts from normal tissues carry out tissue repair functions under certain physiologic conditions (5). Fibroblasts of the tumor stroma have received various names: tumor-associated fibroblasts, carcinoma-associated fibroblasts (CAF), or myofibroblasts (6). They are similar to those found in the wound-healing process, although CAFs are constantly activated (6). In this state of permanent activation, fibroblasts promote tumor growth and tumor progression, favoring a variety of tumor-specific mechanisms (7). Thus, CAFs are involved in various tumor mechanisms, such as extracellular matrix remodeling, release of soluble factors, regulation of tumor cell motility, and the

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Translational Relevance

Findings reveal, for the first time, the heterogeneity, among different patients, of activated cancer-associated fibroblasts effect on cancer cells. This fibroblast heterogeneity defines a gene expression signature able to classify patients with colon cancer into high- and low-risk groups, with important implications: first, in the ongoing efforts to identify colon cancer cell prognostic markers in colon cancer progression; and, second, emphasizes the search of new treatments to arrest fibroblast–epithelial interactions to interfere colon cancer progression.

specialization of tumor metabolism or tumor cell implantation (8–12).

CAFs' activated phenotype is characterized by expression of different proteins, such as α -smooth muscle actin (α -SMA), fibroblast activation protein (FAP), fibroblast-specific protein 1 (FSP1) growth and angiogenic factors. However, CAFs comprise a heterogeneous population of cells and are derived from different sources (13). The CAF heterogeneity observed may reflect the variation of CAF involvement in cancer progression.

In this study, we established experiments to test the capability of primary colon CAFs to promote tumorigenesis of colon cancer cells and found that CAFs work in paracrine fashion in this direction to augment cancer cell migration. The differential promigratory capacity of CAFs identified a gene expression signature that allows classification of patients with colon cancer into high- and low-risk groups.

Materials and Methods

Establishment of primary colon CAFs and normal fibroblasts

Fresh tissue from 15 patients operated for colorectal primary tumors at the Puerta de Hierro-Majadahonda University Hospital was used for the propagation of primary CAFs and normal fibroblasts (NF). Informed written consent was obtained from all participants, as required and approved by the Research Ethics Board of the Puerta de Hierro-Majadahonda University Hospital. For CAF and NF establishment and growth details, see Supplementary Information.

Determination of fibroblast senescence and immunofluorescence

Immunofluorescence analyses followed standard procedures with monoclonal anti-pan cytokeratin (ab6401; Abcam), monoclonal anti-vimentin (ab92547; Abcam), and monoclonal anti- α -SMA (ab7817; Abcam). For details, see Supplementary Information.

To characterize colon fibroblasts, their senescence status was evaluated by the use of the Senescence β -Galactosidase Staining Kit (Abcam), in line with the manufacturer's instructions (details in Supplementary Information).

Collagen gel contraction assay

Collagen gels were prepared with Type I collagen (1.5 mg/mL; Rat Tail Tendon Collagen, RTTC; BD Biosciences), Dulbecco modified Eagle's medium (DMEM; supplemented with penicillin and streptomycin, 200 U/mL, and 2 mmol/L L-glutamine), gel contraction buffer (20 mmol/L HEPES, 0.22% NaHCO₃, and 0.005N NaOH), 20% FBS and CAF cells. The mixture (500 μ L) was cast into each well of a 24-well culture plate. The solution was then allowed to polymerize at 37°C for 30 minutes. After polymerization, the gels were gently released from the plate and transferred into 100-mm tissue culture dishes. After 24 hours, the surface area of the gels was measured. The values of the fibroblast gels were normalized to that of the control gel without cells.

Coculture of tumor colon cells and human colon primary CAFs or NFs for migration assays

Colorectal tumor cell lines LIM1215 or SW480-ADH were cocultured with colon primary CAFs or NFs for migration assays. Cells were cocultured with physical separation, in a setting in which primary CAFs or NFs were seeded in the lower compartment of a Transwell system and colon cells in the upper one (Corning Incorporated; Costar; #3428). After 48 hours, epithelial cells that had reached the lower surface of the filter were measured by fluorescence.

Osteoblast differentiation assay

CAFs were differentiated by adding human Mesenchymal Stem Cell Osteogenic Differentiation Medium (Lonza; PT-3002) to a confluent culture of fibroblasts for 16 days, following the manufacturer's protocol, whereas CAF control cells were maintained with fibroblast basal medium (FBM). Osteoblast differentiation is marked by the formation of mineralized nodules composed of inorganic hydroxyapatite and other organic components. Thus, mineralization was evaluated with the OsteoImage Mineralization Assay (Lonza; PA1503), in line with the manufacturer's instructions, and quantitatively measured by a plate reader (Tecan Infinite 200Pro.).

Affymetrix gene chip hybridization

Total RNA was extracted from CAF samples with TRIzol reagent (TRIzol, Invitrogen-Gibco), in line with the manufacturer's protocol. In accord with the Affymetrix protocol (Affymetrix expression manual), all samples were processed and 2 μ g of fragmented and labeled cDNA was hybridized to the Affymetrix GeneChip arrays (Human Gene 1.0 ST). See Supplementary Information for further details.

Microarray data analysis

Microarray data were normalized and differential expression analyses were conducted, using Bioconductor (14) packages "affy" (ref. 15; version 1.28.1) and Limma (16), respectively. Raw data and normalized microarray expression data were submitted to the GEO database under the accession number GSE51257. To determine the differential

expression profiles of CAF, the expression value from two NF sample averages was subtracted from every CAF sample. For "significant" differential expression genes, we set the P value cutoff to 0.05 or less and the Log₂ fold change (Log₂FC) cutoff at 0.5. Gene Ontology (ref. 17; www.geneontology.org) functional enrichment analysis and heat-map viewing of distinctly expressed genes were based on the Gtools program (ref. 18; www.gitools.org). Using microarray expression data, we determined with ANOVA statistics the CAF gene signatures that were significantly altered ($P \leq 0.05$) in "gene-normalized" (see Supplementary Information for details) expression data, when comparing higher versus lower migratory groups.

Public microarray data and survival analysis

A cohort of 232 patients with colon cancer from the GEO database (GEO accession GSE17538) was used to assess the prognostic value and clinical relevance of "CAF signatures" in colon cancer. Public microarray data on colon cancer were normalized as described earlier. We then used Gtools for sample level enrichment analysis (SLEA; ref. 19) with modular Z -score enrichment statistics (18). Positive and negative z scores indicated significantly higher or lower expression levels of genes in the module (CAF signature and Golgi genes), respectively. We grouped the colon cancer samples according to their modular expression; and Kaplan–Meier survival curves and the Cox HR were then calculated with the R statistical program package, "survival" and "survplot."

qRT-PCR

RNA extraction, target gene mRNA quantification, and synthesis of cDNA were conducted as described elsewhere (20).

The sequences for PCR primers are listed in Supplementary Table S1.

At the end of the PCR cycles, melting curve analyses were conducted to confirm the specificity of the amplification reactions. The PCR products were sequenced in an ABI Prism 377 DNA sequencer apparatus (PE Applied Biosystems).

Statistical analysis

Comparisons between gene expression levels, collagen contractile abilities in different promigratory CAFs, and between CAF and NF migration effects on cancer cells were contrasted using the Student t test. All t tests were performed after evaluation of equality of variance with the Levene test. Two-tailed P values of 0.05 or less were taken as giving statistical significance.

Patients and analysis of "CAF signature" validation

The study is based on a consecutive series of 142 patients undergoing surgery for colon cancer between January 2002 and December 2006. Informed written consent was obtained from all participants, as approved by the Research Ethics Board of Puerta de Hierro Majadahonda University Hospital. Colon cancer–derived samples were obtained

immediately after surgery, snap-frozen in liquid nitrogen and stored at -80°C until processing.

The number of patients in which the detection of the target gene was possible and percentile data of each gene mRNA quantification are detailed in Supplementary Table S2. mRNA expression data of these genes were divided by quartiles (25%, 50%, and 75%) to get four groups of patients and develop the statistical analysis of Kaplan–Meier survival curves and the Cox HR, as previously described. Statistical analysis revealed similar behavior of different gene expression data quartiles of each gene. Therefore, to simplify the analysis, those quartiles were grouped according to mathematical behavior and thus, the variables were dichotomized into "High" and "Low" gene expression levels.

Results

Primary CAFs isolated from colon tumors show activated fibroblast features

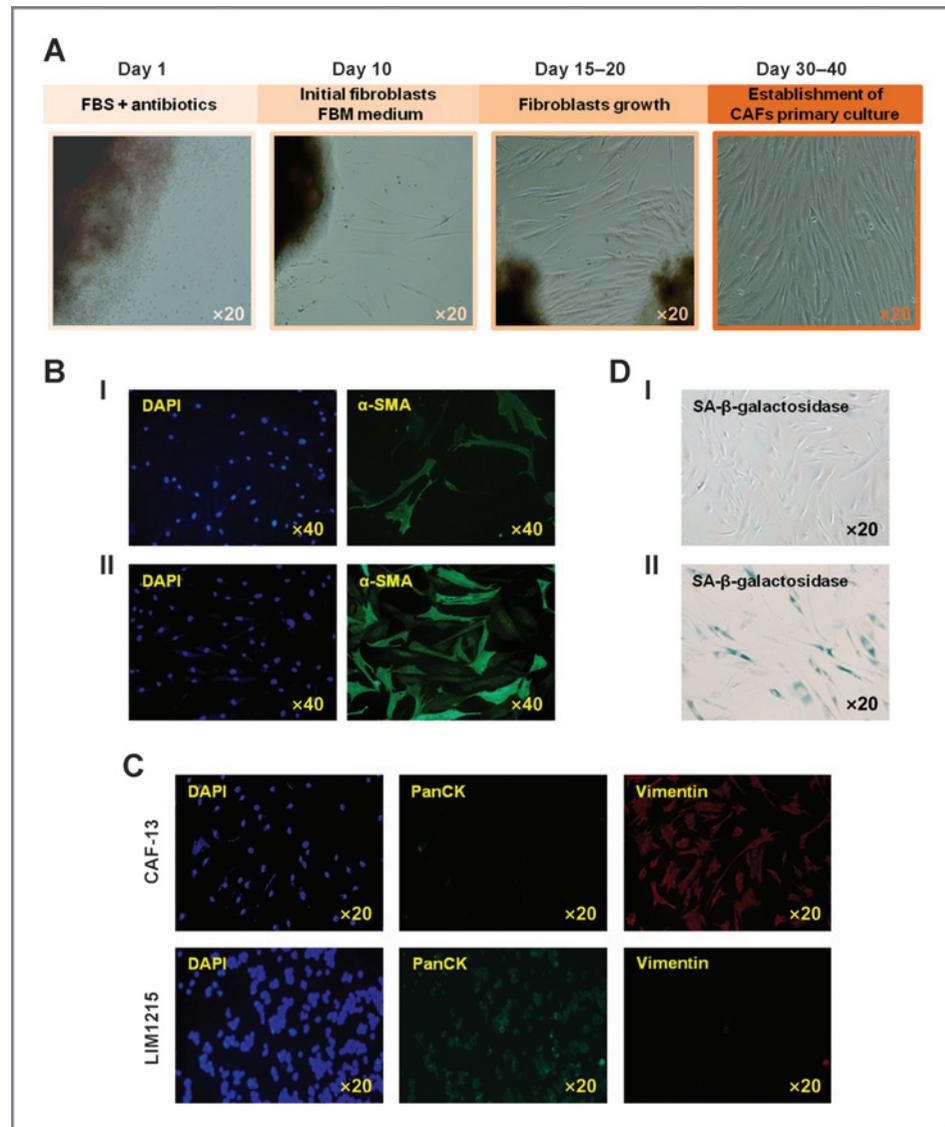
Fresh tissue from primary tumors of 15 patients operated on for colorectal cancer in our hospital was used for the propagation of primary CAFs, as described in the Materials and Methods section (Fig. 1A). Then, morphology and immunohistochemistry were characterized to assess fibroblast characteristics. Fibroblast cultures maintained the phenotypic properties of activated fibroblast or myofibroblast even in absence of interaction with carcinoma cells. Primary CAFs initially showed bi- and/or multipolar morphology and then acquired a uniform spindle-shaped morphology and formed parallel arrays and whorls at confluence. Moreover, expression of α -SMA in primary CAFs was also confirmed (Fig. 1B). To evaluate the CAF enrichment of the culture, Vimentin and Pan Cytokeratin (PanCK) were analyzed for immunofluorescence (Fig. 1C). CAFs were studied and maintained in culture for up to seven passages to evaluate their senescence status, so as to avoid studies with CAFs under replicative senescence. Two CAFs had a high degree of senescence and were excluded from the study (Fig. 1D).

Primary colon CAFs induce different paracrine promigratory effects on cancer cells

A set of coculture experiments with CAFs and two colon cancer lines was carried out to assess the migration-promoting effects of 15 human primary CAFs on colon cancer cells.

Primary CAFs induced a significant increase of cocultured colon cancer cell migration in a setting in which colon cells are seeded in the upper chamber of the Transwell culture system and fibroblasts in the lower one. After 48-hour coculture, epithelial cells that had reached the lower surface of the filter were recovered by trypsinization and counted by fluorescence to assess cell migration. Interestingly, we observed significantly large differences of fibroblast-derived paracrine promigratory effects on cancer cells in CAFs from different patients. Thus, primary CAFs induced LIM1215 cancer migration between 5- and 140-fold for LIM1215 migration without CAF coculture stimulation. On the

Figure 1. Establishment and characterization of primary CAFs from patients with colorectal cancer. Schedule of primary CAF establishment from fresh tissue of primary tumors from patients with colorectal cancer (A). Although at different levels, all CAFs showed α -SMA expression. In the images, examples of a low and a high α -SMA-expressing CAF are given (BI and BII, respectively). The absence of epithelial cells in the established primary cultures was confirmed by PanCK and Vimentin immunostaining. LIM1215 colon cells were used as positive control for PanCK immunostaining (C). Senescence-associated β -galactosidase staining was used to evaluate the degree of senescence of primary CAF cultures (DI showed a negative CAF culture and DII, a positive CAF culture for senescence-associated β -galactosidase staining).



basis of these data, CAFs were classified into four subtypes as follows: "Low" (L) promigratory group, which includes those CAFs unable to increase LIM1215 migration more than 10-fold; "Medium" (M) promigratory group, with CAFs showing a 10- to 25-fold increase in colon cell migration; "High" (H) includes CAFs with a 25- to 50-fold induction of colon cell migration; and the "Extra-high" (EH) promigratory group contains those CAFs able to enhance colon cell migration more than 50-fold (Fig. 2A).

No association was observed between changes in CAFs' subculture passages/population doubling number and CAFs' derived paracrine promigratory effects on cancer cells (Supplementary Fig. S1A).

The differences in the fibroblast-derived paracrine promigratory effects of CAFs on cancer cells were validated with randomized CAFs from 4 different patients using SW480-ADH cells. Similar results to those previously observed with LIM1215 colon cells were found (Fig. 2B). Moreover, a clear

correlation between CAF-derived migration induction of LIM1215 and SW480-ADH was observed (Fig. 2C).

Finally, the promigratory effects on cancer cells of six human primary NFs were also analyzed. Although cancer cell migration was induced by NFs about migration without fibroblasts' coculture stimulation, promigratory CAF-induced effects were higher than NF effects (Supplementary Fig. S1B).

Taken together, these experiments demonstrated that the promigratory effects of human CAFs on colon cancer cells varied in different patients.

Enhanced promigratory capacity of CAFs correlates with activated fibroblast features

Various analyses were performed to evaluate whether most promigratory CAFs showed activated fibroblast features.

The expression levels of α -SMA were evaluated in the human primary CAFs. Those CAFs that increased colon

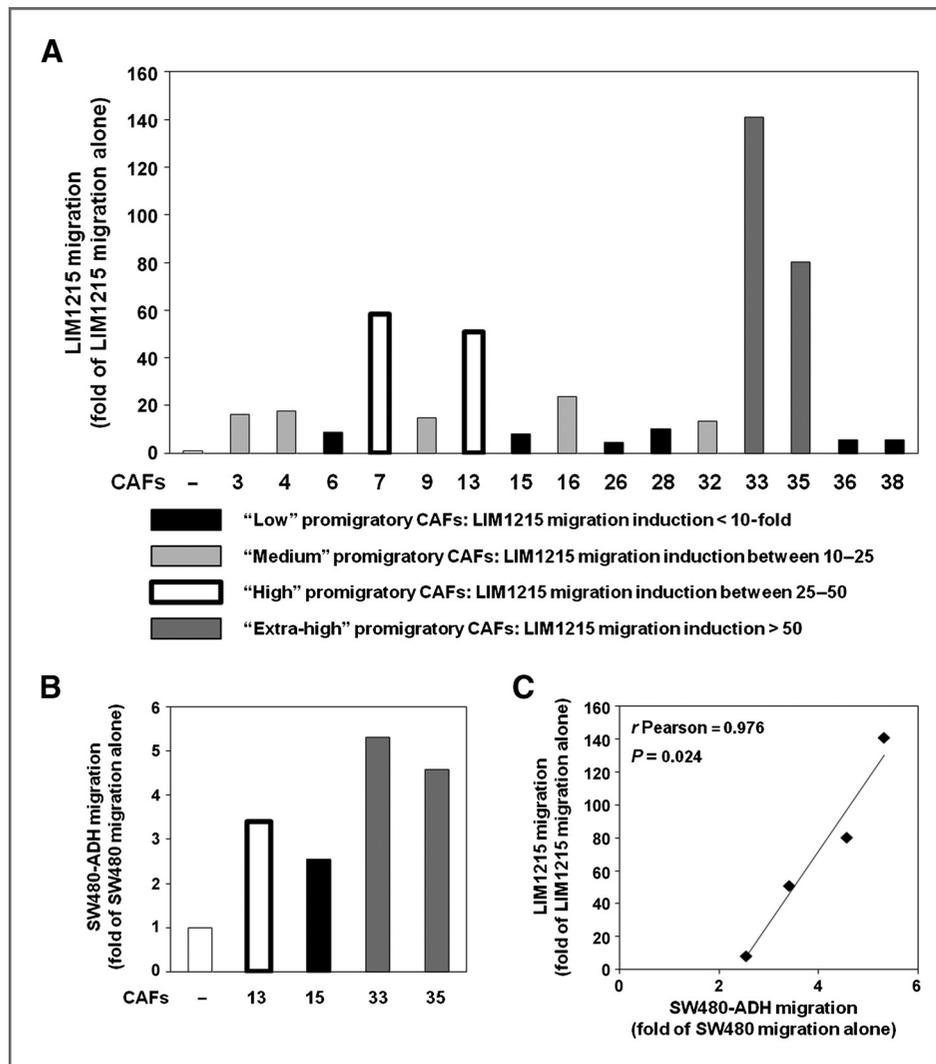


Figure 2. Differences of human primary CAF-derived paracrine promigratory effects on colon cancer cells. Primary CAFs were classified for their ability to stimulate LIM1215 colon cell migration as "Low," "Medium," "High," and "Extra-high" migration groups. The number in the X-axis denotes internal colon patient IDs (A). Promigratory enhancement of colon cell migration was also validated in SW480-ADH colon cells with four randomized CAFs (B). Migratory stimulation of LIM1215 and SW480-ADH cells, depending on cocultured CAFs, showed statistical correlation (C).

cancer cell migration more than 25-fold ("H" and "EH" CAFs) had higher α -SMA mRNA expression levels than CAFs from the "L" or "M" promigratory group (Fig. 3A).

In parallel, the collagen contractile abilities of primary CAFs were also evaluated as a marker of activated fibroblasts. Therefore, the collagen contraction assay was performed in one selected representative CAF from each migration group. A clear correlation was observed between the induction of collagen gel contraction and promigratory effects on cancer cells (Fig. 3B).

These data together indicate a clear association between CAFs' promigratory stimulation of colon cancer cells and the activation grade of fibroblasts.

Most promigratory CAFs showed stem cell markers

The possibility that most promigratory CAFs had a stem cell-like phenotype was evaluated.

SOX2, *OCT3*, *KLF4*, *LIN28*, and *NANOG* genes were used as stem cell markers and their expressions were determined in colon CAFs. Quantitative analysis showed higher expres-

sion levels of all analyzed genes in the "EH" and "H" promigratory groups than in the "M" and "L" subtypes. It should be noted that a significant statistical association between *OCT3* levels and CAFs' promigratory effects on colon cancer cells was observed (Fig. 3C). Moreover, a trend toward statistical significance was observed for *SOX2* and *LIN28* gene expression and CAFs' promigratory abilities. For the genes *KLF4* and *NANOG*, the same trend was observed, but statistical association was not reached, probably because of the small number of samples analyzed and the large difference within CAF groups indicated by SD (Fig. 3C).

The pluripotency of different CAFs was also analyzed by osteoblast differentiation experiments in one selected representative CAF from each migration group.

Interestingly, CAFs showed osteoblast differentiation under osteogenic induction medium, assessed by a mineralization quantification assay. However, no association between osteoblast induction and CAF promigratory abilities was found (Supplementary Fig. S1C).

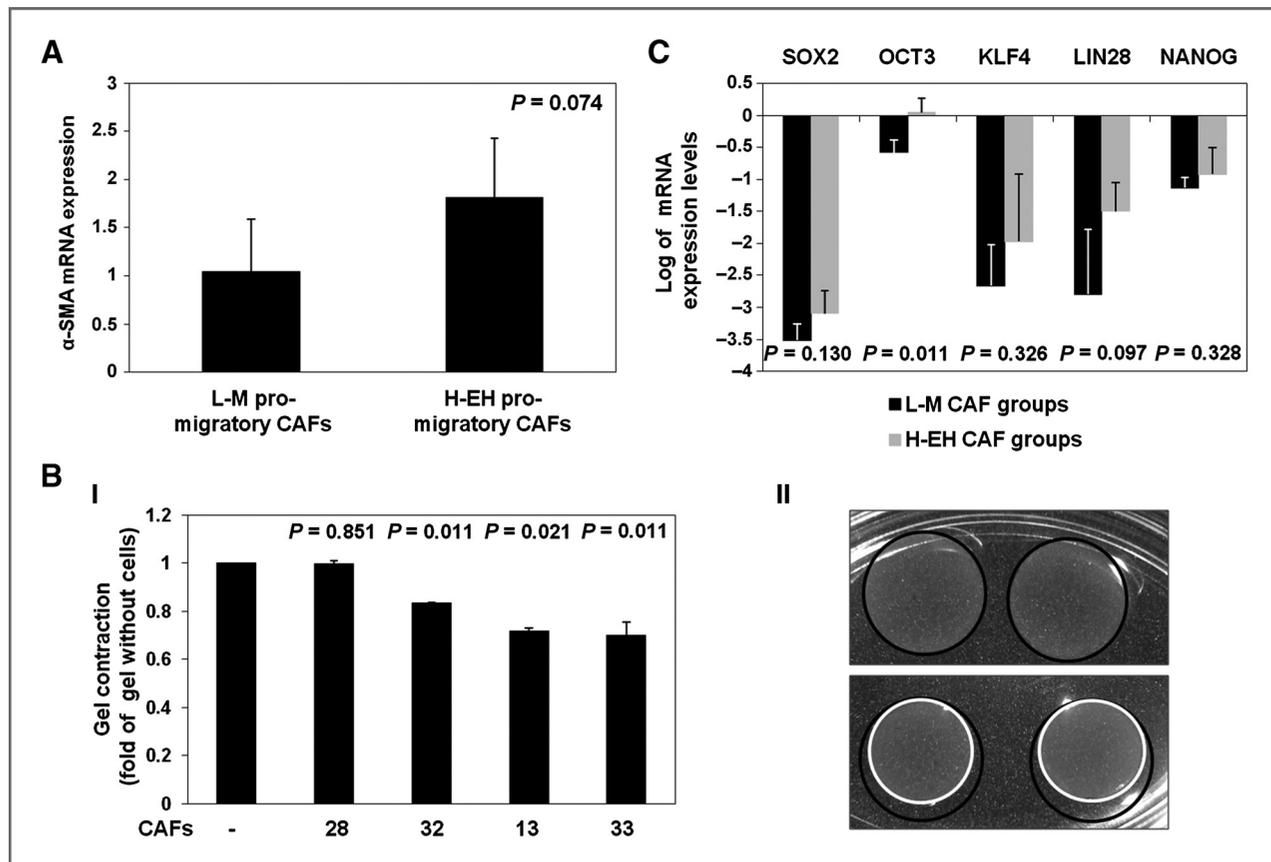


Figure 3. Colon CAF-derived paracrine promigratory effects are associated with activated fibroblast features and stemness markers. Most promigratory CAFs ("H" and "EH") showed higher α -SMA mRNA expression levels (A). Association between primary colon CAF promigratory effects and collagen gel contraction (B). BII showed an example of contraction measurement: black circles and upper pictures are CAFs embedded in collagen gel at 0 hours. Lower pictures show previous measurements (black circles) and new measurements (white circles) after 24 hours (both pictures are shown by replicates; BII). Generally, an increase in stemness markers, measured by RT-PCR, was seen in most promigratory CAFs. Remarkably, there was significant statistical association with *OCT3* expression levels and a trend was also observed with *SOX2* and *LIN28* expression levels (C).

Together, these data suggest an association between CAFs and stemness characteristics. However, no conclusive evidence was obtained for the association between the degree of CAF stemness and their promigratory effect on colon cancer cells.

Identification of differentially expressed genes in CAF promigratory subtypes

To identify differences between fibroblasts with varying promigratory impact on colon cancer cells, representative CAFs from each promigratory subtype were selected for gene expression profiles by gene microarray analysis.

Four CAF samples, Low (L; id = 28), Medium (M; id = 32), High (H; id = 13), and Extra-High (EH; id = 33), and two NFs (id = 36 and 13) as control samples were analyzed.

To determine the differential expression profiles of CAF promigratory subtypes, the expression value from two NF sample averages was subtracted from four migratory group samples (L, M, H, and EH).

An analysis of the samples showed that "L & M" CAF subtypes clustered together and "H & EH" formed another

group. Therefore, we considered L and M (LM) as one replicated group and H and EH (HEH) as another (Fig. 4A). Significant differential expression genes were determined by a P value cutoff at 0.05 and Log2FC by a cutoff at 0.5. A detailed gene list with expression values is given in Supplementary Table S3. These genes were used for further downstream analysis.

The expression of genes in "LM" and "HEH" CAFs was used to generate an initial "CAF signature," to be used for further analysis including classification of patients with cancer and prognostic effects. "Gene-normalized" values with ANOVA statistics (P value cutoff 0.05) were used to determine this signature. Supplementary Table S4 contains a detailed list of CAF signature genes.

Functional clustering of CAF promigratory subtypes shows involvement of Golgi among other overrepresented processes

To better understand the biology behind the differential expression genes in each LM and HEH group, we explored Gene Ontology Biological Process (GOBP) enrichment analysis with binomial statistics.

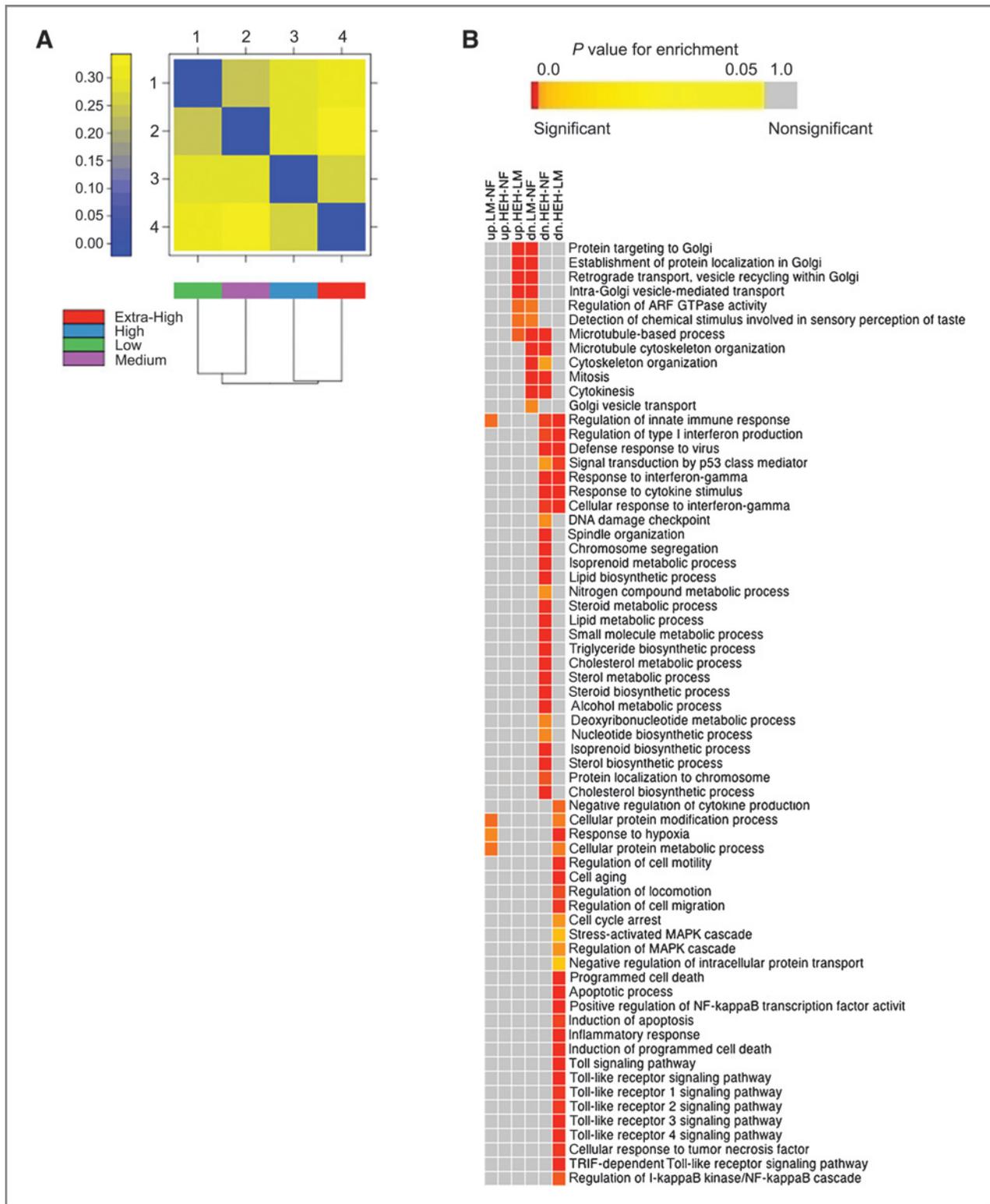


Figure 4. CAFs clustering and functional enrichment analysis of differentially expressed genes. False color heat map of the distances between arrays (CAF samples; A). The color scale was chosen to cover the range of distances encountered in the dataset. Patterns in this plot indicate clustering of the arrays for intended biologic similarities. With expression values of each of the four samples, distant similarity matrices were calculated by the program arrayQualityMetrics (see Materials and Methods for description). Enrichment of selected GOBP categories under different experimental conditions is seen (B). Columns give different comparison conditions between two experimental conditions. With differentially expressed genes in these conditions, functional overrepresentation is shown in rows on the color-coded heat map. Color represents corrected (FDR) P values. The reddish color indicates a more significant P value, whereas yellow indicates a less significant one and gray indicates not significant.

Selected enriched GOBP categories are shown in Fig. 4B. A full list of enriched terms and statistical details is given in Supplementary Table S5. Curiously, differential expression genes in the "protein targeting to Golgi" GOBP module were observed among CAF subtypes. Thus, "Golgi" -related processes were significantly overrepresented in downregulated genes of the "LM" group, whereas the same processes were enriched in upregulated genes in the "HEH" group. Differential expression genes in the "protein targeting to Golgi" GOBP category in migratory groups are shown in Supplementary Fig. S2A.

Notably, differential expression genes in the "negative regulation of cytokine production" (Supplementary Fig. S2B) GOBP category were significantly enriched for downregulated genes in the "HEH" group. In addition, p53 signal transduction and other biologic process related to apoptosis and cell death, such as "signal transduction by p53 class mediator," "induction of apoptotic genes," or "apoptotic process," were all also downregulated in the "HEH" group (Supplementary Fig. S2C–S1E, respectively).

Finally, different GOBP modules relating to immune response, significantly enriched for downregulated genes in the "HEH" group, were also indicated, such as "*toll-signaling pathways*" (Supplementary Fig. S2F), "regulation of innate immune response," "cellular response to interferon-gamma," and "regulation of type I interferon production" (Fig. 4B).

In general terms, gene ontology analysis revealed that "differentially expressed CAF genes" represent functions or processes of immediate relevance to the tumor microenvironment and include regulators of cytokine production or modulation of the immune system.

"CAF signature" is a prognostic marker of survival of patients with colon cancer

To assess the prognostic value and clinical relevance of "CAF signatures" in colon cancer, publicly available microarray datasets (a cohort of 232 patients with colon cancer from the GEO database) were used. Patients were split into two groups based on the expression difference from median. Using Gitoools (Perez-Imaz C and Lopez-Bigas N, 2011), Z-score enrichment was analyzed in the "CAF signature gene module." This analyzed patients with colon cancer who showed significant up- and downregulation for "CAF signature genes." Clinical survival information was then correlated with this modular expression to determine (Kaplan–Meier survival analysis) whether "CAF gene signature" had a role in disease prognosis. The detailed gene list used for "CAF signatures" is shown in Supplementary Table S4 and a heat map of the top 50 "CAF signature" genes appears in Fig. 5A.

The survival analysis showed increased risk factors for disease-free survival (DFS) for patients with high "CAF signature scores" in the data set [HR = 2.2; confidence interval (CI), 1.2–4.1; Fig. 5B]. Similarly, a clear trend, almost statistically significant, was observed for overall survival (OS; HR = 1.8; CI, 1.0–3.3; Fig. 5C). Therefore, patients with a similar expression pattern to "CAF signa-

ture" showed significantly poorer survival in an independent dataset of patients with colon cancer.

On the basis of these results, the prognostic value of "CAF signature" was analyzed for the different tumor stages. The analysis revealed that "CAF signature" showed prognostic value at advanced stages for DFS and OS, but not at early stages (Fig. 5D and E; Supplementary Fig. S3A and S3B). Therefore, the signature studied behaves as a better predictive variable for survival in advanced stages.

Together, these analyses show that higher-expression "CAF signature" genes is a marker for poor prognosis. Moreover, the stage subgroup analysis indicates that the "CAF signature" variable is associated with poor prognosis predominantly in advanced-stage patients.

Validation of "CAF signature" genes as prognostic markers of survival of patients with colon cancer

The deregulated expression of seven randomly selected genes from the "CAF signature" was confirmed by qRT-PCR analysis (Supplementary Table S4 and Fig. 6A).

Among these genes, a set of four genes, *IGFBP3*, *OAS2*, *MX1*, and *ROBO2*, was selected for validation as prognostic markers in an independent series of patients with colon cancer. The mRNA expression of these genes was analyzed by qRT-PCR in colon cancer-derived samples from 142 patients.

IGFBP3 and *ROBO2* showed upregulated expression levels, whereas *OAS2* and *MX1* showed downregulated levels in the "HEH" CAF group. Thus, deregulated gene expression was considered in patients with higher mRNA expression levels of *IGFBP3* and *ROBO2* or patients with lower expression levels of *OAS2* and *MX1*.

Individual survival analysis of each gene showed different associations between deregulated expression mRNA levels and patients' DFS or OS. However, significant statistical difference was not achieved in all cases (Supplementary Fig. S4A and S4B). Nevertheless, the "CAF signature" included the expression of many genes. Thus, the mRNA expression levels of the gene set were combined to study their possible additive effect on patient survival. Interestingly, a clear association was observed between the combination of deregulated gene expressions and DFS or OS in patients with colon cancer (Fig. 6B and C).

Together, these results support the involvement of "CAF signature" genes in survival of patients with colon cancer.

Golgi process as predictor marker of survival of patients with colon cancer

As we found overrepresentation of Golgi-related GOBP (Fig. 4B), we determined to analyze this further. Therefore, GOBP Golgi process-related genes were grouped as a single category/module and their suitability for survival prediction and disease prognosis was assessed.

First, all genes associated with Golgi and the related GOBP process (229 Golgi-related gene modules) were used to classify patients into two groups, using Z-score enrichment analysis on expression data: those who showed a higher expression of the Golgi module (including patients

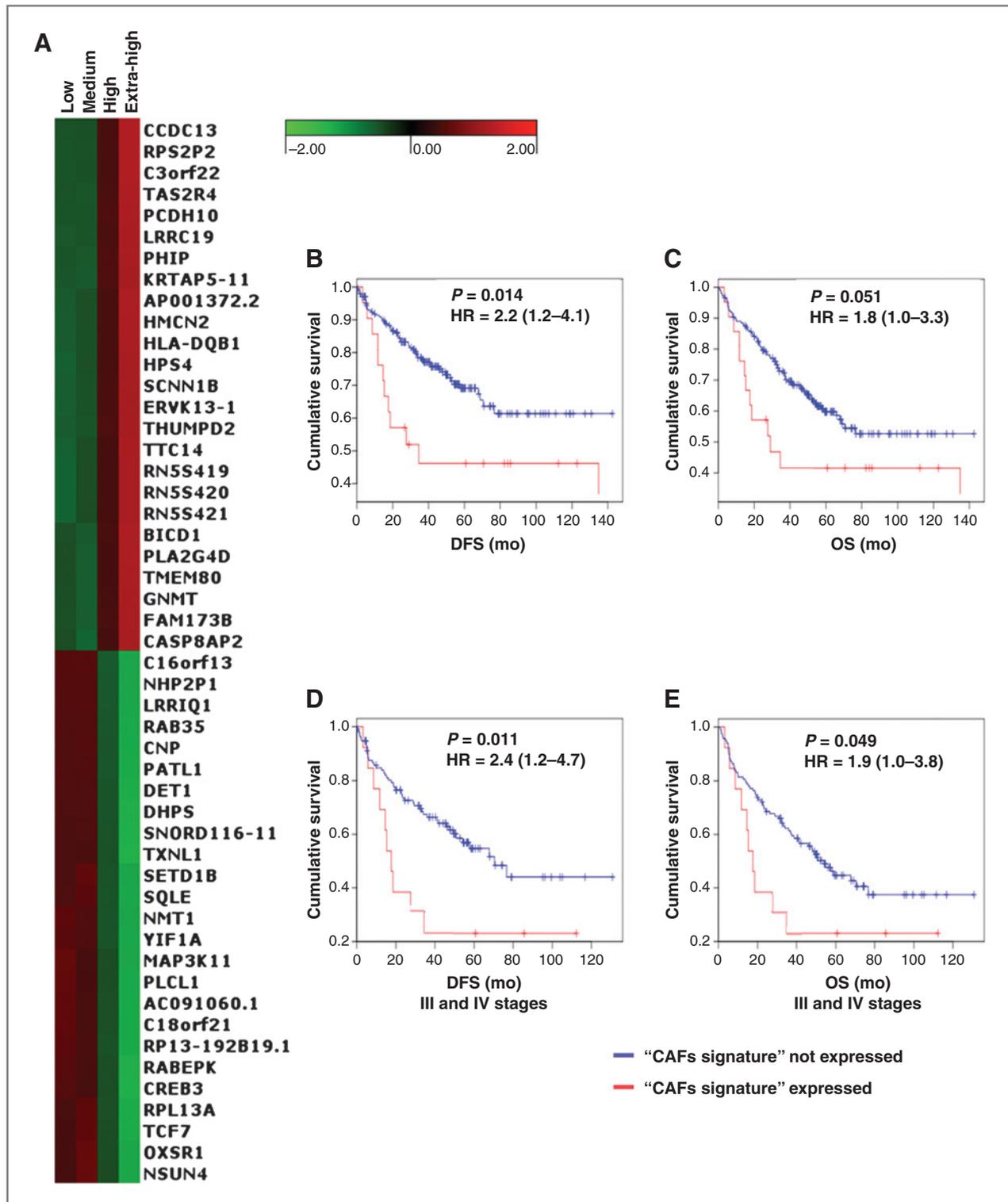


Figure 5. CAF gene signature and its capacity to identify prognostic outcome. The top 25 upregulated and top 25 downregulated CAF signature genes in the color-coded heat map (A). Survivability of colon patients expressing higher and lower expression of CAF signature genes (patients grouped on the basis of the Z score of the CAF signature gene module on median centered expression colon dataset; see Materials and Methods for details). The HR was based on the Cox model (B and C). Similarly, the ability to predict the stage-prognostic capacity of CAF signature was tested with the Kaplan–Meier plot on colon patients' microarray expression data. Patient samples were grouped in stages I to II and III to IV (D and E), on the basis of information provided by authors of these public data (see Materials and Methods for dataset reference). Survivability of patients expressing or nonexpressing CAF signature was tested as described earlier.

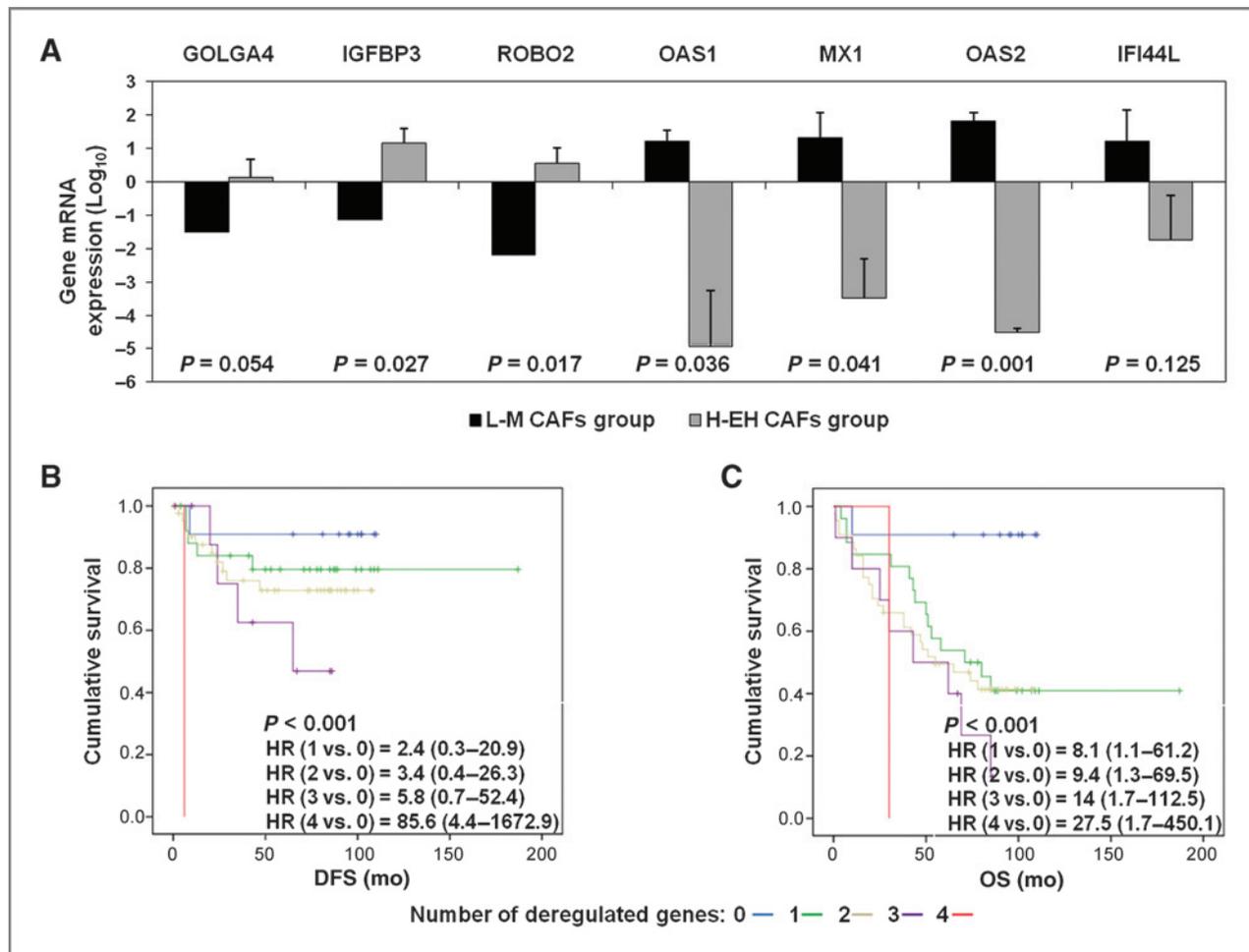


Figure 6. Validation of deregulated genes and prognostic value of "CAF signature" by qRT-PCR. The mRNA expression of seven randomly selected genes from the "CAF signature" was analyzed by qRT-PCR. Six of the seven genes showed statistical differences for mRNA expression between the "Low plus Medium" and "High plus Extra-high" migration groups (A). A clear association was observed between the combination of four gene expression data, including *IGFBP3*, *OAS2*, *MX1*, and *ROBO2*, and DFS (B) or OS (C) in patients with colon cancer.

with most of their Golgi genes upregulated) and patients with a lower expression of the Golgi module. Interestingly, Golgi process genes showed a significant predictive value for DFS and OS in patients with colon cancer. Therefore, as shown in Supplementary Fig. S5A to S5C, patient survival is significantly worse in those with a high score of Golgi genes.

Discussion

This study identifies for the first time, different CAF subtypes in patients with primary colon carcinomas. Thus, CAFs were functionally classified for their ability to promote migration in cancer cells, their activated fibroblast features, and their distinct molecular expression profiles. Interestingly, the association between typical stem-cell markers of CAFs and their promigratory effect on tumor cells was also seen. The clinical relevance of these findings is established by the definition of a gene expression signature, derived from the most protumorigenic CAFs, which showed a remarkable prognostic value for the clinical outcome of patients with colon cancer.

Growing evidence suggests that primary normal fibroblasts and cancer-associated fibroblasts have distinct gene expression signatures in several tumor types. Thus, in breast cancer, significant differences between CAFs and normal counterparts or CAFs derived from different clinical subtypes have been described previously (21–23). In non-small cell lung cancer, genes relating to the TGF- β signaling pathway are differently expressed by NFs and CAFs (24). The transcripts expressed by Tag profiling in CAFs, normal human prostate fibroblast, and fetal cells were also compared in 1 patient with prostate cancer (25). Identified clusters of CAF gene signatures characterized genetically unstable and stable Oral Squamous Cell Carcinoma (OSCC) in relation to each other and to fibroblasts from normal oral mucosa (26). Furthermore, distinct expressions of regulatory microRNA (miRNA) in CAFs derived from CAFs versus paired NFs were determined in endometrial and bladder cancer (27, 28).

Despite the abovementioned findings, CAFs from primary colon cancer and paired NFs have not yet been fully

studied. The growth-promoting effects on colon cancer cells *in vitro* of fibroblast cultures from metastatic colon cancer in liver or from liver away from metastatic lesions and skin from 3 patients were determined, as was the molecular profile expression of CAFs and skin fibroblasts (29). Similar efforts were made to identify protein expression patterns in CAFs and NFs from patients with colon cancer (30). These two studies described differences between CAFs and NFs, but no clinical study to back up their findings was undertaken.

In this scenario, this is the first study that demonstrates the promigratory impact of colon CAFs, from different patients, on cancer cells and shows that they have greater impact than that of NFs. The migration experiments with two cell lines suggest that CAF promigratory effects are not restricted to a particular cell line. The phenotypic characterization of activated fibroblast markers, studied by α -SMA staining and collagen contractility assays, suggests a link between the different activation level of CAFs and their potential to enhance tumorigenesis. In general terms, CAFs collectively share the same activation state, but their expression of activation markers, such as α -SMA, FSP1, FAP, or others, may differ (31). Supporting our data, a recent study demonstrated that the silencing of the *FAP* gene, another classic marker of fibroblast activation, in CAFs drives the reduction of tumor growth *in vivo* and of tumorigenesis in ovarian cancer (32). In the same study, *FAP* silencing and its effects are mediated in some way by downregulation of the stem cell markers in CAFs. Thus, it cannot be ruled out that the acquisition of the stem cell characteristics of CAFs could determine their protumorigenic abilities. Supporting this idea, preliminary findings of the present study showed associations between CAF stem cell markers and their promigratory abilities in colon cells. Moreover, experiments showed osteoblast cell differentiation as a CAF stemness characteristic. However, no association was observed between the degree of CAF differentiation and CAFs' promigratory effect on cancer cells. The potential involvement of stemness in the CAF-dependent migration of colon cells needs to be studied by direct approaches. Such studies should particularly explore the stemness characterization of CAFs. Future studies should also address the possibility that CAF promigratory abilities could be linked to a non-differentiated stemness phenotype.

CAFs are heterogeneous populations and their relative composition differs greatly between tumors, within a given tumor type and even in individual tumors (33). This wide heterogeneity in CAFs may be explained by their possibly miscellaneous origin. Indeed, CAFs are variously reported to derive from resident fibroblast, bone marrow-derived progenitor cells, endothelial or cancer cells through endothelial/epithelial-mesenchymal transition, smooth muscle cells, pericytes, adipocytes, or inflammatory cells (34). The effect of different CAF subpopulations on different CAF promigratory abilities should be further analyzed by expanded analysis of cell origin traceability.

As expected, gene ontology analysis revealed that CAF gene expression represents functions or processes of

immediate relevance to the tumor microenvironment, including regulators of cytokine production or modulation of the immune system. The downregulation of genes relating to "negative regulation of cytokine production" was a particular feature in most protumorigenic CAF groups. This finding suggests that genes that negatively regulate cytokine production could be switched off and hence more cytokines may be produced by this CAF population subtype. Downregulation of immune response pathways for this CAF subpopulation could be associated with induced immune response suppression by primary tumor. Finally, p53 signal transduction and other biologic processes relating to apoptosis and cell death were all also downregulated in this CAF group. The activation state of these cells may explain the downregulation of these pathways.

Surprisingly, differences in pathways relating to "protein targeting to Golgi" between CAF subtypes were observed. In line with the data presented, recent studies have shown that overexpression of Golgi proteins, such as Golgi phosphoprotein 3 in cancer cells, correlated with their proliferation and tumorigenicity *in vitro* (35–39). In addition, descriptions of the molecular network, connecting the Golgi to other organelles, demonstrate the essential roles of the Golgi in different cellular activities, indicating their possible role in prostate tumors (40). In inflammatory bowel disease, altered glycosylation, which occurs in the Golgi apparatus, may be a factor in colon cancer development (41).

A few studies identified a prognostic "CAF signature," compared with NFs, that was associated with poor patient prognosis. In non-small cell lung cancer, analysis of CAFs and NFs identified a subset of differentially expressed genes with prognostic value in multiple independently published expression datasets of primary non-small cell lung cancer (24). In OSCC, the gene expression signature of fibroblasts from genetically unstable OSCC was associated with poor clinical outcome in an independent microarray database of patients with head and neck cancer (26). On microdissecting total stroma cells from Barrett esophagus tumors, a gene signature discriminates preinvasive from invasive disease in digestive cancers, including colon cancer. However, the prognosis value of this stromal signature was not confirmed in any tumor (42). The data reported here imply the clinical relevance of the specific "CAF signature" in colon cancer. Supporting this finding, an association between the expression levels of a four-gene set, included in the "CAF signature," and prognostic value in an independent series of 142 patients with colon cancer was observed. Because of a lack of available public stromal/epithelial ratio data, data extracted from public datasets were applied to whole tumor tissue, contrasting with the identified "CAF signature." Better characterization of the impact of epithelial contamination is also required. Preliminary studies with our CAFs and whole tumor sample counterparts were not sufficient to reach any conclusive results (data not shown). A large array analysis with many samples would be required, and although this issue could be a limitation of the study, the "*in silico*" analysis, in an independent dataset of patients

with colon cancer, confirmed the prognostic value of most protumorigenic "CAF signatures." Similar procedures were developed in the few studies in which prognosis was associated with a prognostic "CAF signature," when compared with NFs (24, 26). It should be noted that the signature studied behaves as a better predictive variable for patient outcome in patients with advanced-stage tumors. This confirms the view that development of new therapies, targeting microenvironment components, would help to improve the medical treatment of those patients usually with worse outcome. Finally, the involvement of the Golgi process suggested by gene ontology analysis is also clinically supported by the specific predictive value of Golgi process genes for clinical outcome in patients with colon cancer. Better characterization of Golgi effects in colon tumors will be needed.

The findings of this study demonstrate the important role of CAF gene expression changes in patients with colon cancer. These changes involve enhancement of colon cell migration. The findings contribute to the identification of colon cancer cell prognostic markers. They emphasize the ongoing efforts to discover the real involvement of CAF changes in cancer progression and encourage the search for new stromal treatment targets that would combine effectively with current systemic therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Functional Heterogeneity of Cancer-Associated Fibroblasts from Human Colon Tumors Shows Specific Prognostic Gene Expression Signature

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