

# Cancer-associated fibroblasts affect breast cancer cell gene expression, invasion and angiogenesis

Noemi Eiro, <sup>1</sup>✉

Phone: +34 985320050

Email: [investigacion@hospitaldejove.com](mailto:investigacion@hospitaldejove.com)

AQ1 AQ2

Lucía González, <sup>1</sup>

Anxo Martínez-Ordoñez, <sup>2</sup>

Belen Fernandez-Garcia, <sup>1</sup>

Luis O. González, <sup>1,3</sup>

Sandra Cid, <sup>1</sup>

Francisco Dominguez, <sup>4</sup>

Román Perez-Fernandez, <sup>2</sup>

Francisco J. Vizoso, <sup>1,5</sup>✉

Phone +34 985320050

Email [investigacion@hospitaldejove.com](mailto:investigacion@hospitaldejove.com)

<sup>1</sup> Unidad de Investigación, Fundación Hospital de Jove, 33290 Gijón, Asturias, Spain

<sup>2</sup> Department of Physiology-CIMUS, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain

<sup>3</sup> Servicio de Anatomía Patológica, Fundación Hospital de Jove, 33290 Gijón, Asturias, Spain

<sup>4</sup> Servicio de Anatomía Patológica, Hospital de

## Abstract

### Purpose

It has been reported that stromal cell features may affect the clinical outcome of breast cancer patients. Cancer associated fibroblasts (CAFs) represent one of the most abundant cell types within the breast cancer stroma. Here, we aimed to explore the influence of CAFs on breast cancer gene expression, as well as on invasion and angiogenesis.

### Methods

qRT-PCR was used to evaluate the expression of several cancer progression related genes (S100A4, TGF $\beta$ , FGF2, FGF7, PDGFA, PDGFB, VEGFA, IL6, IL8, uPA, MMP2, MMP9, MMP11 and TIMP1) in the human breast cancer-derived cell lines MCF-7 and MDA-MB-231, before and after co-culture with CAFs. Stromal mononuclear inflammatory cell (MIC) MMP11 expression was used to stratify primary tumors. In addition, we assessed the in vitro effects of CAFs on both MDA-MB-231 breast cancer cell invasion and endothelial cell (HUVEC) tube formation.

### Results

We found that the expression levels of most of the genes tested were significantly increased in both breast cancer-derived cell lines after co-culture with CAFs from either MMP11<sup>+</sup> or MMP11<sup>-</sup> MIC tumors. IL6 and IL8 showed an increased expression in both cancer-derived cell lines after co-culture with CAFs from MMP11<sup>+</sup> MIC tumors. We also found that the invasive and angiogenic capacities of, respectively, MDA-MB-231 and HUVEC cells were increased after co-culture with CAFs, especially those from MMP11<sup>+</sup> MIC tumors.

# Conclusions

Our data indicate that tumor-derived CAFs can induce up-regulation of genes involved in breast cancer progression. Our data additionally indicate that CAFs, especially those derived from MMP11+ MIC tumors, can promote breast cancer cell invasion and angiogenesis.

---

## Keywords

Cancer-associated fibroblasts

Breast cancer

Tumor invasion

Angiogenesis

MMP11

Noemi ~~Eiró~~Eiro and Lucía González contributed equally to this work.

---

## 1. Introduction

The acquisition of a malignant phenotype does not depend exclusively on intrinsic cancer cell properties, but also on stromal features [1]. Cancer associated fibroblasts (CAFs), one of the most abundant cell types within the breast cancer stroma, contribute to tumor progression through the recruitment of immune cells [2, 3], the degradation and remodeling of extracellular matrix (ECM) components, the promotion of epithelial-mesenchymal transition (EMT) processes and the induction of stem cell traits [4, 5, 6, 7]. These actions are mediated by growth factors and cytokines secreted by CAFs [8, 9, 10, 11]. Mononuclear inflammatory cells (MICs) represent another stromal cell component that may affect both cancer cell and stromal cell behavior. Previously, we found that, depending on the expression of matrix metalloprotease 11 (MMP11) by MICs, breast cancers may exhibit a highly inflammatory profile [12] and a poor prognosis [13, 14, 15, 16]. Indeed, it has been found that CAFs derived from MMP11+ MIC tumors may over-express factors related to tumor progression, such as TIMP-1, VEGFA and S100A4 and, in addition, that their co-culture with breast cancer cells may induce a more aggressive behavior [10]. So, different MIC tumors may differentially modulate CAF phenotypes. As yet, however, little is known about the effects of CAFs on breast cancer cell characteristics.

In order to better understand the dynamic interactions between CAFs and breast cancer cells, the objectives of the present study were (i) to investigate the effects of CAFs on breast cancer cells by analyzing the expression of a panel of genes implicated in tumor progression, (ii) to compare the resulting gene expression profiles after co-culture with CAFs derived from MMP11+ or MMP11- MIC tumors and (iii) to explore its effects on the invasive and tube forming capacities of breast cancer and endothelial cells, respectively.

## 2. Materials and methods

### 2.1. Patient selection and characteristics

In this non-randomized prospective study 20 cases with a confirmed diagnosis of invasive breast carcinoma were included, as previously reported [10]. Specifically, we selected consecutive T1 or T2 invasive ductal carcinoma cases yielding enough material for cell culture during the period July 2011 to August 2013. All patients included underwent tumor resection as first therapeutic approach. The exclusion criteria were: prior history of a malignant tumor, excluding non-melanoma skin cancer, uterine cervical carcinoma in situ, ductal carcinoma in situ or lobular carcinoma in situ breast cancer, and having received any type of therapy prior to surgery. The clinical and pathological features of the 20 patients included in this study are listed in Table 1. The patients were treated according to the guidelines used in our Institution (Fundación Hospital de Jove). Written informed consent was obtained from all patients. The study adheres to national regulations and has been approved by the Fundación Hospital de Jove Ethics and Research Committee.

**Table 1**

Clinicopathological characteristics of the 20 patients with invasive breast carcinoma according to the expression of MMP11 by intratumoral MICs

<b>Characteristics</b>	<b>Tumors MICs MMP11 negative N (%)</b>	<b>Tumors MICs MMP11 positive N (%)</b>
All patients	12 (60.0)	8 (40)
Median age (years)		
<62	6 (50.0)	3 (37.5)
>62	6 (50.0)	5 (62.5)
Tumor size		

Tumor size		
1	2 (16.7)	2 (25.0)
2	8 (66.6)	6 (75.0)
3	2 (16.7)	0 (0)
Histological grade		
Well differentiated (I)	1 (8.3)	1 (12.5)
Moderately differentiated (II)	5 (41.7)	5 (62.5)
Poorly differentiated (III)	6 (50.0)	2 (25.0)
Nodal status		
Negative	6 (50.0)	2 (25.0)
Positive	6 (50.0)	6 (75.0)
Estrogen receptors		
Negative	1 (8.3)	0 (0)
Positive	11 (91.7)	8 (100)
Progesterone receptors		
Negative	2 (16.7)	1 (12.5)
Positive	10 (83.3)	7 (87.5)
HER2		
Negative	10 (83.3)	7 (87.5)
Positive	2 (16.7)	1 (12.5)

## 2.2. CAF isolation, cell lines, and co-culture assays

For the isolation of cancer-associated fibroblasts (CAFs), tumor samples were cut into 1 mm<sup>3</sup> pieces and enzymatically dissociated, as previously reported [10]. Fibroblast purities were analyzed by flow cytometry after incubation with an anti-CD90 antibody (clone AS02, Dianova, Hamburg, Germany) at 1:50 for 45 min. The specificity of the anti-CD90 antibody used was confirmed by immunohistochemistry [10].

The estrogen-independent human breast cancer-derived cell line MDA-MB-

231, the estrogen-dependent human breast cancer-derived cell line MCF-7 and the primary human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The MCF-7 cells were cultured in DMEM-F12 (Lonza, Visp, Switzerland) and the MDA-MB-231 cells were cultured in high glucose DMEM (Sigma, St. Louis, MO, USA). Both media were supplemented with 10% FBS (PAA, New Jersey, NJ, USA) and 1% penicillin-streptomycin solution (Gibco, Paisley, UK). HUVEC cells were used at passages 4–6, and were cultured in VascuLife basal medium supplemented with LifeFactors VascuLife EnGS (Lifeline Cell Technology, Frederick, MD, USA).

CAFs isolated from the 20 breast tumors included were co-cultured with both MDA-MB-231 and MCF-7 cells. To this end, CAFs ( $2 \times 10^4$ ) were seeded at the bottom of tissue culture wells, whereas the breast cancer-derived cells ( $2 \times 10^4$ ) were seeded in 0.24  $\mu\text{m}$  pore size tissue culture inserts. The cells were co-cultured for 72 h in DMEM F-12 supplemented with 10% FBS and 1% penicillin-streptomycin solution, after which RNA was extracted. Co-cultures of fibroblasts derived from MMP11+ or MMP11-MIC tumors with the respective breast cancer-derived cell lines were performed using VascuLife basal medium supplemented with LifeFactors VascuLife EnGS as described above. These media were collected and used as conditioned media (CM) for the HUVEC tube-formation assays (see below).

### 2.3. Immunohistochemistry

Tumors were classified immunohistochemically according to MMP11 expression by intratumoral mononuclear inflammatory cells (MICs). To this end, 5  $\mu\text{m}$  sections were sliced from formalin-fixed and paraffin-embedded (FFPE) tissue samples using a microtome (Leica Microsystems GmbH, Wetzlar, Germany) and transferred to adhesive-coated slides. The sections were stained using a TechMate TM50 autostainer (Dako, Glostrup, Denmark). Immunohistochemistry (IHC) was performed as previously reported [14] and the antibodies used for IHC are listed in Table 2.

**Table 2**

Antibodies used for immunohistochemistry

Antibody	Antigen retrieval	Dilution	Incubation (min)	Linker	Source (Cat. No.)

MMP11	PH6	1:100	60	Yes	Thermo (MA5-11234)
TIMP1	pH 6	1:50	120	Yes	Thermo (MA5-13688)
IL6	pH 6	1:300	120	Yes	AbNova (H0000369-B01P)
IL8	–	1:20	120	Yes	Thermo (PA1-24797)

## 2.4. qRT-PCR and Western blotting

A RNeasy Mini Kit (Qiagen, Hilden, Germany) was used for total RNA extraction following the manufacturer's instructions. For cDNA synthesis, a Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) was used. Reverse transcription was carried out as previously reported [10]. Quantitative real time-PCR (qRT-PCR) was performed as previously reported [10] using specific primers (listed in Table 3).

**Table 3**

Factors analyzed and main roles

Gene symbol	References	Gene name	Main role
S100A4	110,779	S100 calcium binding protein A4	Invasion
TGFβ	101,210	Transforming growth factor beta	Inflammation
FGF2	118,274	Fibroblast growth factor 2 (basic)	Angiogenesis
FGF7	113,109	Fibroblast growth factor 7	Cell growth / Invasion
PDGFA	110,648	Platelet-derived growth factor alpha	Angiogenesis
PDGFB	110,713	Platelet-derived growth factor beta	Angiogenesis
uPA	109,571	Urokinase-type plasminogen activator	ECM remodeling
IL6	113,614	Interleukin 6	Inflammation
IL8	103,136	Interleukin 8	Inflammation
MMP2	103,899	Matrix metalloproteases 2	ECM remodeling
			ECM

MMP9	139,820	Matrix metalloproteases 9	remodeling
MMP11	103,163	Matrix metalloproteases 11	ECM remodeling
TIMP1	103,847	Tissue inhibitor of metalloproteases 1	ECM remodeling
VEGFA	140,392	Vascular endothelial growth factor A	Angiogenesis
GAPDH	101,128	Glyceraldehyde-3-phosphate dehydrogenase	–
ACTB	101,125	Actin, beta	–
SDHA	102,136	Succinate dehydrogenase complex, subunit A, flavoprotein	–

Western blotting was performed by subjecting 50 µg total protein extracted from HUVEC cells to SDS–PAGE. After electrophoresis, the proteins were transferred to Immobilon-FL membranes (Merck Millipore, Madrid, Spain), blocked and incubated overnight at 4 °C with primary antibodies and, next, incubated with anti-mouse Dylight™ 680-conjugated or anti-rabbit Dylight™ 800-conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA). Quantification was performed using LI-COR Odyssey software (Bad Homburg, Germany). The following antibodies were used: anti-phospho(p)-VEGFR2, anti-p-p38, p-ERK1/2 (Cell Signaling, Danvers, MA, USA) and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

## 2.5. Cell invasion and tube-formation assays

Cell invasion assays were performed using BD BioCoatMatrigel invasion chambers (BD Biosciences, Madrid, Spain). Briefly, MCF-7 or MDA-MB-231 cells ( $5 \times 10^4$ ) were placed into the upper chambers in 0.5 ml serum-free DMEM. In the lower chambers DMEM-F12 (1:1) supplemented with 20% FBS (controls) or CAF-conditioned medium (CM) (2 CAF cultures from MMP11- MIC tumors and 2 CAF cultures from MMP11+ MIC tumors for 48 h) were added. After incubation for 22 h, cells that were migrated to the lower surfaces of the filters were fixed in methanol for 2 min at room temperature, stained using crystal violet for 2 min, visualized and counted. Values for cell invasion were expressed as the mean number of cells per microscopic field over four fields per one filter of duplicate experiments.

Tube-formation assays were performed in Matrigel (BD Biosciences). Briefly, 100  $\mu$ l growth factor-reduced Matrigel matrix was loaded into each well of a 96-well plate and allowed to polymerize for 30 min at 37 °C. Next, HUVEC cells ( $4 \times 10^5$ ) were added to each well and incubated at 37 °C in 5% CO<sub>2</sub> for 5 h with conditioned media as described above. The formation of capillary-like structures was assessed under a fluorescence microscope after CellTracker Red CMTPX Dye (0.5  $\mu$ M; Thermo Fisher Scientific, Waltham, MA, USA) staining for 15 min. Tube formation was evaluated by quantifying vessel lengths and junction numbers using AngioTool software [17].

## 2.6. Statistical analysis

All statistical analyses were performed using SPSS 19. The Kolmogorov-Smirnov test was used to determine whether the sample data were normally distributed. Comparisons between groups were performed using Student's *t* test. Differences were considered significant when  $p \leq 0.05$ .

## 3. Results

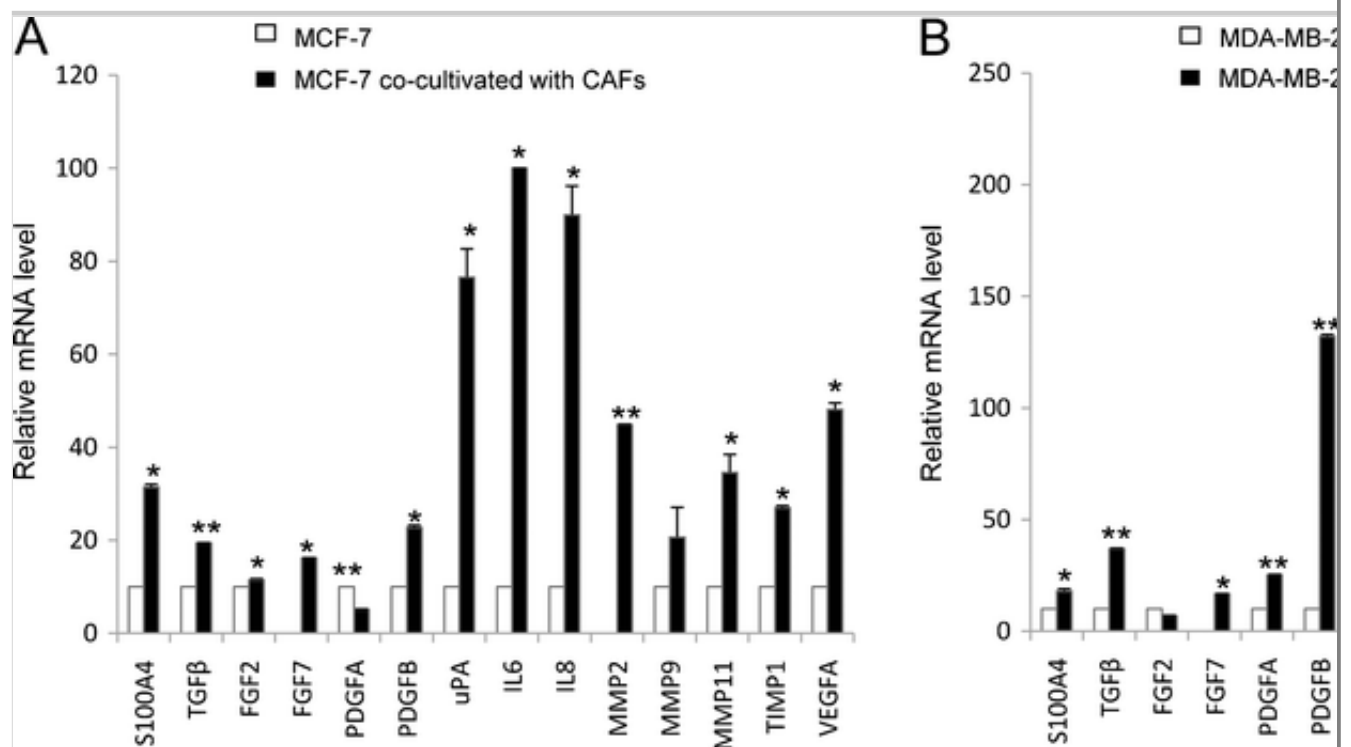
### 3.1. mRNA expression alterations in MCF-7 and MDA-MB-231 cells after co-culture with CAFs

To investigate the influence of stromal fibroblast on breast cancer cells, MCF-7 and MDA-MB-231 cells were co-cultured with 20 different CAF samples, after which the expression levels of 14 genes (Table 3) were assessed by qRT-PCR. We found that after co-culture the S100A4, TGF $\beta$ , FGF7, PDGFB, uPA, IL-6, IL-8, MMP2, MMP11, TIMP-1 and VEGFA mRNA expression levels were upregulated in both MCF-7 and MDA-MB-231 cells (Fig. 1). In contrast, we found that the PDGFA mRNA expression level in MCF-7 cells was decreased after co-culture with the CAFs ( $p < 0.001$ ). Several mRNAs showed increases of at least 5-fold compared to basal levels after co-culture, including uPA in MCF-7 cells and PDGFB, MMP-2 and VEGFA in MDA-MB-231 cells. The IL-6 and IL-8 mRNA levels were found to be increased at least 10-fold in both cell lines after co-culture (Fig. 1a–b).

#### **Fig. 1**

qRT-PCR analysis of 14 factors in (a) MCF-7 cells alone or after co-culture with CAFs and (b) MDA-231 cells alone or after co-culture with CAFs. CAFs

from all 20 patients were included. Data represent mean  $\pm$  SD (\* $p \leq 0.05$ , \*\*  $p \leq 0.001$ )

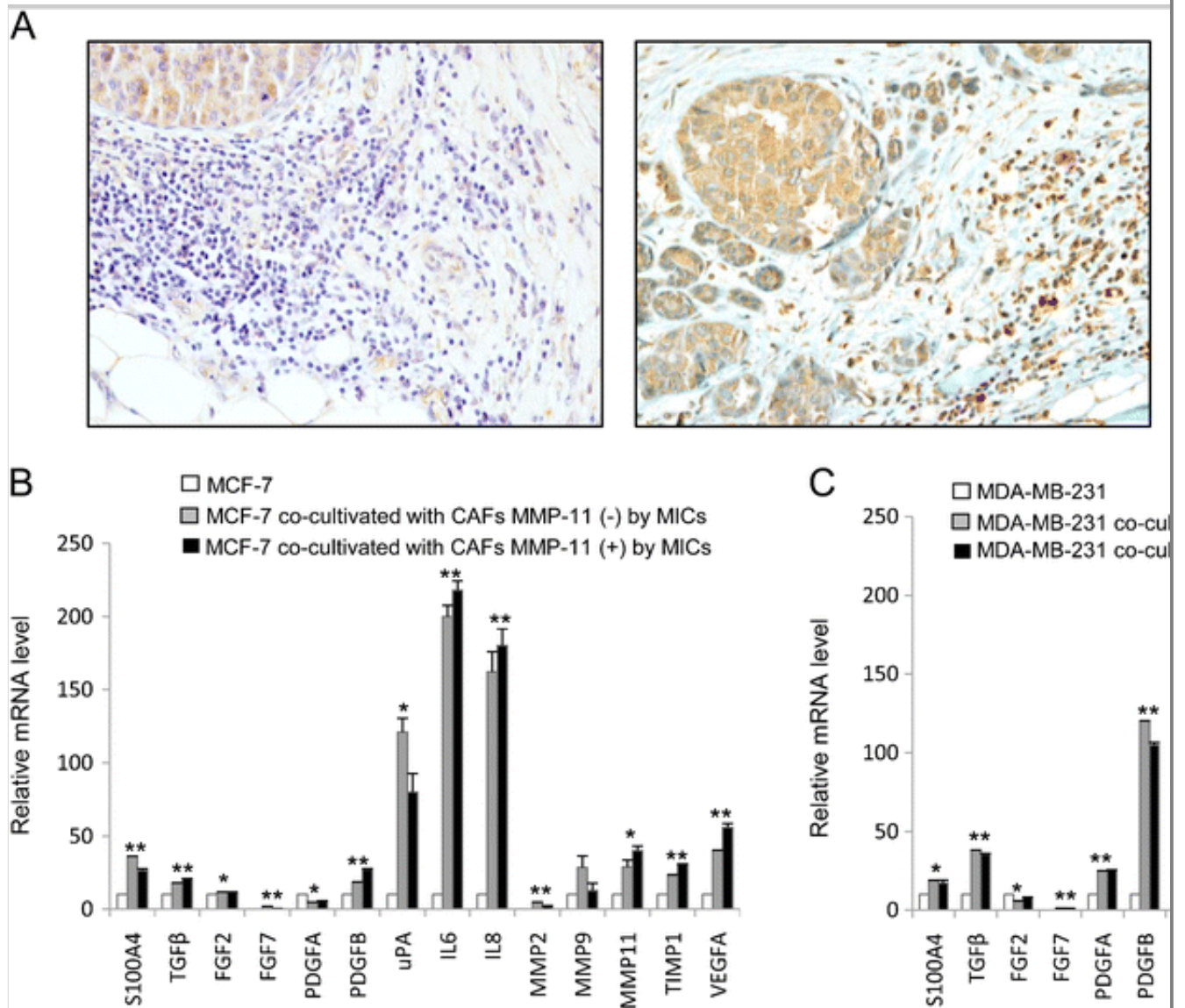


### 3.2. mRNA expression alterations in MCF-7 and MDA-MB-231 cells after co-culture with CAFs from MMP11- or MMP11+ MIC tumors

CAFs were classified according to MMP-11 expression by MICs using IHC of FFPE tumor tissues. We found that of the 20 tumors included in the present study, 8 showed MMP11 positive (+) MICs and 12 showed MMP11 negative (-) MICs (Fig. 2a). In the MMP11+ tumors, at least 70% of the MICs showed positive immunostaining in each evaluated field.

**Fig. 2**

**a** MMP11 immunostaining of primary tumors, showing a negative (left) and a positive (right) example. **b** qRT-PCR analysis of 14 factors in MCF-7 cells alone, after co-culture with CAFs from MMP11- MIC tumors, or after co-culture with CAFs from MMP11+ MIC tumors, (c) MDA-MB-231 cells alone, after co-culture with CAFs from MMP11- MIC tumors, or after co-culture with CAFs from MMP11+ MIC tumors. CAFs from all 20 patients were included. Data represent mean  $\pm$  SD (\* $p \leq 0.05$ , \*\*  $p \leq 0.001$ )



We found that subsequent co-culture of MCF-7 cells with CAFs from MMP11- MIC tumors resulted in uPA mRNA expression upregulation compared to co-culture with CAFs from MMP11+ MIC tumors. Conversely, we found that MCF-7 cells showed increased IL-6 and IL-8 expression levels when co-cultured with CAFs from MMP11+ MIC tumors (Fig. 2b). MDA-MB-231 cells co-cultured with CAFs from MMP11- MIC tumors showed significant ( $p < 0.001$ ) increases in PDGFB, MMP11 and VEGFA mRNA expression levels. Conversely, we found that MDA-MB-231 cells co-cultured with CAFs from MMP11+ MIC tumors showed significantly higher IL-6 ( $p < 0.05$ ), IL-8 and TIMP-1 ( $p < 0.001$ ) mRNA expression levels than MDA-MB-231 cells co-cultured with CAFs from MMP11- MIC tumors.

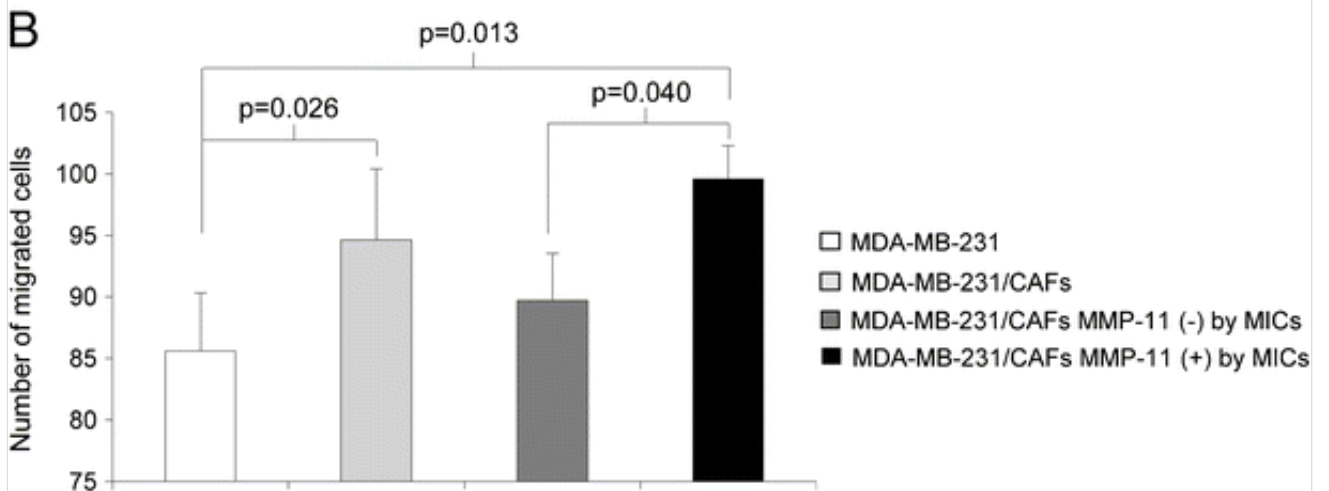
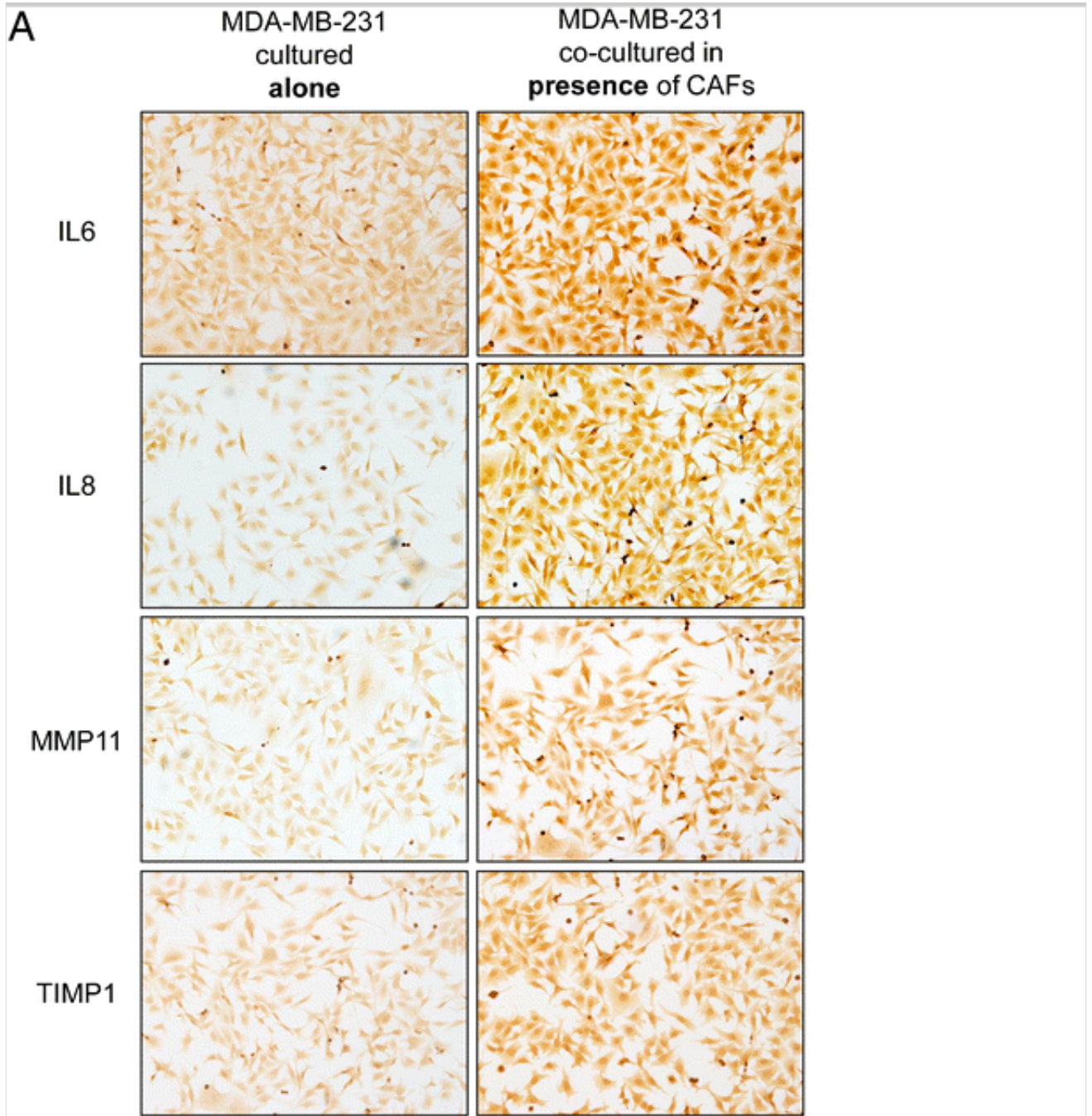
### 3.3. Protein expression alterations in MDA-MB-231 cells after co-culture with CAFs

Next, an IHC study was performed on the aggressive breast cancer cell line

MDA-MB-231 to assess the expression of the IL-6, IL-8, MMP11 and TIMP-1 proteins in the presence or absence of CAFs. An expert pathologist blinded to the results (i.e., MDA-MB-231 cells co-cultured or not with CAFs) evaluated the IHC staining intensities using a numerical score ranging from 0 to 3, reflecting the intensities as follows: 0, no staining; 1, weak staining; 2, intense staining. By doing so, we found that the IHC staining intensities, and thus the protein levels, were higher of IL-6, IL-8, MMP-11 and TIMP-1 when MDA-MB-231 cells were co-cultured with CAFs (Fig. 3a), thereby validating the qRT-PCR data. No cases without IHC staining were found.

**Fig. 3**

**a** Immunostaining of selected factors (IL6, IL8, MMP11 and TIMP1) differentially expressed in MDA-MB-231 cells alone (left) or after co-culture with CAFs (right). **b** Invasive capacity of MDA-MB-231 cells in Matrigel after 48 h without or with CAF-conditioned media from MMP11- MIC or MMP11+ MIC tumors as chemoattractants. Ten randomized fields (magnification 20×) were counted. Data represent the mean  $\pm$  SD. Three independent experiments were performed in each case



### 3.4. Increased invasion of MDA-MB-231 cells after co-

## culture with CAFs

Since MCF-7 cells exhibit a poor invasive capacity, they are usually employed as negative control for invasive studies. Concordantly, we found that the MCF-7 cells showed a poor invasive capacity, even after co-culture with CAFs. Conversely, we found that MDA-MB-231 cells, when co-culture with CAFs, showed a significant ( $p = 0.026$ ) increase in invasive capacity (Fig. 3b). Significant differences with regard to CAF populations also observed, i.e., when MDA-MB-231 cells were co-cultured with CAFs from MMP11+ MIC tumors a significantly higher invasive capacity ( $p = 0.013$ ) was observed compared to MDA-MB-231 cells co-cultured with CAFs from MMP11- MIC tumors.

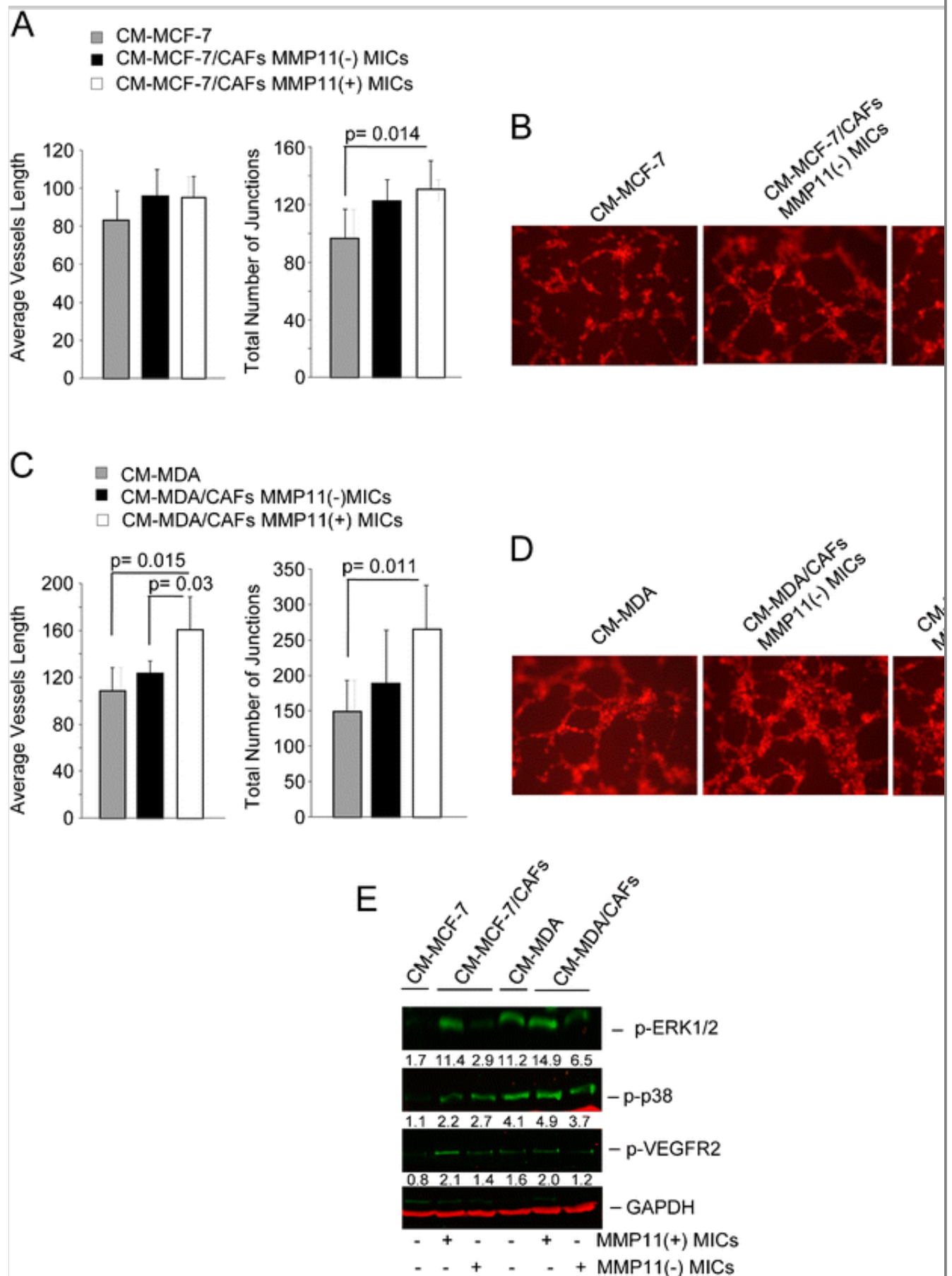
### 3.5. Increased tube formation by HUVECs after co-culture with CAFs

In order to assess the angiogenic capacity induced by CAFs we carried out tube formation assays using HUVEC cells. We found that HUVEC cells showed significant increases in average vessel length when incubated in with conditioned media derived from MCF-7 and MDA-MB-231 cells co-cultured with CAFs ( $p = 0.024$  and  $p = 0.007$ , respectively), as well as increased numbers of capillary-like junctions ( $p = 0.006$  and  $p = 0.021$ , respectively) (Fig. 4a–b). No significant differences were observed in average vessel length when HUVEC cells were incubated in conditioned media derived from MCF-7 cells co-cultured with CAFs from either MMP11- MIC tumors or MMP11+ MIC tumors (Fig. 4a–b), whereas significant ( $p = 0.014$ ) increases in the numbers of capillary-like junctions were observed after incubation of the HUVEC cells in conditioned medium derived from MCF-7 cells co-cultured with CAFs from MMP11+ MIC tumors compared to conditioned medium derived from MCF-7 cells alone ( $96.7 \pm 20.3$ ) (Fig. 4a–b). On the other hand, we found that both the average vessel lengths and the number of junctions in HUVEC cells significantly increased ( $p = 0.015$  and  $p = 0.011$ , respectively) when they were incubated in conditioned medium derived from MDA-MB-231 cells co-cultured with CAFs from MMP11+ MIC tumors compared to conditioned medium derived from MDA-MB-231 cells alone (Fig. 4c–d). Finally, we assessed the expression levels of several angiogenesis related proteins by Western blotting. By doing so, we observed an increased expression of phosphorylated (p)-VEGFR2, p-ERK1/2, and p-p38 in the HUVEC cells after incubation with conditioned media from both MCF-7 and MDA-MB-231 cells co-cultured with CAFs from MMP11+ MIC

tumors (lanes 2 and 5), compared the other conditions tested (Fig. 4e).

**Fig. 4**

Human umbilical vein endothelial cell (HUVEC) tube formation assay. **a** HUVEC cells were cultured for 5 h with conditioned media (CM) from MCF-7 cells (CM-MCF-7), CM-MCF-7/CM-CAFs from MMP11- MIC tumors, or CM-MCF-7/CM-CAFs from MMP11+ MIC tumors, after which average vessel lengths and total numbers of junctions were measured. **b** Representative images of A. **c** HUVEC cells were cultured for 5 h with CM from MDA-MB-231 cells (CM-MDA), CM-MDA/CM-CAFs from MMP11- MIC tumors, or CM-MDA/CM-CAFs from MMP11+ MIC tumors, after which average vessel lengths and total numbers of junctions were measured. **d** Representative images of C. Average vessel lengths and numbers of junctions were quantified using Angiotool software. Data represent mean  $\pm$  SD from three independent experiments. **e** Western blot analysis of phosphorylated (p)-ERK1/2, p-p38, and p-VEGFR2 levels in HUVEC cells after treatment for 30 min with CM from MCF-7 and MDA-MB-231 cells alone or after co-culture with CAFs from MMP11+ or MMP11- MIC tumors. GAPDH expression was used as internal control. Data represent mean  $\pm$  SD from three independent experiments



## 4. Discussion

First, we assessed the effect of CAFs on the expression of 14 stroma-derived

factors, including the calcium-binding protein S100A4, the growth factors TGF $\beta$ , FGF2, FGF7, PDGFA, PDGFB and VEGFA, the inflammatory cytokines IL6 and IL8, the proteases uPA, MMP2, MMP9 and MMP11 and the metalloproteases inhibitor TIMP1 in MCF-7 and MDA-MB-231 breast cancer-derived cells. Secondly, we assessed the effect of CAFs, stratified by the MMP11 status of stromal MICs of the tumors from which they were isolated, on the expression of the respective factors in MCF-7 and MDA-MB-231 cells. Finally, we assessed the effect of CAFs (stratified or not) on the invasive and angiogenic capacities of MDA-MB-231 and HUVEC cells, respectively.

Previously, we observed different CAF-induced expression patterns depending on the tumor cell line with which they were co-cultured [10]. In addition, we found that both breast cancer-derived cell lines used in this study exhibited TGF $\beta$  expression upregulation after co-culture with CAFs [10], which is known to induce EMT in malignant mammary epithelial cells, resulting in the acquisition of highly migratory, invasive, and metastatic phenotypes [18]. Additionally, others have reported that TGF- $\beta$  can induce fibroblasts to secrete a variety of growth factors, cytokines and ECM proteins that can promote tumor development [19, 20]. Here, we show that several of the factors studied (S100A4, TGF $\beta$ , FGF7, PDGFB, uPA, IL-6, IL-8, MMP2, MMP11, TIMP-1 and VEGFA) were increased in each of the two breast cancer-derived cell lines tested after co-culture with CAFs. Taken together, we conclude that reciprocal influences exist between mammary cancer cells and CAFs.

Of the factors that were found to be induced by CAFs after co-culture with MCF-7 and MDA-MB-231 cells, the upregulation of IL-6 and IL-8 was especially relevant, since both factors are cytokines that are known to promote, through autocrine or paracrine signalling, breast cancer growth, invasion [21, 22] and angiogenesis [23]. Additionally, both cytokines have been found to be associated with advanced breast cancer stages and a poor clinical outcome [24, 25, 26]. In addition, we noted some differences in gene expression changes between MCF-7 and MDA-MB-231 cells after co-culture with CAFs. Whereas MCF-7 cells were found to overexpress uPA, MDA-MB-231 cells were found to overexpress (more than 5-fold increases) PDGFB, VEGFA, TIMP1 and MMP11, which are key factors involved in invasion and angiogenesis. PDGFB can promote tumor growth and invasion [27] and, through autocrine signalling, metastasis [28]. In addition, PDGFB

can induce macrophage recruitment, CAF proliferation [29] and angiogenesis [30]. VEGFA can also stimulate angiogenesis and has been found to be significantly associated with a poor survival [31, 32, 33]. TIMP-1, like others TIMPs, is known to be a multifunctional protein, i.e., in addition to its main role as MMP inhibitor, it can also promote tumor cell growth and angiogenesis, and inhibit apoptosis [34]. Previously, associations between high TIMP-1 expression levels and tumor aggressiveness have been reported for breast cancer [35, 36].

MMP11 expression by MICs is considered to serve as a useful biomarker for breast cancer prognosis [12, 13, 14]. We previously reported that the expression of 22 factors related to inflammation and tumor progression (especially IL-1, -5, -6, -17, IFN $\beta$  and NF $\kappa$ B), is associated with MMP11 expression by MICs [12, 37]. In addition, we previously observed an association between MMP11 expression by MICs and a high CD68/(CD3+CD20) ratio in macrophages (CD68<sup>+</sup>), T-cells (CD3<sup>+</sup>) and B-cells (CD20<sup>+</sup>) [38]. Indeed, if there is a high CD68/(CD3+CD20) ratio at the invasive front, most intratumoral MMP11+ MICs are macrophages, suggesting that a high CD68/(CD3+CD20) ratio at the invasive front contributes to the polarization of macrophages in the tumor center to achieve a higher metastatic phenotype. As a result, MMP11 expression by MICs characterizes tumors with a poor prognosis [13, 14], a high inflammatory profile [12, 16, 37], a different stroma composition and a CAF phenotype that contributes to tumor progression [10].

Here, we found that the effects of CAFs from primary MMP11+ or MMP11- MIC tumors on the expression profiles of MCF-7 and MDA-MB-231 cells showed some differences. Interestingly we found that, although conditioned media from both CAF populations increased the invasive phenotype of MDA-MB-231 cells, this effect was more pronounced when the conditioned media were derived from CAFs from MMP11+ MIC tumors. This finding suggests that this CAF population may contribute to a higher invasive capacity of relatively more aggressive breast cancer cells (i.e., MDA-MB-231). In accordance with this notion, we also found that co-culture of MDA-MB-231 cells with CAFs from MMP11+ MIC tumors led to a significantly higher expression of IL-6, IL-8 and TIMP-1, which may be related to an increased tumor cell invasiveness. In addition, we found that conditioned media from MDA-MB-231 cells co-cultured with CAFs from MMP11+ MIC tumors significantly increased angiogenesis and p-ERK1/2, p-

p38 and p-VEGFR2 expression in HUVEC cells, suggesting that CAFs from MMP11+ MIC tumors act on MDA-MB-231 cells to modify their phenotype and increase their malignancy. Interestingly, previous studies have shown that blocking IL-8 [39] and VEGF-A [40] (both up-regulated in breast cancer cells after co-culture with CAFs from MMP11+ MIC tumors) may induce the diminution of both invasive and angiogenic capacities of cancer cells.

In summary, we found that CAFs can induce the expression of several factors related to inflammation and tumor progression, as well as increases in invasive and angiogenic capacities in breast cancer and HUVEC cells, respectively. As such, our data support the existence of crosstalk between CAFs and breast cancer cells to promote tumor growth and invasiveness. The various factors that are produced by CAFs and breast cancer cells may include new therapeutic targets. The identification of CAF populations differentially enhancing the malignant phenotype of cancer cells from primary tumors with a high inflammatory profile contributes to our understanding of the clinical heterogeneity of breast cancer.

## Acknowledgments

This work was supported by grants to F. V from the Instituto de Salud Carlos III (PI13/02745) and from the Consejería de Economía y Empleo del Principado de Asturias (GRUPIN14-116), and to R.P-F. from the Ministerio de Economía y Competividad (SAF2015-69221-R). A.M-O. is a predoctoral fellow from the Ministerio de Educación (FPU14/00548). S.C. was recipient of a predoctoral fellowship financed by the Gobierno del Principado de Asturias “Severo Ochoa” PhD Program (BP14-128).

## Compliance with Ethical Standards

The study adheres to national regulations and was approved by our Institution’s Ethics and Investigation Committee.

*Conflict of interest* The authors have no conflicts of interests to disclose.

## References

1. T. Marsh, K. Pietras, S.S. McAllister, Fibroblasts as architects of cancer pathogenesis. *Biochim. Biophys. Acta* **1832**, 1070–1078 (2013)
2. I. Kogan-Sakin, M. Cohen, N. Paland, S. Madar, H. Solomon, A.

Molchadsky, R. Brosh, Y. Buganim, N. Goldfinger, H. Klocker, J.A. Schalken, V. Rotter, Prostate stromal cells produce CXCL-1, CXCL-2, CXCL-3 and IL-8 in response to epithelia-secreted IL-1. *Carcinogenesis* **30**, 698–705 (2009)

3. M.P. Protti, L. De Monte, Cross-talk within the tumor microenvironment mediates Th2-type inflammation in pancreatic cancer. *Oncoimmunology*. **1**, 89–91 (2012)

4. S. Koontongkaew, The tumor microenvironment contribution to development, growth, invasion and metastasis of head and neck squamous cell carcinomas. *J. Cancer* **4**, 66–83 (2013)

5. S. Madar, I. Goldstein, V. Rotter, ‘Cancer associated fibroblasts’-- more than meets the eye. *Trends Mol. Med.* **19**, 447–453 (2013)

6. N. Erez, M. Truitt, P. Olson, S.T. Arron, D. Hanahan, Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an NF-kappaB-dependent manner. *Cancer Cell* **17**, 135–147 (2010)

7. P.S. Soon, E. Kim, C.K. Pon, A.J. Gill, K. Moore, A.J. Spillane, D.E. Benn, R.C. Baxter, Breast cancer-associated fibroblasts induce epithelial-to-mesenchymal transition in breast cancer cells. *Endocr. Relat. Cancer* **20**, 1–12 (2013)

8. R. Kalluri, M. Zeisberg, Fibroblasts in cancer. *Nat. Rev. Cancer* **6**, 392–401 (2006)

9. M. Allen, J. Louise Jones, Jekyll and Hyde: the role of the microenvironment on the progression of cancer. *J. Pathol.* **223**, 162–176 (2011)

10. L. Gonzalez, N. Eiro, B. Fernandez-Garcia, L.O. Gonzalez, F. Dominguez, F.J. Vizoso, Gene expression profile of normal and cancer-associated fibroblasts according to intratumoral inflammatory cells phenotype from breast cancer tissue. *Mol. Carcinog.* (2015)

11. M.M. Koczorowska, C. Friedemann, K. Geiger, M. Follo, M.L. Biniowski, O. Schilling, Differential effect of TGFbeta on the proteome of cancer associated fibroblasts and cancer epithelial cells in a co-culture approach - a short report. *Cell. Oncol.* **40**, 639–650 (2017)
12. N. Eiro, B. Fernandez-Garcia, L.O. Gonzalez, F.J. Vizoso, Cytokines related to MMP-11 expression by inflammatory cells and breast cancer metastasis. *Oncoimmunology.* **2**, e24010 (2013)
13. L.O. Gonzalez, I. Pidal, S. Junquera, M.D. Corte, J. Vazquez, J.C. Rodriguez, M.L. Lamelas, A.M. Merino, J.L. Garcia-Muniz, F.J. Vizoso, Overexpression of matrix metalloproteinases and their inhibitors in mononuclear inflammatory cells in breast cancer correlates with metastasis-relapse. *Br. J. Cancer* **97**, 957–963 (2007)
14. N. Eiro, B. Fernandez-Garcia, J. Vazquez, J.M. Del Casar, L.O. Gonzalez, F.J. Vizoso, A phenotype from tumor stroma based on the expression of metalloproteases and their inhibitors, associated with prognosis in breast cancer. *Oncoimmunology* **4**, e992222 (2015)
15. L.O. Gonzalez, S. Gonzalez-Reyes, L. Marin, L. Gonzalez, J.M. Gonzalez, M.L. Lamelas, A.M. Merino, E. Rodriguez, I. Pidal, J.M. del Casar, A. Andicoechea, F. Vizoso, Comparative analysis and clinical value of the expression of metalloproteases and their inhibitors by intratumour stromal mononuclear inflammatory cells and those at the invasive front of breast carcinomas. *Histopathology* **57**, 862–876 (2010)
16. B. Fernandez-Garcia, N. Eiro, M.A. Miranda, S. Cid, L.O. Gonzalez, F. Dominguez, F.J. Vizoso, Prognostic significance of inflammatory factors expression by stroma from breast carcinomas. *Carcinogenesis* **37**, 768–776 (2016)
17. E. Zudaire, L. Gambardella, C. Kurcz, S. Vermeren, A computational tool for quantitative analysis of vascular networks. *PLoS One* **6**, e27385 (2011)
18. M.A. Taylor, Y.H. Lee, W.P. Schiemann, Role of TGF-beta and the tumor microenvironment during mammary tumorigenesis. *Gene Expr.* **15**, 117–132 (2011)

19. B. Bierie, H.L. Moses, Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. *Nat. Rev. Cancer* **6**, 506–520 (2006)
20. A. Orimo, P.B. Gupta, D.C. SgROI, F. Arenzana-Seisdedos, T. Delaunay, R. Naeem, V.J. Carey, A.L. Richardson, R.A. Weinberg, Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* **121**, 335–348 (2005)
21. A.W. Studebaker, G. Storci, J.L. Werbeck, P. Sansone, A.K. Sasser, S. Tavolari, T. Huang, M.W. Chan, F.C. Marini, T.J. Rosol, M. Bonafe, B.M. Hall, Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6-dependent manner. *Cancer Res.* **68**, 9087–9095 (2008)
22. J.M. Wang, X. Deng, W. Gong, S. Su, Chemokines and their role in tumor growth and metastasis. *J. Immunol. Methods* **220**, 1–17 (1998)
23. D.E. Hu, Y. Hori, T.P. Fan, Interleukin-8 stimulates angiogenesis in rats. *Inflammation* **17**, 135–143 (1993)
24. T. Bachelot, I. Ray-Coquard, C. Menetrier-Caux, M. Rastkha, A. Duc, J.Y. Blay, Prognostic value of serum levels of interleukin 6 and of serum and plasma levels of vascular endothelial growth factor in hormone-refractory metastatic breast cancer patients. *Br. J. Cancer* **88**, 1721–1726 (2003)
25. R. Salgado, S. Junius, I. Benoy, P. Van Dam, P. Vermeulen, E. Van Marck, P. Huget, L.Y. Dirix, Circulating interleukin-6 predicts survival in patients with metastatic breast cancer. *Int. J. Cancer* **103**, 642–646 (2003)
26. I.H. Benoy, R. Salgado, P. Van Dam, K. Geboers, E. Van Marck, S. Scharpe, P.B. Vermeulen, L.Y. Dirix, Increased serum interleukin-8 in patients with early and metastatic breast cancer correlates with early dissemination and survival. *Clin. Cancer Res.* **10**, 7157–7162 (2004)
27. Y. Cao, Multifarious functions of PDGFs and PDGFRs in tumor

growth and metastasis. *Trends Mol. Med.* **19**, 460–473 (2013)

28. M. Jechlinger, A. Sommer, R. Moriggl, P. Seither, N. Kraut, P. Capodiecci, M. Donovan, C. Cordon-Cardo, H. Beug, S. Grunert, Autocrine PDGFR signaling promotes mammary cancer metastasis. *J. Clin. Invest.* **116**, 1561–1570 (2006)

29. M.M. Mueller, N.E. Fusenig, Friends or foes - bipolar effects of the tumour stroma in cancer. *Nat. Rev. Cancer* **4**, 839–849 (2004)

30. D. Engelmann, D. Mayoli-Nussle, C. Mayrhofer, K. Furst, V. Alla, A. Stoll, A. Spitschak, K. Abshagen, B. Vollmar, S. Ran, B.M. Putzer, E2F1 promotes angiogenesis through the VEGF-C/VEGFR-3 axis in a feedback loop for cooperative induction of PDGF-B. *J. Mol. Cell Biol.* **5**, 391–403 (2013)

31. P. Manders, L.V. Beex, V.C. Tjan-Heijnen, J. Geurts-Moespot, T.H. Van Tienoven, J.A. Foekens, C.G. Sweep, The prognostic value of vascular endothelial growth factor in 574 node-negative breast cancer patients who did not receive adjuvant systemic therapy. *Br. J. Cancer* **87**, 772–778 (2002)

32. H. Bando, H.A. Weich, M. Brokelmann, S. Horiguchi, N. Funata, T. Ogawa, M. Toi, Association between intratumoral free and total VEGF, soluble VEGFR-1, VEGFR-2 and prognosis in breast cancer. *Br. J. Cancer* **92**, 553–561 (2005)

33. S. Ghosh, C.A. Sullivan, M.P. Zerkowski, A.M. Molinaro, D.L. Rimm, R.L. Camp, G.G. Chung, High levels of vascular endothelial growth factor and its receptors (VEGFR-1, VEGFR-2, neuropilin-1) are associated with worse outcome in breast cancer. *Hum. Pathol.* **39**, 1835–1843 (2008)

34. S.O. Wurtz, A.S. Schrohl, N.M. Sorensen, U. Lademann, I.J. Christensen, H. Mouridsen, N. Brunner, Tissue inhibitor of metalloproteinases-1 in breast cancer. *Endocr. Relat. Cancer* **12**, 215–227 (2005)

35. A. Neri, T. Megha, F. Bettarini, D. Tacchini, M.G. Mastrogiulio, D.

Marrelli, E. Pinto, P. Tosi, Is tissue inhibitor of metalloproteinase-1 a new prognosticator for breast cancer? An analysis of 266 cases. *Hum. Pathol.* **43**, 1184–1191 (2012)

36. A. Dechaphunkul, M. Phukaoloun, K. Kanjanapradit, K. Graham, S. Ghosh, C. Santos, J.R. Mackey, Prognostic significance of tissue inhibitor of metalloproteinase-1 in breast cancer. *Int J Breast Cancer* **2012**, 290854 (2012)

37. N. Eiro, L. Gonzalez, L.O. Gonzalez, B. Fernandez-Garcia, M.L. Lamelas, L. Marin, S. Gonzalez-Reyes, J.M. del Casar, F.J. Vizoso, Relationship between the inflammatory molecular profile of breast carcinomas and distant metastasis development. *PLoS One* **7**, e49047 (2012)

38. N. Eiro, I. Pidal, B. Fernandez-Garcia, S. Junquera, M.L. Lamelas, J.M. del Casar, L.O. Gonzalez, A. Lopez-Muniz, F.J. Vizoso, Impact of CD68/(CD3+CD20) ratio at the invasive front of primary tumors on distant metastasis development in breast cancer. *PLoS One* **7**, e52796 (2012)

39. Y. Lin, R. Huang, L. Chen, S. Li, Q. Shi, C. Jordan, R.P. Huang, Identification of interleukin-8 as estrogen receptor-regulated factor involved in breast cancer invasion and angiogenesis by protein arrays. *Int. J. Cancer* **109**, 507–515 (2004)

40. R.K. Jain, Molecular regulation of vessel maturation. *Nat. Med.* **9**, 685–693 (2003)