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Title: Carcinoma-associated fibroblasts derive from mesothelial cells via mesothelial to mesenchymal transition in peritoneal metastasis.

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ABSTRACT

Peritoneal dissemination is a frequent metastatic route for cancers of the ovary and gastrointestinal tract. Tumor cells metastasize by attaching to and invading through the mesothelial cell (MC) monolayer that lines the peritoneal cavity. Metastases are influenced by carcinoma-associated fibroblasts (CAFs), a cell population that derives from different sources. Hence, we investigated whether MCs, through mesothelial to mesenchymal transition (MMT), were a source of CAFs during peritoneal carcinomatosis and whether MMT affected the adhesion and invasion of tumor cells. Biopsies from patients with peritoneal dissemination revealed the presence of myofibroblasts expressing mesothelial markers in the proximity of carcinoma implants. Prominent new vessel formation was observed in the peritoneal areas harboring tumor cells when compared with tumor-free regions. The use of a mouse model of peritoneal dissemination confirmed the myofibroblast conversion of MCs and the increase in angiogenesis at places of tumor implants. Treatment of omentum MCs with conditioned media from carcinoma cell cultures resulted in phenotype changes reminiscent of MMT. Adhesion experiments demonstrated that MMT enhanced the binding of cancer cells to MCs in a β1-integrin-dependent manner. Scanning electron microscopy imaging showed that the enhanced adhesion was mostly due to increased cell-cell interaction and not to a mere matrix exposure. Invasion assays suggested a reciprocal stimulation of the invasive capacity of tumor cells and MCs. Our results demonstrate that CAFs can derive from mesothelial cells during peritoneal metastasis. We suggest that MMT renders the peritoneum more receptive for tumor cell attachment/invasion and contributes to secondary tumor growth by promoting its vascularization.

Keywords: Carcinoma-associated fibroblasts; Mesothelial cells; Mesothelial-to-mesenchymal transition; Peritoneal metastasis.

INTRODUCTION

Neoplasias originated adjacent to the peritoneal cavity, such as ovarian or colorectal cancers, frequently disseminate via transcoelomic route to develop peritoneal metastases, which evolve very rapidly and correlate with poor prognosis [1, 2]. Surgery is inefficient to render patients free of disease, resulting in low survival rates [3, 4]. In peritoneal metastasis, cancer cells detached from the primary tumor are transported by peritoneal fluid and spread locally to colonize the peritoneum [1, 2]. The organization of the peritoneum is simple; a single layer of mesothelial cells (MCs) lines a compact region that is composed of connective tissue with few fibroblasts, mast cells, macrophages, and vessels. MCs may acquire a myofibroblast-like phenotype, through an epithelial to mesenchymal transition (EMT)-like process, during inflammatory and repair responses (e.g. peritoneal damage induced by dialysis fluid) [5, 6]. MCs share characteristics with both epithelial and endothelial cells, which may undergo EMT and endothelial to mesenchymal transition (EndMT), respectively. Thus, recently we and other authors have proposed renaming the myofibroblast conversion of MCs, that takes place in different organs such as lung, liver or peritoneum, with a more appropriate term: mesothelial to mesenchymal transition (MMT) [7-11]. MMT is characterized by an increase in the invasive capacity of MCs, which allows them to invade the peritoneal compact zone. Mesenchymal MCs acquire the capacity to synthesize inflammatory and angiogenic factors, as well as extracellular matrix (ECM) components, thereby contributing to the deterioration of the peritoneum during chronic peritoneal injury [5, 6].

The first contact of metastasizing tumor cells with the peritoneum is provided by the MC monolayer. Inflammatory factors released by tumor and immune cells, as well

as the formation of ascites, may alter the tissue architecture of the peritoneal membrane making it more receptive to further metastatic implantation [12, 13]. Inflammation promotes the mesenchymal conversion of MCs [6, 14], however, the possible role of the MMT process in the attachment of tumor cells to the peritoneum has been overlooked.

Carcinoma-associated fibroblasts (CAFs) are a prominent cell type within the tumor stroma and participate in most stages of tumor progression [15]. CAFs share characteristics with myofibroblasts, including the expression of α-smooth muscle actin (α-SMA), and are considered activated fibroblasts capable of producing a wide array of growth factors, as well as ECM components, thereby contributing to the growth and vascularization of solid tumors [16]. There is emerging evidence that the origin of CAFs may vary between different cancers and within different areas of individual tumors [17]. The activation of resident fibroblasts has been considered the main origin of CAFs in the tumor microenvironment [18, 19]. More recent studies point to cells recruited from the bone marrow as a possible source of CAFs. In addition, it has been shown that endothelial cells, through an EndMT, may also be a source of CAFs [14, 17, 20]. Epithelial cells are considered an important source of activated fibroblasts during fibrosis [21], but it is unclear if normal epithelial cells convert into CAFs in tumors [18, 22].

Herein, we show that CAFs located within or nearby peritoneal carcinoma implants expressed mesothelial markers. Cell culture experiments demonstrated that carcinoma cells secreted MMT-promoting factors and that the mesenchymal conversion of MCs favored cell-cell interaction with tumor cells. The results showed that mesenchymal MCs and tumor cells mutually stimulate their invasive capacity. We propose that the MMT of MCs plays an important role both in the initial steps of

peritoneal metastasis and in the growth of secondary tumor implants.

MATERIALS AND METHODS

Analysis of biopsies from patients with peritoneal metastasis

Peritoneal metastases from 5 serous ovarian carcinomas, 4 colonic adenocarcinomas, 3 pancreatic adenocarcinomas, 1 endometrial adenocarcinoma, and pleural metastases from 3 pulmonary adenocarcinomas were considered in this study. Informed written consent to use surgical samples was obtained from the patients, with the approval of the Ethics Committee of the Hospital de la Princesa (Madrid, Spain). Tissue samples were fixed in neutral-buffered 3.7% formalin and embedded in paraffin to obtain serial sections 3µm thick. Haematoxylin-eosin (H&E) and immunohistochemical (IHC) staining were performed as is described in Supplementary materials and methods.

Mouse model of peritoneal carcinomatosis

Swiss nu/nu 2-month-old mice were used in this study (Charles River Laboratories, Barcelona, Spain). The experimental protocol followed was in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and was approved by the Animal Ethics Committee of the "Unidad de Experimentación Animal" del Centro de Biología Molecular "Severo Ochoa" (Madrid, Spain).

A total of $5x10^6$ SKOV-3 cells were inoculated into the peritoneal cavity of mice. Mice were sacrificed at different time points (4 to 8 weeks) and then parietal and visceral peritoneal samples were collected for IHC and immunofluorescence analysis. Peritoneal specimens were routinely fixed in neutral-buffered 3.7% formalin and embedded in paraffin. Deparaffinized sections (3µm) were stained with H&E. IHC and immunofluorescence procedures are described in Supplementary materials and methods.

Carcinoma cell lines, primary peritoneal MCs and induction of MMT

The cell lines SKOV-3 and HT29, derived from human ovarian carcinoma and colorectal adenocarcinoma, respectively, were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS).

Human peritoneal MCs were obtained from omentum samples of patients undergoing elective surgery as previously described [5, 7, 8]. Procedures are detailed in Supplementary materials and methods. This study adjusts to the Declaration of Helsinki and it was approved by the Ethics Committee of Centro de Biología Molecular "Severo Ochoa" (Madrid, Spain). Informed written consent to use surgical samples was obtained from omentum donors.

To induce MMT with conditioned media, omentum MCs were treated with 75% of carcinoma cell supernatants for 72 hours or 6 days as is described in Supplementary materials and methods. As a control of MMT *in vitro*, MCs were treated with 0.5 ng/mL of transforming growth factor-β1 (TGF-β1) plus 2.5 ng/mL of interleukin-1β (IL-1β) (R&D Systems) for 72 hours or 6 days, which has been proven to be a suitable MMT model [7, 8, 23]. To interfere with the MMT process, omentum MCs were treated for 72 hours with SKOV-3 conditioned media or with TGF-β1 (1ng/mL) in the presence or absence of a selective inhibitor of the TGF-β Type I Receptor GW788388 (see Supplementary materials and methods). The analysis of MMT-related molecules was achieved by quantitative RT-PCR or western blot in mesothelial and SKOV-3 cells as described in Supplementary materials and methods.

Adhesion assays and scanning electron microscopy

Adhesion assays of SKOV-3 cells labeled with Carboxyfluorescein diacetate Succinimidyl Ester (CFSE; Molecular Probes, Invitrogen) on MCs either treated or not with TGF-β1 plus IL-1β for 72 hours, were performed as is described in Supplementary materials and methods.

For scanning electron microscopy analysis (see Supplementary materials and methods), a parallel experimental procedure was carried out in which coverslips were fixed in 3% glutaraldehyde.

To characterize the adhesion molecules involved in tumor-mesothelium interaction, SKOV-3 cells labeled with the fluorescent probe BCECF-AM (Sigma-Aldrich) were added to MCs, treated or not with TGF-β1 plus IL-β1, in presence of the monoclonal antibodies indicated in Supplementary materials and methods [24, 25].

Invasion assays

Invasion assays were performed in 12-mm diameter transwell units (3.0 μm pore size; Corning, MA, USA). First, we analyzed the effect of MCs, with either epithelial-like or mesenchymal-like phenotypes, embedded in the collagen matrix on SKOV-3 cells invasion capacity. Next, we analyzed the effect of carcinoma SKOV-3 cells embedded in the matrix on MCs invasion capacity. The protocols for the invasion assays are detailed in Supplementary materials and methods.

Statistical analysis

Results are presented as 25th and 75th percentiles, median, minimum and maximum values in box plots graphics; and as mean ± SEM in bar graphics. The data

groups were compared with the non-parametric Mann–Whitney rank sum U-test using the SPSS statistic package version 15.0 (Chicago, IL, USA) and GraphPad Prism version 5.0 (La Jolla, CA, USA). $P<0.05$ was considered statistically significant.

RESULTS

Human biopsies analysis shows that CAFs originate from MCs and collaborate in tumor angiogenesis

Histologic observation of peritoneal biopsies from human ovarian carcinoma implants showed the localization of metastatic foci in the submesothelial area surrounded by spindle-like cells. Immuno-staining of serial sections showed overlapped expression of α-SMA and mesothelial markers, such as cytokeratins and calretinin, in the stromal tissue surrounding the tumor implants. As expected, cytokeratin staining also revealed the epithelial nature of metastasizing cells (**Figure 1A**). Additional mesothelial markers, like Wilm's tumor protein-1 (WT1) and mesothelin, were also expressed in fibroblast-like cells proximal to peritoneal micrometastases derived from an endometrial adenocarcinoma (**Figure 1B**). The co-expression of mesothelial and mesenchymal markers suggested that in peritoneal metastases CAFs may derive from MCs via MMT.

To confirm the mesothelial origin of CAFs, we took advantage of the differential expression of cytokeratins. Colon carcinoma cells express cytokeratin-20 but not cytokeratin-7 and, conversely, MCs express cytokeratin-7 but not cytokeratin-20. The analysis of peritoneal metastases of colon carcinoma in humans demonstrated that the expression of cytokeratin-20 was confined to tumor implants, whereas the expression of cytokeratin-7 was observed in the preserved mesothelium of tumor-free regions and in fibroblast-like cells located in the close proximity of tumor micrometastases (**Figure 1C**). To further verify that CAFs may originate from MCs, we analyzed metastatic implants of lung cancers in the pleura, another anatomic cavity lined by mesothelium. We observed CAFs expressing calretinin close to cancer implants (**Suppl. Figure S1**).

In addition, we did not observe expression of mesothelial makers in CAFs of tumors located outside the coelomic cavities (**Suppl. Figure S2**).

It was shown that during the MMT process, MCs acquired the ability to produce large amounts of VEGF [26]. Thus, we hypothesized that CAFs derived from MCs could play a role in tumor vascularization. Quantification of the number of vessels in biopsies from peritoneal implants derived from different human cancers revealed a significant increase ($p=0.002$) in CD34-positive vessels in the areas of tumor micrometastasis compared with tumor-free zones from the same biopsies (**Figures 2A to 2C**). To confirm that CAFs derived from MCs participate in tumor angiogenesis, we took advantage of the fact that some tumors express low levels of VEGF. As shown in **Figure 2D**, an endometrial adenocarcinoma expressed low amounts of VEGF. In contrast, MC-derived CAFs (cytokeratin positive) appeared as high producers of VEGF (**Figure 2E**).

CAFs originate from MCs in a mouse model of peritoneal dissemination

Parietal peritoneal tissues stained with H&E showed a fibro-proliferative response at the sites of small tumor implants, with submesothelial accumulation of spindle-like cells and fibrosis, which were absent in tumor-free zones of the same samples (**Figures 3A and 3B**). IHC analysis showed intense cytokeratin staining of metastasizing cells and a weak cytokeratin staining in MCs located nearby, indicating that these MCs were undergoing a MMT process, which implied the down-regulation of cytokeratin expression. Surrounding the micrometastatic nodules there was a prominent accumulation of α-SMA-positive fibroblasts. In the peritoneal region without metastatic implants there was no expression of α-SMA other than that in the smooth muscle cells

of the vessel walls, and the expression of cytokeratin was limited to the preserved mesothelium (**Figures 3C to 3F)**. To confirm the origin of CAFs, we used WT1 as an additional mesothelial marker. IHC analysis showed nuclear staining of WT1 in the preserved mesothelium of tumor-free regions and in submesothelial CAFs surrounding tumor implants (**Figures 3G and 3H)**. Immunofluorescence techniques confirmed the presence of fibroblast-like cells that were double-positive for WT1 and α-SMA (**Figure 4**). Western blot analysis demonstrated that SKOV-3 cells were negative for WT1 and α-SMA, and confirmed the down-regulation of cytokeratin and the induction of α-SMA during the MMT process (**Suppl. Figure S3**).

Blood vessels of parietal tissues were stained with anti-CD31 antibody. There was a dramatic increase in the number of vessels in adjacent areas to micrometastases when compared with tumor-free regions (**Figures 5A and 5B)**. The new vessels were mainly located in the upper submesothelial zone, where MC-derived CAFs expressing high levels of VEGF (**Figures 5C and 5D**) tended to accumulate, suggesting that MCs could, indeed, play an important role in tumor vascularization.

Analysis of peritoneal specimens with larger implants showed that at advanced stages of peritoneal dissemination, the expression of cytokeratin was restricted to carcinoma cells. Nevertheless, WT1 and α-SMA-positive cells could be observed not only in the proximity of carcinoma implants, but also integrated within the tumor stroma (**Suppl. Figure S4**).

Peritoneal metastases were also observed in the omental tissue, where cytokeratin and α-SMA-positive spindle cells were found in the interstitial stroma surrounding tumor implants (**Suppl. Figure S5**).

Conditioned media from carcinoma cells induces MMT in vitro

Incubation of omentum MCs with SKOV-3 cells conditioned media induced the acquisition of a spindle-like morphology that was evident at 72 hours and more pronounced at 6 days, with a similar appearance to that of MCs treated with TGF-β1 plus IL-1β (**Figure 6A**). To verify the mesenchymal conversion of MCs, the expression patterns of MMT markers were analyzed by quantitative RT-PCR in cells treated with conditioned media for 72 hours. Under these conditions, the expression of E-cadherin was repressed and conversely, the expression of Snail was induced when compared with control media-treated cells (**Figures 6B and 6C**). The expression of other MMT markers (fibronectin, collagen I, VEGF and TGF-β1) were up-regulated in MCs treated with conditioned media compared to control cells (**Figures 6D to 6G**). The molecular reprogramming after 6 days of treatment with SKOV-3 conditioned media was more profound (**data not shown**).

Similar experiments were performed using the colorectal adenocarcinoma cell line HT29. Treatment of MCs with supernatant from HT29 cultures induced the acquisition of a spindle-like morphology, but this was evident only at 6 days (**Suppl. Figure S6A**). Repression of E-cadherin and the up-regulation of fibronectin and collagen-I also reached statistical significance at 6 days (**Suppl. Figures S6B to S6D**).

TGF-β1 is a key molecule in the mesenchymal conversion of MCs. Accordingly, experiments blocking the TGF-β type I receptor were performed. Quantitative RT-PCR analysis revealed that the selective inhibitor GW788388 blocked the induction of Snail, collagen I and fibronectin mediated by TGF-β1 or SKOV-3 conditioned media (**Figure 7**).

Mesenchymal conversion of MCs enhances the adhesion of tumor cells

Adhesion experiments demonstrated that SKOV-3 cells had a significantly higher interaction capacity with mesenchymal-like MCs (stimulated with TGF-β1 plus IL-1β) than with untreated MCs (**Figures 8A and 8B**). We investigated whether β1-integrins mediated the increased adhesion of tumor cells to MCs undergoing MMT. Treatment of cells with the blocking anti-β1 antibody Lia1/2 disrupted both the basal adhesion of SKOV-3 cells to untreated MCs and the increased adhesion to transdifferentiated MCs. Conversely, treatment with the activating anti-β1 antibody TS2/16 enhanced dramatically the adhesion of tumor cells to untreated MCs and to MCs treated with TGF-β1 plus IL-1β. Other blocking antibodies specific for the adhesion molecules VLA-1 (5E8D9) and β2-integrin (Lia3/2) did not affect the adhesion of tumor cells to MCs (**Figure 8C**). Our data demonstrated that the MMT promoted the adhesion of tumor cells and suggested that activated β1-integrin mediated, at least partially, the tumor cell-mesothelium interaction.

To explore whether the enhanced adhesion of tumor cell to mesenchymal-like MCs was due to exposure of underlying matrix or to an increased cell-cell interaction, we carried out scanning electron microscopy imaging analysis. The images showed that SKOV-3 cells, maintaining a round-shaped morphology, laid on the monolayer of MCs with cobblestone features (**Figure 8D**). In contrast, SKOV-3 cells spread on transdifferentiated MCs loosing their round shape. Tumor cells emitted pseudopodial protrusions across the cytoplasmic extensions of mesenchymal-like MCs (**Figure 8E**). Detailed analysis of the images revealed that the enhanced adhesion of tumor cells was mostly due to increased cell-cell interaction and not to the underlying matrix exposed through the intercellular spaces.

MCs and tumor cells mutually stimulate their invasive capacity

We next investigated if mesenchymal MCs could promote the invasion of tumor cells. SKOV-3 cells seeded on a collagen I matrix did not show invasive capacity. On the contrary, carcinoma cells seeded on matrix harboring MCs with an epithelial-like phenotype showed an increase in their invasive capacity. This effect was much more evident when MCs with a mesenchymal phenotype were embedded in the matrix (**Figures 9A and 9B).** The number of invading SKOV-3 cells increased significantly ($p=0.003$) when mesenchymal MCs were present in the matrix, compared with embedded epithelial-like MCs (**Figure 9C**).

The histological analysis revealed that the MC monolayer was preserved in tumor-free regions but it was disrupted in areas with micrometastases, where CAFs expressing mesothelial markers could be observed (**Figure 1C, Inset**). These data suggested that tumor cells might, in turn, promote the invasion of the stroma by MCs. We performed *in vitro* invasion assays to analyze the effect of carcinoma cells on MC invasion capacity. SKOV-3 cells embedded in a collagen I matrix were able to attract the MCs from the monolayer into the matrix. In contrast, MCs seeded on collagen I matrix without cancer cells remained mainly as a monolayer (**Figures 10A to 10C**). The number of invading MCs showed a significant increase ($p=0.03$) when SKOV-3 cells were present in the matrix (**Figure 10D**).

DISCUSSION

The origin of CAFs has not been clearly established and it seems very likely that these activated fibroblasts are derived from various sources [16-18, 27, 28]. Herein we show, for the first time, that CAFs found in peritoneal metastatic implants derive from the mesothelium through a mechanism implicating tumor-induced MMT of MCs. The mesenchymal transition of MCs, in turn, favors the adhesion and invasion of metastasizing tumor cells and promotes the growth of secondary tumor implants (**Summarized in Figure 11**).

The presence of MCs that have undergone MMT was first observed in the peritoneum of peritoneal dialysis patients [5, 29]. Afterwards, emerging evidence has suggested that MMT is an important event for peritoneal structural alteration, including fibrosis and angiogenesis [26, 30]. In this work, we show the presence of myofibroblasts expressing mesothelial markers, both in human biopsies and in mouse peritoneal specimens, suggesting that a subpopulation of CAFs stems from MCs during peritoneal tumor dissemination. In addition, we show a marked fibro-proliferative response and a prominent angiogenesis at the places of tumor implants, where MC-derived CAFs accumulate. These results suggest that MMT plays important roles in tumor stroma formation and vascularization. The fact that MCs that have undergone MMT produce large amounts of VEGF [26] suggests that these cells may favor tumor angiogenesis and growth [31]. In this respect, it has been demonstrated that the expression of a soluble form of the VEGF receptor Flt-1 in the mesothelium suppresses the peritoneal metastasis of gastric tumors [32]. Experimental animal models of peritoneal fibrosis have demonstrated that treatments directed against MMT or against the MMT-promoting stimuli reduce the accumulation of myofibroblasts, ameliorate

submesothelial fibrosis and angiogenesis and improve peritoneal function [6-8]. Thus, it is tempting to speculate that MMT may also be a putative therapeutic target to arrest peritoneal metastasis.

Metastasizing cancer cells have two alternatives for attaching to the peritoneum: the surface of the MC monolayer or the exposed submesothelial matrix. It has been proposed that tumor cells prefer the ECM for first attachment. In this context, it has been shown that during the initial stages of peritoneal metastasis tumor cells bind predominantly to "milky spots" in which the ECM is exposed. Nevertheless, it is important to notice that secretions from tumor cells, as well as from MCs and immune cells, generate an inflammatory environment, which may influence peritoneal metastasis transforming the initial pattern of "milky spot" attachment into a widespread pattern of dissemination [33]. It has been hypothesized that tumor-associated inflammatory environment induces damage to the MC monolayer, leading to the exposure of the submesothelial connective tissue and providing a more favorable surface for tumor cell binding. Another important effect of inflammation is the triggering of myofibroblastic conversion of MCs [6, 34]; however, the involvement of the MMT process in the binding of tumor cells to the peritoneum had not been explored yet.

The mesenchymal transition of MCs may play important roles in rendering the peritoneum more receptive for further metastatic implants. In this regard, our *in vitro* adhesion experiments demonstrate that tumor cells adhere better to mesenchymal than to epithelial-like MC monolayers. Scanning electron microscopy analysis reveals that the enhanced adhesion of tumor cells to mesenchymal MCs is not due to a mere exposure of underlying matrix but rather to an increased cell-cell interaction. The tumor cell-mesothelium interaction is mediated, at least in part, by β1-integrins [35, 36], which

could reflect that cancer cells bind to mesothelium-associated ECM and/or to the MC adhesion molecule VCAM-1 [37]. In agreement with this notion, it has been described that cleavage of MC-associated matrix proteins fibronectin and vitronectin by MMP-2 enhances integrin-mediated carcinoma-mesothelium attachment [38, 39]. Furthermore, it has been shown that blocking antibodies or siRNA directed against VCAM-1 or its ligand α4β1-integrin significantly decrease the adhesion and transmigration of SKOV-3 cells through the MC monolayer [40]. Besides β1-integrins, other adhesion molecules, including CD44, CA125/MUC16 or ICAM-1, may also participate in the binding of cancer cells to mesothelium [41-46]. Determining the role of these molecules in tumor cell-mesothelium attachment along the mesenchymal conversion of MCs would require further analysis.

Our histological analysis shows that numerous MC-derived CAFs accumulate in areas with micrometastases, but not in tumor-free regions, suggesting that tumor cells promote the invasion of adjacent MCs. Indeed, our *in vitro* invasion assays demonstrate that carcinoma cells embedded in the matrix enhance the invasive capacity of MCs. The enhanced invasion triggered by tumor cells could be a consequence of the acquisition of a mesenchymal phenotype by MCs. In fact, we have previously demonstrated that during the MMT process, MCs increase their migration/invasion capacity [23]. MCs that have invaded the matrix could, in turn, further promote the invasion of carcinoma cells. In this context, we demonstrate that MCs with mesenchymal phenotype embedded in the matrix markedly stimulate the invasion of SKOV-3 cells. Thus, these results suggest that mesenchymal MCs and carcinoma cells establish a feed-forward cycle by mutually stimulating their invasive capacity.

Interestingly, MCs behave in a similar fashion to resident fibroblasts in terms of the induction of carcinoma cell invasion *in vitro*. It was shown that normal omentum-derived fibroblasts induce the adhesion and invasion of carcinoma cells in 3D culture models [38, 47]. On the other hand, Cai et al, reported that activated fibroblasts (either CAFs isolated from malignant ovary carcinomas or normal omentum fibroblasts stimulated with tumor conditioned media) have much higher effects on carcinoma cell attachment and invasion than normal fibroblasts [19, 48]. We have also observed that MCs with a mesenchymal phenotype embedded in the matrix stimulate the invasion of SKOV-3 cells more than that of MCs with an epithelial-like phenotype.

TGF-β1 is a key molecule controlling MMT and peritoneal fibrosis [8]. The measurement of TGF-β1 in the conditioned media from carcinoma cells showed that SKOV-3 cells produced higher levels of this cytokine than HT-29 (1141.6 ± 169.3 vs. 369.4 ± 27.2 pg/mL). This might explain the stronger effect exerted by SKOV-3 cells on the mesenchymal conversion of MCs, when compared with HT29 cells. In addition, blockade of TGF-β1 receptor prevented the mesenchymal conversion of MCs treated with conditioned media from SKOV-3 cells. TGF-β1 is a prototypical inducer of EMT and is also a key factor in the myofibroblastic differentiation of recruited fibrocytes and in the mesenchymal conversion of endothelial cells via EndMT [8, 27, 49, 50]. Thus, it can be speculated that accumulation of CAFs, regardless of their origin, could be interfered by targeting the TGF-β1 pathway [51].

The most important limitation of our study is that we have not performed lineage-tracing studies to formally demonstrate that the CAFs derived from MCs. The data do not exclude the possibility of *de novo* expression of mesothelial markers in activated fibroblasts. However, recent cell linage studies have demonstrated the myo-

fibroblast conversion of MCs during embryogenesis and in liver fibrogenesis, reinforcing our findings [10, 52].

In conclusion, this work shows that myofibroblasts derived from MCs may adopt roles attributed classically to CAFs, forming a suitable metastatic niche to promote cancer adhesion/invasion and growth. Thereby, CAFs derived from adjacent mesothelium could constitute an alternative target in the treatment of metastases that disseminate via the peritoneum.

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STATEMENT OF AUTHOR CONTRIBUTIONS

PS and ML-C conceived and carried out the study design. JAJ-H supplied, analyzed and interpreted the biopsies from patients. PS, AR-V, MLP-L, VR-C, AG and RR carried out *in vitro* experiments. JG-B performed scanning electron microscopy procedures. PS, AR-V, AG, RR and KS carried out *in vivo* experiments. PS, JD, PLM, MF, CC and ML-C were involved in data analysis and interpretation, literature search, generation of figures, writing the paper and had final approval of the submitted and published versions.

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FIGURE LEGENDS

Figure 1: Analysis of human peritoneal carcinoma implants reveals the mesothelial origin for CAFs. A variable number of CAFs expressing calretinin were present in all the cases analyzed, not only in ovarian carcinomas metastases, but also in intestinal, pancreatic and endometrial ones. **(A)** H&E staining shows an ovarian cancer micrometastasis in the submesothelial area surrounded by spindle-like cells (**panel a**). IHC analysis of serial sections reveals overlapped expression of α-SMA and the mesothelial markers calretinin and cytokeratin in fibroblastic cells surrounding the tumor implant (**panels b to d**). Cytokeratins also stain the metastasizing cells, revealing their epithelial nature (**panel d**). Scale bars: 25μm. **(B)** Serial sections of a peritoneal implant of an endometrial cancer biopsy reveal the presence of fibroblastic cells expressing nuclear WT1 (**panel a**) and mesothelin (**panel b**). Mesothelin is also detected within the endometrial tumor mass. Scale bars: 100μm. **(C)** Biopsy of peritoneal metastasis from a patient diagnosed with colon carcinoma. Multiple tumor nodules express cytokeratin-20 while stromal cells close to metastatic implants are negative for this marker (**panel a**). In contrast, fibroblast-like cells surrounding tumor implants express the mesothelial marker cytokeratin-7. The inset shows a higher magnification of the delimited area (**panel b**). Scale bars: 100μm. T: Tumor.

Figure 2: The number of vessels increases in areas adjacent to micrometastases, where MC-derived CAFs accumulate and express large amounts of VEGF. (A) Representative specimen of ovary cancer metastasis in peritoneum. The "Non-Tumor" image shows a submesothelial area from the biopsy without evidence of tumor implants

and with a low number of vessels, which were mostly confined to the deeper compact zone. The "Tumor" image depicts a submesothelial area with micrometastases and elevated number of CD34-positive vessels. Scale bars: 100μm. **(B)** Separate analysis of three ovarian, two pancreatic, one colorectal and one endometrial-derived metastases consistently shows the differences. **(C)** Quantification of CD34-positive vessels observed in tumor implant areas compared to distant zones without evidence of peritoneal metastases. Box plot graphic depicts 25th and 75th percentiles, median, minimum and maximum values. Differences were statistically significant (p=0.002). **(D)** Representative case of endometrial adenocarcinoma metastasis in peritoneum. The tumor implant shows low expression levels of VEGF, which is mostly limited to endothelial cells in angiogenesis areas **(panel a)**. Spindle-shaped CAFs surrounding the tumor mass express high levels of VEGF **(panel b)**. Scale bars: 25μm. **(E)** Serial sections of the same case confirm that MC-derived CAFs (cytokeratin positive) proximal to the tumor produce large amounts of VEGF (**panels a and b**). Scale bars: 25μm. T: Tumor.

Figure 3: CAFs originate from MCs in a mouse model of peritoneal dissemination. Parietal peritoneum samples were analyzed by IHC four weeks after i.p. injection of SKOV-3 cells. **(A and B)** H&E staining shows that areas distant from tumor implants (Non-Tumor) have a conserved histological structure, without evidence of fibrosis and with a preserved MC monolayer. At the places of tumor implants there is a fibro-proliferative response, with the accumulation of spindle-like cells. **(C and D)** IHC analysis shows cytokeratin expression in the preserved mesothelium of Non-Tumor regions. In the proximity of tumor implants, there is an intense cytokeratin staining in

metastasizing tumor cells and a weak cytokeratin staining in MCs and in some fibroblast-like cells. **(E and F)** In the Non-Tumor areas there is no expression of α-SMA other than that in the smooth muscle cells of the vessel walls. Surrounding the tumor implants there is accumulation of α-SMA-positive fibroblasts. **(G and H)** WT1 is used as an additional MCs marker to confirm the mesothelial origin of CAFs. IHC analysis reveals nuclear staining of WT1 in the preserved mesothelium of tumor-free regions and in submesothelial CAFs surrounding tumor implants. Arrows point to MC-derived CAFs expressing cytokeratin, α-SMA or WT1 in the proximity of sub-mesothelial tumor implants. The insets show higher magnifications of the delimited areas. Scale bars: 25μm. T: Tumor; Pan-CK: Pan-cytokeratin.

Figure 4: Mesothelial-derived CAFs co-express WT1 and α-SMA in the mouse model of peritoneal dissemination. (A) Distant areas from tumor micro-implants show MCs forming a monolayer and expressing nuclear WT1 (green). MCs lining the peritoneum are exposed to factors produced by the tumor and, as a consequence, MCs with an apparently preserved structure begin to express low levels of α-SMA (red). **(B)** In areas close to the micrometastasis, MCs co-expressing WT1 (green) and α-SMA (red) change their original localization on the peritoneal surface and start to invade the sub-mesothelial compact zone. **(C)** CAFs co-expressing WT1 and α-SMA are embedded in the parenchyma of a micrometastasis cohabiting with tumor cells negative for both markers. Arrows point to MC-derived CAFs co-expressing WT1 and α-SMA. Scale bars: 10μm.

Figure 5: Increased angiogenesis is found in sites where MC-derived CAFs expressing high amounts of VEGF accumulate. (A and B) CD31 staining reveals that non-tumor zones do not possess a high number of vessels. At the sites of tumor implants, there is a dramatic increase of vessels density, particularly located in the upper compact zone where MC-derived CAFs tend to accumulate. **(C and D)** VEGF is expressed in a preserved mesothelium distant from tumor implants. At tumor zones, MCs and MC-derived CAFs, as well as cancer cells, proximal to vascularized areas express high levels of VEGF. Arrows point to angiogenesis zones. Scale bars: 25μm. T: Tumor.

Figure 6: Conditioned media from SKOV-3 cells induces MMT *in vitro*. (A) Treatment of omentum MCs with culture media from SKOV-3 cells induces the acquisition of a spindle-like phenotype that is evident at 72 hours and more pronounced at 6 days. The morphology alteration of MCs is similar to that observed in cells treated with TGF-β1 plus IL-1β. Scale bars: 100μm. **(B and C)** The mesenchymal conversion of MCs treated with conditioned media is verified at the molecular level by qRT-PCR analysis of recognized MMT markers. The expression of E-cadherin is repressed in MCs treated for 72h with SKOV-3-derived conditioned media when compared with control media-treated MCs. On the contrary, the expression of Snail is up-regulated in MCs treated with conditioned media. **(D to G)** The expression of other MMT-associated markers including the matrix components fibronectin and collagen I, as well as the growth factors VEGF and TGF-β1, are also significantly up-regulated in MCs treated with conditioned media. Bar graphics represent mean ± SEM. Symbols represent the

statistic differences between groups. F.I.: Fold Induction; SKOV-3 CM: SKOV-3 Conditioned Medium; T+I: TGF-β1 plus IL-1β.

Figure 7: TGF-β Type I Receptor blocking interferes with MMT induced by SKOV-3 conditioned media. Omentum-derived MCs were treated for 72 hours with TGF-β (1 ng/mL) or SKOV-3 conditioned media in the presence or absence of 2μM or 5μM of GW788388. **(A and B)** Quantitative RT-PCR shows that GW788388 blocks the induction of fibronectin and collagen I mediated by TGF-β1 or SKOV-3 conditioned media. **(C)** GW788388 also blocks the up-regulation of the MMT-associated molecule Snail. Bar graphics represent mean ± SEM. Symbols represent the statistic differences between groups. F.I.: Fold Induction; SKOV-3 CM: SKOV-3 Conditioned Medium.

Figure 8: Tumor cells adhere mainly to the transdifferentiated MC monolayer through cell-cell interactions. (A) Representative images show CFSE-labeled SKOV-3 cells (green) adhered on omentum-derived MC monolayers (DAPI) that have been pre-treated or not with TGF-β1 plus IL-1β. Scale bars: 100μm. **(B)** Tumor cells adhere more efficiently to mesenchymal-like MCs than to MCs with epithelial morphology. Results are presented as the number of adhered SKOV-3 cells per field. Box plot graphic depicts 25th and 75th percentiles, median, minimum and maximum values. Numbers above boxes depict mean ± SEM. Differences are statistically significant ($p=0.04$). **(C)** β1-integrins mediate the increased adhesion of tumor cells to transdifferentiated MCs. Treatment of MCs with the blocking anti-β1 antibody Lia1/2 reduces significantly both the basal adhesion of SKOV-3 cells to control MCs and the increased adhesion to

transdifferentiated MCs. Conversely, treatments with the activating anti-β1 antibody TS2/16 markedly enhance the adhesion of tumor cells to control and transdifferentiated MCs. Other blocking antibodies specific for the adhesion molecules VLA-1 (5E8D9) and β2-integrin (Lia3/2) do not affect the adhesion of tumor cells to MCs. Results are depicted as percentage of adhered cells, considering the number of cells prior to wash as 100%. Bar graphics represent mean ± SEM. Symbols represent the statistical differences between groups. T+I: TGF-β1 plus IL-1β. **(D)** High resolution images of co-cultures were obtained by scanning electron microscopy techniques. The image analysis shows SKOV-3 cells laying on a monolayer of MCs with epithelial-like morphology. Tumor cells adhered to control monolayers maintain a round-shaped morphology. **(E)** Transdifferentiated MC monolayers show a disorganized structure on which numerous cancer cells spread out and lose their round aspect. At high magnification SKOV-3 cells display pseudopodial protrusions across the cytoplasmic extensions of mesenchymal-like MCs. Detailed analysis of the images suggests that the enhanced adhesion of tumor cells to transdifferentiated MCs was mostly by means of direct cell-cell contacts. Scale bars: 10μm. T+I: TGF-β1 plus IL-1β.

Figure 9: Mesenchymal-like MCs strongly stimulate the invasive capacity of carcinoma cells. (A and B) Transversal projection and 3D reconstructions of Confocal Laser Scanning Microscopy show that SKOV-3 cells (red) seeded on a collagen I matrix do not invade after five days. In contrast, carcinoma cells seeded on matrix harboring CFSE- and propidium iodide-labelled MCs (green + red) with either epithelial-like (MCs) or mesenchymal-like (MCs+T+I) phenotype show significant increase in the invasive capacity. This effect is much more evident when MCs with a mesenchymal

phenotype are embedded in the matrix. Scale bars: 100μm. **(C)** Quantification analyses of SKOV-3 invading cells were performed subtracting CFSE-labelled MCs (green) from the total propidium iodide-stained cells (red) that invaded the matrix. Thus, the invasion of SKOV-3 corresponds to red cells in the merge panel, since embedded MCs are yellow (green + red). Results show the percentage of invading SKOV-3 cells in gels containing MCs versus gels containing MCs pre-treated with TGF-β1 plus IL-1β. Bar graphics represent mean ± SEM. The difference is statistically significant (p=0.003). T+I: TGF-β1 plus IL-1β.

Figure 10: Tumor cells embedded in the matrix induce MC invasion. (A) H&E sections of paraffin embedded gels show the disruption of the MC monolayer when SKOV-3 cells are present in a collagen I matrix, mimicking a metastatic submesothelium. **(B)** Confocal images display transversal projections of CFSE- and propidium iodide-labelled SKOV-3 cells (green + red) embedded in a collagen I matrix. Carcinoma cells are able to attract MCs (red) from the monolayer into the matrix. However, MCs seeded on collagen I matrixes without cancer cells remain mainly as a monolayer. **(C)** Reconstructions in 3D of Confocal Laser Scanning Microscopy. **(D)** Quantification analyses of invading MCs were performed subtracting CFSE-labelled SKOV-3 cells (green) from the total propidium iodide-stained cells (red) that invaded the matrix. Thus, the invasion of MCs corresponds to red cells in the merge panel, since embedded SKOV-3 cells are yellow (green + red). Results show the percentage of invading MCs in gels containing SKOV-3 cells versus gels without tumor cells. Bar

graphics represent mean ± SEM. The difference is statistically significant (p=0.03). Scale bars: 100μm.

Figure 11: Tumor peritoneal dissemination model. (A) First contact and early MMT. Tumor cells dispersed in the peritoneal cavity initially attach to milky spots of mesothelium where they trigger an early MMT. The mechanisms behind cancer anchoring to the peritoneal barrier seem to involve the regulation of, at least, the β1-integrin molecule by the mesothelium. **(B) MMT progression. Mesothelial and cancer cell invasion.** Cancer cell attachment is more apparent as the MMT advances. MCs acquire invasive capacity, providing cancer cells direct access to colonize the submesothelium. **(C) Fibrosis, angiogenesis and CAF accumulation.** The accumulation of submesothelial myofibroblasts via MMT (CAFs) provides the tumor of the adequate blood support and ECM components to survive. During disease progression, intraperitoneal tumor implants could nurture new CAFs recruited from the adjacent mesothelium, contributing to the colonization of new metastasis areas.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1: Analysis of human lung cancer-derived metastasis in the pleura. Two representative slides show calretinin-positive fibroblasts coexisting with large-cell lung carcinoma implants in pleura. These results support the mesothelial origin of CAFs. T: Tumor. Scale bars represent 100μm.

Supplementary Figure S2: MC markers are not expressed in CAFs of tumors located outside the coelomic cavities. (**A and B**) Serial sections of a cutaneous basal cell carcinoma show CAFs negative for mesothelial markers (cytokeratin AE1/AE3 and calretinin) accompanying the tumor. (**C**) An infiltrating breast carcinoma positive for cytokeratin AE1/AE3 shows proximal CAFs negative for this mesothelial marker. Scale bars represent 25μm. T: Tumor.

Supplementary Figure S3: Expression pattern of mesothelial markers in primary MCs, either treated or not with TGF-β1 plus IL-1β, and in SKOV-3 cells. Western blot analysis shows that SKOV-3 cells are negative for WT1, α-SMA and calretinin. The expression of pan-cytokeratin in SKOV-3 cells reveals its epithelial origin. On the contrary, omental-derived MCs express WT1, pan-cytokeratin and calretinin. The levels of these markers are dramatically down-regulated in MCs treated with TGF-β1 + IL-1β. The molecule α-SMA is increased in MCs transdifferentiated *in vitro*. Expression of β-actin is employed as a loading control.

Supplementary Figure S4: Large tumor implants coexist with fibrotic areas where CAFs express mesothelial markers. H&E analysis of advanced peritoneal disseminations (8 weeks after i.p. SKOV-3 cell injection) shows larger metastatic masses coexisting with widespread areas of fibrosis. IHC studies reveal the existence of CAFs expressing mesothelial markers not only in the proximity of carcinoma implants, but also integrated within the tumor stroma. The expression of cytokeratin is restricted exclusively to carcinoma cells. However, extended WT1 and α-SMA staining can be observed cohabiting with the large peritoneal tumors. The inset shows a higher magnification of the WT1 nuclear staining. Scale bars represent 100μm. T: Tumor.

Supplementary Figure S5: Analysis of omental metastasis in the mouse model of peritoneal dissemination. (A) Visceral macroscopic metastases are observed in mice i.p. inoculated with SKOV-3 cells. **(B)** Immunohistochemical analyses of omental tissues reveal that MCs positive for cytokeratin are lining omentum in tumor-free areas. **(C)** Fibroblastic cells (CAFs) express pan-cytokeratin in the stroma interstices between cancer cells. **(D)** α-SMA is negative in MCs lining tumor-free omental tissue. **(E)** A higher magnification of the tumor mass shows spindle-like cells positive for α-SMA embedded in the tumor parenchyma. Scale bars represent 100μm (**B and D**) and 25μm (C and E). Arrows point to MCs expressing cytokeratin or α-SMA. T: Tumor.

Supplementary Figure S6: Conditioned media from HT29 cells induces MMT *in vitro*. (A) Conditioned media obtained from the colorectal adenocarcinoma cell line HT29 was applied during 6 days to omentum-derived MCs to induce MMT. The

treatment induced the acquisition of a spindle-like morphology in MCs. Scale bars represent 100μm. (**B to D**) MMT molecular reprogramming, including the repression of E-cadherin and the up-regulation of mesenchymal markers such as fibronectin and collagen-I, reach the statistical significance at 6 days of exposition to HT29 conditioned media. Bar graphics represent mean ± SEM. Symbols represent the statistic differences between groups. F.I.: Fold Induction; HT29 CM: HT29 Conditioned Medium; T+I: TGF-β1 plus IL-1β.