ANTIOXIDANTS & REDOX SIGNALING
Volume 14, Number 12, 2011

DOI: 10.1089/ars.2010.3727

ORIGINAL RESEARCH COMMUNICATION

Cancer Associated Fibroblasts Exploit Reactive Oxygen Species Through a Proinflammatory Signature Leading to Epithelial Mesenchymal Transition and Stemness

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Abstract

Cancer-associated fibroblasts (CAFs) are key determinants in the malignant progression of cancer, supporting tumorigenesis and metastasis. CAFs also mediate epithelial mesenchymal transition (EMT) of tumor cells and their achievement of stem cell traits. We demonstrate that CAFs induce EMT and stemness through a proinflammatory signature, which exploits reactive oxygen species to drive a migratory and aggressive phenotype of prostate carcinoma cells. CAFs exert their propelling role for EMT in strict dependence on cycloxygenase-2 (COX-2), nuclear factor-κB, and hypoxia-inducible factor-1. CAF-secreted metalloproteases elicit in carcinoma cells a Rac1b/COX-2-mediated release of reactive oxygen species, which is mandatory for EMT, stemness, and dissemination of metastatic cells. Tumor growth is abolished, and metastasis formation is severely impaired by RNA interfering-mediated targeting of the proinflammatory signature, thereby supporting the therapeutic targeting of the circuitry COX-2/nuclear factor-κB /hypoxia-inducible factor-1 as a valuable antimetastatic tool affecting cancer cell malignancy. *Antioxid. Redox Signal.* 14, 2361–2371.

Introduction

Although tumorigenesis is largely acknowledged as a cell-autonomous process involving genetically transformed cancer cells, the importance of stromal cell types populating the tumoral microenvironment is now well established (4, 41). Reactive stroma is composed by several heterotypic cells, including cancer associated fibroblasts (CAFs), macrophages, and endothelial cells. CAFs are phenotypically and functionally distinguishable from their normal counterparts for their augmented proliferation and differential expression of extracellular matrix (ECM) proteins and soluble factors (21). Conversely to resting fibroblasts, CAFs acquire an activated phenotype and can be recognized by their expression of fibroblast-specific protein 1, desmin, vimentin, and α-smooth-muscle actin. To date, fibroblasts in tumor tissues are recognized as a diverse population of myofibroblastic cells intermixed with other fibroblasts that do not express α-smooth-muscle actin, although they exert tumor promoting activity (12, 17, 26, 39).

CAFs communicate among themselves as well as with cancer cells and inflammatory and immune cells directly through cell contact and indirectly through paracrine/exocrine signaling, proteases, and modulation of ECM. This complex communications network is pivotal to providing the appropriate microenvironment to support tumorigenesis, angiogenesis, and metastasis. Normal fibroblasts play a helpful role in maintaining epithelial homeostasis by suppressing proliferation of adjacent epithelia (2, 3, 43). After epithelial neoplastic transformation, CAFs undergo profound changes, promote tumor growth, induce angiogenesis, and recruit bone marrow–derived endothelial progenitor cells (1, 30, 31). Interestingly, CAFs can even mediate resistance to antiangiogenic therapy (11). In addition, CAFs act on motility of cancer cells through remodeling the ECM (35) and inducing epithelial mesenchymal transition (EMT) of adiacent carcinoma cells (17). EMT is an epigenetic transcriptional program by which epithelial cells gain mesenchymal features as reduced cell-cell contact and increased motility, thereby escaping the primary tumor and allowing dissemination of metastases at a distance (15, 40). A hallmark of EMT is the loss of *E-cadherin* expression, correlated with the tumor grade and stage (9, 10, 14). Several transcription factors have been implicated in the control of EMT, including Snail, Slug, Twist, Goosecoid, ZEB1, and SIP1 (29).

Conversely to embryonic EMT, EMT serving tissue repair or metastatic dissemination is often correlated with tissue inflammation and persists until the provoking spur/injury is

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removed. In keeping, treatment of mammary carcinoma cells with tumor necrosis factor-α leads to stabilization of Snail through activation of nuclear factor-κB (NF-κB) pathway (46). As in developing tumors, CAFs have been shown to orchestrate macrophage recruitment and neovascularization in strict dependence on NF-κB (13). We reasoned that CAFs may exert their propelling role for EMT by eliciting proinflammatory pathways in metastatic cells. We, therefore, examined the role of proinflammatory, pro-oxidant, and promigratory pathways elicited in EMT undergoing prostate carcinoma (PCa) cells due to CAF exposure.

Materials and Methods

Materials

Unless specified, all reagents were obtained from Sigma. All antibodies were from Santa Cruz Biotechnology, except for anti-hypoxia inducible factor-1α (HIF-1α) and Rac1 (BD Biosciences), anti-Rac1b (Millipore), and FITC conjugated anti-CD133 antibodies (Abcam). The FITC mouse anti human CD44 (clone G44-26) and PE mouse anti human CD24 (clone ML5) antibodies were from BD Bioscience. shRNA against cycloxygenase-2 (COX-2), HIF-1α, NF-κB, and negative control shRNA were from Santa Cruz Biotechnology. 2′,7′-dihydrochlorofluorescein diacetate (H_2DCF-DA) was from Molecular Probes. Ilomastat and Rac1 inhibitor were from Calbiochem. Tumor growth factor β1 (TGFβ1) and interleukin-6 (IL-6) were from PeproTech.

Cell culture and transfections

PC3 cells were obtained from European Collection of Cell Cultures. Human prostate fibroblasts (HPFs) and CAFs were isolated from surgical explantation from patients affected by benign prostatic hyperplasia (HPFs) or from cancer regions of patients bearing PCa (Gleason 4+5) (CAFs) (17). Four different surgical explantations were used for both CAFs and HPFs, and their ability to induce EMT resulted comparable. All the isolated human prostate primary fibroblasts, as well as the other unique renewable resources used in this article, will be available on request for the scientific community. PC3 cells and fibroblasts were grown in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, 10 U/ml penicillin, and 10 mg/ml streptomycin. CAFs and HPFs have been used for 15 passages without significant modification of their ability to elicit EMT.

For fibroblast activation, cells were grown to sub-confluence and treated for 24 h with 10 ng/ml TGF-β1 or 50 ng/ml IL-6. Fresh serum-free medium was added for an additional 24 h before collection of conditioned medium (CM), in order to obtain CM free from TGF-β1 or IL-6 (but conditioned by their earlier administration). PC3 cells were then incubated with the obtained CM for 72 h and then used for different analyses.

Transient transfection with p[NF-κB]Luc was performed with Lipofectamine (Invitrogen), following manufacturer's instructions, and its efficiency has been evaluated to 60% using a co-transfection with pEGFP-N1 (ratio pEGFP-N1/p[NF-κB]Luc 1:10). Silencing with shRNA was performed with Lipofectamine (Invitrogen) following manufacturer's instructions. Silenced cells were selected by puromycin treatment, and a pool of stably transfected cells was generated in order to avoid clonal selection.

Western blot analysis

PC3 cells (1×10^6) were lysed for 20 min on ice in 500 μl RIPA lysis buffer (50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 1% Triton X-100, 2 m*M* EGTA, 1 m*M* sodium orthovanadate, 1 m*M* phenylmethanesulphonyl-fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). 20 μg of total proteins were loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, separated, and transferred onto nitrocellulose. The immunoblots were incubated in 3% bovine serum albumin, 10 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA, and 0.1% Tween 20 for 1 h at room temperature; probed first with specific antibodies and then with secondary antibodies.

NF-κB activity assay

About 1×10^6 PC3 cells were transiently transfected with 2 μg of the luciferase reporter plasmid p[NF-κB]Luc, kept in growth medium for 24 h, and then exposed to fibroblast CM. Determination of luciferase activities was performed using the Luciferase Assay System (Promega), following the manufacturer's instructions.

In vitro *Boyden invasion assay*

Transwells (Corning), equipped with an 8-μm-pore polyvinylpyrrolidone-free polycarbonate filters, were coated with 50 μg/cm^2 of reconstituted Matrigel. About 5×10^4 PC3 cells were loaded into the upper compartment in serum-free growth medium. Cells were allowed to migrate toward complete growth medium. After 24 h of incubation at 37°C, non invading cells were removed mechanically using cotton swabs, and the membrane was stained with DiffQuick solution.

Assay of intracellular reactive oxygen species

Intracellular production of H_2O_2 was assayed as previously described (8). Five minutes before the end of the incubation time, cells were treated with 5 μg/ml H_2DCF-DA. After phosphate-buffered saline washing, cells were lysed in 1 ml of RIPA buffer and analyzed immediately by fluorimetric analysis at 510 nm. Data have been normalized to total protein content.

Prostaspheres formation and clonogenicity assay

PC3 cells were incubated for 72 h with CM and then detached using Accutase (Sigma). Single PC3 cells were plated at 150 cells/cm^2 on low attachment 100 mm plate in Dulbecco's modified Eagle's medium/F12 supplemented with B27 and N2 media, 5 μg/ml insulin, 20 ng/ml fibroblast GF-2, and 20 ng/ml epidermal GF. PC3 cells were grown under these conditions for 15-20 days and formed non-adherent P0 spheres termed prostaspheres. For the evaluation of self-renewal, a single prostasphere was dissociated in single cells with Accutase, and a diluition of one cell per well into 96-well low attachment plates was performed in order to isolate individual P1 prostaspheres. Single-cell cloning was confirmed by microscopic analysis, and single clones were counted.

Flow cytometry

1×10^6 PC3 cells were labeled with FITC-anti-CD44 (clone G44-26) and PE-anti-CD24 (clone ML5) antibodies for 1h at

4°C in the dark. Cells were washed, and flow cytometry was performed using an FACSscan (BD Biosciences).

Rac1 activity assay

To quantify the amount of Rac1/Rac1b-GTP, 1×10^6 PC3 cells were lysed in RIPA buffer, and the lysates were incubated with 10 μg of p21 activated kinase-GST fusion protein, absorbed on glutathione-Sepharose beads for 1 h at 4°C. Immunoreactive Rac1 and Rac1b were then quantified by Western blot analysis. Lysates were normalized for Rac1 and Rac1b content by immunoblotting.

Xenograft experiments

In vivo experiments were carried out in accordance with national guidelines and approved by the ethical committee of Animal Welfare Office of Italian Work Ministry and conform to the legal mandates and Italian guidelines for the care and maintenance of laboratory animals. Six- to 8-week-old male (six per group) SCID-bg/bg mice (Charles River Laboratories International) were injected subcutaneously with 1×10^6 PC3 cells or with 1×10^6 PC3 cells plus 0.5×10^6 fibroblasts, as described in (17).

Lung colonization assays

Six- to 8-week old female SCID-bg/bg mice were injected with PC3 cells treated for 72 h with CM from CAFs or with serum-free medium as a control. Mice (six animals per group) were injected into the lateral tail veins with differently treated-PC3 cells (1×10^6 in 0.2 ml of phosphate-buffered saline). They were monitored every 3 days and sacrificed after 8 weeks. Lungs were inspected for metastatic nodules by histological analyses.

Statistical analysis

Data are presented as means ± standard deviation from at least three independent experiments. Statistical analysis of the data was performed by Student's *t*-test. *p*-Values ≤ 0.05 were considered statistically significant.

Results

CAFs activates a proinflammatory response to elicit EMT in PC3 cells

To assess the role of proinflammatoty signals elicited by CAFs in EMT undergoing cells, we used PC3 cells, a line isolated from a bone metastasis of human PCa, and CAFs or *in vitro* activated HPFs. HPFs were isolated from prostate from patients affected by benign prostatic hyperplasia, whereas CAFs were obtained from the surgically explanted prostate of patients bearing PCa (Gleason 4 + 5). Expression of E-cadherin and cytokeratin was performed to exclude epithelial contamination of both CAFs and HPFs. We have already reported that CAFs and HPFs activated *in vitro* with TGFβ1 or IL-6 elicits a clear EMT in PC3 cells (17).

In order to study the molecular pathways driving CAF-induced EMT response in PC3 cells, we analyze the proinflammatory pathways in PC3 cells treated with CM from CAFs or from HPFs activated with TGFβ1 or IL-6. PC3 cells respond to CAF-induced EMT with activation of the NF-κB/COX-2 pathway, showing similar responses to CAFs or *in vitro* activated HPFs (Fig. 1A). In addition, CAF exposure leads to activation of HIF-1 (Fig. 1A), a known transcription factor normally engaged by hypoxia but already reported to be activated in normoxia by inflammation in a COX-2-dependent manner (38). Silencing of NF-κB, COX-2, and HIF-1 by shRNA in PC3 cells after exposure to CAFs or *in vitro* activated HPFs reveals that this proinflammatory axis plays a mandatory role in orchestrating CAF-induced EMT in PC3 cells. Indeed, silencing of each COX-2, NF-κB, and HIF-1 is able to block invasiveness and expression of EMT markers (Snail, Twist, Met, E-Cad) (Fig. 1B, C and Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/ars).

We have already correlated CAF-induced EMT with achievement of stem cell traits in PC3 cells, reporting increased expression of CD133 and CD44/CD24 ratio, P1 prostasphere formation, and tumor initiating cells in SCID mice xenografts (17). To validate our data on the proinflammatory response elicited by CAFs on PC3 cells, we analyzed expression of stem cell markers and anchorage independence growth of PC3 cells silenced for NF-κB, COX-2, and HIF-1. In keeping with the idea that stemness of PC3 cells is intimately linked to activation of their EMT after exposure to CAFs, silencing of NF-κB, COX-2, or HIF-1 blocks the increase in CD133 or CD44/CD24 expression and P1 prostasphere formation (Fig. 1D–F).

PCa cells activate reactive oxygen species production on CAF exposure

Inflammation and oxidative stress have already been linked to cancer progression and claimed as responsible for enhanced malignancy (25, 32). Oxidative stress, due to deregulated intracellular reactive oxygen species (ROS) production, has been correlated to PCa carcinogenesis, androgen resistance, anchorage independence, and resistance to *anoikis* (18, 22). We observed that exposure to CAFs, or *in vitro* activated HPFs, leads to enhancement of ROS content of PC3 cells (Fig. 2A). ROS delivery appears to be mainly due to arachidonic acid metabolism deregulation, as indicated by the ability of COX-2 inhibitors to block CAF-induced ROS generation and the invasive phenotype (Fig. 2A, B and Supplementary Fig. S2). In keeping, silencing of COX-2 is able to dramatically reduce CAF-mediated ROS production (Fig. 2C).

EMT has already been correlated with oxidative stress in mouse breast carcinoma cells, a mandatory event for carcinogenesis due to DNA oxidative damage. In this model, EMT has been elicited by treatment with soluble metalloprotease-3 (MMP-3), and ROS production is due to activation of Rac1b, an alternatively spliced isoform of Rac1 (36). We observed that treatment of CAFs with the MMP broad range inhibitor ilomastat severely decreases COX-2, HIF-1, and NF-κB activation in PC3 cells (Fig. 3A) as well as ROS production and EMT (Fig. 3B, C) due to exposure to CAFs or to HPFs activated *in vitro* by IL-6. In addition, we observed a clear activation of Rac1 and a *de novo* expression of Rac1b isoform (Fig. 3D, E). In keeping with the reported constitutive activation of Rac1b on its transcriptional activation (33), we detect a clear linearity between expression and activity of Rac1b isoform (Fig. 3E). Finally, the role of Rac1 activation in eliciting a proinflammatory response in PC3 cells is stressed by the ability of Rac-1 inhibitor to downregulate the NF-κB, COX-2, and HIF-1 pathway (Fig. 3F).

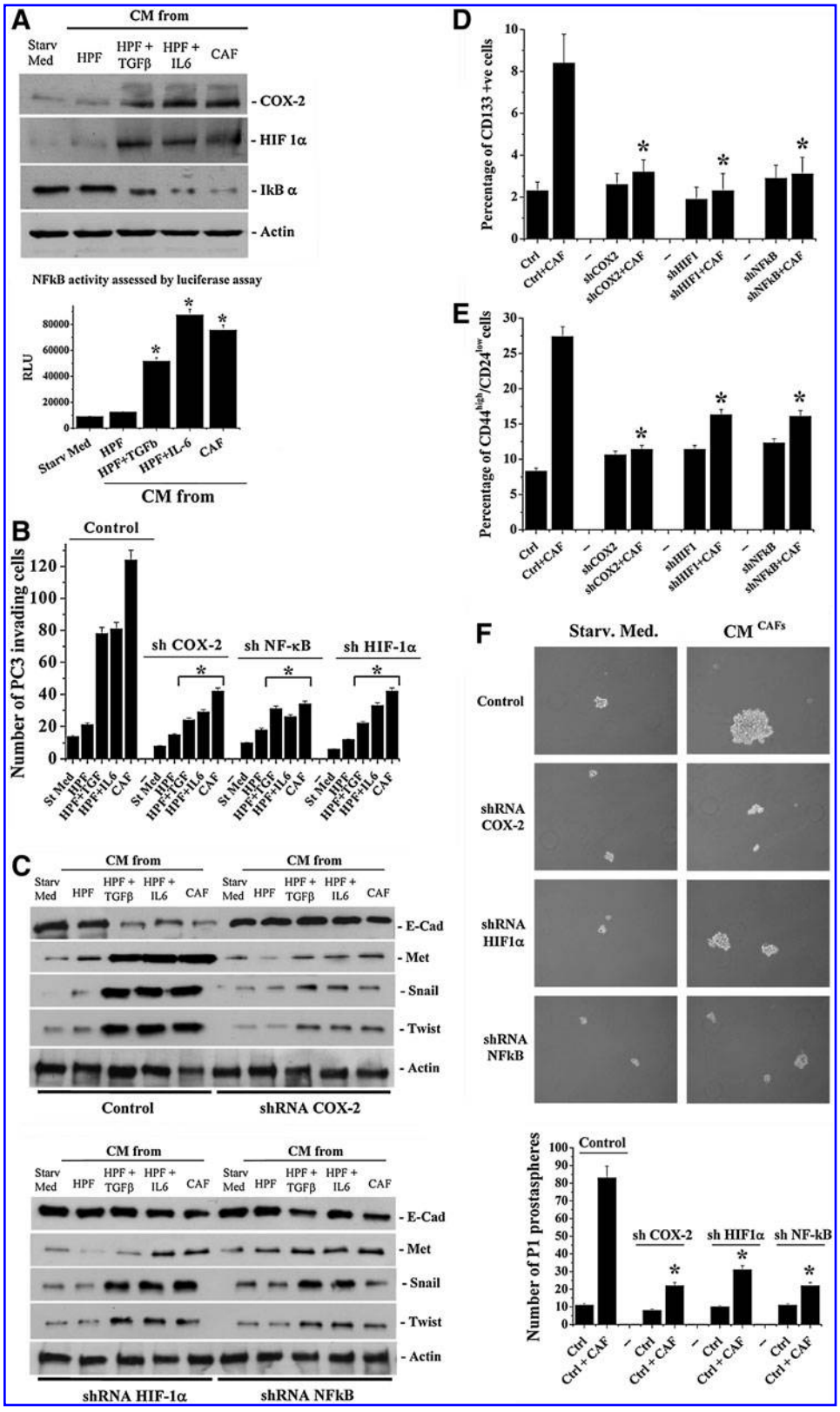


FIG. 1. CAFs promote EMT and stemness by triggering a proinflammatory pathway. **(A)** PC3 cells were incubated with the indicated CM and the levels of COX-2, HIF-1α, and IkBα were assessed by immunoblotting. NF-κB activity was also evaluated by a luciferase assay. *$p < 0.001$ *versus* St Med. **(B)** PC3 cells stably silenced with shRNA for COX-2, HIF-1α, and NF-κB were treated as in **A,** and their invasion was evaluated. Invading cells were counted and a bar graph, representative of six randomly chosen fields, is shown. *$p < 0.005$ *versus* Control, HPFs + TGFβ1, HPFs + IL-6, and CAFs, respectively. **(C)** PC3 cells were stimulated with CM as in A, and the expression of EMT markers was analyzed by immunoblotting. **(D, E)** PC3 cells, silenced as in B, were stimulated with CM from CAFs, and the percentage of CD133 positive cells **(D)** and $CD44^{high}/CD24^{low}$ cells **(E)** was analyzed by means of FACS analysis. *$p < 0.001$ *versus* Control + CAF. **(F)** Cells, silenced as in **B**, were treated as in D, and P1 individual spheres derived from PC3 cells treated as indicated were counted, photographed, and plotted. *$p < 0.001$ *versus* Control + CAFs. CAF, cancer-associated fibroblasts; EMT, epithelial-mesenchymal transition; CM, conditioned medium; COX-2, cycloxygenase-2; HIF-1α, hypoxia inducible factor-1α; NF-κB, nuclear factor-κB; HPFs, human prostate fibroblasts; TGFβ1, tumor growth factor-β1; IL-6, interleukin-6.

CAF-mediated ROS production in PC3 cells is mandatory for EMT and stemness

COX-2-mediated ROS mainly affects the pre-malignant condition and carcinogenesis of high-grade prostate intraepithelial neoplasia, but its role in tumor progression is unknown (34). We, therefore, analyzed the role of ROS produced on CAF exposure, mainly due to COX-2 activation, in EMT and invasiveness. We used both N-acetyl-cysteine (NAC) or permeable-catalase treatments and observed an

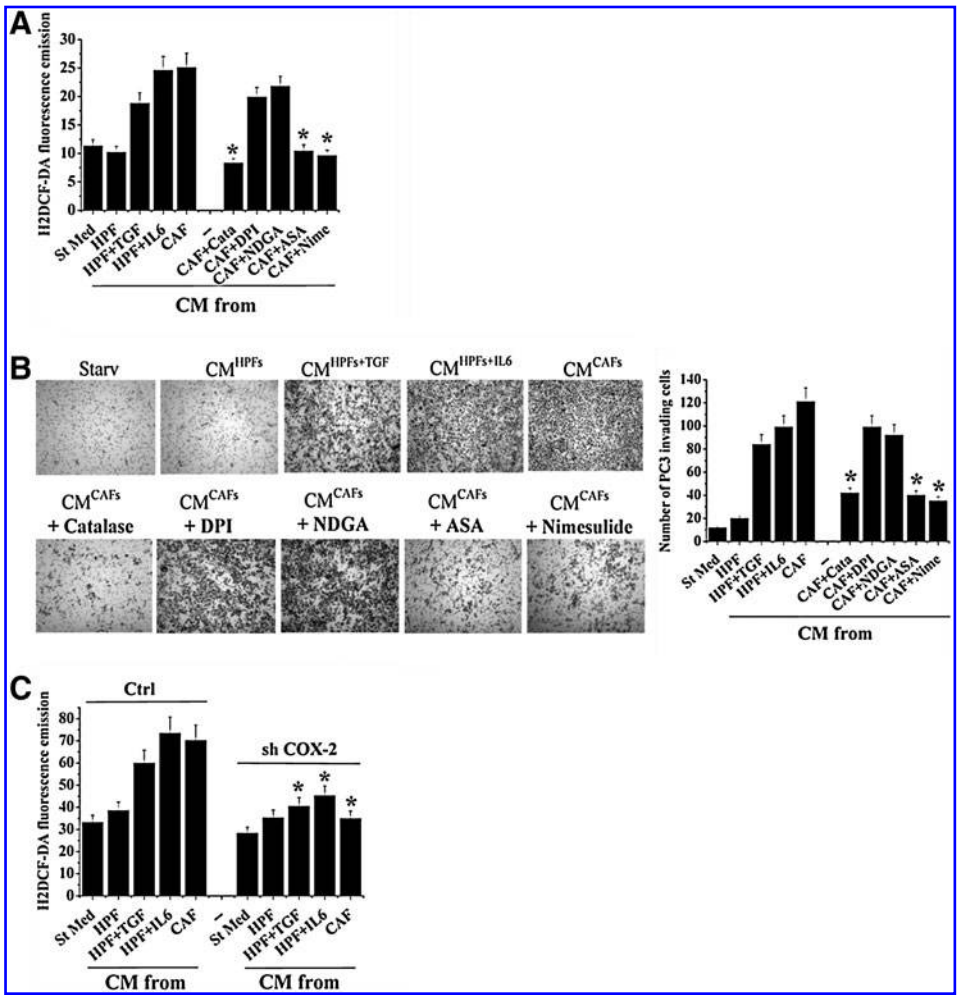


FIG. 2. CAFs promote a COX-2-dependent oxidative response. (A) PC3 cells were incubated with the indicated CM, in the presence or not of antioxidant compounds (for 24 h): 1 μg/ml catalase as a general H_2O_2 scavenger, 5 μM DPI specific for NADPH oxidase, 10 μM NDGA for 5′-lipoxigenase, 10 μM ASA for COX-1 and COX-2, and 75 μM Nimesulide selective for COX-2. H_2O_2 production was evaluated by means of H_2DCF-DA incubation and spectrophotometric analysis, normalized on total protein content, and plotted. *$p < 0.001$ *versus* CAFs. **(B)** PC3 cells were treated as in **A,** and cell invasion was evaluated. Invading cells were counted, photographed, and a bar graph, representative of six randomly chosen fields, is shown. *$p < 0.005$ *versus* CAFs. **(C)** PC3 cells stably silenced with shRNA for COX-2, or with negative control shRNA, were incubated with CM, and H_2O_2 production was evaluated as in A. *$p < 0.001$ *versus* Ctrl HPFs + TGFβ1, HPFs + IL-6, and CAFs, respectively. DPI, diphenyl-iodide; NDGA, Nordihydroguaiaretic acid; ASA, acetyl-salicilyc acid; H_2DCF-DA, 2′,7′-dihydrochlorofluorescein diacetate.

overlapping response: a definite inhibition of the proinvasive effect elicited by exposure to CAFs or *in vitro* activated HPFs, as well as of the relative EMT programme (Fig. 4A, B and Supplementary Fig. S3A, B). The treatment of EMT undergoing PC3 cells with selective ROS source inhibitors (NDGA for lypoxigenases, DPI for NADPH oxidase, acetyl-salicilyc acid for COX-1/2, and nimesulide for COX-2) allows us to confirm that the main source of ROS during CAF-mediated EMT is COX-2 (Fig. 4C). In addition, ROS removal abolishes the activation of HIF-1α and NF-κB (Supplementary Fig. S4). In keeping with a key role of ROS as mediators of EMT signaling, we observed that both NAC and catalase are able to inhibit achievement of stem cell traits in PC3 cells. Indeed, ROS removal greatly impairs CD133 and CD44/CD24 ratio increase due to CAF contact, confirming a role for ROS in EMT-related stemness (Fig. 4D, E and Supplementary Fig. S3C, D).

Besides superoxide, COX-2 also produces prostaglandins (34). We observed that in PC3 cells, after block of COX-2 with nimesulide, the administration of prostaglandin E2 (PGE_2) does not imitate CAF exposure. Indeed, CAFs or *in vitro* activated HPFs are ineffective on PC3 cells treated with nimesulide and PGE_2, which are unable to activate COX-2, HIF-1, and the EMT transcriptional program (Supplementary Fig. S5A, B). Conversely PGE_2 is partly active on NF-κB activation.

CAF-mediated proinflammatory signals enhance tumor formation and metastasis dissemination

To demonstrate that CAF-induced pro-oxidant and inflammatory activation of COX-2/HIF-1 axis is a necessary requirement for tumor growth and metastatic spread, we subcutaneously injected in SCID mice a mixture of CAFs and PC3 cells. We have already reported that 1×10^6 PC3 cells and absence of Matrigel are limiting conditions in which CAFs exert their spur for both xenografts and spontaneous metastases (17). The results indicate that silencing of COX-2 or HIF-1α in PC3 cells completely abolishes the effects exerted by CAFs on xenografts (Fig. 5A). CAFs co-injected with PC3 cells silenced for COX-2 or HIF-1α do not give rise to tumors even at the end point (92 days). In keeping with our previous observations, we detect spontaneous lung metastases in mice injected with PC3 + CAFs (Fig. 5A).

To confirm the involvement of the pro-oxidant and inflammatory COX-2/HIF-1 pathway in metastatic spread of EMT undergoing cells, we carried out an experimental metastasis assay. This assay assesses the ability of tumor cells to survive in the bloodstream, to extravasate, and to address the tumor in a new location. Our experimental design is based on *in vitro* mimic of CAF/PC3 contact and EMT engagement and *in vivo* analysis of lung colonization of conditioned PC3 cells.

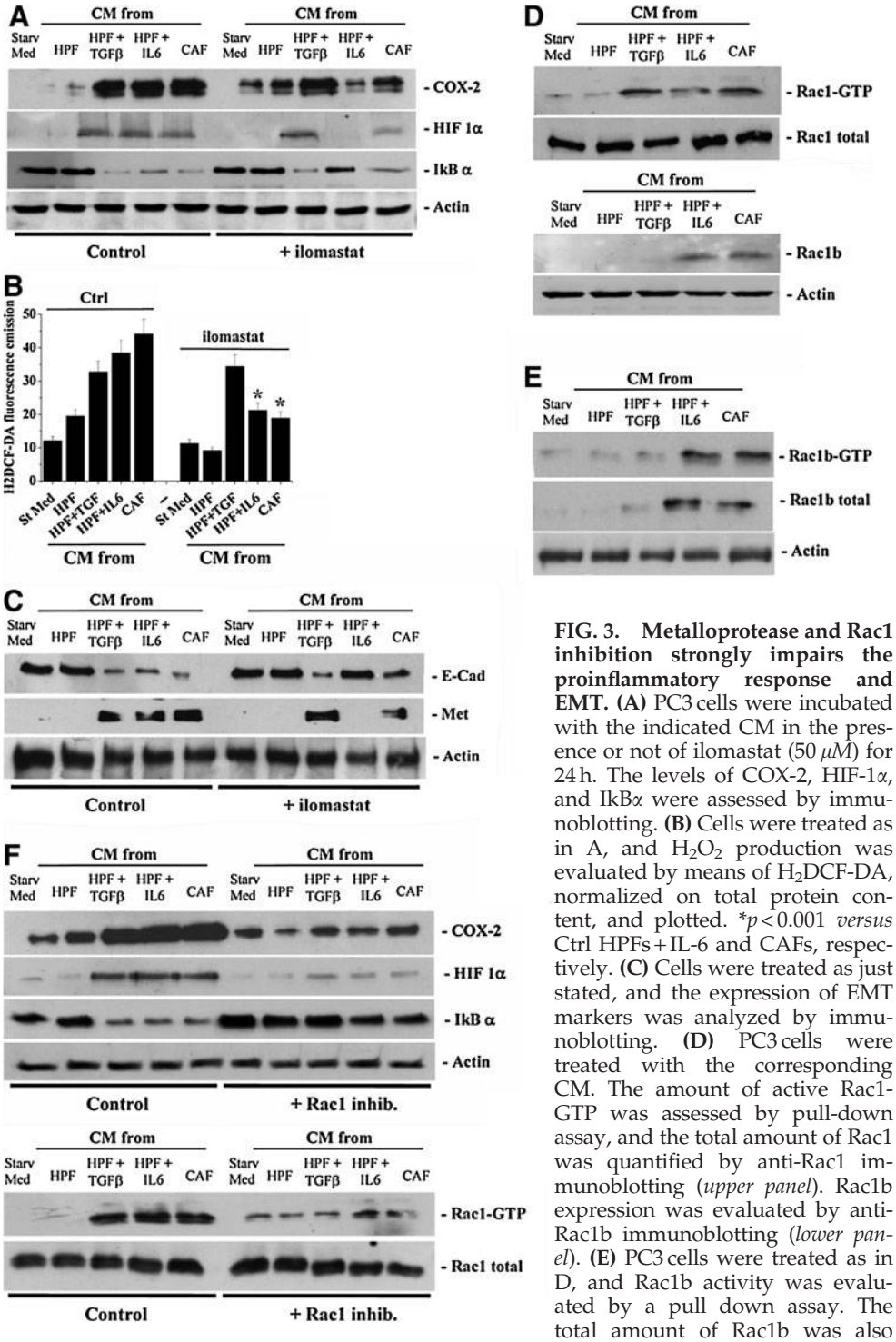


FIG. 3. Metalloprotease and Rac1 inhibition strongly impairs the proinflammatory response and EMT. (A) PC3 cells were incubated with the indicated CM in the presence or not of ilomastat (50 μM) for 24 h. The levels of COX-2, HIF-1α, and IkBα were assessed by immunoblotting. **(B)** Cells were treated as in A, and H_2O_2 production was evaluated by means of H_2DCF-DA, normalized on total protein content, and plotted. *$p < 0.001$ *versus* Ctrl HPFs + IL-6 and CAFs, respectively. **(C)** Cells were treated as just stated, and the expression of EMT markers was analyzed by immunoblotting. **(D)** PC3 cells were treated with the corresponding CM. The amount of active Rac1-GTP was assessed by pull-down assay, and the total amount of Rac1 was quantified by anti-Rac1 immunoblotting (*upper panel*). Rac1b expression was evaluated by anti-Rac1b immunoblotting (*lower panel*). **(E)** PC3 cells were treated as in D, and Rac1b activity was evaluated by a pull down assay. The total amount of Rac1b was also quantified by anti-Rac1b immunoblot. **(F)** Cells were treated as in **D**, with or without Rac1 inhibitor administration (90 μM). The levels of COX-2, HIF-1α, and IkBα were assessed by immunoblotting (*upper panel*). Impairment of Rac1 activation on Rac1 inhibitor treatment was confirmed by a pull down assay (*lower panel*).

We checked that PC3 cells, after *in vitro* contact with CAFs, have undergone EMT and activation of COX-2 and HIF-1 (Fig. 5B). The results reveal that CAF-conditioned cells give rise to a 25-fold increase in formation of lung metastatic nodules (Fig. 5C). In keeping, silencing of COX-2 or HIF-1α dramatically reduces the effect of CAFs on formation of lung metastases, thereby confirming that the spur elicited by CAFs through induction of EMT, which gives rise to a highly aggressive phenotype in PC3 cells able to successfully colonize distant organs with metastatic tumors, is greatly dependent on the activation of proinflammatory signaling through COX-2 and HIF-1 by CAFs.

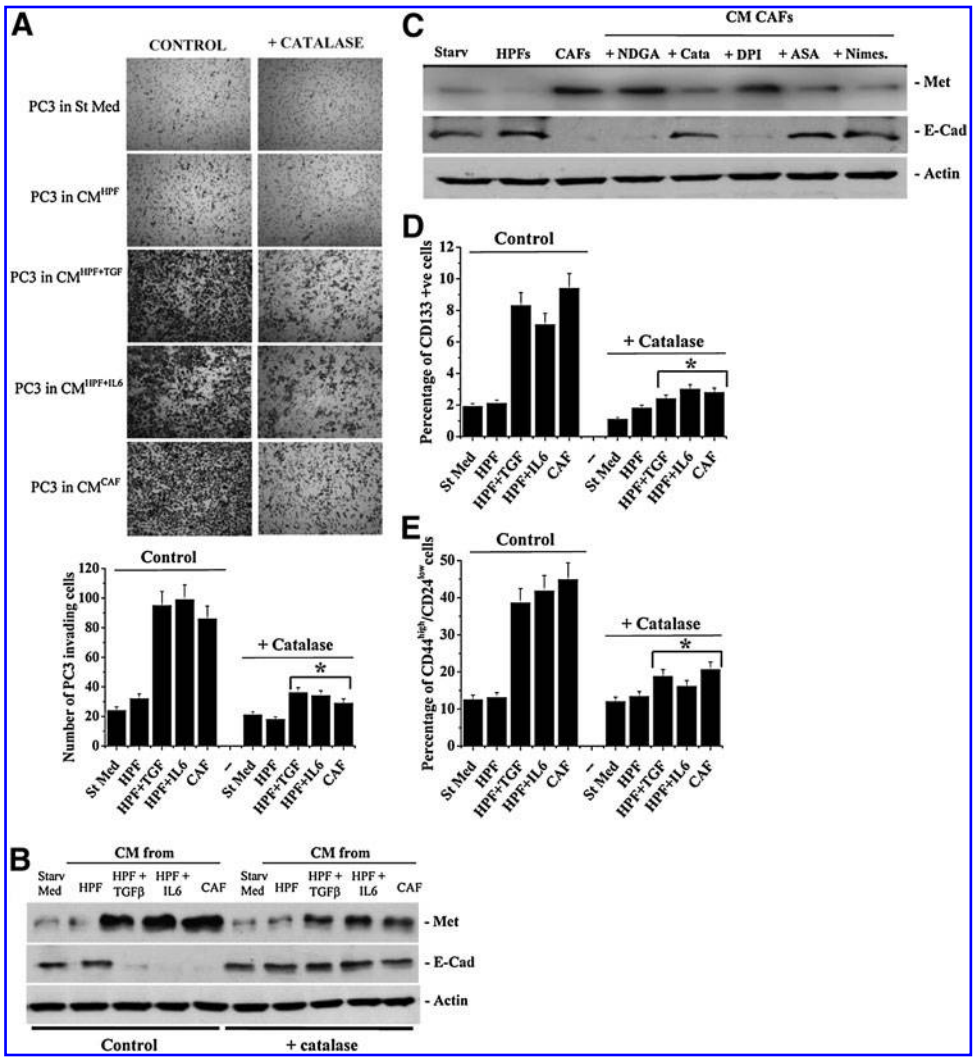


FIG. 4. CAF-induced oxidative stress is mandatory to elicit EMT and stemness. (A) PC3 cells were treated with the indicated CM in the presence or not of catalase (1 μg/ml) for 24 h. Cell invasion was evaluated by Boyden assay. Invading cells were counted, photographed, and a bar graph, representative of six randomly chosen fields, is shown. *$p < 0.001$ *versus* Control HPFs + TGFβ1, HPFs + IL-6, and CAFs, respectively. **(B)** PC3 cells treated as in **A** were checked for the expression of EMT markers. **(C)** PC3 cells were incubated with CM from HPFs or from CAFs with or without antioxidant administration for 24 h: 10 μM NDGA, 1 μg/ml catalase, 5 μM DPI, 10 μM ASA, and 75 μM Nimesulide. The expression of EMT markers was evaluated by immunoblotting. **(D, E)** PC3 cells were treated as in **A,** and the percentage of CD133 positive cells **(D)** and $CD44^{high}/CD24^{low}$ cells **(E)** was analyzed by means of FACS analysis. *$p < 0.001$ *versus* Control HPFs + TGFβ1, HPFs + IL-6, and CAFs, respectively.

Discussion

The results presented in this report lead to three major conclusions: (i) CAFs act on cancer cells by activating a proinflammatory route, thus leading them to achieve a motile and stem-like phenotype through EMT; (ii) the proinflammatory signature activated in cancer cells is mainly driven by the pathway involving NF-κB, COX-2, and HIF-1; and (iii) in response to CAF contact, cancer cells experience a COX-2-mediated ROS delivery, driven by MMP-activated Rac1b, which is mandatory for EMT and dissemination of metastatic cells.

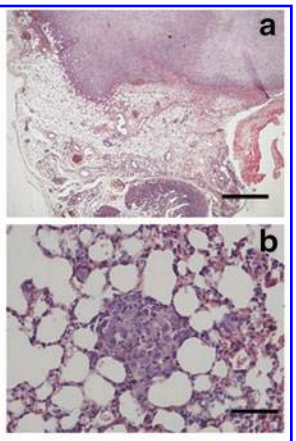
Inflammation has already been correlated with EMT activation. For example, administration of tumor necrosis factor-α is able to stabilize Snail-1, a known transcription factor leading to EMT, through inhibition of its ubiquitination and degradation (46). CAF-mediated proinflammatory signals appear to be mainly mediated by MMPs released directly by activated fibroblasts, as indicated by sensitivity of EMT activation to ilomastat, a broad-range inhibitor of MMPs (17). Although we have reported that CAFs secrete mainly MMP-2 and MMP-9, both of them able to cleave E-cadherin and to elicit EMT in several cancer models (21), further analyses are warranted for the identification of the protease/s specifically involved.

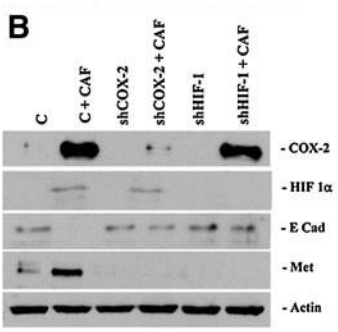
We now involve NK-kB and HIF-1 transcription factors as key players for CAF-mediated EMT activation. NF-κB has already been shown to negatively control the Snail-1 transcription factor ubiquitination and degradation through regulation of COP9 signalosome 2 in response to tumor necrosis factor-α (45). Besides, HIF-1 has been correlated with the EMT process through the activation of both Twist and Snail-1 transcription factors, leading to increased invasivenss (6, 47). We report herein an activation of HIF-1 in response to CAF contact in normoxic conditions, which is mandatory for *in vitro* EMT activation as well as for both xenograft and lung metastasis formation. In keeping with our findings, activation of HIF-1 has been reported also in normoxic conditions, as treatment with several GFs and chemo-attractive agents (5, 38). Interestingly, EMT activation and chemotaxis surely share the activation of a migratory cell behavior, therefore allowing a correlation of non-hypoxic activation of HIF-1 to the induction of a migratory behavior for PCa cells.

We also engage COX-2 expression, as well as its production of ROS, in the activation of EMT by CAFs. COX-2 is dramatically known as a conspirator of prostate cancer progression, although its specific role is uncertain. Early observations indicated a correlation with expression of

A
Subcutaneous transplantation

Cells and treatments	Tumor latency[a] (days)	Growth rate (cm^3/week)	Incidence[b]
PC3	n.d.[c]	n.d.	0/6
PC3 + CAF	14	0.322	6/6
PC3 sh COX-2	n.d.	n.d.	0/6
PC3 sh COX-2 + CAF	n.d.	n.d.	0/6
PC3 sh HIF-1 α	n.d.	n.d.	0/6
PC3 sh HIF-1α + CAF	n.d.	n.d.	0/6





C
Intravenously injection

Cells and treatments	Lung nodules median (range)
PC3	6 (0-42)
PC3 stimulated by CAF	>150 (134->150)
PC3 sh COX-2	6 (0-12)
PC3 sh COX-2 stimulated by CAF	2 (1-22)
PC3 sh HIF-1 α	6 (4-18)
PC3 sh HIF-1α stimulated by CAF	14 (2-38)

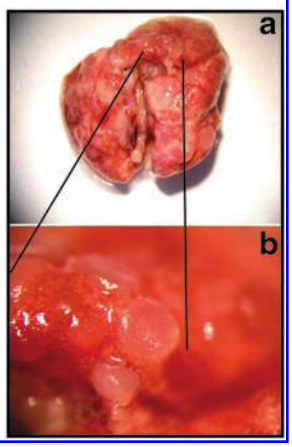


FIG. 5. CAF-induced proinflammatory response promotes tumor formation and lung metastatic colonization. (A) Xenograft growth in SCID bg/bg mice. Stably silenced PC3 cells with shRNA for COX-2, shRNA HIF-1α, or with negative control shRNA were subcutaneously injected in both the lateral flanks of mice with or without CAFs. Tumor latency, growth rate, and incidence are reported in the table. Representative paraffin-embedded tissue sections from primary tumour **(a)** and lung micrometastases **(b)** obtained by subcutaneous injection of PC3 cells and CAFs, stained with hematoxylin and eosin, are shown. Bar 50 μM. [a]Time between tumor cell injection and detection of a palpable tumor in 50% of mice. [b]Number of mice with tumor/number of inoculated mice. [c]Not detected at 3 months from injection of tumor cells. **(B)** Stably silenced PC3 cells with shRNA for COX-2, shRNA HIF-1α, or with negative control shRNA were treated with CM from CAFs. The real EMT of PC3 cells treated with CM was confirmed by expression of EMT markers, and the immunoblots for E-Cad and Met were shown. Silencing of COX-2 and HIF-1α was confirmed by immunoblotting. **(C)** PC3 cells described in **B** were injected into the lateral tail vein of SCID bg/bg mice. After 8 weeks, the animals were sacrificed, and the lungs were examined. Lung nodules were counted, and the median values ± standard deviation are shown in the table. Lungs obtained by CAF-treated PC3, which show a higher number of surface lung colonies, are shown **(a, b)**.

COX-2 and prostate tumor grade (19, 23, 34). In addition, COX-2 has also found increased in high-grade prostate intraepithelial neoplasia in the TRAMP mouse model (37). Interestingly, selective COX-2 inhibitors induce apoptosis in PC3 cells grown in nude mice (24). Conversely, few data correlate COX-2 expression with an invasive/motile phenotype, reminiscent of the EMT process induced by CAFs we are reporting herein. Forced COX-2 overexpression contributes to malignant phenotype by decreased E-cadherin expression and by modulated production of angiogenic factors (44, 48). Between the two products of COX-2, that is, prostaglandins and ROS, our data indicate ROS as the major factor responsible for the activation of the EMT programme. Indeed, PGE_2 administration is not able to rescue the complete block of EMT of PC3 cells induced by COX-2 inhibition. In keeping with a key role of COX-2-mediated ROS delivery, both antioxidant treatment and COX-2/inhibition/silencing totally impedes engagement of the EMT programme.

ROS can act on tumor progression in several ways. They cause oxidative damage to DNA and genomic instability or alter gene expression through modulation of transcription factors. In addition, repeated treatment with H_2O_2 caused a phenotypic conversion from mammary epithelial to fibroblast-like as in malignant transformation (27). In mammary carcinoma cells, ROS have also been correlated with EMT,

through a Rac1b-mediated delivery of mitochondrial ROS (36). In keeping with these findings, CAF-mediated EMT also involves Rac1b expression/activation. Rac1 is a key player of mesenchymal motility, as it drives cell polarization, leading to edge organization and lamellipodia formation (33). The Rac1b isoform is constitutively activated, but its specific role is still unknown. Its involvement in mammary carcinoma EMT has been correlated with mitochondrial ROS delivery, whereas CAF-mediated EMT of PCa cells exploits ROS produced by COX-2 activity, thereby suggesting that different ROS sources can be used for the same plan. Noteworthy, although CAF-induced EMT of PC3 cells is clearly MMP- and Rac1b-dependent, some differences emerge from *in vitro* activation of HPFs. Indeed, although IL-6 induces an activation state in HPFs very similar to CAFs extracted from patients bearing PCas (17), TGF-β1-treated HPFs, commonly referred to as myofibroblasts (17, 21), affect PC3 EMT in an MMP/Rac1b-independent manner. These findings are in agreement with previous observations showing activation of Rac1b in MMP3-dependent EMT of breast carcinoma cells, but not in cells undergoing TGF-β1-dependent EMT (28). In addition, we underline that, although Radisky *et al.* supported ROS as carcinogenic agents acting on genomic instability and classifying their redox-mediated EMT of cancer cells as a source for cancer associated myofibroblasts (36), our data implicate ROS as key mediators of tumor progression, through achievement of a motile and stem-like behaviour, leading cancer cells to spread to distant organs.

HIF-1 transcription factor is a major candidate as target for ROS produced during CAF-induced EMT. Indeed, HIF-1 is stabilized by a redox-dependent pathway, involving inactivation of prolyl hydroxylases, iron-dependent enzymes engaged in degradation of HIFs (20, 32). The key role of redox stabilization of HIF-1 has also been underscored by recent data describing that the antitumorigenic effects of NAC and VitC depend on HIF-1 redox stabilization (16). In a model of mouse breast cancer, a recent article also implicated oxidative stress, due to *JunD* deletion, as the driving force for myofibroblasts differentiation. Again, HIF-1 stabilization due to oxidative stress has been called on for activation of fibroblasts into myofibroblasts, cytokine secretion, and tumor spread (42). We now involve a redox-mediated HIF-1 accumulation for activation of EMT in prostate cancer cells, allowing them to escape the primary tumor and disseminate metastases. In keeping with our observations, early EMT-related events induced by hypoxia, as regulation of glycogen synthase kinase-3β and Snail-1, were dependent on transient intracellular generation of ROS (6).

The final picture of the interplay between CAFs and tumors during cancer progression involves ROS at least at two key levels: (i) fibroblasts may exploit oxidative stress for their activation and for affecting tumor aggressiveness, macrophage recruitment, and angiogenesis (7, 13, 42), (ii) CAF-induced EMT of PCa cells is again redox dependent for allowing achievement of mesenchymal motility and escaping the invasive front of tumors. Both processes exploit similar proinflammatory oxidative molecular signature, involving NF-κB, COX-2, and HIF-1. Considering that metastasis is a phenomenon reminiscent of the migratory/invasive behavior of inflammatory cells, it is not surprising that CAF-mediated EMT undergoing cells share with inflammatory cells the same signals. Indeed, both metastatic and inflammatory cells need to move away, invade/infiltrate the local sites where they are recruited by appropriate signals. Hence, we can propose a new challenge to the paradigm of antioxidants as valuable antimetastatic therapeutic tools, as they can affect both stromal cell reactivity (42) and cancer cells malignancy.

Acknowledgments

This work was supported by the Associazione Italiana Ricerca sul Cancro (AIRC), Istituto Toscano Tumori, and Fondazione Cassa di Risparmio di Lucca. We thank Dr. Sergio Serni for prostate surgical specimens and Eugenio Torre for histological analyses.

Author Disclosure Statement

No competing financial interests exist.

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Date of first submission to ARS Central, October 21, 2010; date of final revised submission, January 05, 2011; date of acceptance, January 14, 2011.

Abbreviations Used

CAFs = cancer-associated fibroblasts
CM = conditioned medium
COX-2 = cycloxygenase-2
ECM = extracellular matrix
EMT = epithelial-mesenchymal transition
GFs = growth factors
H_2-DCF-DA = 2′,7′-dihydrochlorofluorescein diacetate
HIF-1 = hypoxia-inducible factor-1
HPFs = human prostate fibroblasts
IL-6 = interleukin-6
MMP = metalloproteases
NAC = N-acetyl cysteine
NF-κB = nuclear factor-κB
PCa = prostate carcinoma
PGE_2 = prostaglandin E2
ROS = reactive oxygen species
TGF-β1 = tumor growth factor-β1

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