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Tumor and Stem Cell Biology

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Reciprocal Metabolic Reprogramming through Lactate Shuttle Coordinately Influences Tumor-Stroma Interplay

Tania Fiaschi[1], Alberto Marini[1], Elisa Giannoni[1], Maria Letizia Taddei[1], Paolo Gandellini[3], Alina De Donatis[1], Michele Lanciotti[2], Sergio Serni[2], Paolo Cirri[1], and Paola Chiarugi[1]

Abstract

Cancer-associated fibroblasts (CAF) engage in tumor progression by promoting the ability of cancer cells to undergo epithelial–mesenchymal transition (EMT), and also by enhancing stem cells traits and metastatic dissemination. Here we show that the reciprocal interplay between CAFs and prostate cancer cells goes beyond the engagement of EMT to include mutual metabolic reprogramming. Gene expression analysis of CAFs cultured *ex vivo* or human prostate fibroblasts obtained from benign prostate hyperplasia revealed that CAFs undergo Warburg metabolism and mitochondrial oxidative stress. This metabolic reprogramming toward a Warburg phenotype occurred as a result of contact with prostate cancer cells. Intercellular contact activated the stromal fibroblasts, triggering increased expression of glucose transporter GLUT1, lactate production, and extrusion of lactate by *de novo* expressed monocarboxylate transporter-4 (MCT4). Conversely, prostate cancer cells, upon contact with CAFs, were reprogrammed toward aerobic metabolism, with a decrease in GLUT1 expression and an increase in lactate upload via the lactate transporter MCT1. Metabolic reprogramming of both stromal and cancer cells was under strict control of the hypoxia-inducible factor 1 (HIF1), which drove redox- and SIRT3-dependent stabilization of HIF1 in normoxic conditions. Prostate cancer cells gradually became independent of glucose consumption, while developing a dependence on lactate upload to drive anabolic pathways and thereby cell growth. In agreement, pharmacologic inhibition of MCT1-mediated lactate upload dramatically affected prostate cancer cell survival and tumor outgrowth. Hence, cancer cells allocate Warburg metabolism to their corrupted CAFs, exploiting their byproducts to grow in a low glucose environment, symbiotically adapting with stromal cells to glucose availability. *Cancer Res; 72(19); 5130–40. ©2012 AACR.*

Introduction

The skill of cancer cells to invade and metastasize is caused by the genetic changes that these cells have undergone during multistep tumorigenesis. However, cancer cell microenvironment is a strong determinant of whether or not it acquires the potential to metastasize (1, 2). Thus, during primary tumor formation, carcinoma cells engage a multifaceted collection of cells composed by cancer-associated fibroblasts (CAF), endothelial cells and inflammatory cells, the so-called tumor-associated stroma, that engage a molecular cross talk with cancer cells, secreting large amounts of factors/cytokines and influencing invasion and metastasis (3–6).

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Cancer cells undergo profound changes in their metabolism and recently both tumor microenvironment and metabolic reprogramming have been included in the Hallmarks of Cancer (7). In fact, cancer cells undergo aerobic glycolysis coupled with increased glucose uptake caused by incomplete glucose oxidation, that favor cell proliferation through an efficient anabolism of glycolytic intermediates, needed to increase cancer biomass (8, 9). Cancer cells express the M2 splice isoform of pyruvate kinase (PK), an enzyme that shifts glucose metabolism toward aerobic glycolysis, short-circuiting ATP production and avoiding ATP inhibition of glycolysis (10, 11). Hypoxia has been involved in metabolic reprogramming of cancer cells, establishing a functional loop between glycolytic and respiring cells and sustaining survival of cancer cells (12). Indeed, hypoxia leads cancer cells to upload lactate, produced by neighboring hypoxic cells, which feeds aerobic cancer cells through respiration and anabolic functions (12).

A strict link between tumor metabolism and oxidative stress has also been reported. Indeed, the genetic loss of the NAD-dependent deacetylase SIRT3 in breast cancers favors the Warburg phenotype, causing oxidative stress that culminates in hypoxia-inducible factor-1 (HIF1) stabilization (13). Furthermore, PK-M2 has been indicated as a target of oxidative stress (14), a very common feature of several cancers. Redox inhibition of PK-M2 is required to divert glucose flux from glycolysis to the pentose phosphate pathway, thereby

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generating reducing potential through generation of NADPH for detoxification of reactive oxygen species (ROS; 14–16).

CAFs have already been proven to elicit a prooxidant environment in cancer cells, deeply affecting tumor progression and metastasis spread (17). Indeed, CAFs promote epithelial–mesenchymal transition (EMT) in human prostate carcinoma (PCa) cells as well as enhancement of tumor growth, increase of stem cell markers development and spontaneous metastases (18). The ability of CAFs to elicit EMT and stem-like traits is because of activation of a proinflammatory signature involving cycloxygenase-2, NF-κB, and HIF1, which is responsible for a motogen transcriptional program (17). In addition, CAFs themselves undergo oxidative stress during their differentiation into a myofibroblast-like highly reactive and secretory phenotype (19–21).

In this article we report evidence of a reciprocal metabolic reprogramming of CAFs and PCa cells. In particular, we show that following activation CAFs shift their metabolism toward a more glycolytic one, through a HIF1- and oxidative stress-dependent extrusion of lactate. This catabolite shuttles back to cancer cells, which use it for tricarboxylic acid cycle and protein synthesis, fueling cancer cell proliferation.

Materials and Methods

Materials

Unless specified, all reagents were obtained from Sigma. Antibodies anti-actin, MCT1, MCT4 were from Santa Cruz Biotechnology. Antibodies anti-HIF-1α were from Becton Dickinson. Antibodies anti-SIRT3 were from Cell Signaling. Antibodies anti-SOD2 and acetyl-lysine were from Abcam. HIF1-siRNA (sc-35561), MCT1-siRNA (sc-37235), and SIRT3-siRNA (sc-61555) were from Santa Cruz Biotechnology. TGF-β1 was from PeproTech. Mitosox and 2′,7′-dichlorfluorescein-diacetate (DCF-DA) were from Molecular Probe. [U-^{14}C] lactate and [^{3}H]deoxy-glucose were from Perkin Elmer. TaqMan Reverse Transcription Reagents Kit, Lipofectamine 2000 and GLUT1 primers were from Invitrogen.

Cell culture

Human PCa cells (PC3) were from the European Collection of Cell Culture, were authenticated by PCR/short tandem repeat (STR) analysis (European Collection of Animal Cell Cultures) and used within 6 months of resuscitation of original cultures. Healthy human prostate fibroblasts (HPF) and CAFs were isolated from surgical explanation of patients who signed informed consent in accordance with the Ethics Committee of Azienda Ospedaliera Universitaria Careggi. Tissues from patients affected by benign prostatic hyperplasia or aggressive PCa were used for obtaining HPFs or CAFs (12). CAFs between 4 and 10 passages were used, tested by mycoplasma presence, and authenticated by morphology and fibroblast activation protein expression. Conditioned media (CM) were obtained by 48-hour serum-starved cells, clarified by centrifugation and used freshly.

Fibroblasts and PCa cells activation

HPFs were grown to subconfluence and treated for 24 hours with CM^{PCa} (obtained culturing PCa cells in serum-free medium for 48 hours) to obtain PCa-activated fibroblasts (AF). Fresh serum-free medium was added to HPFs for an additional 24 hours before collection of CM^{HPF}.

Western blot analysis

A total of 1×10^6 cells were lysed for 20 minutes on ice in 500 μΛ of complete radioimmunoprecipitation assay (RIPA) lysis buffer (18). Lysates were clarified by centrifugation, separated by SDS-PAGE and transferred onto polyvinylidene difluoride. The immunoblots were incubated in 2% milk and probed with primary and secondary antibodies.

Proliferation assay

PCa cell proliferation has been measured cytometrically by the use of carboxyfluorescein diacetate succinimidyl ester (CFSE). Cells were treated with 10 μmol/L CSFE for 15 minutes at 37°C and then plated alone or in coculture with CAFs and cultured for 48 hours. Cytofluorimetric analysis allows the determination of cell proliferation index (the average number of cell divisions that a cell in the original population has undergone) on the basis of progressive decreasing of cell fluorescence as a function of the number of cell divisions. Alternatively, CAFs+PCa coculture were grown for 7 days and PCa clones were counted under an optical microscope.

Real-time PCR

Total RNA (1 μg) was reverse transcribed using TaqMan Reverse Transcription Reagents Kit. Measurement of gene expression was performed by quantitative real-time PCR (RT-PCR; ABI PRISM 7700 Sequence Detector, Applied Biosystems). The amount of target, normalized to an endogenous reference (eukaryotic 18S RNA, endogenous control, Applied Biosystem) was given by $2^{-\Delta\Delta CT}$ calculation.

Glucose uptake

Cells were treated with CM for 72 hours. 2-deoxy-glucose uptake was evaluated in a buffered solution (140 mmol/L NaCl, 20 mmol/L Hepes/Na, 2.5 mmol/L $MgSO_4$, 1 mmol/L $CaCl_2$, and 5 mmol/L KCl, pH7.4) containing 0.5 μCi/mL [^{3}H]deoxy-glucose for 15 minutes at 37°C. Cells were subsequently washed with cold PBS and lysed with 0.1 mol/L NaOH. Incorporated radioactive was assayed by liquid scintillation counting and normalized on protein content.

ROS evaluation

For total ROS analysis cells were treated for 3 minute with 5 μmol/L DCF-DA and lysed with RIPA buffer. The fluorescence values were normalized for proteins content. Evaluation of mitochondrial ROS was performed adding 5 μmol/L Mitosox to the cells for 15 minutes at 37°C. After washing the cells with PBS, fluorescence was analyzed by cytofluorimeter.

Lactate assay

Lactate was measured in the cultured media with Lactate Assay kit (Source Bioscience Life Sciences) according to the manufacturer's instruction.

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Incorporation of lactate into proteins

Cells were treated with CM for 72 hours and then [U-^{14}C] lactate was added for additional 24 hours. Cells were then resuspended in 20% trichloroacetic acid, placed on ice for 30 minutes and centrifuged. The resuspended pellet was assayed for [^{14}C] labeled proteins by scintillator.

Detection of released CO_2 by radioactive lactate

Cells were treated with CM for 72 hours and then 0.2 μCi/mL D-[U-^{14}C] lactate was added for 15 minutes. Each dish had a taped piece of Whatman paper facing the inside of the dish wetted with 100 μL of phenyl-ethylamine-methanol (1:1) to trap the CO_2. Then 200 μL of 4M H_2SO_4 was added to cells. Finally, Whatman paper was removed, transferred to scintillation vials for counting.

Xenograft experiments

In vivo experiments were conducted 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. Experiments were performed using 6- to 8-week-old male severe combined immunodeficient (SCID)-bg/bg mice (Charles River Laboratories International). Animals (6 per group) were monitored daily and tumor size was measured every 2 to 3 days by a caliper. Tumor volumes were determined by the length (L) and the width (W): $V = (LW^2)/2$ (12).

Statistical analysis

Data are presented as means ± *SD* from at least 3 independent experiments. Statistical analysis of the data was performed by Student t test. P values of ≤ 0.05 were considered statistically significant.

Results

Analysis of HPFs and CAFs from human patients

In colorectal carcinoma histologic analyses suggest that the stroma infiltrating these tumors buffer and recycle products of anaerobic metabolism of cancer cells to sustain invasive cancer growth (22). On the contrary, in breast cancers PK-M2 and lactate dehydrogenase (LDH) are highly expressed in the stromal CAFs (23). In addition, fibroblasts undergoing activation because of deletion of caveolin-1 experience oxidative stress, and a HIF1-mediated shift toward aerobic glycolysis and elimination of mitochondrial activity through mitophagy (24). In this controversial context we would like to clarify the contribution of prostate CAFs to Warburg metabolism of aggressive PCa. Human prostate CAFs were isolated from prostate biopsies from 9 patients with PCa, whose pathologic values are reported in Supplementary Table S1. Our choice has been driven by cancer aggressiveness and prostate-specific antigen (PSA) values. As healthy counterparts, we used fibroblasts isolated from 4 men with benign prostatic hyperplasia (HPFs). First, we observed that prostate CAFs undergo increased anaerobic glycolysis, as revealed by their increased basal glucose uptake (Fig. 1A). In addition, the analysis of gene pathways revealed that glycolysis is activated in CAFs with respect to HPFs, being PK-M2, ALDO-A/B/C, ENO-1/2, TPI-1 the main upregulated genes (Supplementary Fig. 2SA–2SC). We also observed that CAFs show increased expression of MCT4 (Fig. 1B), the passive lactate-proton symporter driving lactate efflux, thereby validating the idea that prostate CAFs increased their lactate production with respect to healthy HPFs. Finally, we observed that prostate CAFs experience a state of oxidative stress in comparison to HPFs (Fig. 1C). This last finding is in keeping with other data reporting that oxidants are important players for fibroblast activation and CAF-dependent tumor invasion (17, 19, 21). Indeed, our data reveal that both cytoplasmic and mitochondrial H_2O_2 are greater in CAFs compared with HPFs (Fig. 1C and D). Gene expression analysis, glucose uptake, as well as their ROS production, indicate that prostate CAFs experience a Warburg metabolism because of their activation in response to cross talk with cancer cells.

Prostate HPFs undergo Warburg effect in response to activation

To investigate the activation of Warburg metabolism due to CAFs differentiation, we used HPFs activated *in vitro* using CM from PCa cells. Indeed, we have already reported that PCa secrete soluble factors able to elicit a mesenchymal–mesenchymal transition in HPFs, leading them to an activation state similar to myofibroblasts (18). We termed these *in vitro* activated HPFs, as PCa-AFs. We observed that exposure of HPFs to PCa-CM elicits a clear increase in their lactate secretion (Fig. 2A), likely because of the increase in MCT4 in PCa-AFs (Fig. 2B). Activation of fibroblasts does not affect their usage of lactate, as revealed by analyses of lactate upload and MCT1 expression level (Fig. 2B and Supplementary Fig. S3). A conversion to a Warburg metabolism in response to fibroblast activation is confirmed by the ability to increase the expression of GLUT1 glucose transporter and the consequent enhanced glucose uptake (Fig. 2C and D).

The Warburg metabolic shift in CAFs is redox and HIF1 dependent

Differentiation of CAFs toward a myofibroblast phenotype has been reported as a redox-dependent event (19, 21). In addition, HIF1 is a master regulator of anaerobic metabolism during exposure to hypoxia, which undergoes redox regulation as well. To address the possibility that the conversion to a Warburg phenotype of HPFs could be dependent on oxidative stress and HIF1-mediated transcription in normoxia, we first observed that PCa-AFs have a higher basal level of ROS with respect to HPFs (Fig. 3A). Pretreatment of HPFs and PCa-AFs with the oxidant scavenger N-acetyl cysteine (NAC) inhibits lactate extrusion by fibroblasts (Fig. 3B), suggesting that their Warburg phenotype depends on ROS content. In agreement, we found that the expression of the MCT4 transporter is decreased in presence of the scavenger (Fig. 3C). Although HPFs are activated to PCa-AFs in normoxia, we observed a clear stabilization of the transcription factor HIF1 in PCa-AFs, in agreement with their high-ROS content (Fig. 3C). The inhibition of HIF1, either transcriptionally by RNA interference

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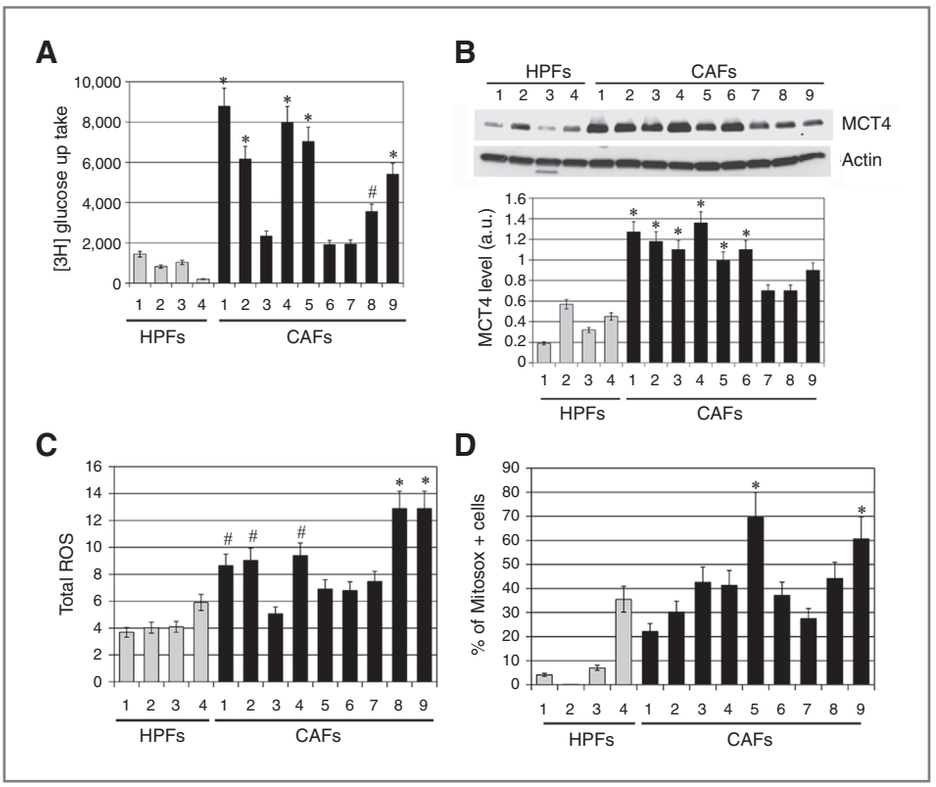

Figure 1. Analysis of human *ex vivo* HPFs and CAFs. A, analysis of [^{3}H]-glucose uptake in HPFs and CAFs. B, MCT4 immunoblot in HPFs and CAFs. Normalization was done by actin immunoblot. The plot below reports densitometric quantitation (ratio MCT4:actin). Oxidative stress in CAFs and HPFs evaluated as level of total ROS released, analyzed using DCF-DA (C) or using the Mitosox probe for the detection of mitochondrial ROS (D). Values of plots C and D were normalized on sample protein content. *, $P < 0.001$ versus HPFs; #, $P < 0.01$ versus HPFs. a.u., arbitrary units.

or functionally with the inhibitor topotecan, confirms that HIF1 plays a mandatory role in both MCT4 upregulation due to fibroblast activation and in lactate efflux (Fig. 3B–D).

SIRT3 is involved in ROS production and HIF1 stabilization in PCa-AFs

Recent evidence showed that the mitochondrial deacetylase SIRT3 is involved in the control of HIF1 expression, and redox signaling has been implicated in such control (13). A decrease in SIRT3 expression leads to increased superoxide dismutase 2 (SOD2) acetylation/inhibition, thereby causing ROS increase and HIF1 stabilization (25, 26). We observed that the contact with PCa cells, while activating HPFs to PCa-AFs, leads in fibroblasts to a downregulation of SIRT3 and an increase in SOD2 acetylation, together with a clear stabilization of HIF1 (Fig. 4A). In agreement with the idea of a redox-mediated stabilization of HIF1 via SIRT3 dowregulation, mitochondrial ROS are strongly increased upon HPFs activation (Fig. 4B and C). SIRT3 acts as a key upstream regulator of ROS production during HPFs activation, leading to HIF1 stabilization. Indeed, when downregulation of SIRT3 was forced by RNA interference in HPFs, we found a strong overproduction of ROS accompanied by a dramatic increase of HIF1 accumulation (Fig. 4D). The role of SIRT3 is also confirmed by treatment with the SIRT-activator kaempferol, leading to ROS and HIF1 downregulation in CAFs (Fig. 4C).

PCa upload lactate produced by CAFs

Because HPFs undergo Warburg effect upon their activation in response to PCa interplay, extruding lactate in the extracellular compartment, we speculate that PCa cells can upload this lactate generated by stromal fibroblasts, using it for different purposes. To address this point PCa cells were treated with CM from HPFs or CAFs (*ex vivo* cultures), or cocultured with HPFs (obtaining PCa-AFs). On the basis of data obtained by our analysis of *ex vivo* fibroblasts (Fig. 1), we used for further studies HPFs 1, 3, and 4 or CAFs 1, 4, and 9. The results show that lactate produced by CAFs is strongly decreased where PCa are present (both treatment with CM or coculture), thus suggesting that lactate has been consumed by PCa (Fig. 5A). Because activated fibroblasts showed unchanged MCT1 expression and unaffected lactate upload compared with HPFs (Fig. 2B and Supplementary Fig. S1), we speculated that PCa in contact with CAFs are also metabolically reprogrammed to upload lactate. In keeping, we found that the MCT1 transporter is upregulated in PCa cells treated with CM from CAFs or in coculture with CAFs (Fig. 5B). In agreement, MCT4 expression in PCa cells is unaffected by coculture with CAFs (Supplementary Fig. S4A). Again, HIF1 and redox signaling play a

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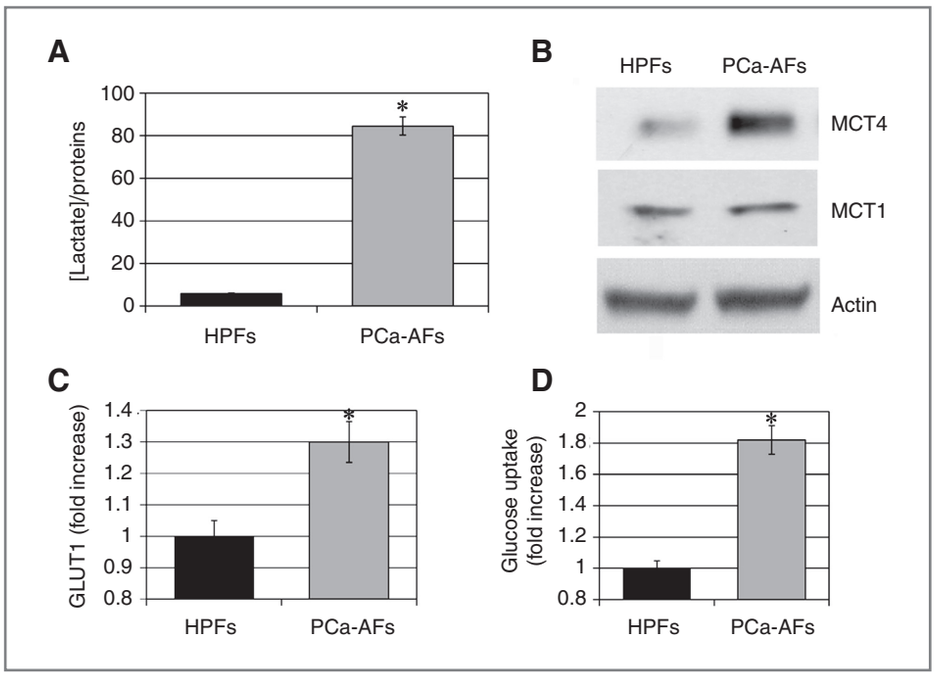

Figure 2. Analysis of Warburg metabolism in HPFs activated *in vitro*. HPFs were treated with CM^{PCa} for 24 hours obtaining PCa-AFs, then serum-free medium was added for an additional 48 hours. A, lactate assay in culture medium. B, MCT4 and MCT1 immunoblots in HPFs and PCa-AFs. Normalization was done by actin immunoblot. C, RT-PCR analysis for GLUT1 mRNA in HPFs and PCa-AFs. D, [^{3}H]-glucose uptake in HPFs and PCa-AFs. *, $P < 0.001$ versus HPFs.

mandatory role in metabolic reprogramming of PCa cells through upregulation of MCT1, as revealed by treatments with NAC or topotecan (Fig. 5B).

To analyze the destiny of lactate extruded by CAFs, we treated PCa cells with [^{14}C]-lactate and analyzed its upload by PCa cells, after treatment with CM or coculture with CAFs (Fig. 5C, top panel). In the same experimental setting, we also evaluated respiration of lactate by PCa, through analysis of released [^{14}C]-CO_2, and lactate reconversion toward anabolic pathways, through analysis of [^{14}C]-proteins (Fig. 5C, middle and bottom panels). The results clearly indicate that both CM or direct contact with CAFs, drive a metabolic reprogramming of PCa cells leading them to upload lactate and to use it both in respiration and anabolism. Final confirmation of a metabolic shift of PCa cells toward an aerobic metabolism, upon contact with CAFs, is given by analyses of both [^{3}H]-glucose uptake and expression of GLUT1 (Fig 5D).

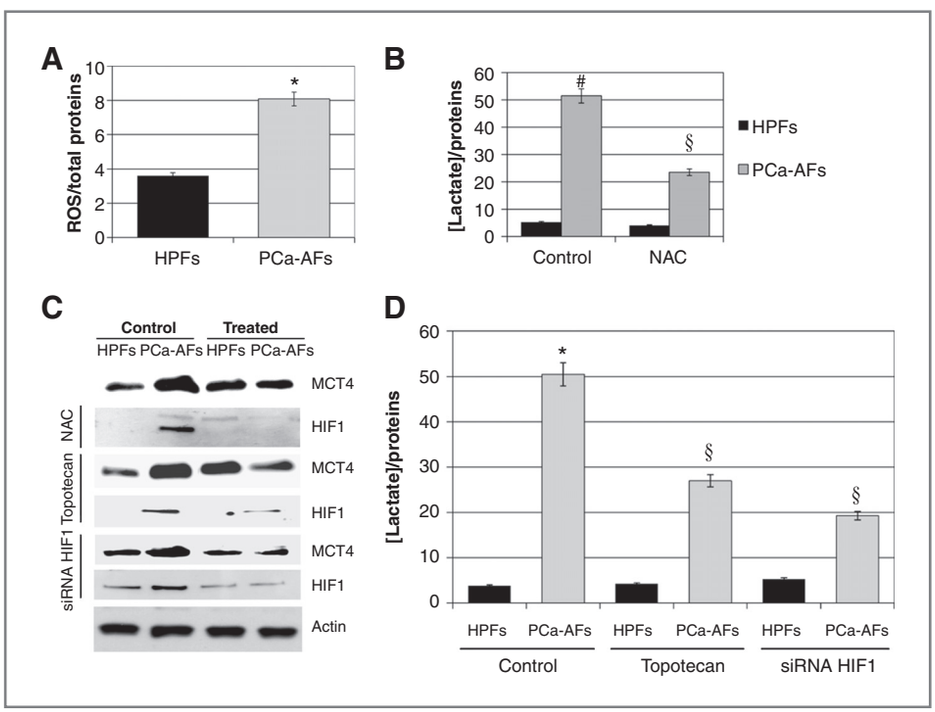

Figure 3. Warburg metabolism of PCa-AFs is ROS- and HIF1-dependent. HPFs were treated with CM^{PCa} for 24 hours obtaining PCa-AFs, then serum-free medium was added for an additional 48 hours. A, analysis of total ROS content in HPFs and PCa-AFs. B, assay of lactate extruded by HPFs and PCa-AFs. A total of 20 mmol/L of NAC was added to serum-free medium for 24 hours. C, immunoblot analysis of redox- and HIF1-dependence of MCT4 expression. HIF1α was silenced in HPFs by RNA interference for 48 hours before adding CM^{PCa}, although 20 mmol/L NAC and 250 nmol/L topotecan were added for 24 hours. HIF1 silencing, topotecan, or NAC treatments are indicated on the left. Actin immunoblot was used for normalization for each treatment. D, assay of lactate extruded in HPFs and PCa-AFs after treatment with topotecan or HIF1α silencing as in C. *, $P < 0.001$ versus HPFs; #, $P < 0.001$ versus control; §, $P < 0.01$ versus HPFs.

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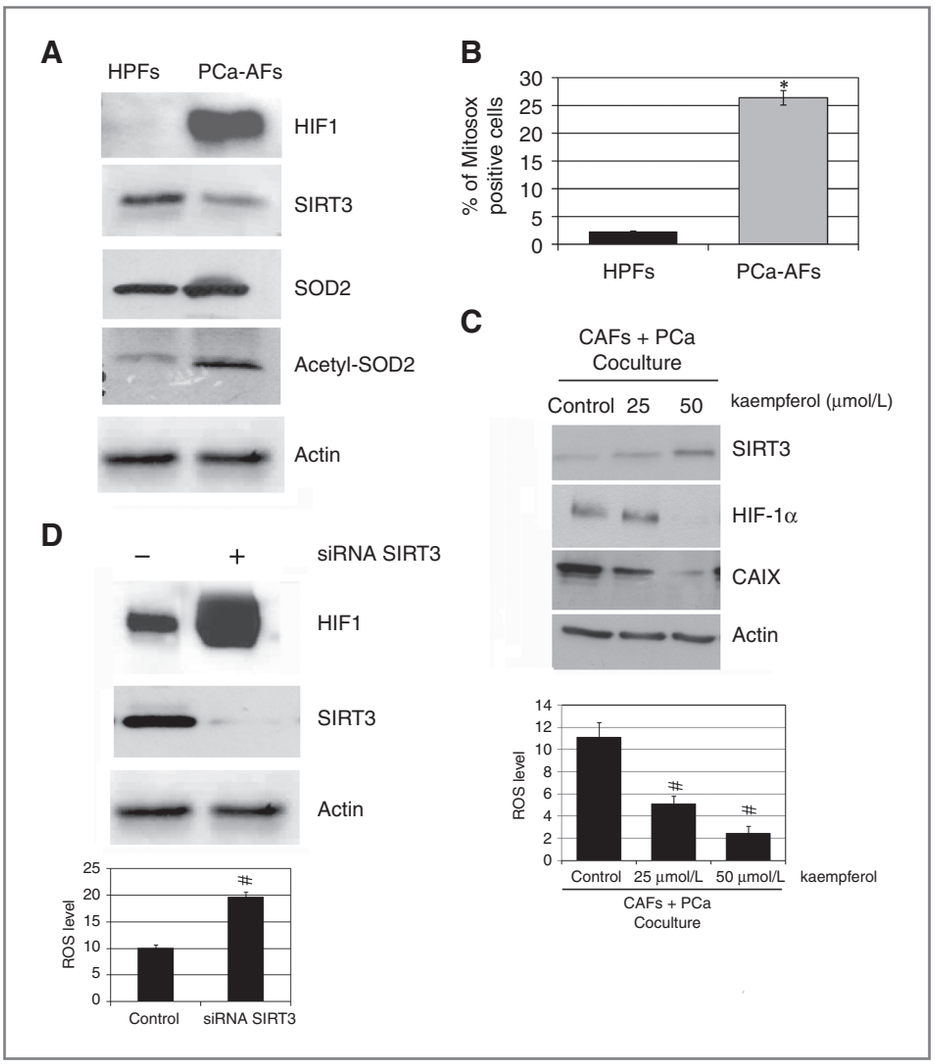

Figure 4. HIF1 accumulation is regulated by SIRT3 in PCa-AFs. HPFs were treated with CM^{PCa} for 24 hours obtaining PCa-AFs, then serum-free medium was added for an additional 48 hours. A, immunoblot analysis of HIF1, SIRT3, SOD2, and acetyl-SOD2. Actin immunoblot was used for normalization. B, cytofluorimetric analysis of mitochondrial ROS using Mitosox probe. C, immunoblots for SIRT3, HIF1, and CAIX (a common target of HIF1) were done on CAFs + PCa coculture. Note that 25 μmol/L and 50 μmol/L kaempferol (SIRT3 activator) was added to serum-free medium for 24 hours. Actin immunoblot was used for normalization. Total ROS production of the same samples is reported in the plot below. D, SIRT3 was silenced in HPFs by RNA interference for 48 hours before adding CM^{PCa}. HIF1 and SIRT3 immunoblots are shown and total ROS production of the same samples is reported in the plot below. *, $P < 0.001$ versus HPFs; #, $P < 0.001$ versus control.

Lactate shuttle is functional for PCa cell growth

With the aim to investigate what is the benefit for PCa cells to upload lactate produced by surrounding CAFs, we first analyzed cell proliferation. PCa cells were labeled with the fluorochrome CSFE, cocultured with CAFs and then assayed for proliferation by flow cytometry. The results indicate that the proliferation index of PCa cells increased by 30%, suggesting an active role of CAFs in sustaining PCa cells proliferation (Fig. 6A). The relevance of lactate upload for PCa cell growth was confirmed using α-cyano-4-hydroxycinnamate (CHC), a specific MCT1 inhibitor, already acknowledged to disrupt lactate shuttle between hypoxic and normoxic cells within tumors (refs. 12, 27; Fig. 6A). Furthermore, PCa cell growth was assayed in a time course coculture experiment, in which cancer cells were seeded with increasing number of CAFs (1:3; 1:5; 1:10). PCa islets developed after some days and we observed a clear trophic effect of CAFs for PCa cell proliferation (Fig. 6B). Again, the advantage given by coculture with CAFs for PCa cell growth was reverted by blocking the function of MCT1 transporter with CHC (Fig. 6B). The effect of CHC is highly specific for PCa cells, which are actively uploading lactate, as it impairs the viability of PCa cells without significantly affecting CAFs viability (Fig. 6C). Similar results were observed after silencing of MCT1 by RNA interference (Supplementary Fig. S4B) or by the use of different metabolism inhibitors. Indeed, the block of the metabolic circuitry established between cocultured PCa cells and CAFs, by treatment with 2-deoxyglucose (2-DG), dichloroacetate (DCA), or Antimycin A, as well as forced reexpression of SIRT3 by kaempferol (28), leads to a remarkable decrease of PCa cell growth (Fig. 6D). We finally validated *in vivo* the idea that CAFs induce a metabolic shift in neighboring PCa cells, leading them to become MCT1-dependent for their growth. Wild type or MCT1 silenced PCa were subcutaneously injected in SCID-bg/bg together with CAFs. As we already reported, coinjection of CAFs with PCa cells strongly enhances the tumor growth rate (17), although we observed that silencing of MCT1 reduces this ability by 50%, thereby underscoring the

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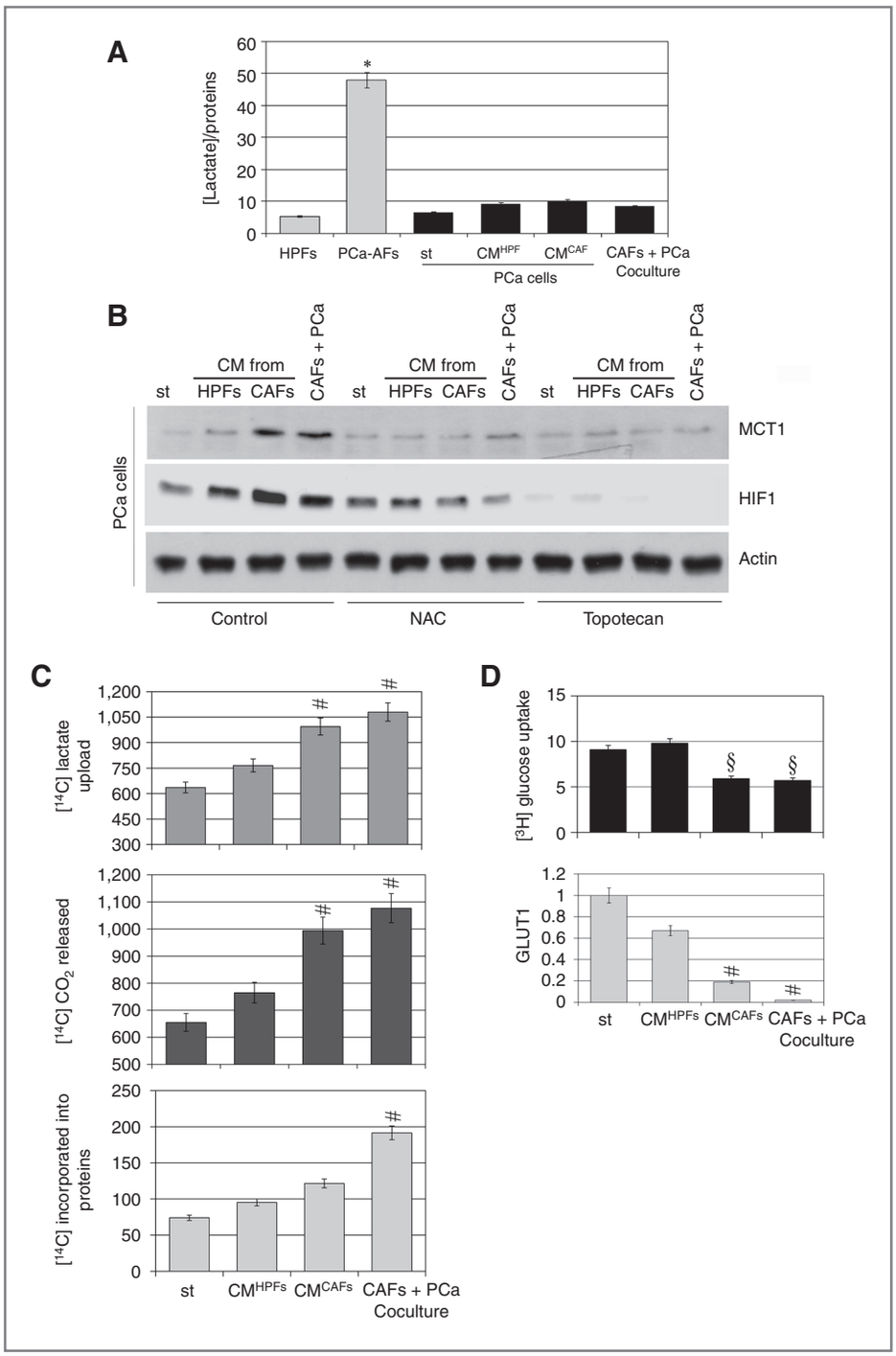

Figure 5. PCa cells upload and use lactate produced by CAFs. PCa cells were cultured with serum-free medium (st) or with CM^{HPF}, CM^{CAF}, or cocultured with CAFs (proportion CAFs:PCa 3:1). A, assay for lactate extrusion. B, MCT1 or HIF1 immunoblots of the same samples. Actin immunoblot was used for normalization. C, $[^{14}C]$-lactate upload (top), respiration of $[^{14}C]$-lactate, evaluated as $[^{14}C]$-CO_2 released (middle) and incorporation of $[^{14}C]$ into proteins (bottom). D, analysis of $[^{3}H]$-glucose uptake (top) and GLUT1 expression by RT-PCR (bottom). *, $P < 0.001$ versus HPFs; #, $P < 0.001$ versus st; §, $P < 0.01$ versus st.

significance of lactate shuttle between CAFs and PCa cells during tumor growth (Fig. 6E).

Discussion

The emergence of new antineoplastic drugs targeting metabolic pathways urgently prompt the scientific community to study metabolic deregulation of tumors, to clarify the differences among different tumor histotypes, as well as of the stromal cells infiltrating growing tumors. Conflicting data are emerging about the role of stromal fibroblasts in metabolic reprogramming of cancer cells. Histopathologic analysis reveals that PK-M2 and LDH are highly expressed in the stroma of breast cancer lacking caveolin-1 expression (23). In addition,

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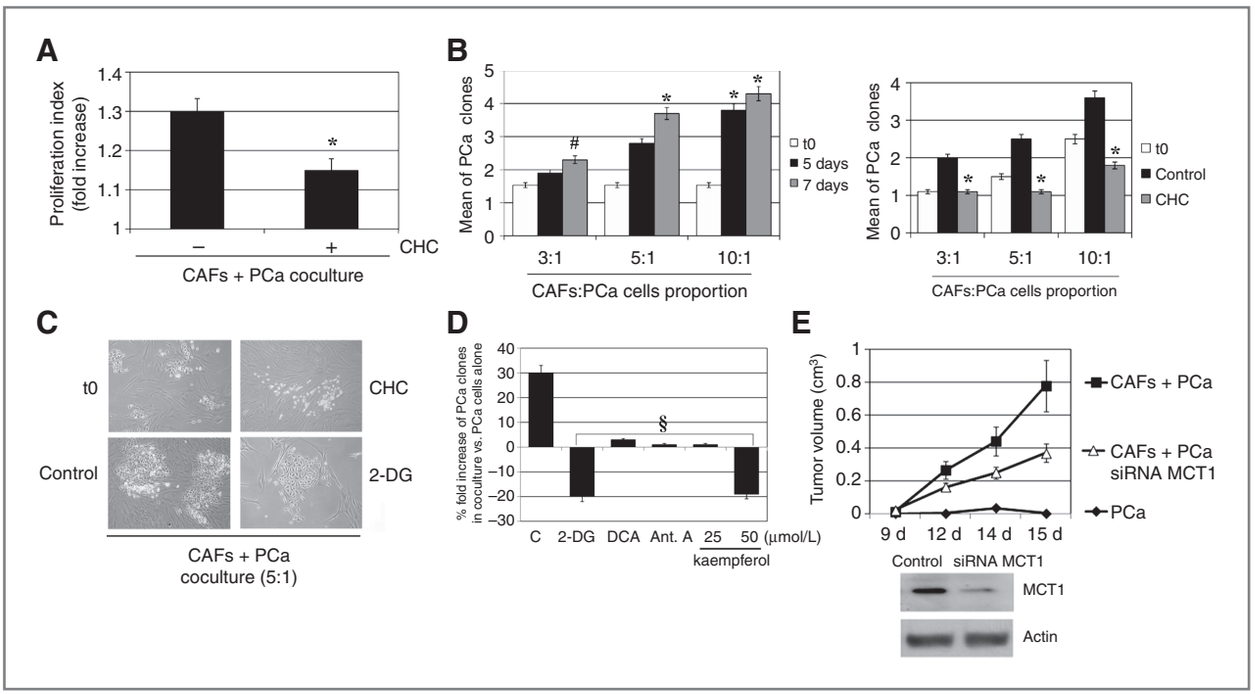

Figure 6. Lactate produced by CAFs is useful for PCa growth. A, analysis of PCa growth in coculture with CAFs. PCa cells were first labeled with CFSE and then cocultured with CAFs for 48 hours, in the presence or not of 2.5 mmol/L CHC, before cytofluorimetric analysis of cell proliferation. Proliferation index has been reported in the bar graph. B, number of PCa cell clones in coculture with CAFs. A total of 3×10^3 PCa cells were seeded with increasing number of CAFs (3:1, 5:1, 10:1) for 5/7 days and PCa cells clones were counted under an optic microscope (left). Note that 2.5 mmol/L CHC was added to coculture for the whole period (right). C, representative images of PCa clones obtained in cocolture with CAFs for 7 days in the presence of 2.5 mmol/L CHC, 1 mg/mL 2-deoxyglucose (2-DG). D, increase in PCa cell clone number, in coculture with CAFs (5:1 ratio), for 7 days with 1 mg/mL 2-DG, 0.5 mmol/L DCA acid, 20 nmol/L Antimycin (Ant. A), 25 and 50 μmol/L kaempferol. E, xenograft growth in SCID-bg/bg mice of wild-type or MCT1-silenced PCa cells injected subcutaneously. PCa cells were injected alone or with activated fibroblasts (5:1 ratio). MCT1 silencing by RNA interference in PCa cells is shown. *, $P < 0.001$ versus (–) or versus t0; #, $P < 0.01$ versus t0; §, $P < 0.001$ versus C.

fibroblasts undergoing activation because of caveolin-1 deletion, or in response to downregulation of caveolin-1 upon oxidative stress induced by contact with cancer cells, show a stabilization of HIF1 (23, 29, 30). Although these fibroblasts are not properly *ex vivo* CAFs, they experience a shift toward aerobic glycolysis and elimination of mitochondrial activity through mitophagy (30). In disagreement with these data, histologic analyses of colorectal carcinoma suggested that the stroma infiltrating these tumors expresses aerobic metabolism enzymes that are involved in recycling products of anaerobic metabolism of cancer cells to sustain invasive cancer growth. In keeping, a very recent study reported that hypoxic breast cancer cells recruit mesenchymal stem cells through their secretion of lactate due to glycolytic Warburg metabolism, allowing stromal cells to use lactate produced by tumor cells (31).

On the basis of these controversial findings, our investigations focused on *ex vivo* CAFs, obtained by patients bearing cancers with clear aggressiveness as revealed by their grading and PSA values, are therefore really warranted. In our model we observed that CAFs behave as metabolic synergistic bystanders of cancer cells, actively participating in the complex metabolism of tumors, by engaging a biunivocal relationship with cancer cells forcing them to respire and overcome energy depletion because of the Warburg effect (Fig. 7A). In particular, fibroblasts in contact with epithelial cancer cells undergo myofibroblast differentiation and produce lactate through aerobic glycolysis and Warburg metabolism (Fig. 7B), which is used by cancer cells for respiration. In addition, cancer cells lactate uptake leads to the reduction of extracellular acidity, which represents an unfavorable environmental factor for tumor survival. The metabolic reprogramming of PCa cells and their CAFs involves also reexpression of MCT4 in CAFs, granting for efficient extrusion of lactate from CAFs, and MCT1 in cancer cells, allowing them to powerfully upload the anabolite to respire and fuel anabolism. Expression of MCT4 is increased in CAFs extracted by human PCa, compared with benign prostate hyperplasia, with higher MCT4 level associated with poorer clinical outcome. In keeping with these data, stromal MCT4 has been correlated with poor clinical outcome in triple-negative breast cancers (32). The upload of lactate by opportunistic cells has been described in other symbiotic systems (33–36). Hypoxic and normoxic areas of tumors are able to engage a sort of Cori Cycle culminating in fueling respiration of normoxic cells with lactate at expenses of the anaerobic metabolism of hypoxic cells (12). In addition, MCT1 expression and lactate upload has been correlated with p53 loss, with higher MCT1 expressed by tumors associated with

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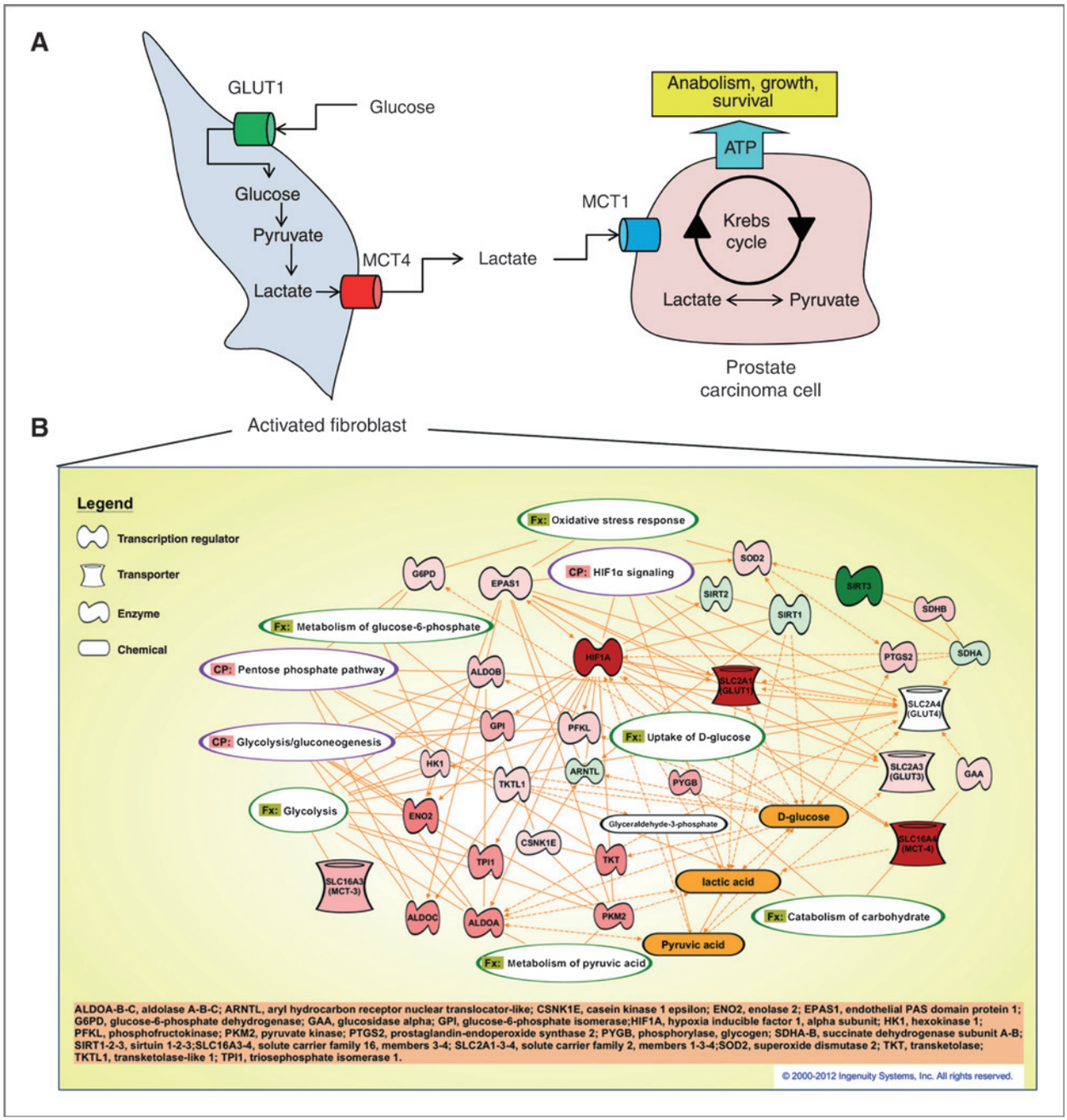

Figure 7. Proposed model of the metabolic loop between CAFs and PCa. A, CAFs and PCa cells establish reciprocal metabolic changes. PCa cells favor in CAFs, a Warburg-like glycolytic metabolism, thus increasing glucose uptake and its conversion into lactate. Lactate extruded by CAFs by MCT4 transporter is uploaded by PCa cells, through their MCT1 transporter, and used for fueling Krebs cycle, as well as anabolic processes and cell proliferation. B, network of molecules involved in lactate metabolism in CAFs. An *in silico* search for connections between genes modulated in prostatic CAFs and involved in lactate metabolism was accomplished using Ingenuity Pathway Analysis Spring Release 2012 (Ingenuity Systems; www.ingenuity.com). The network was grown starting from molecules that were upregulated in CAFs versus HPFs on the basis of our unpublished microarray analysis and related to the catabolism of carbohydrates. Overall, scales of pink indicate upmodulated genes, whereas scales of green are used for downmodulated genes. Additional molecules recognized as relevant for lactate metabolism from our experiments were included and colored in red when upregulated or dark green when downregulated at protein level. Direct or indirect interactions between molecules are indicated with either a solid or a dashed line, respectively, and may consist of activation (→), inhibition (—|), or binding (—). Molecular functions (Fx) and canonical pathways (CP) were finally added to the drawing.

poorer clinical outcome (37). In keeping with these data, we show here that MCT1 expression by PCa is mandatory for tumor outgrowth, as indicated by the efficiency of *in vivo* targeting of MCT1 with CHC or RNA interference.

MCT1 expression due to p53 loss is correlated with exposure to hypoxia of breast cancer cells (37). We observed that exposure of PCa cells to CAFs does not need hypoxia to elicit MCT1 expression and functional lactate dependence of cell

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survival/growth, as well as of tumor outgrowth in SCID mice. These data are in agreement with our previous observations indicating that CAFs are able to mimic hypoxic signaling in PCa cells, inducing HIF1 activation through oxidative stress-mediated stabilization of the transcription factor (18, 38, 39). We now enlarge the value of oxidative stress-mediated HIF1 signaling in tumor microenvironment, as the metabolic reprogramming toward glycolisis of CAFs and toward reverse Warburg metabolism of PCa cells are both redox-dependent. Hence, CAFs and hypoxia are synergistic mediators of tumor microenvironment, both exploiting oxidative stress to signal downstream through HIF1 transcription, to enhance tumor cell plasticity affecting both motility (36) and metabolism (38, 40). In keeping, we show here that oxidative stress in CAFs explanted from tumors correlated with PCa aggressiveness and PSA values.

Oxidative stress and redox-mediated HIF1 stabilization have also been involved in SIRT3 loss and consequent deregulation of oxidative pathways (25, 41). In particular, SIRT3 opposes to Warburg phenotype of cancer cells, mainly acting via destabilization of HIF1, leading to inhibition of glycolysis and activation of oxidative metabolism (13). We now report that the NAD-dependent deacetylase SIRT3 is clearly downregulated during stromal reactivity and myofibroblasts differentiation, thereby leading to SOD acetylation/activation and driving a ROS-dependent HIF1 stabilization. The glycolytic switch evident in breast cancer cells lacking SIRT3 has been proposed to contribute to tumorigenesis (13), but we now enlarge the role played by SIRT3 loss or loss of function by including CAFs as cells undergoing a SIRT3-mediated regulation of HIF1 and Warburg metabolism.

CAFs have already been acknowledged to elicit epigenetic programs leading cancer cells to achieve stem-like features and mesenchymal motility through EMT (18). Both these events are driven by HIF1- and NF-κB-dependent proinflammatory signature, exploiting oxidative stress (17). Now, among CAFs duties, we include metabolic reprogramming of cancer cells toward a reverse Warburg phenotype, allowing carcinoma highly infiltrated by reactive stroma to allocate Warburg metabolism to stromal cells and exploiting their byproducts to survive and grow in a glucose-free milieu. This feature is again an example of adaptive strategies engaged by plastic cancer cells, as these cells can either use Warburg metabolism in high glucose environment, but shift to reverse Warburg metabolism upon CAFs contact, if hypoxic/ischemic conditions lead to glucose starvation.

These data have important pharmacologic implications, as they include stromal fibroblasts as key regulators of metabolic adaptive strategies based on oxidative stress handling, engaged by cancer cells to face the hostile tumor environment, adding a further level of complexity to the system. Problems handling *in vivo* drugs targeting Warburg metabolism, such as DCA or 2-DG, could rely on the multiple adaptations exploited by cancer cells, due or in synergy with its stroma. Indeed, targeting of glycolysis is largely insufficient to inhibit progression toward aggressive behavior, as malignant cells exploit oxidative stress to engage antioxidant responses, involving Nrf-2 transcription (42) and activation of pentose phosphate pathway (15, 43–46). Hence, possible successful anticancer therapies should target both management of oxidative stress and metabolic Warburg reprogramming, addressing both glycolysis and penthose phosphate pathway, in both cancer and stromal cells, thus impairing the ability of cancer cells to exploit oxidative stress but forcing them to succumb to it.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Fiaschi, A. Marini, A. De Donatis, M. Lanciotti, S. Serni

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Fiaschi, A. Marini, E. Giannoni, P. Gandellini, A. De Donatis, M. Lanciotti, S. Serni, P. Chiarugi

Writing, review, and/or revision of the manuscript: T. Fiaschi, A. Marini, P. Gandellini, M. Lanciotti, S. Serni, P. Chiarugi

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