RECI PROCAL METABOLIC REPROGRAMMING THROUGH LACTATE SHUTTLE COORDINATELY INFLUENCES TUMOR-STROMA INTERPLAY

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Non-standard abbreviations: CAFs: cancer-associated fibroblasts; CM: conditioned medium; EMT: epithelial-mesenchymal transition; HPF: human prostate fibroblasts; PCa: prostate carcinoma; PCa-AF: PCa-Activated Fibroblast

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Abstract

Cancer associated fibroblasts (CAFs) 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 (PCa) 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 (HPFs) revealed that CAFs undergo Warburg metabolism and mitochondrial oxidative stress. This metabolic reprogramming towards a Warburg phenotype occurred as a result of contact with PCa 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, PCa cells, upon contact with CAFs, were reprogrammed towards 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 HIF1, which drove redox- and SIRT3-dependent stabilization of HIF1 in normoxic conditions. PCa cells gradually became independent of glucose consumption, while developing a dependence on lactate upload to drive anabolic pathways and thereby cell growth. In agreement, pharmacological inhibition of MCT1-mediated lactate upload dramatically affected PCa cells 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.
Introduction

The skill of cancer cells to invade and metastasize is caused by the genetic changes that these cells have undergone during multi-step 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 (CAFs), endothelial cells and inflammatory cells, the so called tumor-associated stroma, that engage a molecular crosstalk with cancer cells, secreting large amounts of factors/cytokines and influencing invasion and metastasis (4-6).

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 due to incomplete glucose oxidation, that favour 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 which shifts glucose metabolism towards 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 neighbouring 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 favours the Warburg phenotype, causing oxidative stress which 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 generating reducing potential through generation of NADPH for detoxification of reactive oxygen species (ROS) (14-16).

CAFs have already been proven to elicit a pro-oxidant environment in cancer cells, deeply affecting tumor progression and metastasis spread (17). Indeed, CAFs promote 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 due to activation of a pro-inflammatory signature involving cyclooxygenase-2, nuclear factor-kB (NF-kB) 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 paper we report evidence of a reciprocal metabolic reprogramming of CAFs and PCa cells. In particular, we demonstrate that following activation CAFs shift their metabolism towards 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 TCA cycle and protein synthesis, fueling cancer cell proliferation.
Material 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'-dichloro-Fluorescein-diacetate (DCF-DA) were from Molecular Probe. [U-14C] lactate and [3H]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/STR (short tandem repeat) analysis (ECACC) and used within 6 months of resuscitation of original cultures. Healthy human prostate fibroblasts (HPFs) and CAFs were isolated from surgical explantation 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 (FAP) 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 sub-confluence and treated for 24 hour with CMPCa (obtained culturing PCa cells in serum-free medium for 48 hours) to obtain PCa-AFs. Fresh serum-free medium was added to HPFs for an additional 24 hours before collection of CMHPF.
Western blot analysis. 1x10^6 cells were lysed for 20 minutes on ice in 500 μl of complete radioimmunoprecipitation assay (RIPA) lysis buffer (18). Lysates were clarified by centrifugation, separated by SDS-PAGE and transferred onto PVDF. 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 μM CSFE for 15 min at 37°C and then plated alone or in co-culture 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 co-culture were grown for 7 days and PCa clones were counted under an optical microscope.

Real Time RT-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 (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^{ΔΔCT} calculation.

Glucose up-take. Cells were treated with CM for 72 hours. 2-deoxy-glucose uptake was evaluated in an buffered solution (140 mM NaCl, 20 mM Hepes/Na, 2.5 mM MgSO_4, 1 mM CaCl_2 and 5 mM KCl, pH7.4) containing 0.5 μCi/ml [3H] deoxy-glucose for 15 minutes at 37°C. Cells were subsequently washed with cold PBS and lysed with 0.1 M 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 μM DCF-DA and lysed with RIPA buffer. The fluorescence values were normalized for proteins content. Evaluation of mitochondrial ROS was performed adding 5 μM 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.

**Incorporation of lactate into proteins.** Cells were treated with CM for 72 hours and then [U-14C] 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 [14C] labelled proteins by scintillator.

**Detection of released CO₂ by radioactive lactate.** Cells were treated with CM for 72 hours and then 0.2 μCi/ml D-[U-14C] 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 CO₂. Then 200 μl of 4M H₂SO₄ 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 six- to 8-week-old male severe combined immunodeficient (SCID)-bg/bg mice (Charles River Laboratories International). Animals (six 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 three independent experiments. Statistical analysis of the data was performed by Student's *t* test. *P* values of ≤0.05 were considered statistically significant.
Results

1. Analysis of HPFs and CAFs from human patients. In colorectal carcinoma histological analyses suggest that the stroma infiltrating these tumors buffer and recycle products of anaerobic metabolism of cancer cells in order to sustain invasive cancer growth (22). On the contrary in breast cancers PK-M2 and lactate dehydrogenase are highly expressed in the stromal CAFs (23). In addition, fibroblasts undergoing activation due to deletion of caveolin-1 experience oxidative stress, and a HIF1-mediated shift towards 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 nine patients with PCa, whose pathological values are reported in Suppl. Tab. I. Our choice has been driven by cancer aggressiveness and PSA values. As healthy counterparts, we used fibroblasts isolated from four men with benign prostatic hyperplasia (human prostate fibroblasts, HPFs). Firstly, we observed that prostate CAFs undergo increased anaerobic glycolysis, as revealed by their increased basal glucose up-take (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 (Suppl. Fig. 2A, B and C). 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$_2$O$_2$ are greater in CAFs compared to HPFs (Fig. 1C and 1D). Gene expression analysis, glucose uptake,
as well as their ROS production, indicate that prostate CAFs experience a Warburg metabolism due to their activation in response to cross-talk with cancer cells.

2. Prostate HPFs undergo Warburg effect in response to activation. To investigate the activation of Warburg metabolism due to CAFs differentiation, we used HPFs activated \textit{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 \textit{in vitro} activated HPFs, as PCa-activated fibroblasts (PCa-AFs). We observed that exposure of HPFs to PCa-CM elicits a clear increase in their lactate secretion (Fig. 2A), likely due to the increase in MCT4 in PCa-AFs (Fig. 2B). Activation of fibroblasts does not affect their utilization of lactate, as revealed by analyses of lactate up-load and MCT-1 expression level (Fig. 2B and Suppl. Fig. 3). 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, D).

3. The Warburg metabolic shift in CAFs is redox-and HIF1-dependent. Differentiation of CAFs towards 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 firstly 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 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. 3 B, C and D).

4. **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 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 downregulation, mitochondrial ROS are strongly increased upon HPFs activation (Fig. 4B, C). SIRT3 acts as a key upstream regulator of ROS production during HPFs activation, leading to HIF1 stabilization. Indeed, when down-regulation 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).

5. **PCa up-load lactate produced by CAFs.** Since 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 co-cultured 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 co-culture), thus suggesting that lactate has been consumed by PCa (Fig. 5A). Since activated fibroblasts showed unchanged MCT1 expression and unaffected lactate up-load compared to HPFs (Fig. 2B and Suppl. 1), we speculated that PCa in contact with CAFs are
also metabolically reprogrammed to upload lactate. In keeping, we found that the MCT1 transporter is up-regulated in PCa cells treated with CM from CAFs or in co-culture with CAFs (Fig. 5B). In agreement, MCT4 expression in PCa cells is unaffected by co-culture with CAFs (Suppl. Fig. 4A). Again, HIF1 and redox signaling play a mandatory role in metabolic reprogramming of PCa cells through up-regulation 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 \(^{14}\text{C}\)-lactate and analyzed its upload by PCa cells, after treatment with CM or co-culture with CAFs (Fig. 5C, upper panel). In the same experimental setting, we also evaluated respiration of lactate by PCa, through analysis of released \(^{14}\text{C}\)-CO\(_2\), and lactate reconversion towards anabolic pathways, through analysis of \(^{14}\text{C}\)-proteins (Fig. 5C, middle and lower 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 towards an aerobic metabolism, upon contact with CAFs, is given by analyses of both \(^{3}\text{H}\)-glucose uptake and expression of GLUT1 (Fig 5D).

6. Lactate shuttle is functional for PCa cell growth. With the aim to investigate which is the benefit for PCa cells to upload lactate produced by surrounding CAFs, we firstly analyzed cell proliferation. PCa cells were labeled with the fluorochrome CSFE, co-cultured 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 up-load 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 (12, 27) (Fig. 6A). Furthermore, PCa cell growth was assayed in a time course co-culture 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 co-culture 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 up-loading lactate, since 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 (Suppl. Fig. 4B) or by the use of different metabolism inhibitors. Indeed, the block of the metabolic circuitry established between co-cultured PCa cells and CAFs, by treatment with 2-deoxyglucose (2-DG), dichloroacetate (DCA) or Antimicin A, as well as forced re-expression 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, co-injection of CAFs with PCa cells strongly enhances the tumor growth rate (17), while we observed that silencing of MCT1 reduces this ability by 50%, thereby underscoring the 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. Histopathological analysis reveals that PKM2 and lactate dehydrogenase are highly expressed in the stroma of breast cancer lacking caveolin-1 expression (23). In addition, fibroblasts undergoing activation due to caveolin-1 deletion, or in response to down-regulation 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 towards aerobic glycolysis and elimination of mitochondrial activity through mitophagy (30). In disagreement with these data, histological 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 in order 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 utilize 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 due to 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 lead to the reduction of extracellular acidity, which represents a not favourable environmental factor for tumor survival. The metabolic reprogramming of PCa cells and their CAFs involves also re-expression 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 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 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 towards glycolysis of CAFs and towards 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 down-regulated 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-KB dependent pro-inflammatory signature, exploiting oxidative stress (17). Now, among CAFs duties, we include metabolic reprogramming of cancer cells towards 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 pharmacological 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, like di-chloro-acetate or 2-deoxyglucose, 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 towards 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 pentose 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.
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FIGURE LEGENDS

Fig. 1. Analysis of human ex vivo HPFs and CAFs. A) Analysis of [3H]-glucose up-take in HPFs and CAFs. B) MCT4 immunoblot in HPFs and CAFs. Normalization was performed 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 have been normalized on sample protein content. *p< 0,001 vs HPFs; #p< 0,01 vs HPFs.

Fig. 2. Analysis of Warburg metabolism in HPFs activated in vitro. HPFs were treated with CMPCa for 24 hours obtaining PCa-AFs, then serum-free medium was added for additional 48 hours. A) lactate assay in culture medium. B) MCT4 and MCT1 immunoblots in HPFs and PCa-AFs. Normalization was performed by actin immunoblot. C) Real Time PCR analysis for GLUT1 mRNA in HPFs and PCa-AFs. D) [3H]-glucose up-take in HPFs and PCa-AFs. * p< 0,001 vs HPFs.

Fig. 3. Warburg metabolism of PCa-AFs is ROS- and HIF1-dependent. HPFs were treated with CMPCa for 24 hours obtaining PCa-AFs, then serum-free medium was added for additional 48 hours. A) Analysis of total ROS content in HPFs and PCa-AFs. B) Assay of lactate extruded by HPFs and PCa-AFs. 20 mM of NAC has been added to serum free medium for 24 h. C) Immunoblot analysis of redox- and HIF1-dependence of MCT4 expression. HIF1α was silenced in HPFs by RNA interference for 48 hours, before adding CMPCa, while 20 mM NAC and 250 nM topotecan were added for 24 hours. Actin immunoblot has been used for normalization. D) Assay of lactate extruded in HPFs and PCa-AFs after treatment with topotecan or HIF1α silencing as in C. *p<0,001 vs HPFs, #p< 0,001 vs control, §p< 0,1 vs HPFs.
**Fig. 4. HIF1 accumulation is regulated by SIRT3 in PCa-AFs.** HPFs were treated with CM<sub>PCa</sub> for 24 hours obtaining PCa-AFs, then serum-free medium was added for additional 48 hours. A) Immunoblot analysis of HIF1, SIRT3, SOD2 and acetyl-SOD2. Actin immunoblot has been used for normalization B) Cytofluorimetric analysis of mitochondrial ROS using Mitosox probe. C) Immunoblots for SIRT3, HIF1, CAIX (a common target of HIF1) has been performed on CAFs+PCa co-culture. 25 μM and 50 μM kaempferol (SIRT3 activator) has been added to serum free medium for 24 h. Actin immunoblot has been used for normalization. Total ROS production of the same samples is reported in the plot below. D) SIRT3 has been silenced in HPFs by RNA interference for 48 hours, before adding CM<sub>PCa</sub>. HIF1 and SIRT3 immunoblots are show and total ROS production of the same samples is reported in the plot below. *p< 0.001 vs HPFs, #p< 0.001 vs control.

**Fig. 5. PCa cells up-load and use lactate produced by CAFs.** PCa cells were cultured with serum-free medium (st), or with CM<sub>HPF</sub>, CM<sub>CAF</sub> or co-cultured with CAFs (proportion CAFs:PCa 3:1). A) Assay for lactate extrusion. B) MCT1 or HIF1 immunoblots of the same samples. Actin immunoblot has been used for normalization. C) [14C]-lactate up-load (upper panel), respiration of [14C]-lactate, evaluated as [14C]-CO₂ released (central panel) and incorporation of [14C] into proteins (bottom panel). D) Analysis of [3H]-glucose uptake (upper panel) and GLUT1 expression by Real Time-PCR (bottom panel). *p< 0.001 vs HPFs, #p< 0.001 vs st, §p< 0.01 vs st.

**Fig. 6. Lactate produced by CAFs is useful for PCa growth.** A) Analysis of PCa growth in co-culture with CAFs. PCa cells were first labeled with CSFE and then co-cultured with CAFs for 48 h, in the presence or not of 2,5 mM CHC, before cytofluorimetric analysis of cell proliferation. Proliferation index has been reported in the bar graph. B) Number of PCa cells clones in co-culture with CAFs. 3x10³ 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 panel). 2,5 mM CHC has
been added to co-culture for the whole period (right panel). C) Representative images of PCa clones obtained in co-culture with CAFs for 7 days in the presence of 2.5 mM CHC, 1mg/ml 2-deoxyglucose (DG). D) Increase in PCa cell clone number, in co-culture with CAFs (5:1 ratio), for 7 day with 1mg/ml 2-DG, 0.5 mM di-cloro acetic acid (DCA), 20 nM Antimicyn (Ant. A), 25 and 50 μM kaempferol. E) Xenograft growth in SCID bg/bg mice of wild-type or MCT1-silenced PCa cells injected s.c.. PCa cells have been injected alone or with activated fibroblasts (5:1 ratio). MCT1 silencing by RNA interference in PCa cells is shown. *p< 0.001 vs (-) or vs t0, #p< 0.01 vs t0, §p< 0.001 vs C.

Fig. 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 up-take 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 up-regulated in CAFs vs HPFs on the basis of our unpublished microarray analysis and related to the catabolism of carbohydrates. Overall, scales of pink indicate up-modulated genes, whereas scales of green are used for down-modulated genes. Additional molecules recognized as relevant for lactate metabolism from our experiments were included and colored in red, when up-regulated, or dark green, when down-regulated 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.
Figure 1

A

[3H] glucose uptake

HPFs | CAFs

1 2 3 4 1 2 3 4 5 6 7 8 9

B

MCT4 level (a.u.)

HPFs | CAFs

1 2 3 4 5 6 7 8 9

MCT4

actin

C

total ROS

HPFs | CAFs

1 2 3 4 1 2 3 4 5 6 7 8 9

D

% of MitoSox + cells

HPFs | CAFs

1 2 3 4 1 2 3 4 5 6 7 8 9
Figure 2

A

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Figure 3

A

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[Graph A: Comparison of ROS/total proteins between HPFs and PCa-AFs.]

[Graph B: Comparison of lactate/proteins between control and NAC conditions for HPFs and PCa-AFs.]

[Graph C: Western blot analysis showing expression levels of MCT4, HIF1, and actin under control, topotecan, and siRNA HIF1 conditions.]

[Graph D: Bar chart showing lactate/proteins for HPFs and PCa-AFs in control, topotecan, and siRNA HIF1 conditions.]
Figure 5

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**Figure 7**

A

- GLUT1
- glucose
- glucose → pyruvate
- MCT4 → lactate

B

Activated fibroblast

Prostate carcinoma cell

**Krebs cycle**

**anabolism, growth, survival**

**ATP**

**Legend**

- **transcription regulator**
- **transporter**
- **enzyme**
- **chemical**

**ALDOA-A,B,C: aldolase A-B-C; ARNTL, aryl hydrocarbon receptor nuclear translocator-like; CSNKIE: casein kinase 1 epsilon; ENO2, enolase 2; EPAS1, endothelial PAS domain protein 1; G6PD, glucose-6-phosphate dehydrogenase; GAA, glucocerebrosidase; GPI, glucose-6-phosphate isomerase; HIF1A, hypoxia inducible factor 1; HK1, hexokinase 1; PKM2, phosphofructokinase; PTGS2, prostaglandin-endoperoxide synthase 2; PFKP, phosphofructokinase; SDHA-B, succinate dehydrogenase subunit A-B; SIRT1-3, sirtuins 1-3; SLC2A1-3, solute carrier family 2, members 1-3; SLC2A4-6, solute carrier family 2, members 4-6; SOD2, superoxide dismutase 2; TKT, transketolase; TKTL1, transketolase-like 1; TP1, treosphosporolase isomerase 1.**

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RECIPIROCAL METABOLIC REPROGRAMMING THROUGH LACTATE SHUTTLE COORDINATELY INFLUENCES TUMOR-STROMA INTERPLAY

Tania Fiaschi, Alberto Marini, Elisa Giannoni, et al.

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