UNIVERSITÀ DEGLI STUDI DI SASSARI

Scuola di Dottorato di Ricerca in
Scienze Biomolecolari e Biotecnologiche

Indirizzo: Biochimica e Biologia molecolare
XXVI ciclo

Role of stromal fibroblasts in prostate carcinoma progression and metabolic reprogramming of cancer cells

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Anno Accademico 2012-2013

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INTRODUCTION

TUMOR MICROENVIRONMENT

It is now well established that tumor growth, progression and metastasis is not just determined by malignant cells themselves, but also require the tumor microenvironment. The latter includes extra-cellular matrix (ECM) components, hypoxia and stromal cells, either resident or recruited from the circulation, represented by fibroblasts, pro-inflammatory cells (e.g. macrophages), endothelial cells and pericytes (Fig.1).

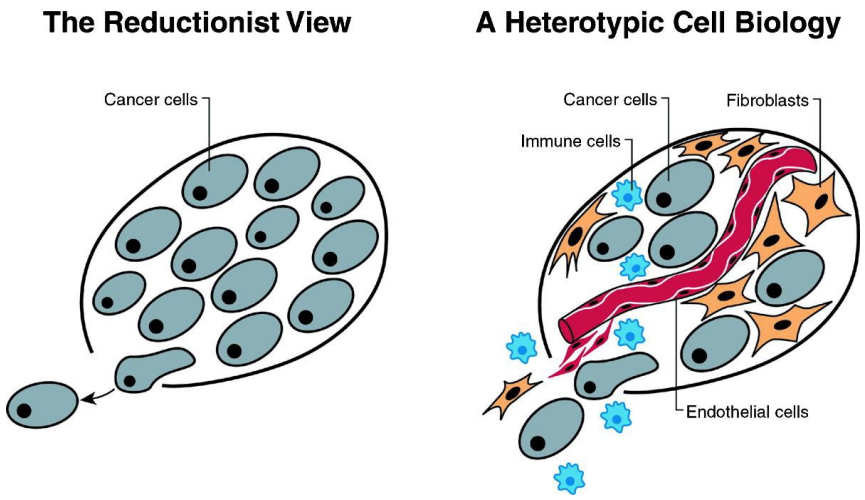

Fig. 1.Tumor as complex tissue. Cancer is not single cell disease and tumor initiation and progression is highly influenced by tumor stroma that includes fibroblasts, immune cells and endothelial cells.

Tumor stroma is similar to that observed during wound healing and it is commonly referred to as "reactive stroma", which is associated with an increased number of fibroblasts, enhanced capillary density and collagen and fibrin deposition (Kalluri and Zeisberg, 2006). Cancer cells create a complex and continuative “cross-talk” with surrounding, non-malignant cells and/or with the extracellular architecture, made of direct cell-to-cell contacts and paracrine/exocrine signals. Tumor-stroma interactions at both the primary and secondary tumor sites allow and support tumor survival and outgrowth, organ homing and invasion. The role of tumor support played by stromal

cells spans from growing of new vessels, with the recruitment of endothelial progenitors and their activation to form functional vessels, to secretion of a large amount of cytokines and soluble factors affecting cancer cell behavior (Taddei et al., 2013).

Neoplastic cells and stromal cells around them change progressively during the multistep transformation of normal tissues into high-grade malignancies and tumor-stroma interactions evolve along with tumor progression. Incipient neoplasia begin the interplay by recruiting and activating stromal cell types that assemble into an initial preneoplastic stroma, which in turn responds reciprocally by enhancing the neoplastic phenotypes of the nearby cancer cells. The cancer cells, which may further evolve genetically, again feed signals back to the stroma, continuing the reprogramming of normal stromal cells to serve the budding neoplasm; ultimately signals originating in the tumor stroma enable cancer cells to invade normal adjacent tissues and disseminate (Hanahan and Weinberg, 2011). In this context, several evidences have identified fibroblasts as key mediators in promoting tumor progression.

Fibroblasts and Cancer Associated Fibroblasts

Fibroblasts are elongated cells, characterized by extensive cellular processes, with fusiform and tapered shape (Tarin and Croft, 1969). They can be easily isolated from tissues and cultured *in vitro*. Their fusiform morphology makes them identifiable and, despite the paucity of specific markers, some molecules can be related to a fibroblastic phenotype, although none of these is exclusive of fibroblasts and/or expressed in all fibroblasts. Among these markers, the fibroblasts specific protein-1 (FSP-1) seems to provide the best specificity for the identifications of fibroblasts *in vivo*, but other markers can be considered site-specific, like desmin, a specific marker for skin fibroblasts.

The important functions of fibroblasts include the deposition of ECM, regulation of epithelial differentiation, regulation of inflammation and involvement in wound healing (Kalluri and Zeisberg, 2006). Fibroblasts synthesize many of the constituents of ECM, such as type I, III and V collagen and fibonectin. They also contribute to the formation of basement membranes by secreting type IV collagen and laminin. Fibroblasts are also

an important source of ECM-degrading proteases such as matrix metalloproteinases (MMPs), which highlights their crucial role in maintaining an ECM homeostasis by regulating ECM turnover. In addition, fibroblasts are important in maintaining the homeostasis of adjacent epithelia through the secretion of growth factors and direct mesenchymal-epithelial cell interactions (Kalluri and Zeisberg, 2006).

As well as their function in healthy organs, fibroblasts have a prominent role in wound repair. They invade lesions, generate ECM to serve as a scaffold for other cells, and possess cytoskeletal elements that facilitate contractions of healing wounds (Kalluri and Zeisberg, 2006). When tissue injury occurs, fibroblasts undergo an activate state named "myofibroblast", characterized by the *de novo* expression of α-SMA protein, the actin isoform typical of smooth muscle cells, and the ability to synthesize increased levels of ECM components and ECM-degrading proteases such as matrix metalloproteinase-2 (MMP-2), MMP-3 and MMP-9, thus facilitating increased ECM turnover and altering ECM composition (Rodemann H.P. and Muller G.A., 1991) (Fig.2). Activated fibroblasts often secrete increased amounts of growth factors such as Hepatocyte Growth Factor (HGF), Insulin-like Growth Factor (IGF), Nerve Growth Factor (NGF), WNT1, Epidermal Growth Factor (EGF) and Fibroblast Growth Factor-2 (FGF2), which can induce proliferative signals within adjacent epithelial cells (Bhowmick et al., 2004). Activated fibroblasts also have an important role as modulators of the immune response following tissue injury, through the secretion of cytokines such as interleukin-1 and chemokines such as monocyte chemotactic protein 1 (MCP1) (Strieter et al., 1989; Rollins et al., 1989). Activated fibroblasts are found in healing wounds and sclerosing tissues, and are also associated with tumors. Different stimuli induce this activation, including growth factors such as Transforming Growth Factor-β (TGF-β), EGF, Platelet-Derived Growth Factor (PDGF) and FGF2, which are released from injured epithelial cells and infiltrating mononuclear cells such as monocytes and macrophages. In addition, fibroblasts are activated by direct cell-cell communication and contacts with leukocytes through adhesion molecules such as Intercellular-Adhesion Molecule-1 (ICAM1) or Vascular-Cell Adhesion Molecule-1 (VCAM1) (Clayton et al., 1998). Fibroblast activation can also be achieved through reactive oxygen species, complement factor C1 or altered ECM composition (Zeisberg et al., 2000).

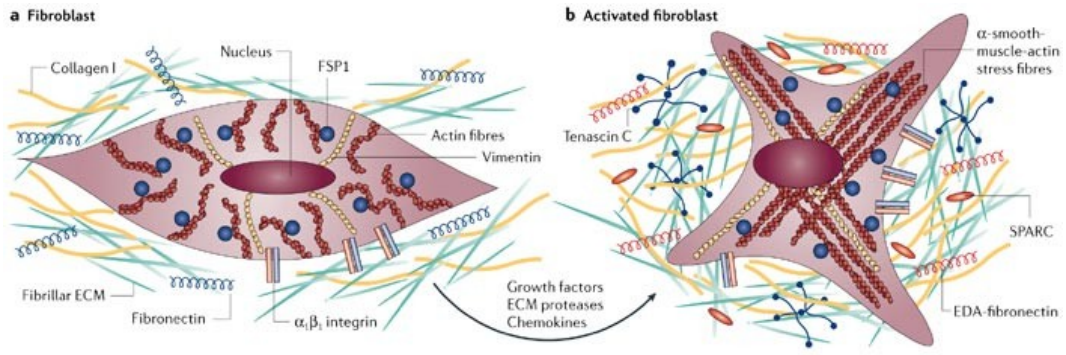

Fig.2. Fibroblasts activation. Fibroblasts can acquire an activated phenotype, which is associated with an increased proliferative activity and enhanced secretion of ECM. Phenotypically, activated fibroblasts are often characterized as expressing α-smooth-muscle actin.

Most of knowledge about tumor stromal cells stem from the studies of carcinomas. In the early growth of tumors, cancer cells form a neoplastic lesion that is embedded in the microenvironment of a given tissue (Hanahan and Weinberg, 2000) but separated from the surrounding tissue and contained within the boundary of a basement membrane. This is called the carcinoma *in situ* (CIS). CIS is associated with "reactive stroma", similar to that observed during wound healing. Vascular Endothelial Growth Factor (VEGF), produced mainly by fibroblasts and inflammatory cells, represents a key molecule for the development of the stroma (Brown et al., 1999). VEGF induces microvascular permeability, thus allowing the extravasation of plasma proteins such as fibrin, which attracts fibroblasts, endothelial cells and inflammatory cells (Senger et al. 1983; Dvorak et al., 1984; Brown et al., 1999). These cells produce ECM that is rich in fibronectin and type I collagen, both implicated in the development of tumour angiogenesis (Leung et al., 1989; Brown et al., 1999; Feng et al., 2000). During tumor progression from carcinoma *in situ* to invasive carcinoma, tumor cells invade the reactive stroma (Dvorak et al., 2011; Ronnov-Jessen et al. 1996). Basement membrane and stroma are degraded, and myofibroblast come into direct contact with the tumor cells. Invasive cancer is usually associated with the expansion of tumor stroma and to an increase of the deposition of ECM, (Shekhar et al. 2003; Ronnov-Jessen et al. 1996; Van Kempen et al. 2003). This phenomenon appears to be very similar to the changes that take place during fibrosis, but while fibrosis is associated with a decrease of vascularization (Brown et al. 1999), solid tumors are more vascularized (Folkman 1971).

Activated fibroblasts associated with reactive stroma are called Cancer Associated Fibroblasts (CAFs) and they are characterized by contractile and secretory features (Cirri and Chiarugi, 2012; Kalluri and Zeisberg, 2006) (Fig.3). CAFs become activated in response to tumor-delivered factors through a mesenchymal-mesenchymal transition (MMT). They were first identified by immunocytochemistry using a combination of different markers such as α-SMA (α-smooth-muscle-actin), vimentin, desmin and Fibroblast Activation Protein (FAP), a serine protease located on the cell surface of tumor stromal fiboblasts (Garin-Chesa et al., 1990; Lazard et al., 1993; Mueller and Fusenig, 2004). Unlike what happens for myofibroblasts, CAF activation is not reversed once the activating stimulus is attenuated, so their presence persists in tumor stroma (Tomasek et al., 2002; Hinz et al., 2001). Indeed, CAFs represent the most prominent cell type within the reactive stroma of many cancers, such as breast, prostate and pancreatic carcinoma (Pietras and Ostman, 2010; Kalluri and Zeisberg, 2006). CAFs are associated with cancer cells at all stages of cancer progression by promoting tumor growth, angiogenesis and the metastatic process (Tlsty and Coussens, 2006; Kalluri and Zeisberg, 2006; Orimo et al., 2005).

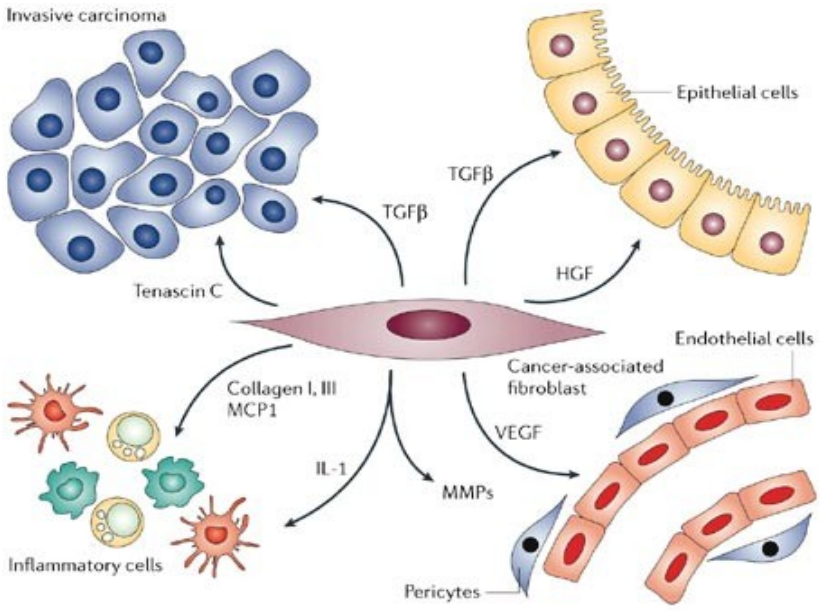

Fig. 3. Cross-talk between CAFs and different types of cells of tumor microenvironment.

CAFs originate through different ways. *In primis,* CAFs derive from resident fibroblast activated by cancer-derived growth factors such as TGF-β, PDGF and FGF2 (Elenbaas

and Weinberg, 2001). TGF-β is associated with an increase of fibrotic tissue, tumor progression and fibroblasts recruitment (Siegel and Massague, 2003). In addition, TGF-β is the most important factor of the tumor microenvironment promoting Epithelial-Mesenchymal Transition (EMT) in tumor cells and leading fibroblasts to a CAF phenotype expressing α-SMA *in vitro* (Siegel and Massague 2003). EMT is an epigenetic program that leads epithelial cells to lose their cell–cell and cell-ECM interactions to undergo cytoskeleton reorganization and to gain morphological and functional characteristics of mesenchymal cells, thus generating an invasive cell, able to secrete proteases, to deeply change the surrounding ECM and to move away from the site of the primary tumor (Friedl, 2004; Kalluri and Weinberg, 2009; Nieto and Cano, 2012).

Several data indicate that CAFs activation is a redox dependent process (Cirri and Chiarugi, 2011); indeed, stimulation of CAFs with TGF-β elicits a burst of reactive oxygen species (ROS) which causes the achievement of the activated phenotype, the down regulation of gap junctions as well as their tumor promoting activity in skin tumor (Cat et al., 2006; Stuhlmann et al., 2004). In addition, the importance of oxidative stress in CAFs activation has been highlighted by Toullec et al.; in particular, ROS promote conversion of fibroblasts into highly migrating myofibroblasts through accumulation of the hypoxia-inducible factor (HIF)-1α transcription factor and the CXCL12 chemokine (Toullec et al., 2010) (Fig.4).

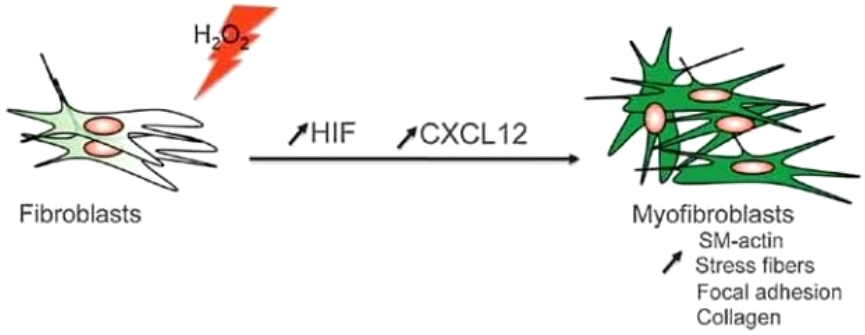

Fig.4. In carcinoma, chronic oxidative stress promotes the conversion of fibroblasts into myofibroblasts.

Recent studies conducted in our laboratory have shown that IL-6, secreted by prostate carcinoma PC3 cells isolated from a bone metastasis of prostate carcinoma (PCa) cells, promotes a particular phenotype named PCa-activated fibroblast (PCa-AF). In contrast

to the TGF-β-dependent phenotype, these cells do not express α-SMA, but their activated state is confirmed by the expression of the FAP protein and production of ECM. The PCa-AFs strongly activate the process of EMT and therefore PC3 cells invasiveness (Giannoni et al., 2010) (Fig. 5). According to our observations, production of IL-6 by tumor cells has been correlated to higher carcinomas aggressiveness (Royuela et al., 2004; Chung et al., 2005; Niu et al., 2009).

A second source of CAFs are represented by bone marrow-derived mesenchymal stem cells (MSCs) which are recruited at tumor site by cytokines and growth factors produced by tumor cells (Dwyer et al., 2007; Spaeth et al., 2008; Feng and Chen, 2009). Moreover, emerging evidence indicates that also EMT involving normal epithelial cells flanking to the tumor, is a source of activated fibroblasts in both fibrosis and cancer (Selman and Pardo, 2006). In addition, CAFs may originate directly from carcinoma cells through EMT (Kalluri and Zeisberg, 2006; Radisky et al., 2007), allowing cancer cells to adopt a mesenchymal cell phenotype, with increased migratory and invasive capacities (Kalluri and Weinberg, 2009). Indeed, mainly in breast cancers, it has been reported CAF somatic mutations in p53 and PTEN, as well as gene copy number alteration at other loci in tumor stroma (Kurose et al., 2002; Moinfar et al., 2000). Finally, CAFs may derive from proliferating endothelial cells via endothelial to mesenchymal transition (EndMT) under TGF-β stimulation (Zeisberg et al., 2007).

Role of CAFs in tumor progression

Cancer cells and CAFs establish a close cross-talk based on mutual stimulation: carcinoma cells elicits a reactive response in the stroma and ,conversely, the activated fibroblasts located in the microenvironment affect cancer cell responses, thus influencing tumor progression.

Several findings underline the role of CAF in tumor growth. It has long been known that only human prostatic CAFs co-cultured with initiated human prostatic epithelial cells strongly stimulate growth and altere histology of the epithelial population, while normal prostatic fibroblasts are unable to elicit this effect (Olumi et al., 1999). Accordingly, it has been demonstrated that only tumor prostate fibroblasts, but not

normal prostatic fibroblasts, stimulate proliferation and malignant transformation of epithelial cells derived from SV40-T antigen immortalized benign prostate hyperplasia (Hayward et al., 2001). Beside, a direct involvement of resident fibroblasts in the initiation of cancer has been elucidated: in a mouse model, the overexpression of TGF-β and/or HGF in fibroblasts induces the initiation of breast cancer within the normal epithelium (Kuperwasser et al., 2004).

Alongside the role of CAFs in the initiation of neoplasia, CAFs play a mandatory role in progression of tumors towards malignancy by the regulation of motility and invasion of cancer cells, thus positively affecting their metastatic spread towards distant organs (Joyce et al., 2009) (Fig. 5). The contribution of CAFs to the invasive process of cancer cells is mainly due to the induction of EMT (Brahimi-Horn et al., 2011; Pani et al., 2010). This feature is primarily dependent on CAFs' ability to remodel the ECM. Indeed, in addition to secreting growth factors that directly affect cell motility, CAFs are sources of ECM degrading proteases as MMPs, which allow cancer cells to escape the primary tumor site (Kalluri and Zeisberg, 2006). It has been demonstrated that treatment of mammary epithelial cells with MMP3, which is highly expressed in fibroblasts, results in cleavage of E-cadherin and EMT induction, thus sustaining a progressive phenotypic conversion from normal mammary epithelial cells to an invasive mesenchymal phenotype (Lochter et al., 1997). In addition, EMT has been correlated with the induction of a cancer stem phenotype both in breast and in prostate cancer (Giannoni et al., 2010; Mani et al., 2008; Blick et al., 2010). This ability confers to cancer cells the self renewal capability that is crucial for clonal expansion during metastatic dissemination (Polyak and Weinberg, 2009). In particular, recent studies conducted by our research group showed that CAFs exposure promotes EMT of prostate carcinoma cells associated with enhanced expression of stem cell markers, ability to form prostaspheres and to self-renew, leading to an increase of aggressiveness and metastatic spread (Giannoni et al., 2010) (Fig. 5).

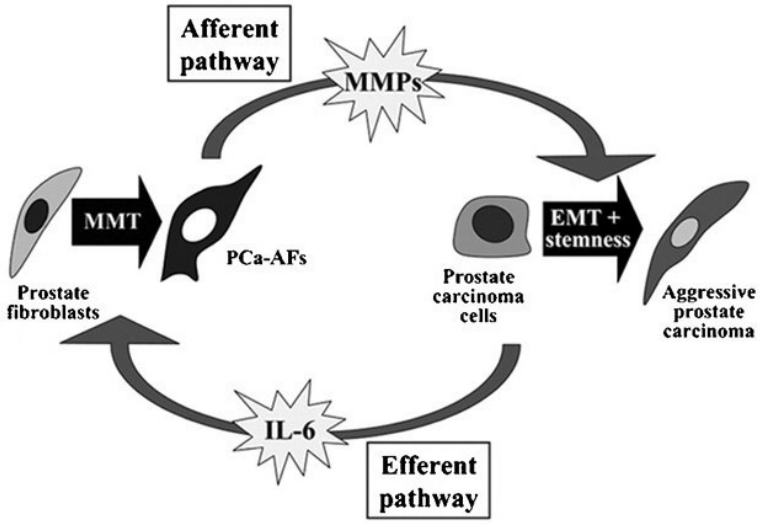

Fig. 5 Reciprocal interplay between stromal fibroblasts and PCa cells through secretion of IL-6 (efferent pathway) and MMP-dependent EMT in PCa cells (afferent pathway).

Moreover, in pancreatic cancer it has been highlighted a key role of the microenvironment in the induction of stemness: indeed, co-cultures of tumor and pancreatic stellate cells, the major profibrogenic cell type in the pancreas, enhance the cancer stem cell-like phenotypes of tumor cells (Hamada et al., 2012). Beside, the chemokine (C-C motif) ligand 2 (CCL2) secreted by CAFs, stimulates sphere formation and cancer stem cells self-renewal in breast cancer cells (Tsuyada et al., 2012). Finally, myofibroblasts, through hepatocyte growth factor secretion, activate β-catenin-dependent transcription and subsequently colon cancer cells clonogenicity (Vermeulen et al., 2010).

MicroRNAs are emerging as potential regulators of the relationship between EMT and stemness. Indeed, breast cancer stem cells show a miRNA expression profile very similar with respect to breast cancer cells undergoing EMT (i.e., high levels of miR-155 and low levels of miR-200 family) (Blick et al., 2010; Shimono et al., 2009). In agreement, manipulation of miRNAs able to revert the EMT phenotype, suppresses cancer stem cell properties, as demonstrated by the lower tumorigenic potential of miR-200c-expressing CD44high/CD24low cells. Furthermore, the achievement of EMT correlates to an increase in the resistance to apoptosis, allowing cells to survive along their route from the primary tumor to the site of dissemination (Gal et al., 2008).

Recent evidences suggests that EMT is a redox-dependent phenomenon. In mammary epithelial cells, exposure to MMP3 induces EMT, through a Rac1b-mediated release of

mitochondrial ROS (Radisky et al., 2008). Accordingly, CAFs promote EMT of cancer cells by exploiting the intrinsic or extrinsic oxidative stress and producing cytokines and proteases (Cirri and Chiarugi, 2011). In keeping, our research group demonstrated that prostate CAFs release cytokines and MMP2 and MMP9, which in turn activate the small GTPase Rac1, cyclooxygenase-2 (COX-2) and a consequent COX-2-mediated ROS production (Giannoni et al., 2011). The establishment of a pro-oxidant environment in prostate cancer cells is also necessary for the redox dependent stabilization of HIF-1α and NF-kB (Comito et al., 2011; Gloire and Piette, 2009; Hamanaka and Chandel, 2009). These factors sustain then the activation of the transcription factors Snail and Twist, promoting the EMT program in prostate carcinoma cells (Fig. 5).

Furthermore, it is useful to mention that even senescent fibroblasts are able to induce EMT in nearby epithelial cells, thus influencing tumor progression (Laberge et al., 2012). Indeed, cellular senescence is associated with an increased oxidative stress and secretion of several pro-inflammatory cytokines which collectively generate the so-called SASP (Senescence Activated Secretory Pathway) phenotype (Bavik et al., 2006). Induction of EMT by SASP has been reported for breast cancer where the SASP component factors IL-6 and IL-8 enhance the invasiveness of a panel of cancer cell lines in culture (Badache and Hynes, 2001; Coppe et al., 2008; Yuan et al., 2005).

CAFs have a primary role in the guidance of tumor cell movement. Indeed, it has been demonstrated that stromal fibroblasts co-cultured with squamous cell carcinoma collectively move across the ECM; in particular cancer cells use Cdc42 and MRCK (Myotonic Dystrophy Kinase-Related CDC42-binding protein kinases) mediated regulation of myosin light chain (MLC) activity, exploiting the tracks generated by fibroblasts (Gaggioli et al. 2007). The contractile force in stromal fibroblasts to remodel the ECM for the creation of tracks for the collective migration is dependent on the activation of the kinase JAK1 (Sanz-Moreno et al., 2011). The same pathway is also responsible for the actomyosin contractility of melanoma cells that migrate with an amoeboid motility style (Sanz-Moreno et al., 2011).

The pro-invasive role of CAFs during tumor progression has been shown in several types of tumors, such as breast cancer, prostate cancer (Giannoni et al., 2010; Orimo et al., 2005) and head and neck squamous carcinoma (Hinsley et al., 2012). In ovarian

carcinoma has been underlined that CAFs play a vital role in promoting angiogenesis, lymphangiogenesis and cancer cell invasion with respect to normal fibroblasts (Giannoni et al., 2010; Orimo et al., 2005; Zhang et al., 2011). Moreover, CAFs expressing FAP increase the migration and invasion of colorectal cancer cells through a Fibroblasts Growth Factor-1/Fibroblast Growth Factor Receptor 3 (FGF1/FGFR-3) signaling (Henriksson et al., 2011). Finally, in a model of human pancreatic cancer, the overexpression of FAP in fibroblasts directly modifies the stromal ECM through its enzymatic activity. The authors show that FAP alters the architecture and the composition of the ECM promoting tumor invasion along characteristic parallel fiber orientation (Lee et al., 2011).

Alongside their pro-invasive role, CAFs are able to recruit endothelial precursor cells (EPCs) from bone marrow, thereby inducing *de novo* angiogenesis (Orimo et al., 2005) and participate in the preparation of the metastatic site in which the secondary tumor will grow up. Recent data demonstrated that the metastatic cells can bring their stromal components from tumor primary site to the metastatic niche in the lungs. The stromal cells, co-traveling with metastatic cancer cells, provide them an early growth advantage and protect them in the bloodstream from anoikis (the apoptotic cell death due to lack or improper cell adhesion to ECM), thus ensuring long-term survival and proliferation in the metastatic sites (Duda et al., 2010).

Beside MMPs, which remodel ECM, activated fibroblasts produce several growth factors and cytokines that sustain tumor progression. Indeed, CAFs produce paracrine diffusible signals, including TGF-β, HGF, VEGF, FGF, Stromal cell-Derived Factor-1 (SDF-1) as well as cathepsins and plasminogen activators (Joyce and Pollard, 2009; De et al., 2008). In particular, it has been shown that CAFs promote the growth of breast carcinoma cells significantly more with respect to normal mammary fibroblasts derived from the same patients. These CAFs, play a central role in promoting directly the growth of tumor cells through their ability to secrete SDF-1 as well as in promoting angiogenesis through the recruitment of EPCs (Orimo et al., 2005). Moreover, fibroblast-derived SDF-1 enhanced the invasion of pancreatic cancer cells as well as their production of CXCL8. The cooperation between CXCL8 and SDF-1 enhances also the proliferation/invasion of human umbilical vein endothelial cells, thus sustaining tumor progression (Matsuo et al., 2009). Furthermore, in the same cellular model,

cancer cell-derived IL-1α significantly promotes HGF expression by fibroblasts. As a consequence, HGF enhances not only the invasiveness and proliferation of pancreatic cancer cells, but also enhances migration and proliferation of human umbilical vein endothelial cells, thus positively influencing the metastatic potential of pancreatic cancer cells (Xu et al., 2010).

Anyway, the role of CAFs in tumor progression goes beyond the engagement of pro-invasive features in cancer cells and embrace a real metabolic reprogramming of both cell types. This topic will be extensively described later.

Role of other microenvironmental components in tumor progression

Among components of tumor microenvironment, both hypoxia and acidity require adaptive response that influence cancer cells behaviors and involve metabolic pathway and invasive properties.

HYPOXIA

Solid tumors frequently outgrow the blood supply, resulting in nutrient and oxygen insufficiency. In this state, commonly referred as hypoxia, tumors can experience decreased oxygen pressure to 1% or below. Hypoxia is considered as an independent negative prognostic indicator and contributes to cancer progression affecting the behavior of both cancer and stromal cells (Taddei et al., 2013). Intratumoral hypoxia causes:

- activation of a glycolytic metabolism to circumvent lack of oxygen;
- acquisition of invasive features, mainly through EMT, in order to escape from the hostile environment;
- activation of pathways for survival to stressful conditions;
- secretion of soluble growth factors eliciting *de novo* angiogenesis allowing nutrient/oxygen supply.

The ability of cells to sense and adapt to hypoxia is mainly mediated by hypoxia inducible factor (HIF) family of transcription factors. Targets of HIFs span from

glucose, glutamine and fat metabolism, oxygen homeostasis, tissue remodeling and motility, angiogenesis, erythropoiesis, proliferation, and survival to stress (Keith et al., 2012). HIF1 and HIF2 complexes are the major responsible from gene expression changes during hypoxia. They are heterodimers composed of the constitutively expressed HIF-1β (or ARNT) subunit and either the HIF-1α or HIF-2α subunits. Under normoxic conditions, the HIF-1α subunits undergo oxygen-dependent hydroxylation by prolyl-hydroxylases (PHDs) on Pro402 and Pro564, which results in their recognition by von Hippel-Lindau tumor suppressor (VHL), an E3 ubiquitin ligase, and subsequent degradation (Chan et al., 2002) (Fig. 6). Of note, PHDs catalyze the hydroxylation reaction requiring oxygen and α-ketoglutarate as substrates, iron and ascorbate as cofactors. When hypoxia is occurring, HIFα are stabilized by lack of hydroxylation and functionally dimerizes with its partner HIF-1β to bind to the core sequence 5'-RCGTG-3' and enhancing the transcription of target genes involved in promoting all adaptations to hypoxia (Fig. 7). Levels of HIF-1α are also influenced in normoxic conditions by genetic alterations, including mutations of VHL gene, succinate dehydrogenase and fumarate hydratase, or growth factors, hormones and cytokines produced by both tumor and stromal cells (Dery et al., 2005) (Fig. 6).

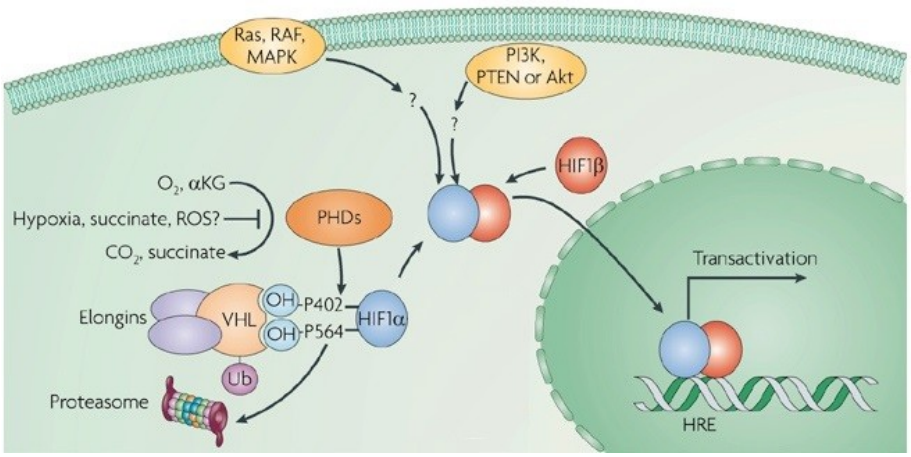

Fig. 6. Regulation of HIF-1 stabilization. HRE: Hypoxia response element.

Hypoxic cells undergo a glycolytic switch from aerobic to anaerobic metabolism (Wheaton et al., 2011), allowing maintenance of metabolic activities under limited oxygen avaibility. Other adaptive metabolic features include, engagement of pentose

phosphate pathway (PPP) and addiction to glutamine use. Of note, several of these adaptations have been acknowledged as the biochemical basis for resistance to chemotherapy and radiation (DeClerck and Elble, 2010; Heddleston et al., 2010; Rohwer and Cramer, 2011). Several genes controlling glucose uptake (e.g, glucose transporters GLUT1 and GLUT3), glycolysis (e.g., hexokinase-1/2, enolase, phosphoglycerate kinase-1, aldolase, pyruvate kinase M), lactate fermentation (lactate dehydronase A), inhibition of mitochondrial respiration (e.g., pyruvate dehydrogenase kinase) are directly targeted by HIFs (Stubbs and Griffiths, 2010) (Fig. 7).

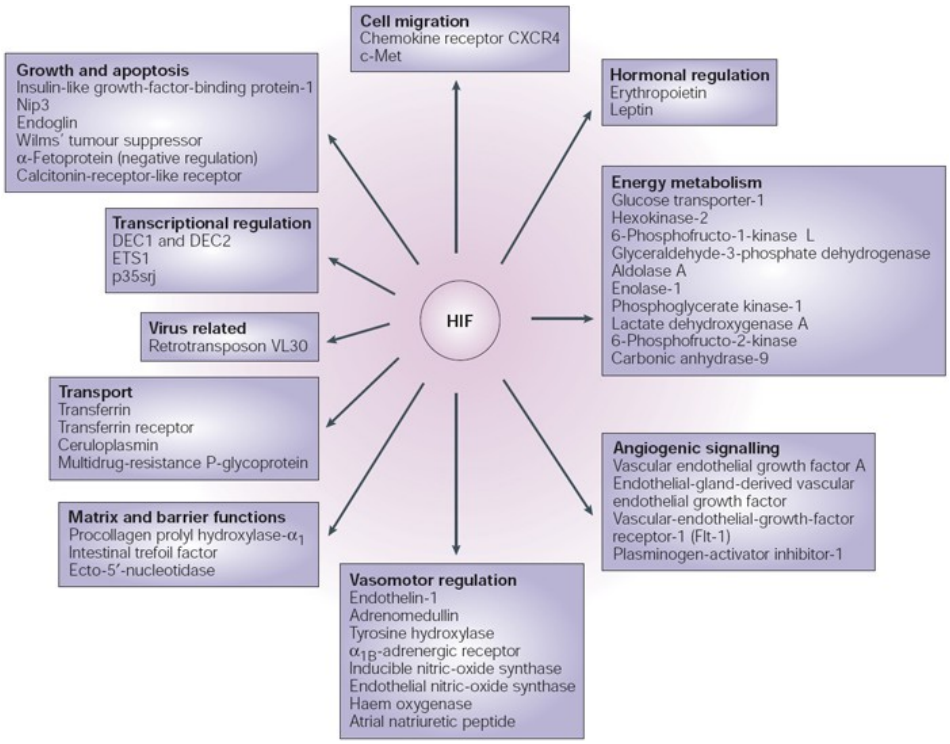

Fig. 7. HIF target genes.

Increased glucose uptake is associated with inhibition of pyruvate kinase. Indeed, all tumors analyzed to date express the M2 variant of pyruvate kinase (PKM2), which is a redox sensitive enzyme undergoing inhibition following cysteine oxidation (Anastasiou et al., 2011; Bayle and Devilee, 2012; Chen et al., 2010). Hypoxia has been correlated with a state of oxidative stress, both due to mitochondrial or NADPH oxidase delivery of ROS causing ROS-mediated inhibition of PHDs through a Fenton reaction, and leading to HIF-1 stabilization. Hypoxic oxidative stress causes oxidation and inhibition

of PKM2, with the consequence of accumulation of glycolysis intermediates, rapidly fueling anabolism of aminoacids and proteins, as well as PPP. The latter increases the pool of NADPH, leads to synthesis of ribose and DNA, allowing to safely handle with oxidative stress and to overcome DNA damage and chemotherapy stress. In keeping with this, hypoxia and PPP reprogramming have been correlated with increased resistance to therapy in several cancer models (Anastasiou et al., 2011; Brahimi-Horn et al., 2011; Gruning and Ralser, 2011).

In hypoxia, cancer cells are unable to catabolize pyruvate into acetyl-CoA, stimulating lipid biosynthesis. This is mainly due to HIF-1-mediated expression of pyruvate dehydrogenase kinase (PDK), which totally blocks decarboxylation of pyruvate through pyruvate dehydrogenase (PDH) (Wheaton et al., 2011). Hypoxic cells solve this problem becoming addicted to glutamine uptake (Dang, 2012). Using a reductive carboxylation, glutamine is converted into isocitric acid by isocitric dehydrogenase-2 (IDH-2), leading to citrate exportation from mitochondria. Citrate is converted by ATP-citrate lyase to acetyl-CoA that is used to lipid synthesis. IDH1/IDH2 acknowledged to produce α-hydroxy-glutarate which is a strong inhibitor of PHDs due to its competition with their cofactor α-ketoglutarate, have been correlated to activation of HIF-1-dependent transcriptional response (Chowdhury et al., 2011; Metellus et al., 2011).

A latere with metabolic changes, during hypoxia tumor cells activate a specific escaping program to run away this hostile environment. In addition, the products of glycolysis cause acidification of the extracellular space and the resulting cellular toxicity acts as a further selective pressure for cells that are resistant to acidic conditions (Pani et al., 2010). Moreover, hypoxic cells release angiogenic factors that stimulate *de novo* vascularisation, which restores O_2 and nutrient supply in order to meet the growing metabolic demands of proliferating cells.

To escape the hypoxic environment, the first strategy is to activate an invasive program and the primary driver engaged by hypoxia is the hepatocyte growth factor-MET receptor (Pennacchietti et al., 2003). In particular, mitochondrial ROS lead to stabilization of HIF-1α and expression and activation of MET, resulting in cell migration towards the blood or lymphatic microcirculation (Comito et al., 2011). Moreover, hypoxia also promotes tumor cell motility and invasion by triggering the EMT. Indeed, HIF-1α is a potent activator of Twist and other EMT inducers (Giannoni

et al., 2011; Cannito et al., 2008; Jiang et al., 2011; Yang et al., 2008). Twist1/2 cause downregulation of E-cadherin, that is concomitant with *de novo* expression of N-cadherin, a trait leading to up-regulation of polarized motility through the small GTPase Rac1. Through both MMPs produced during EMT program and up-regulation of urokinase-type plasminogen activator receptor expression, hypoxia enhances proteolytic activity at the invasive front of cancer cells, stimulating motility by altering integrin/ECM interactions (Sullivan and Graham, 2007; Del Rosso et al., 2002). In addition, hypoxia induced EMT has been correlated to a supplementary adaptation, i.e. resistance to anoikis (Sullivan and Graham, 2007; Nieto, 2011; Gort et al., 2008; Whelan et al., 2010). This adaptation allows survival in the bloodstream of metastatic cancer cells which have experienced activation of the HIF-1 transcriptional program. Hypoxia-driven EMT is reinforced by a reciprocal activation between HIF-1α and NF-kB, underscoring a further link between hypoxia and inflammation (Giannoni et al., 2011; Rius et al., 2008).

In order to permit successful metastases, hypoxia elicits adaptations useful to permit successful metastases, such as *de novo* angiogenesis and lymphangiogenesis (Sullivan and Graham, 2007). HIFs target several angiogenic factors, the masterpiece of which is VEGF, reportedly correlated with sprouting angiogenesis, lymphangiogenesis, as well as with the dynamic tumor-stromal interactions required for the subsequent stages of metastasis (Carmeliet and Jain, 2011). Angiogenesis and lymphangiogenesis within the primary tumor provide the necessary routes for dissemination, and VEGF-induced changes in vascular permeability promote both intravasation and extravasation. Through instruction of these critical pathways and adaptations, hypoxia promotes several step of the metastatic cascade, selecting tumor cell populations able to escape the hostile microenvironment of the primary tumor. In addition, HIFs transcription factors have been involved in the regulation of stemness, both in normal and in cancer cells, through up-regulation of pluripotency genes (Simon and Keith, 2008). Indeed, HIF-2 is an upstream regulator of Oct4, one of the mandatory transcription factors to reprogram differentiated cells towards stemness (the so called induced-stem cells) (Iida et al., 2012; Li et al., 2009). Recent data have demonstrated that even HIF-1 shows a mandatory role in the regulation of stemness of cancer cells induced by hypoxia. Indeed, at least in lymphomas, the HIF-1α inhibitor echinomycin selectively kills c-

$Kit^+Sca\text{-}1^+$ cancer stem cells, and the ablation of HIF-1α in these cancer stem cells severely impedes their self-renewing and tumor-initiating activity (Wang et al., 2011).

ACIDITY

Deregulated pH is emerging as another adaptive response of most cancers. Tumor cells show a 'reversed' pH gradient with a constitutively increased intracellular pH (pHi) that is higher than the extracellular pH (pHe) (Webb et al., 2011). In normal adult cells, pHi is generally ~7.2, while pHe is ~7.4. On the contrary, cancer cells have a higher pHi of >7.4 and a lower pHe of ~6.7–7.1 (Gallagher et al., 2008).

The acid-outside pH of tumor cells is a consequence of the adaptation towards glycolytic metabolism induced by hypoxia; indeed, enhanced glucose uptake and his conversion to lactic acid, as well as the inefficient removal of lactate and CO_2 as a consequence of a poor vasculature within the tumor mass, result in the increase of acid production (Brahimi-Horn and Pouyssegur, 2007; Brahimi-Horn et al., 2007a; Brahimi-Horn et al., 2007b).

Intracellular pH is crucial to normal cell function. Therefore, hypoxic tumor cells have developed key strategies to protect the cytosol from acidosis and allow cells to survive in hypoxia. HIF-1 regulates proton extrusion and pH homeostasis by enhancing expression of plasma membrane ion pumps and transporters (Brahimi-Horn and Pouyssegur, 2007). The most notable pHi regulatory systems of tumor cells are:

- the Na^+/H^+ exchanger (NHE-1), known to play a key role *in vivo* in tumor development, in particular when highly glycolytic cells produce large amounts of lactate (Cardone et al., 2005; Pouyssegur et al., 2001; Shimoda et al., 2006);
- the V-ATPase, which mainly contributes to the maintenance of an aberrant pH gradient between the alkaline cytosol and the acidic extracellular environment (Nishi and Forgac, 2002);
- the monocarboxylate transporters (MCTs), whose increased expression confers a selective advantage to cancer cells owing to the high affinity of these transporters for lactate (Ullah et al., 2006);
- the bicarbonate transporters that import HCO_3^- into the cells through Cl^-/HCO_3^- exchangers, playing an important role in cellular alkalinization, since the

imported HCO_3^- traps intracellular H^+ and thus maintains a permissive pHi that favors cell survival (Izumi et al., 2003; Karumanchi et al., 2001).

Another contribution to intracellular alkalinization is provided by a family of proteins, the carbonic anhydrases (CAs), which have extracellular catalytic domains that accelerate the hydration of extracellular CO_2 to HCO_3^- and H^+. In particular, expression of the membrane-associated CA IX and CA XII isoforms is substantially increased under hypoxic conditions in HIF-1-dependent manner (Chiche et al., 2009; Chiche et al., 2010; Ilie et al., 2010; Loncaster et al., 2001; Pastorekova et al., 2006; Swietach et al., 2009; Wykoff et al., 2000; Kaluz et al., 2009; Supuran, 2008).

Deregulation of pH confers adaptive advantages to cancer cells, allowing the achievement of specific features, such as growth-factor independent proliferation, evasion of apoptosis, metabolic adaptation, migration and invasion and thus facilitating the metastatic dissemination of tumor cells (Cardone et al., 2005; Gatenby et al., 2006; Stock and Schwab, 2009). For example, the polarity of migrating cells, the *de novo* assembly of actin filaments and the dynamics of integrin-ECM attachments are processes facilitated by high pHi. Moreover, the low pH of tumor extracellular microenvironment may promote the degradation and remodeling of ECM through the activation of proteolytic enzymes, including MMPs and tissue serine proteases (Busco et al., 2010; Rofstad et al., 2006). Of note, the activity of MMP3, is higher at acidic pH (Johnson et al., 2000) and the expression and secretion of MMP9 increases at lower pHe and higher pHi (Bourguignon et al., 2004; Putney and Barber, 2004). In addition, there is strong evidence indicating a pivotal role of lysosomal-like vesicles in the degradation of ECM, cell invasion and cell migration (Glunde et al., 2003; Montcourrier et al., 1994; Montcourrier et al., 1997). For example, in breast cancer, acidic pHe causes a significant redistribution of lysosomes from the perinuclear region to the cell periphery (Glunde et al., 2003). Lysosomes displacement to the cell periphery may be a mechanism to facilitate increased secretion of degradative enzymes (Glunde et al., 2003). In malignant tumor cells, vesicles containing cathepsin B also redistributed toward the cell periphery at acidic pHe and constitutive secretion of active cathepsin B allowed for the cleavage of secreted, latent MMPs into active enzymes (Giusti et al., 2008; Rozhin et al., 1994).

TUMOR METABOLISM

The uncontrolled cell proliferation, that represents the essence of neoplastic disease, involves not only deregulated control of cell growth but also corresponding adjustments of energy metabolism, in order to fuel anabolic pathway. One important characteristic of cancer cell metabolism is the consistent switch of the energy production pathway from oxidative phosphorylation to glycolysis (Kondoh et al., 2007) (Fig.8).

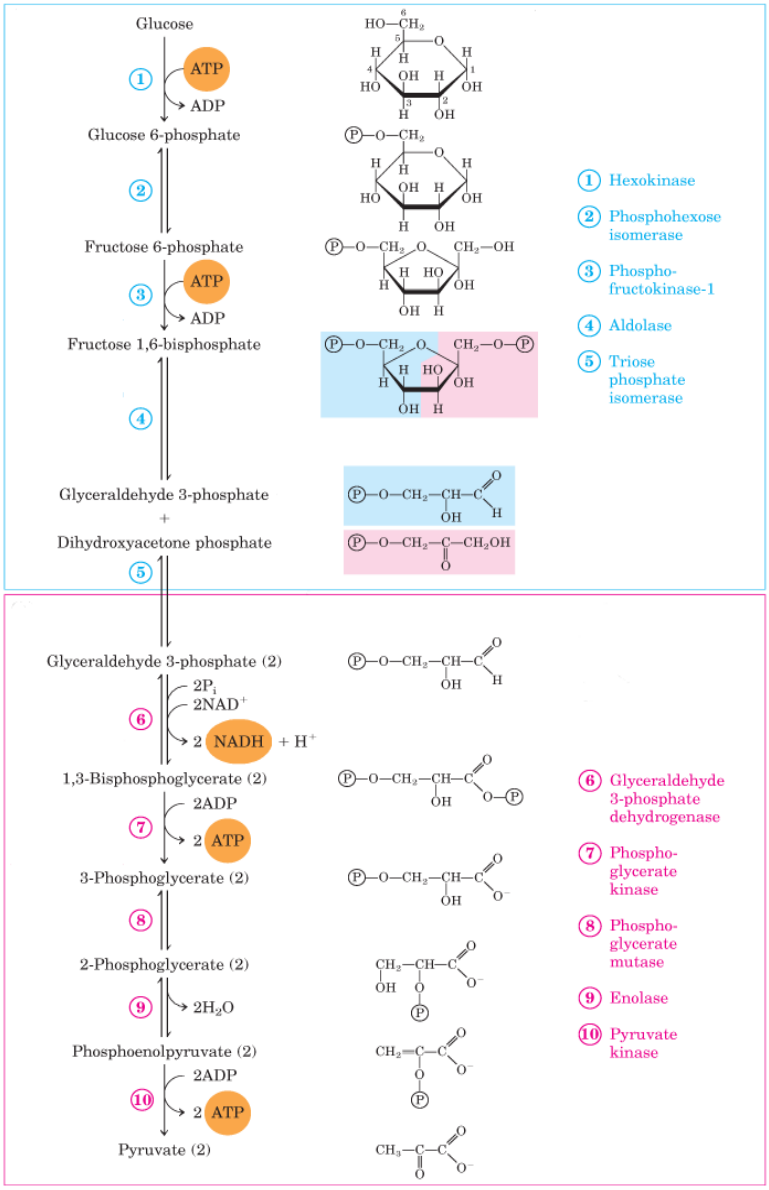

Fig. 8. Reactions of glycolysis.

In 1924, Otto Warburg observed that cancer cells consume much larger quantities of glucose than their normal counterparts and metabolize it predominantly through glycolysis, thus producing high levels of lactate even in oxygen-rich conditions (Warburg, 1956, Warburg et al., 1924). This process is called aerobic glycolysis, or "Warburg effect". In general, through the glycolysis process, one molecule of glucose generates two molecules of pyruvate and produces two molecules of ATP. In mammals, pyruvate has diverse fates. In an environment with limited oxygen, pyruvate is converted into lactate by lactate dehydrogenase (LDH), a process which is called anaerobic glycolysis. In the presence of oxygen, pyruvate is oxidized into acetyl-CoA by the pyruvate dehydrogenase (PDH) complex (Fig. 9).

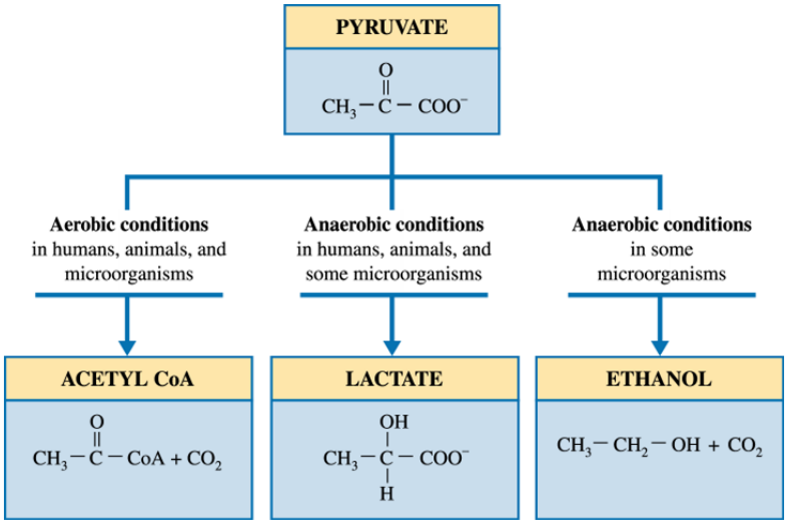

Fig. 9. Fates of pyruvate.

Acetyl-CoA is oxidized in the citric acid cycle (also known as Krebs cycle or trocarboxylic acid cycle) to generate electrons -in the form of NADH and $FADH_2$- and CO_2 in the mitochondria. The electron transport chain generates a pH gradient across the inner mitochondrial membrane. The force motive produced by the pH gradient generates ATP by ATP synthase. This process is called oxidative phosphorylation. In the latter, one molecule of glucose can be completed degraded into H_2O and CO_2 to produce 36 molecules of ATP. Overall, the oxidative phosphorylation process is more efficient in producing ATP than glycolysis. The reason why cancer cells prefer consuming more glucose by aerobic glycolysys to produce ATP is not well defined yet.

Further studies by Warburg itself hypothesized that the Warburg effect was mainly related to a mitochondrial deficiency developed by tumor cells, for example, through genetic mutations, thereby leading to a low energy delivery from mitochondrial respiration. Finally, aerobic glycolysis had to be the only possible metabolic mechanism that allowed cells to survive (Warburg, 1956). Anyway, subsequent studies demonstrated that mitochondrial deficiency was a rare condition in cancer cells (Weinhouse, 1956). Today, several authors highlight that the importance of aerobic glycolysis for tumor cells, as well as for proliferating cells in multicellular organisms, extends beyond ATP production, to allow nutrient assimilation into biosynthetic precursor and facilitate biomass accumulation (Lunt and Vander Heiden, 2011). Therefore, the main function of up-regulated glycolysis is to maintain the levels of glycolytic intermediates needed to support biosynthesis. Indeed, glucose provide the precursors for the chemical constituents (nucleotides, amino acids, lipids) that are used to build macromolecules essential for cell division. For example:

- glucose-6-phosphate can be oxidized into PPP in order to produce NADPH and ribose-5-phosphate, which in turn are used to synthesize nucleotides for DNA and RNA;
- dihydroxyacetone phosphate is the precursor to glycerol-3-phosphate, that is crucial for the biosynthesis of the phospholipids and triacylglycerols that serve as major structural lipids in cell membranes;
- 3-phosphoglycerate provides the carbons for cysteine, glycine and serine, whereas pyruvate provides the carbons for alanine.

Besides aerobic glycolysis, cancer cells exhibit substantial alterations in several metabolic pathway including tricarboxylic acid (TCA) cycle, glutaminolysis, mitochondrial respiratory chain and PPP. The metabolic changes can be attributed to the activation and/or malfunction of oncogenes, and/or loss of tumor suppressors (Chen and Russo, 2012) (Fig. 10).

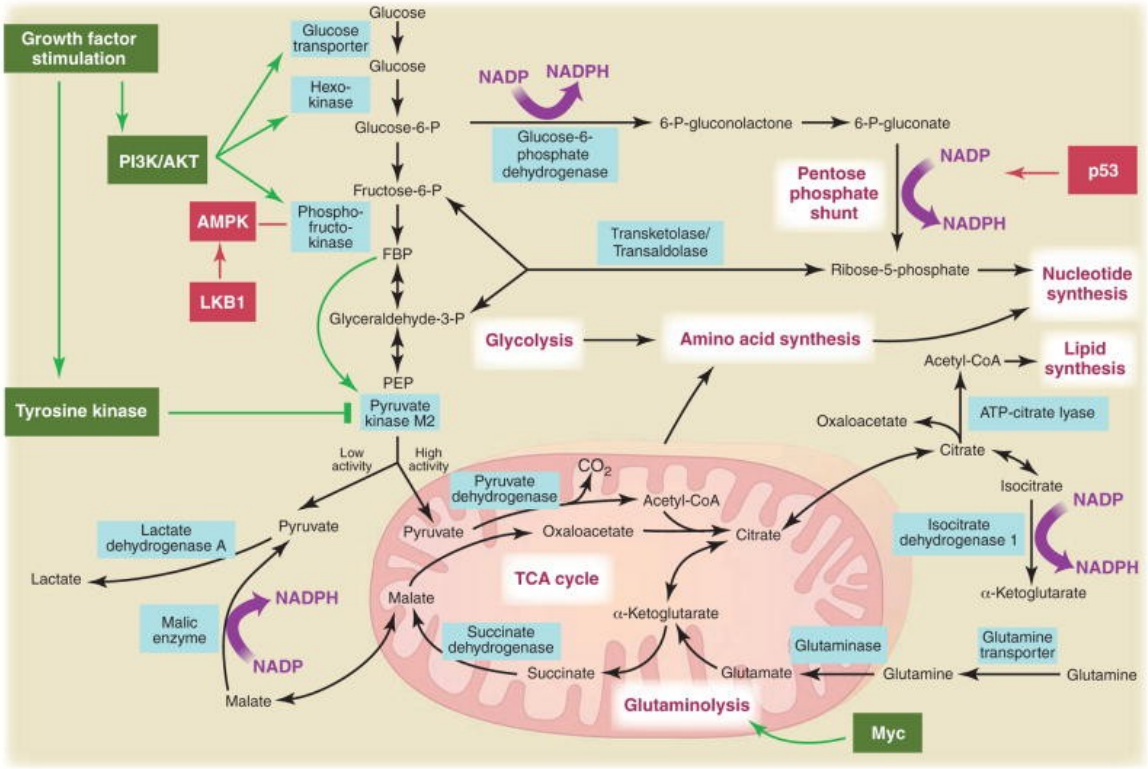

Fig. 10. Metabolic pathways active in proliferating and cancer cells are directly controlled by signaling pathways involving known oncogenes (green) and tumor suppressor genes (red).

Regulation of cancer cell metabolism: the Warburg effect

Aerobic glycolysis has been observed in a wide variety of tumors that originate from different cell types, but most normal cells in adult tissues from which cancer cells arise generally do not utilize this kind of metabolism. Thus, cancer cells revert to a metabolic phenotype that is characteristic of rapidly dividing cells, which suggests that aerobic glycolysis must provide advantages during proliferation.

GLUCOSE TRANSPORTERS AND GLYCOLYTIC ENZYMES

Glucose import into mammalian cells is facilitated primarily by five transmembrane transporters, GLUT1–5. Although GLUT2, GLUT4, and GLUT5 are found only in specific tissues, GLUT1 and GLUT3 are expressed in nearly all mammalian cells and have the lowest K_M values (1mM) of the five, which allow them to transport glucose

into the cell at a nearly constant rate from serum in which the glucose concentration ranges from 4mM to 8mM. Tumor cells frequently overexpress GLUT1 and GLUT3 and have an increased capacity for glucose uptake (Au et al., 1997; Suzuki et al., 1999; Yamamoto et al., 1990; Younes et al., 1996). Suppression of GLUT1 expression in a human gastric cancer cell line can decrease the number of cells in S phase and inhibit tumor growth (Noguchi et al., 2000).

Once in the cell, glucose must be converted to glucose-6-phosphate by hexokinase (HK) to prevent its transport out of the cell and to prime it for metabolism in subsequent reactions (Berg et al., 2007). Four mammalian HK isoforms (HKI–IV) are known, and HKI is expressed in most normal cells and at particularly high levels in brain tissue (Wilson, 2003). HKII expression is more limited and is normally found mainly in skeletal muscle and adipose tissue. However, cancer cells frequently overexpress HKII (Mathupala et al., 2006), and at least some glioblastoma cells are specifically dependent on HKII over other isoforms of the enzyme (Wolf et al., 2011). Both HKI and HKII are associated with the voltage-dependent anion channel (VDAC) on the cytosolic side of the outer mitochondrial membrane. This VDAC-HK association may inhibit mitochondria-induced apoptosis (Majewski et al., 2004) and give HK preferential access to mitochondria generated ATP (Arora and Pedersen, 1988). Why mitochondria-bound HKII appears to be selected for in cancer cells remains unclear.

Although HK traps glucose inside the cell, phosphofructokinase-1 (PFK1) controls its commitment to glycolysis and is therefore highly regulated. PFK1, which irreversibly converts fructose-6-phosphate to fructose-1,6-bisphosphate (FBP), is overexpressed in various human cancer cell lines (Vora et al., 1985). PFK1 is allosterically inhibited by high levels of ATP (Berg et al., 2007), and relieving this ATP inhibition is an important means to increase glucose metabolism in proliferating cells (Fang et al., 2010; Israelsen and Vander Heiden, 2010; Scholnick et al., 1973). PFK1 inhibition by ATP is diminished by fructose-2,6-bisphosphate, a metabolite synthesized from fructose-6-phosphate by PFK2. Regulation of PFK2 expression or activity has been proposed as an important way to couple growth signals with regulation of glucose metabolism in proliferating cells (Christofk et al., 2008; Marsin et al., 2000; Telang et al., 2006); moreover, PFK2 is expressed constitutively in several human cancer cell lines and is found to be required for tumor cell growth (Chen and Russo, 2012). In human tissues,

PFK1 subunit composition, a complex mixture of homotetramers or heterotetramers composed of up to three different subunits, can vary depending on tissue type. Each subunit (C, L, M) differs in sensitivity to allosteric effectors; thus, the kinetic and regulatory properties of PFK1 are determined by subunit composition (Dunaway et al., 1988). PFK1 subunits overexpressed in rat thyroid carcinomas and human gliomas are less sensitive to the allosteric inhibitors ATP and citrate (Meldolesi et al. 1976, Oskam et al. 1985, Staal et al. 1987). In addition to controlling glucose commitment to glycolysis, PFK1 may regulate the amounts of glucose-6-phosphate available for nucleotide biosynthesis (Lunt and Vander Heiden, 2011).

Among glycolytic enzymes, pyruvate kinase (PK) has received particular attention during the last few years. Indeed, recent findings have shown that many cancer cells exclusively express the M2 isoform of PK (Mazurek et al., 2005), and PKM2 expression is important for tumor growth (Christofk et al., 2008). PK catalyzes the reaction generating pyruvate and ATP from phosphoenolpyruvate (PEP) and ADP. Four isoforms of PK (L, R, M1, and M2) are present in mammals. The L and R isotypes are encoded by the PKLR gene. Their expression is tissue specific and is regulated by different promoters. The L isotype is expressed in the liver, kidney, and intestine, and the R isotype is expressed in red blood cells (Mazurek et al., 2005; Mazurek, 2011; Clower et al., 2010; Noguchi et al., 1987). PKM1 and PKM2 are encoded by the PKM gene and are the products of two mutually exclusive alternatively spliced exons (exon 9 and exon 10, respectively) (Clower et al., 2010; David et al., 2010; Noguchi et al., 1986): M1 is expressed in most adult differentiated tissues such as brain and muscle, whereas M2 is expressed in embryonic cells, adult stem cells, and cancer cells (Christofk et al., 2008, Mazurek et al., 2005; Mazurek, 2011; Bluemlein et al., 2011; Lee et al., 2008; Cairns et al., 2011). Splicing of PKM is controlled by the splicing repressors, heterogeneous nuclear ribonucleoprotein (hnRNP) A1 and A2, as well as polypyrimidine tract binding protein (PTB, also known as hnRNPI), and the expression of those repressors is up-regulated by c-Myc (Clower et al., 2010; David et al., 2010). These proteins bind to exon 9 and repress PKM1 mRNA splicing, resulting in the inclusion of exon 10 and thereby contributing to the high levels of PKM2 expression (Clower et al., 2010; David et al., 2010; Chen et al., 2012) (Fig. 11).

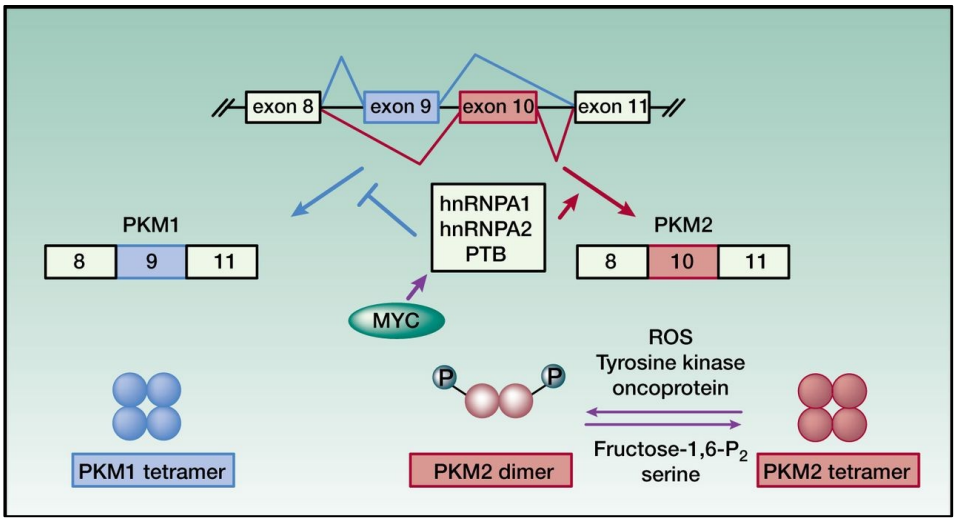

Fig. 11. PKM1 and PKM2 expression after alternative splicing.

A number of regulators of PKM2 expression have been reported (Clower et al., 2010; Mazurek, 2011; David et al., 2010; Chen et al., 2012; Luo et al., 2011; Lv et al., 2011; Panasyuk et al., 2012): in particular, a recent study showed that PKM2 expression is induced by activated mTOR (mammalian target of rapamycin), which transactivates HIF-1 and promotes the c-Myc-hnRNPs–mediated alternative splicing, leading to the aerobic glycolysis in tumor cells (Sun et al., 2011). The expression and lower glycolytic enzyme activity of PKM2 are necessary for the Warburg effect, which provides cancer cells with selective advantages, including tumor growth and suppression of ROS (Christofk et al., 2008; Vander Heiden and Cantley, 2009; Cairns et al., 2011; Anastasiou et al., 2011) for the following reasons. First is that the glycolytic pathway generates ATP more rapidly than the oxidative phosphorylation (Pfeiffer et al., 2011), allowing faster incorporation of carbon into its biomass (Vander Heiden and Cantley, 2009; Hamanaka and Chandel, 2012). Between yield and rate of ATP production, a trade-off has been reported to be present in sugar degradation by glycolysis and mitochondrial respiration. Then, glycolysis generates ATP at a high rate but low yield via massive consumption of glucose (Pfeiffer et al., 2011; Vazquez et al., 2010). The second reason is that lower activity of PKM2 facilitates the production of glycolytic intermediates to enter the glycolysis branch pathways, such as glycerol synthesis, amino acid syntesis and the pentose phosphate pathway, which generates NADPH to suppress ROS production and is also involved in nucleotide synthesis (Vander Heiden and

Cantley, 2009; Cairns et al., 2011; Hamanaka and Chandel, 2012; Jiang et al., 2010; Boxer et al., 2010). In other words, the increase in glycolysis induced by the lower activity of PKM2 can supply cancer cells with varied resources of substrates necessary for their rapid proliferation (Fig 12).

PKM2 exists as either a low-activity dimeric or high-activity tetrameric form, whereas PKM1 constantly exists as a high-activity tetrameric form (Mazurek, 2011; Mazurek et al., 2005; Dang, 2009). Cancer cells predominantly express the low-activity dimeric form of PKM2 (Christofk et al., 2008; Christofk et al., 2008a; Hitosugi et al., 2009). Christofk and colleagues (Christofk et al., 2008) and Vander Heiden and colleagues (Vander Heiden et al., 2010) reported that PKM1-expressing cells showed much higher PK activity than PKM2-expressing cells; these cells consumed more oxygen, produced less lactate, and were highly sensitive to the mitochondrial ATP synthesis inhibitor, oligomycin (Christofk et al., 2008). In addition, Hitosugi and colleagues reported that tyrosine phosphorylation (Tyr 105) of PKM2 disrupts the active tetrameric form of PKM2, leading to the suppression of its activity. Furthermore, PKM2-mutated cells, in which tyrosine residue 105 is replaced with a phenylalanine, had increased PK activity as observed in PKM1-expressing cells (Hitosugi et al., 2009). Therefore, the low activity of dimeric PKM2 is a very important driver for glycolysis. In contrast, the high activity of PKM2 and PKM1 tetramers drives the TCA cycle (Christofk et al., 2008; Christofk et al., 2008a; Hitosugi et al., 2009) (Fig. 11, Fig. 12).

Various factors have been reported to control the switch between the dimeric and tetrameric forms of PKM2 (Fig. 11, Fig. 12). For example, fructose-1,6-bisphosphate binds allosterically to PKM2 and facilitates the formation of the active tetramer (Tamada et al., 2012). Serine is also a positive regulator of PKM2 (Mazurek, 2011; Ward and Thompson, 2012; Ashizawa et al., 1991; Eigenbrodt et al., 1983; Ye et al., 2012). In contrast, tyrosine phosphorylation of PKM2 induces the release of fructose-1,6-bisphosphate, which causes PKM2 to convert from tetrameric form to less active dimeric form (Christofk et al., 2008a; Hitosugi et al., 2009). In addition, oncoproteins such as HPV-16 E7 and activated pp60v-src kinase dissociate the tetrameric form to yield the dimeric form (Mazurek et al., 2002). Furthermore, recent studies show that oxidative stress causes dissociation of the tetramer and a subsequent reduction in PKM2 activity (Anastasiou et al., 2011), and that acetylation of lysine residue within PKM2

suppresses its catalytic activity and induces the degradation by chaperone-mediated autophagy (Lv et al., 2011). In addition, it has been reported that mucin 1 phosphorylated by EGF receptor (EGFR) interacts with PKM2 and suppresses its activity (Kosugi et al., 2011). As previously described, PKM2 activity is inhibited by oxidative stress as well as tyrosine phosphorylation (Anastasiou et al., 2011). Oxidative stress induces the oxidization of Cys358 within PKM2, which promotes glycolysis and PPP flux, leading to the production of glutathione (GSH) and consequent ROS depletion (Anastasiou et al., 2011; Gruning and Ralser, 2011; Hamanaka and Chandel, 2011). Thus, cancer cells have multiple mechanisms for avoiding ROS accumulation, which gives them a survival advantage in terms of tumor growth and therapeutic resistance (Anastasiou et al., 2011; Tamada et al., 2012; Ishimoto et al., 2011).

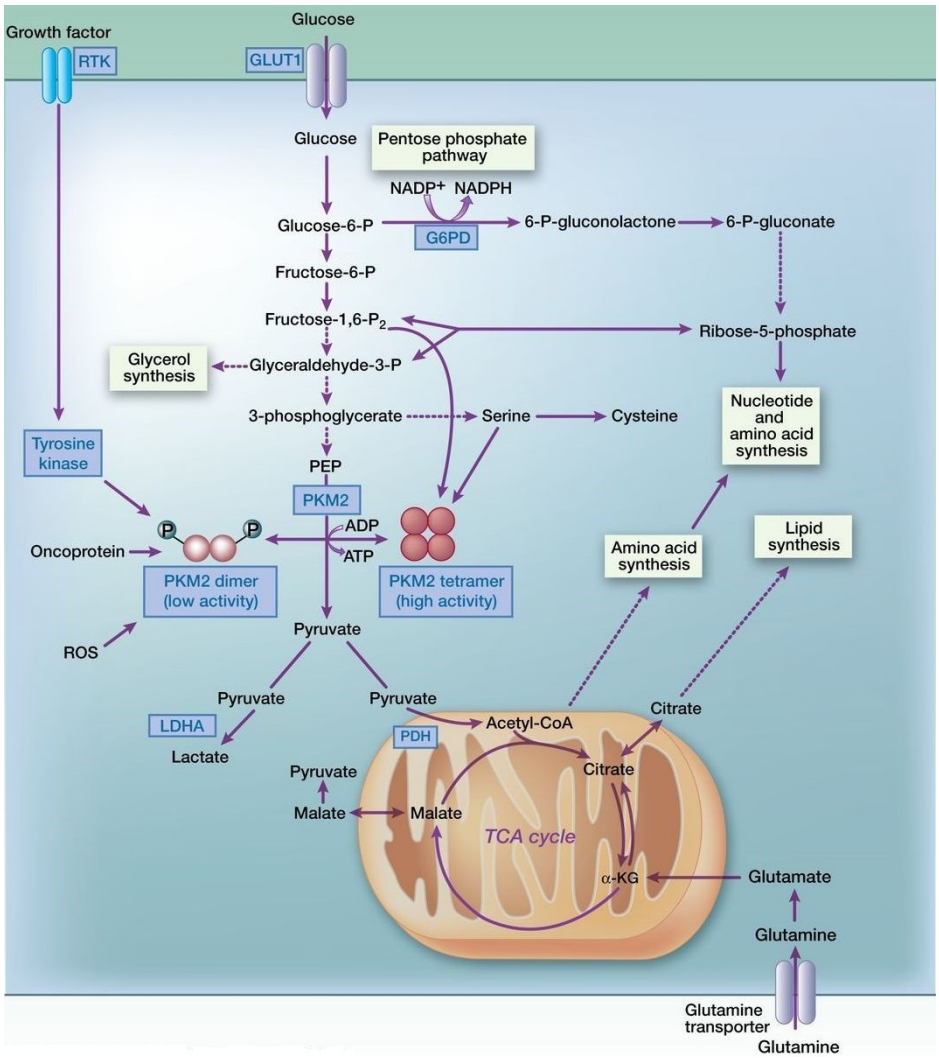

Fig. 12. Metabolic pathway regulated by PKM2 in cancer cells.

Finally, an increasing number of reports document the non-glycolytic functions of dimeric PKM2. In particular, the role of PKM2 in transcription is attracting attention. It has been reported that PKM2 interacts directly with the HIF-1 subunit and promotes transactivation of HIF-1 target genes (Luo et al., 2011). As HIF-1 also activates the transcription of the genes encoding PKM2, cancer cells may have the positive feedback loop between PKM2 and HIF-1, which contributes to the characteristic metabolism in cancer cells.

Nuclear PKM2 has been shown to activate gene transcriptions and cell proliferation (Lee et al., 2008; Hoshino et al., 2007; Gao et al., 2012; Luo et al., 2011; Yang et al., 2011; Ignacak and Stachurska, 2003). Translocation of PKM2 into the nucleus induced by EGFR activation was reported to promote β-catenin transactivation, leading to expression of cyclinD1 and c-Myc (Yang et al., 2011). Given that c-Myc upregulates transcription of hnRNPs contributing to the high PKM2/PKM1 ratio (Clower et al., 2010; David et al., 2010), and that c-Myc promotes glycolysis by driving the expression of GLUT1 and LDHA (Munoz-Pinedo et al., 2012; Dang et al., 2009), the events induced by the translocation of PKM2 into the nucleus may be connected with a feed-forward loop to drive glycolysis.

MOLECULAR PATHWAYS IN AEROBIC GLYCOLYSIS

The metabolic changes induced by cell growth signals are largely conserved between normal and cancer cells; however, cancer cells activate signaling pathways in the absence of normal extracellular stimuli, thus promoting a metabolic phenotype that allows inappropriate cell proliferation (Fig. 10, Fig. 13).

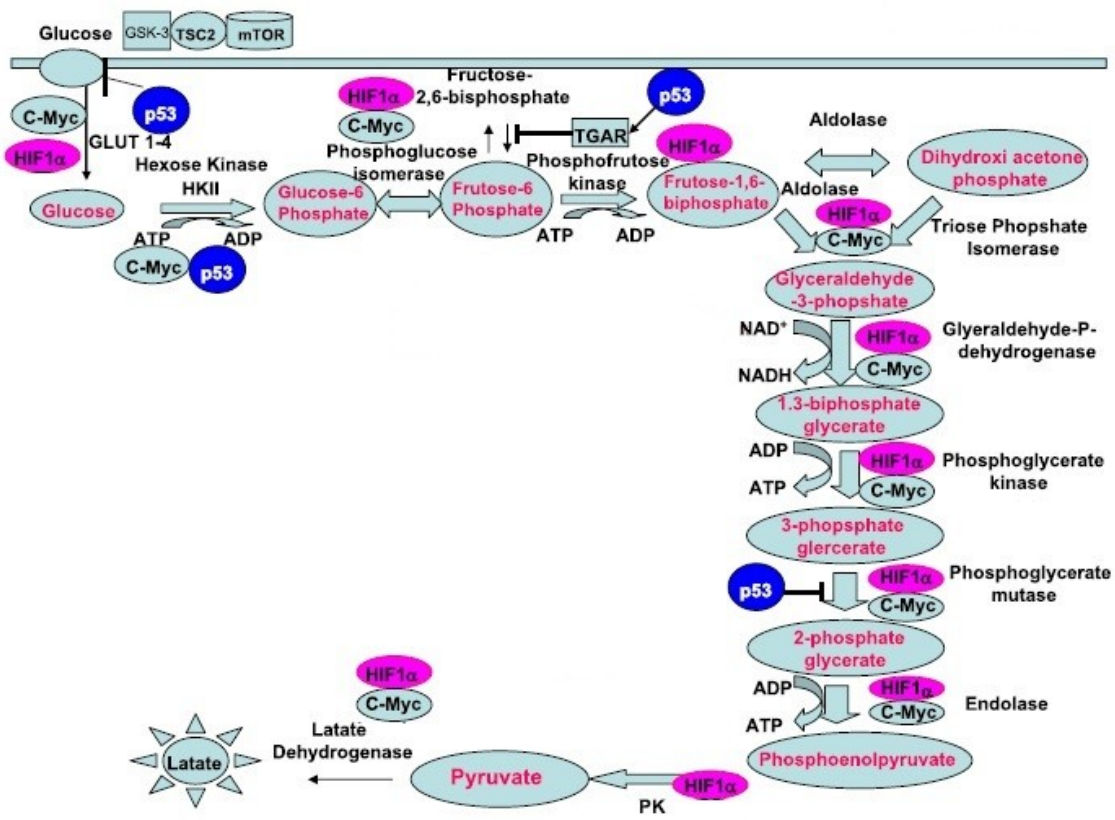

Fig. 13. Regulation of glucose transporters and glycolysis by c-Myc, HIF-1α and p53.

A major regulator of glucose metabolism is the phosphoinositide 3-kinase (PI3K) signaling pathway. PI3K signaling through the protein kinases AKT and mTOR can increase uptake of glucose by increasing expression of the glucose transporter GLUT1 (Barthel et al., 1999; Frauwirth et al., 2002; Vander Heiden et al., 2001) and maintaining GLUT1 levels on the cell surface by preventing internalization (Wieman et al., 2007) (Fig. 13). AKT activation enhances flux through glycolysis (Elstrom et al. 2004) in part by maintaining HK association with mitochondria (Gottlob et al., 2001) and PFK2 through phosphorylation, which generates the allosteric activator of PFK1 fructose-2,6-bisphosphate (Deprez et al., 1997). In normal cells, the PI3K pathway is tightly controlled to increase glucose uptake and metabolism in response to growth signals (Cantley, 2002). However, in cancer cells, various mutations activate PI3K in the absence of growth signals, which suggests that inappropriate activation of this pathway may be a major driver of aerobic glycolysis in cancer cells (DeBerardinis et al., 2008).

Rapidly proliferating cells require close proximity to blood vessels for access to oxygen and nutrients. As tumors grow, cells may encounter hypoxic conditions that lead to induction of HIF-1 transcription factor. HIF-1 increases the expression of VEGF to facilitate the growth of new blood vessels. HIF-1 also increases the transcription of glucose transporters (GLUT1, GLUT3), many glycolytic enzymes, and LDHA (O'Rourke et al., 1996; Semenza et al., 1994) (Fig. 13). During periods of fast growth and rapid biomass synthesis, such as embryogenesis and tumorigenesis, local hypoxic conditions may arise and stimulate HIF-1 to enhance glycolytic gene expression. HIF-1 is required for embryogenesis, as mice homozygous for a loss-of-function mutation in HIF-1α or HIF-1β die at mid gestation (Iyer et al., 1998; Maltepe et al., 1997). Loss of HIF-1α in cancer cells also dramatically slows their growth as xenograft tumors in nude mice (Jiang et al., 1997; Maxwell et al., 1997; Ryan et al., 1998). Even under normoxic conditions, HIF-1α can be induced by the glycolytic metabolites pyruvate and lactate (McFate et al., 2008), mTOR activation, NAD^+ levels, reactive oxygen species, nitric oxide, many TCA cycle metabolites (Semenza, 2010b), and oncogene gain of function or tumor suppressor gene loss of function (Semenza, 2010a). Of note, recent findings have highlighted the role of mitochondrial NAD-dependent deacetylase SIRT3 in destabilizing HIF-1α. Indeed, SIRT3-loss increases ROS production, leading to HIF-1α stabilization. SIRT3 expression is reduced in human breast cancers and several other malignancies and its loss correlates with the up-regulation of HIF-1α target genes. Loss of SIRT3 results in aberrant mitochondrial metabolism and genomic instability, while its over-expression represses glycolysis and proliferation in breast cancer cells, providing a metabolic mechanism for tumor suppression (Kim et al., 2010, Finley et al., 2011). Moreover, knocking down SIRT3 increases tumorigenesis in mouse xenograft models, and this is abolished by the antioxidant N-acetyl cysteine (NAC) (Bell et al., 2011) (Fig. 14).

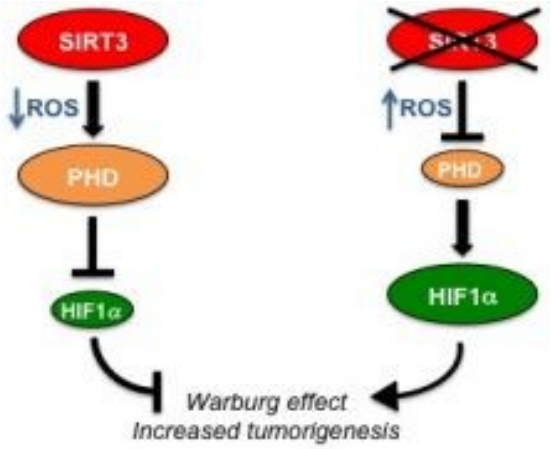

Fig. 14. Regulation of HIF-1α stabilization by SIRT3.

Activation of the PI3K pathway appears to be an important way to increase HIF-1 transcription in cancer (DeBerardinis et al., 2008; Majumder and Sellers, 2005). In addition to increasing the expression of glycolytic enzymes, increased HIF-1 activity promotes aerobic glycolysis by upregulating the expression of pyruvate dehydrogenase kinase (PDK). HIF-1-induced PDK activity inhibits PDH, which converts pyruvate to acetyl-CoA (Kim et al., 2006; Papandreou et al., 2006). Inhibition of PDH activity decreases pyruvate flux into the TCA cycle, which promotes pyruvate conversion to lactate. Thus, activation of HIF resulting from hypoxia, PI3K activation, or other mechanisms can promote glucose metabolism by aerobic glycolysis.

The transcription factor c-Myc promotes expression of glucose transporters and glycolytic enzymes (Ahuja et al., 2010; Osthus et al., 2000) (Fig. 13). c-Myc regulates the expression of LDHA (Shim et al. 1997), and c-Myc-dependent tumors cannot proliferate when LDHA expression is knocked down (Le et al., 2010). Furthermore, c-Myc regulates enzymes in the nucleotide biosynthesis pathway, including thymidylate synthase, inosine monophosphate dehydrogenase 1 and 2, and phosphoribosyl pyrophosphate synthetase-2 (Tong et al., 2009b). Additional transcription factors related to c-Myc, ChREBP and MondoA, control glycolytic enzyme expression and can promote anabolic metabolism in some contexts (Sloan and Ayer, 2010; Tong et al., 2009a). Ras, another oncogene widely implicated in human cancer, also promotes glucose metabolism by enhancing glucose uptake (Yun et al., 2009). Mutation of p53 is another common genetic event in human cancer, and the p53 protein prevents tumor growth by suppressing metabolic pathways conducive to proliferation of stressed or

damaged cells. p53 promotes mitochondrial respiration through expression of SCO2 protein (Synthesis of Cytochrome c Oxidase), while inhibiting glycolysis by repressing glucose transporters, inhibiting the glycolytic enzyme phosphoglycerate mutase and decreasing the activity of PFK1 through TIGAR, that functions to lower fructose-2,6-bisphosphate levels (Cheung and Vousden, 2010; Levine and Puzio-Kuter, 2010). Thus, glycolysis can be promote by loss of p53 function in cancer (Fig. 13).

Regulation of cancer cell metabolism: the TCA cycle

TCA cycle (also known as citric acid cycle or the Krebs cycle) (Fig. 15) is a series of chemical reactions used by all aerobic living organism to generate energy through the oxidization of pyruvate derived from carbohydrates, lipids and proteins into carbon dioxide and water. In addition, TCA cycle provides precursors for the biosynthesis of compounds including certain amino acids as well as the reducing agent NADH that is used in oxidative phosphorilation (Chen and Russo, 2012).

It has been shown that a number mutations in genes that encode enzymes including aconitase, isocitrate dehydrogenase 1-2 (IDH1, IDH2), succinate dehydrogenase (SDH) and fumarate hydratase (FH) in TCA cycle lead to some types of cancer (Briere et al, 2006).

ACONITASE

Aconitase is a mitochondrial enzyme that catalyzes the stereospecific isomerization of citrate to isocitrate via cis-aconitate in the TCA cycle (Lauble et al., 1992; Lauble et al., 1994). Aconitase is regarded as the key enzyme in citrate oxidation in human prostate epithelial cells, a unique organ that produces and releases large amounts of citrate. The abnormal expression and activity of aconitase have been implicated in tumorigenesis of the prostate (Mycielska et al., 2006). It has been observed that the mRNA levels of mitochondrial and cytosolic aconitases and aconitase activity, as well as fatty acid synthase, are significantly higher in metastatic PC3 cells than in normal human prostate cells (PNT2-C2) (Mycielska et al., 2006). Using immunohistochemical analysis of prostate cancer tissue sections and malignant prostate cell lines, Singh et al. (Singh et

al., 2006) detected the presence of mitochondrial-aconitase (m-aconitase) in the mitochondrial compartment in PC3, LNCaP, and DU-145 malignant prostate cell lines and prostate tissue sections from prostate cancer subjects where mitochondrial aconitase enzyme is present in the glandular epithelium of normal glands, hyperplastic glands, adenocarcinomatous glands, and prostatic intraepithelial neoplastic foci. Moreover, Tsui et al. (Tsui et al., 2011) observed that p53 down-regulated the gene expression of m-aconitase in human prostate carcinoma cells (Fig. 15).

ISOCITRATE DEHYDROGENASE

IDH1 converts isocitrate to α-ketoglutarate with generation of NADH. Up to 12% of glioblastoma tumors have spontaneous point mutations in IDH1 genes (Parsons et al., 2008). Mutations that affected IDH1 have also been identified in grades II and III astrocytomas, oligodendrogliomas and glioblastomas that developed from these lower-grade lesions (Yan et al., 2009). Similar mutations in IDH2 at residue Arg172 in the active site have been identified in acute myeloid leukemia (Ward et al., 2010) and other diseases (Kranendijk et al., 2010). These mutations disable the enzyme's normal ability to convert isocitrate to α-ketoglutarate and confer on the enzymes a new function, i.e. the ability to convert α-ketoglutarate to d-2-hydroxyglutarate (D-2-HG) (Kranendijk et al., 2010; Dang et al., 2010). The elevated levels of 2HG *in vivo* are thought to contribute to the formation and malignant progression of gliomas (Dang et al., 2010).

SUCCINATE DEHYDROGENASE

SDH, an enzyme complex consisting of four subunits A, B, C and D, catalyzes the oxidation of succinate to fumarate with the reduction of ubiquinone to ubiquinol. SDH is bound to the inner membrane of mammalian mitochondria. It is the only enzyme that participates in both the TCA cycle and the electron transport chain (Oyedotun and Lemire, 2004). Inherited or somatic mutations in subunits B, C, or D of SDH have been associated with several types of cancers including pheochromocytoma, paraganglioma, renal cell carcinoma and papillary thyroid cancers (Baysal, 2003; Neumann et al., 2004; Pollard et al., 2003; Eng et al., 2003; Rustin and Rotig, 2002). Reduced expression and loss of heterozygosity of SDH gene are observed in gastric and colon carcinoma (Habano et al., 2003). The R22X mutation of SDH gene in hereditary paraganglioma

abolishes the enzymatic activity of complex II of mitochondrial respiratory chain and activates HIF-1α, leading to increased expression of HIF-1α-regulated genes (Gimenez-Roqueplo et al., 2001, 2003, 2002). Selak et al. (Selak et al., 2005) have shown that succinate inhibits PHDs, leading to HIF-1α stabilization under normoxic conditions. Thus, succinate can increase expression of genes that facilitate angiogenesis, metastasis, and glycolysis, ultimately leading to tumor progression.

FUMARATE HYDRATASE

FH catalyzes the reversible hydration/dehydration of fumarate to malate. Mutations in the FH gene have been identified in chromosome 1q42.3-43 mapped to the genetic locus for multiple cutaneous and uterine leiomyoma syndrome (MCL), which is inherited in an autosomal dominant pattern (Tomlinson et al., 2002). Germline mutations in the FH gene predispose to multiple MCL and MCL-associated renal cell cancer.

Reduced FH expression has been seen in clear cell renal cancer, the most common histologic variant of kidney cancer, leading to the accumulation of HIF-2α, which is known to promote renal carcinogenesis, migration and invasion whereas over-expression of FH in renal cancer cells inhibits cellular migration and invasion (Sudarshan et al., 2011). These data provide novel insights into the tumor suppressor functions of FH in sporadic kidney cancer.

FH-deficient cells and tissues accumulate high levels of fumarate, which may act as an oncometabolite and contribute to tumorigenesis. Fumarate has been proposed to have a role in the covalent modification of cysteine residues to S-(2-succinyl) cysteine (2SC) (termed protein succination). Bardella et al. (Bardella et al., 2005) assessed 2SC levels in the models of hereditary leiomyomatosis and renal cell cancer (HLRCC) syndrome. They observed robust detection of 2SC in Fh1 (murine FH)-deficient renal cysts and in a retrospective series of HLRCC tumors with established FH mutations. Importantly, 2SC was undetectable in normal tissues and tumor types not associated with HLRCC. In another study, Sullivan et al. showed that accumulated fumarate directly binds the antioxidant glutathione *in vitro* and *in vivo* to produce the metabolite succinated glutathione (GSF). GSF acts as an alternative substrate to glutathione reductase to decrease NADPH levels and enhance mitochondrial ROS and HIF-1 activation

(Sullivan et al., 2013). Increased ROS also correlates with hypermethylation of histones in these cells. Thus, fumarate serves as a proto-oncometabolite by binding to glutathione which results in the accumulation of ROS.

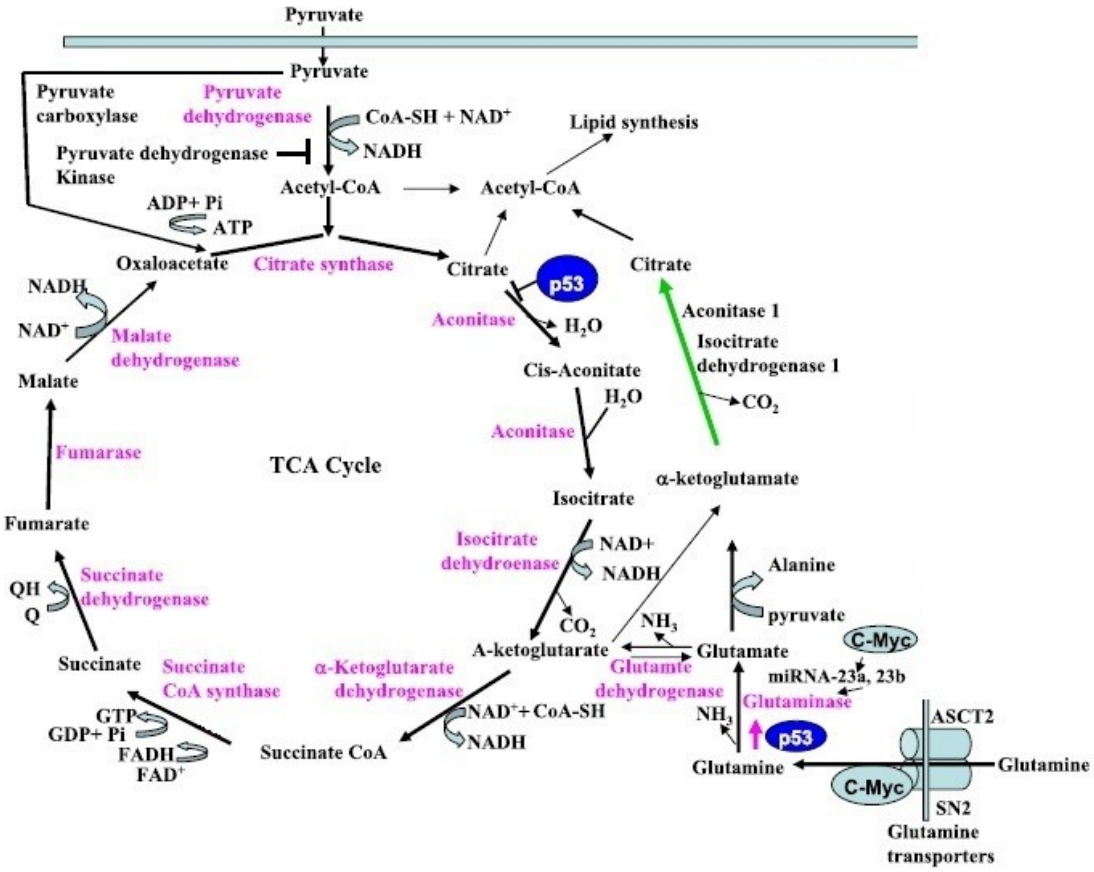

Fig. 15. Regulation of TCA cycle and glutaminolysis by c-Myc and p53.

Regulation of cancer cell metabolism: glutaminolysis

Glutamine is the most abundant free amino acid in the circulation and in intracellular pools. It is not only a precursor for the synthesis of amino acids, proteins, nucleotides, and a number of biologically important molecules, but also play a regulatory role in several cell specific processes including metabolism (e.g. oxidative fuel, gluconeogenic precursor, and lipogenic precursor), cell integrity (apoptosis and cell proliferation), protein synthesis and degradation, redox potential, respiratory burst, insulin resistance, insulin secretion and extracellular matrix synthesis. Glutamine is also an essential

nutrient for cancer cell proliferation (Smith, 1990; Newsholme et al., 2003; Curi et al., 2005; Cassago et al., 2012).

Glutaminolysis (Fig. 15) is a series of biochemical reactions by which glutamine is degraded to glutamate, aspartate, CO_2, pyruvate, lactate, alanine and citrate. Glutamine is imported into cells through high affinity surface glutamine importers such as ASCT2 and SN2 (Wise et al., 2008). Once it enters the cells, the majority of glutamine either donates nitrogen to macromolecules or is deamidated by glutaminases (GLS), which converts glutamine to glutamate (Glu). Glu has several fates. It can be converted directly to glutathione (GSH) by glutathione cysteine ligase (GCL). The reduced GSH, one of the most abundant anti-oxidants present in mammalian cells, is vital to controlling the redox state of the subcellular compartments (Wu et al., 2004). Glu can also be catabolized through removal of α-nitrogen by aminotransferases or glutamate dehydrogenase (GDH) producing α-ketoglutarate. The latter can be oxidized through the TCA cycle for the production of ATP or serves as substrate for biosynthesis of the polyglutamated folic acid or processed further in mitochondria (Fig. 15). The cyclization of glutamate produces proline, an amino acid important for synthesis of collagen and connective tissue. Alternatively, some tissues e.g. rat liver and brown adipocyte cell lines can reductively carboxylate α-ketoglutarate to generate citrate (Des Rosiers et al., 1995; Yoo et al., 2008). There is evidence indicating that a fraction glutamine-derived carbon can exit the TCA cycle as malate and serve as substrate of malic enzymes 1, which produces NADPH (DeBerardinis et al., 2007). More recently, it has been demonstrated (Metallo et al., 2012) that human cells use reductive metabolism of α-ketogluatare to synthesize acetyl-CoA for lipid synthesis (Fig.15). This IDH1-dependent pathway is active in most cell lines under normal culture conditions, but cells grown under hypoxia rely almost exclusively on the reductive carboxylation of glutamine-derived α-ketoglutarate for *de novo* lipogenesis. Furthermore, renal cell lines deficient in the von Hippel–Lindau tumor suppressor protein preferentially use reductive glutamine metabolism for lipid biosynthesis even at normal oxygen levels.

In order to compensate for Warburg effect and to help maintain a functioning TCA cycle, cancer cells often rely on elevated glutamine metabolism through a marked elevation of glutaminase activity (Erickson and Cerione, 2010). Several studies (Wise et al., 2008; Gao et al., 2009; Le et al., 2012) have revealed that c-Myc plays a major role

in regulating glutaminolysis. Le et al. (Le et al., 2012) investigated the metabolic responses of a c-Myc inducible human Burkitt lymphoma model P493 cell line to aerobic and hypoxic conditions, and to glucose deprivation, using radiolabeled glucose as the tracer isotope for resolving metabolomics. They observed that glutamine import and metabolism through the TCA cycle persisted under hypoxia and that glutamine contributed significantly to citrate carbons. Under glucose deprivation, glutamine-derived fumarate, malate, and citrate were significantly increased, thus demonstrating an alternative energy-generating glutaminolysis pathway involving a glucose-independent TCA cycle.

There are three isoforms of glutaminases, GLS1, GLS2 and GLSC (a splice variant of GLS1) (Cassago et al., 2012). GLS1 is required for cell cycle progression through S phase (Colombo et al., 2011). GLS2 gene encodes a mitochondrial glutaminase, a key enzyme that catalyzes the hydrolysis of glutamine to glutamate and thereby a regulator of GSH synthesis and energy production (Perez-Gomez et al., 2003; Campos-Sandoval et al., 2007). It has been shown that glutamine importers and GLS1 expression are up-regulated by c-Myc (Wise et al., 2008; Gao et al., 2009; Wise et al., 2010) and that GLS2 expression is up-regulated by p53 (Hu et al., 2010; Suzuki et al., 2010; Rajagopalan and DeBerardinis, 2011) (Fig. 15).

Wise et al. (Wise et al., 2008) reported that c-Myc activates the transcription of glutamine importers ASCT2 and SN2 by selectively binding to the promoter regions of both genes that are required for glutamine uptake and metabolism (Fig. 15). A consequence of this c-Myc-dependent glutaminolysis is the reprogramming of mitochondrial metabolism to depend on glutamine catabolism to sustain cellular viability and TCA cycle anaplerosis. The ability of c-Myc-expressing cells to engage in glutaminolysis does not depend on concomitant activation of PI3K or AKT. The stimulation of mitochondrial glutamine metabolism resulted in reduced glucose carbon entering the TCA cycle and a decreased contribution of glucose to the mitochondrial-dependent synthesis of phospholipids. Gao et al. (Gao et al., 2009) reported that the c-Myc up-regulates the expression of mitochondrial GLS1 in human P-493 B lymphoma cells and PC3 prostate cancer cells through repressing microRNAs miR-23a and miR-23b that target the GLS's 3′ untranslating regions and that glutamine and glutaminase are necessary for Myc-mediated cancer cell proliferation and survival. Together, these

studies suggest that oncogenic levels of c-Myc induce a transcriptional program that promotes glutaminolysis and triggers cellular addiction to glutamine as a bioenergetic substrate.

On the other hand, we have to mention that GLS2 is a p53-target gene and contributes to its role in tumor suppression. GLS2 regulates cellular energy metabolism by increasing production of glutamate and α-ketoglutarate, which in turn results in enhanced mitochondrial respiration and ATP generation (Fig. 15). Furthermore, GLS2 also regulates antioxidant defense function in cells by increasing GSH levels and decreasing ROS levels, which, in turn, protects cells from oxidative stress-induced apoptosis. It has been shown that GLS2 expression induced by p53 under both non-stressed and stressed conditions leading to:

- increased levels of glutamate and α-ketoglutarate, mitochondrial respiration rate, and GSH levels
- decreased ROS levels in cells, resulting in an overall decrease in DNA oxidation (Hu et al., 2010; Suzuki et al., 2010; Eto et al., 1994).

In agreement with this data, the expression of GLS2 is loss in human liver tumors whereas GLS2 over-expression reduces tumor cell colony formation abilities.

Deregulated tumor cell metabolism provides an opportunity to selectively target this disease and to improve cancer therapy. Therefore, a number of small molecules targeting transporters, enzymes and proteins involved in metabolic deregulation of tumor cells have been developed and some of these are in pre-clinical or clinical studies (Zhao et al., 2013; Ramsay et al., 2011).

Role of tumor microenvironment in regulation of cancer cell metabolism

Several studies during the last years has demonstrated that tumor microenvironment modifies cancer cell metabolism. As previously reported, hypoxia induces a glycolytic switch in tumor metabolism as a result of HIF activation, in order to circumvent lack of oxygen. However, in solid tumors, tumor mass is composed by normoxic regions, closed to blood vessels, and hypoxic regions, as a results of disorganized vasculature. In

this regard, metabolic coupling was demonstrated between well-oxygenated and hypoxic cancer cells, whereby lactate produced by glycolysis of hypoxic tumor cells fuels mitochondrial oxidative phosphorylation in well-oxygenated tumor cells (Sonveaux et al., 2008). Thus, lactate produced by glycolytic tumor cells within hypoxic regions is used as an oxidative substrate by other tumor cells in which the oxygen supply is higher (Fig. 16). Moreover, in this way, normoxic subpopulation avoids extracellular glucose depletion, as glucose is metabolized through glycolysis by hypoxic cancer cells.

Lactate used for mitochondrial metabolism gives several advantages to cancer cells:

- pyruvate oxidation to lactate by LDH leads to production of reducing power that prevents harmful intracellular oxidative stress, thereby favouring cells survival (Lee et al., 2003; Pelicano et al., 2006);
- lactate oxidation do not requires ATP consumption;
- each molecule of lactate generates 18 ATP molecules, thereby allowing cells to save energy for glycolytic enzymes activity.

In this model, lactate up-take in tumor cells is mediated by monocarboxylate transporter-1 (MCT1) (Fig. 16). In agreement, inhibition of MCT-1 with α-cyano-4-hydroxycinnamate (CHC) or siRNA induces a switch from lactate-fueled respiration to glycolysis. A similar switch in oxygenated tumor cells in both a mouse model of lung carcinoma and xenotransplanted human colorectal adenocarcinoma cells is observed after administration of CHC. MCT1 inhibition delays tumor growth, induces tumor core necrosis, and decreases tumor hypoxia (Sonveaux et al., 2008).

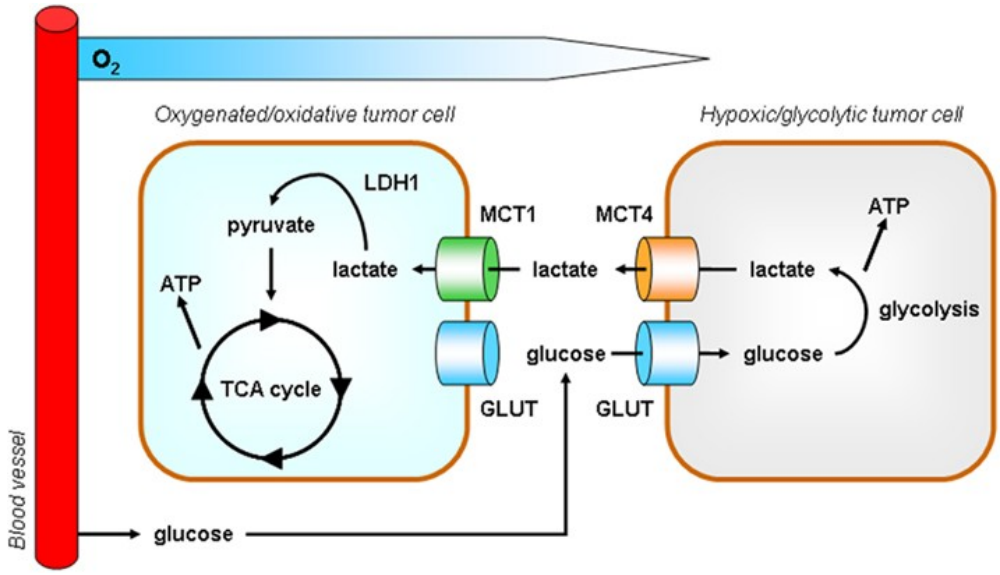

Fig. 16. Model of metabolic symbiosis between hypoxic and respiring cells.

Besides hypoxia, stromal cells are emerging as important players in metabolic reprogramming of cancer cells (Koukourakis et al., 2006). In this regard, Lisanti and colleagues have developed a model of metabolic coupling between tumor stroma and cancer cells (Fig. 17). In this model, loss of caveolin-1 (Cav-1) causes the metabolic reprogramming of stromal cells to support the growth of adjacent epithelial tumor cells. Caveolins are a family of scaffolding proteins that function in endocytosis, signal transduction, and cholesterol transport (Okamoto et al., 1998). Interestingly, loss of Cav-1 in fibroblasts is sufficient to induce a CAF phenotype (upregulation of α-SMA, calponin, vimentin, increased deposition of MMP9 and ECM components, and hyperactivation of the TGF-β signaling pathway) (Sotgia et al., 2009; Martinez-Outschoorn et al., 2010a; Martinez-Outschoorn et al., 2010b). Witkiewicz et al. have identified a loss of stromal Cav-1 as single independent predictor of clinical outcome in human breast cancer patients. More specifically, loss of stromal Cav-1 (in cancer associated fibroblasts) strictly correlates with early tumor recurrence, lymphonode metastasis, increased tumor stage, tamoxifen-resistance, and overall poor clinical outcome (Witkiewicz et al., 2009; Sloan et al., 2009). In agreement, loss of stromal Cav-1 in prostate cancer patients correlate with advanced prostate cancer and the presence of metastatic disease, as well as high Gleason score, another indicator of poor prognosis (Di Vizio et al., 2009).

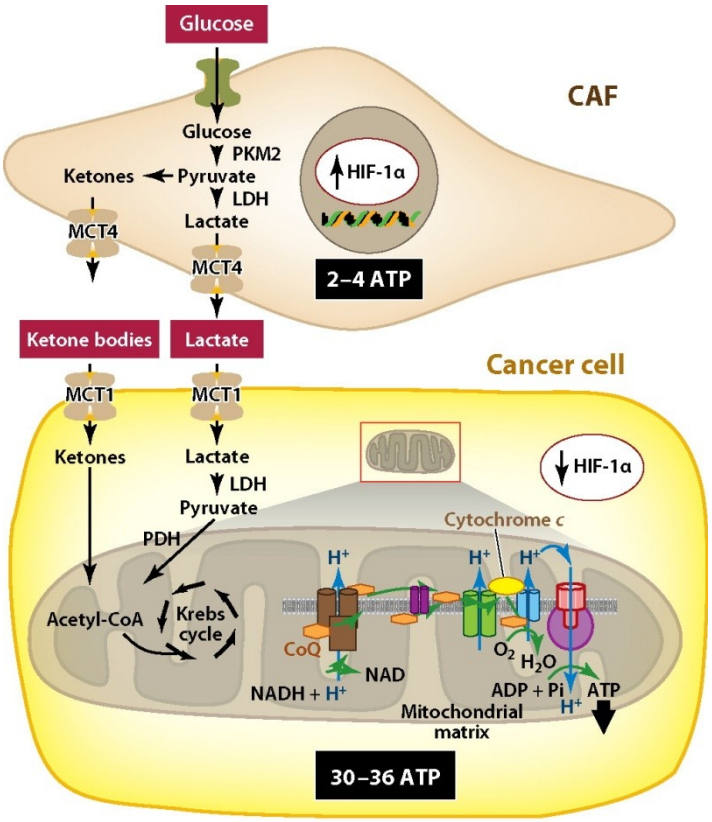

Fig. 17. Model of metabolic symbiosis between cancer cells and CAFs.

Lisanti's group performed unbiased proteomic analysis on mesenchymal stem cells derived from WT and Cav-1 (−/−) deficient mice, as CAFs are thought to originate from MSCs of the bone marrow. They showed that a loss of stromal Cav-1 expression up-regulates the protein expression of: 8 myofibroblast markers (such as vimentin, calponin, and collagen), 8 glycolytic enzymes (LDHA and PKM2, as well as phosphoglycerate kinase-1 and triose-phosphate isomerase), 2 anti-oxidants which are markers of oxidative stress (catalase and peroxiredoxin-1) (Pavlides et al., 2009). Based on this and other unbiased transcriptional profiling studies, they proposed that a loss of stromal Cav-1 in CAFs is associated with ROS production and oxidative stress. This, in turn, is sufficient to activate HIF-1α and NF-kB, leading to the induction of aerobic glycolysis in CAFs under normoxic conditions (Martinez-Outschoorn et al., 2010). Lisanti and colleagues also proposed that the glycolytic metabolism in CAFs then drives the production of excess lactate and/or pyruvate; these high-energy metabolites could then be transferred to adjacent cancer cells where they enter the TCA cycle, resulting in increased oxidative phosphorylation and efficient ATP production (Fig. 17, Fig 18).

Secretion and re-uptake of lactate or pyruvate would be mediated by the monocarboxylate family of transporters, such as MCT1/4. This model was termed "The Reverse Warburg Effect", which is just the opposite of the conventional Warburg effect, in which cancer cells are thought to undergo aerobic glycolysis. Importantly, fibroblast oxidative stress leads to up-regulation of the antiapoptotic protein TIGAR (TP53-induced glycolysis and apoptosis regulator) in tumor cells (Martinez-Outschoorn et al., 2010) (Fig. 18). TIGAR is a known inhibitor of both autophagy and apoptosis, and functionally shifts cancer cells away from aerobic glycolysis towards oxidative mitochondrial metabolism. (Bensaad et al., 2009; Li and Jogl, 2009; Bensaad et al., 2006; Green et al., 2006).

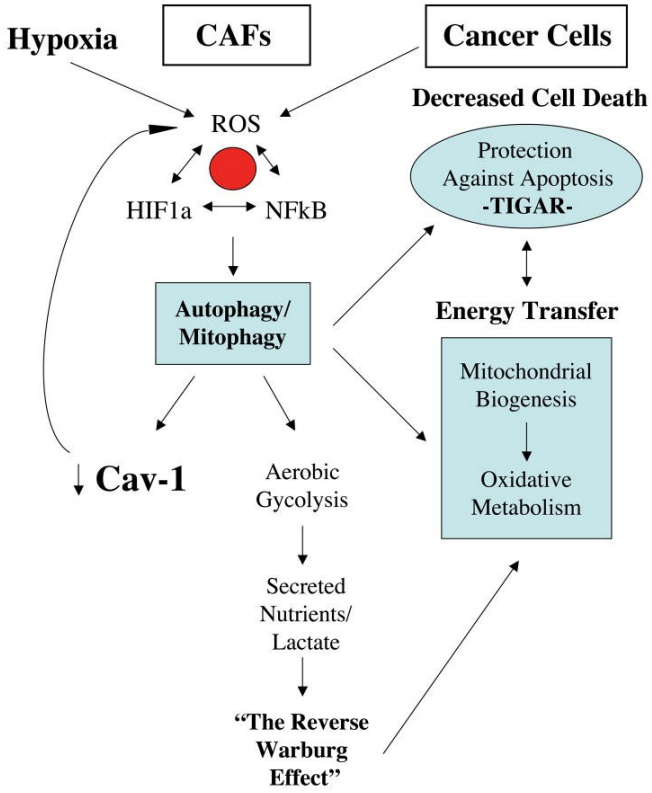

Fig. 18. The autophagic tumor-stroma model of cancer. In this model, cancer cells use oxidative stress as a "weapon" to extract recycled nutrients from cancer-associated fibroblasts, via the induction of autophagy. Stromal autophagy, in turn, provides energy-rich recycled nutrients (such as lacatate, ketones, and glutamine) to fuel oxidative mitochondrial metabolism in cancer cells.

In addition, Cav-1-deficient CAFs undergo a catabolic process of autophagy/mitophagy (Fig. 18). Indeed, under conditions of oxidative stress, Cav-1 is target for autophagic/lysosomal degradation, which is efficiently blocked using either, anti-

oxidants (N-acetyl cysteine, metformin, or quercetin) or lysosomal inhibitors (chloroquine) (Martinez-Outschoorn et al., 2010b). This process leads to generate chemical building blocks such as amino acids (glutamine), nucleotides, and lipids, that can sustain and support the mitochondria-dependent growth of tumor cells (Pavlides et al., 2009; Pavlides et al., 2010; Martinez-Outschoorn et al., 2010c; Martinez-Outschoorn et al., 2011) (Fig. 18). In this regard, Lisanti's group proposed that autophagic fibroblasts may serve as a key source of energy-rich glutamine to fuel cancer cell mitochondrial activity, driving a vicious cycle of catabolism in the tumor stroma and anabolic tumor expansion (Fig. 19). Indeed, MCF7 cancer cells/fibroblasts co-culture promotes glutamine catabolism and mitochondrial biogenesis, and decreases glutamine synthesis in MCF7 cancer cells. Furthermore, glutamine increases the expression of autophagy markers in fibroblasts, but decreases expression of autophagy markers in MCF7 cells, indicating that glutamine regulates the autophagy program in a compartment-specific manner. Functionally, glutamine protects MCF7 cells against apoptosis, via the upregulation of the anti-apoptotic and anti-autophagic protein TIGAR. Also, they show that glutamine cooperates with stromal fibroblasts to confer tamoxifen-resistance in MCF7 cancer cells (Ko et al., 2011).

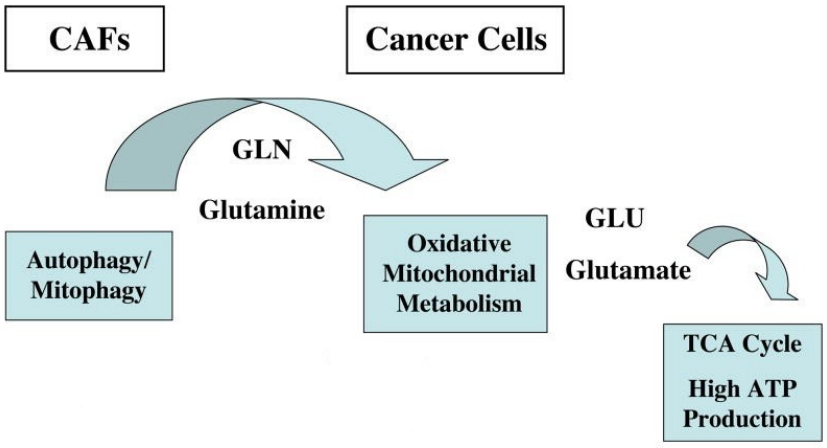

Fig. 19. Glutamine shuttle between CAFs and tumor cells.

Further studies have also demonstrated that a highly catabolic tumor microenvironment supports tumor formation. Metabolomic analysis of colon and stomach cancer tissues, versus matched normal tissues, demonstrated the up-regulation of metabolites involved

in glycolysis, PPP, TCA and urea cycles, and the significant accumulation of amino acids and nucleotides in cancer tissues. These results indicate that cancer tissues rely upon glycolytic and mitochondrial metabolism for energy generation and display a highly catabolic microenvironment, which points to the augmented autophagic degradation of proteins (Hirayama et al., 2009). Consistent with the compartment-specific effects of autophagy, clinical studies have indicated that the elevated expression of autophagy markers in cancer cells correlates with a favorable prognosis, whereas increased levels of autophagy markers in stromal cells predict poor clinical outcome (Futreal et al., 1992; Saito et al., 1993; Nicotra et al., 2010; Li et., al., 2009; Ding et al., 2008; Tan et al., 2007).

In agreement with Lisant's group results, other studies have similarly demonstrated the tumor-promoting effects of glycolytic enzymes in stromal cells. Laser capture microdissection and transcriptional profiling of human prostate cancer-associated stroma revealed the up-regulation of phosphoglycerate kinase-1, one of the two ATP-generating enzymes of the glycolytic pathway. Phosphoglycerate kinase-1 overexpression in normal primary human fibroblasts induces a CAF phenotype and, in a xenograft model, greatly supports the growth of coinjected prostate cancer cells via induction of CXCL12/SDF-1 (Wang et al., 2010). Conversely, overexpression of phosphoglycerate kinase-1 in lung tumor cells abrogates tumor growth and greatly prolongs survival in mice (Tang et al., 2008), which suggests that acute induction of glycolysis may differently affect tumor growth, depending on the cellular compartment.

The model of coupling metabolism within tumor stroma, involving "direct" and "reverse Warburg" metabolism, could explain the controversial data concerning the role of mitochondria in cancer progression. Indeed, although the traditional view of cancer metabolism is that cells undergo aerobic glycolysis, it has been shown that cancer cells have a broad spectrum of bioenergetic states ranging from predominance of aerobic glycolysis to predominance of oxidative phosphorylation. Several data indicate a mandatory role of mitochondria in cancer cells, ranging from lactate respiration, to TCA cycle fueling with ketone bodies and glutamine, to citrate exportation to fuel fatty acids synthesis (Moreno-Sánchez et al., 2007). Thus, mitochondrial functional disruption may have positive effects on cancer progression and, to date, a number of mitochondrial metabolism inhibitors (e.g., oxidative phosphorilation inhibitors, mitochondrial ATP

transport inhibitors) are in pre-clinical or clinical development (Ramsay et al., 2011). In this regard, the "reverse Warburg model" could explain the antiproliferative effects of metformin. The latter is a widely used oral antidiabetic drug, endowed with promising effects for cancer prevention and treatment (Bost et al., 2012). Metformin is reported to inhibit mitochondrial complex I activity, thereby disrupting oxidative mitochondrial metabolism, mandatory for cancer "reverse Warburg" metabolism and granting survival and growth. Sanchez-Alvarez and colleagues (Sanchez-Alvarez et al., 2013) although not directly using metformin, reported that uncoupling protein-mediated mitochondrial dysfunction actually has compartment-specific effects. Indeed, mitochondrial dysfunction in stromal CAFs enhances their metabolic reprogramming to production of energy-rich metabolites, increasing tumor growth. On the contrary, disruption of mitochondrial function through mitochondrial uncoupling proteins (UCPs) overexpression in cancer cells leads to the opposite effect, restraining tumor growth.

Finally, in contrast with the model of glycolytic and catabolic stroma, immunohistochemical data from evaluation of colorectal adenocarcinomas showed that cancer cells share common enzyme/transporter activities suggestive of an anaerobic metabolism (high HIF-1α and GLUT1 levels) with high ability for glucose absorption. The tumor-associated fibroblasts expressed proteins involved in lactate absorption (high MCT1/MCT2), lactate oxidation and reduced glucose absorption (low GLUT1 level). The expression profile of the tumor-associated endothelium indicated aerobic metabolism, and resistance to lactate intake (lack of MCT1). Thus, these results show that stroma express complementary metabolic pathways, buffering and recycling products of anaerobic metabolism to sustain cancer cells survival (Koukourakis et al., 2006).

Tumor metabolism and chemoresistance

Drug resistance of tumor cells is recognized as the primary cause of failure of chemotherapeutic treatment of most human tumors. Drug resistance can be described as a lack of meaningful response or a partial response to therapy, or regrowth of tumor after an initial response. Tumor cell population is characterized by subclones resistant to

anticancer drugs, which are responsible for the regrowth of the tumor mass after or during therapy. Although pharmacological factors including inadequate drug concentration at the tumor site can contribute to clinical resistance, cellular factors play a major role in intrinsic or acquired chemoresistance of several tumors (Zhao et al., 2013).

In general, anticancer agents work by interrupting critical events within the cellular lifecycle resulting in either irreversible damage to the cell or induction of apoptotic pathways. DNA replication is directly or indirectly a feature targeted by a wide variety of compounds. Both the cisplatin and temozolomide families of compounds modify the DNA, either by forming bulky adducts or by alkylating the bases, preying on the limited or compromised DNA repair ability that is common within many cancers. Similarly, nucleoside mimics such as gemcitabine and 5-FU (5-fluorouracil) disrupt replication by inhibiting the synthesis of deoxynucleotides through the inhibition of ribonucleotide reductase or thymidylate synthase, respectively. More indirect methods of disrupting DNA replication target the topoisomerases, using families of compounds such as adriamycin and doxorubicin. These intercalating drugs stop DNA replication by stabilizing topoisomerase II, which prevents progression of the replication fork and ultimately leads to cellular death. Similarly, taxol class drugs indirectly target replication by stabilizing tubulin. This blocks progression of the cellular cycle, as metaphase chromosomes can no longer achieve the correct configuration, ultimately resulting in cell checkpoint activation and/or stalling of the cell cycle. Although DNA replication is a common target for current clinical anticancer drugs, it is not the only clinically effective target. Other classes of drugs affect the deregulated signaling pathways within cancer. The unchecked activation of these signaling networks often results in increased angiogenesis and unregulated growth. Selective estrogen receptor modulators, such as tamoxifen and raloxifen, modulate signaling through the estrogen receptor-mediated pathways and have been particularly effective for patients with certain types of breast cancer. Similarly, the EGFR family has been effectively targeted using drugs such as lapatinib (tyrosine kinase inhibitor active against EGFR and HER2) and trastuzumab (a humanized antibody targeting the HER2 receptor). The EGFR family, and specifically HER2, are aberrantly active in many types of cancers and

initiate signaling pathways that lead the cells to grow aggressively and often results in a less positive outcome than seen in non-HER2 expressing tumors (Zhao et al., 2013).

The relevant mechanisms that can contribute to chemoresistance in cancer include: increased expression of defense factors involved in reducing intracellular drug concentration; alterations in drug-target interaction; and changes in cellular response, in particular increased cell ability to repair DNA damage or tolerate oxidative stress conditions, and defects in apoptotic pathways. In most cases, such alterations promote the development of resistant cell clones that are associated with a trans-differentiation of cancer cell machinery that enables more aggressive and invasive phenotype, induces the activation of EMT programme, increases stemness-like features and proliferative rate (Gal et al., 2008; Iseri et al., 2011; Kajiyama et al., 2007; Wang et al., 2009; Yang et al., 2006; Puhr et al., 2012).

Increasing evidences support the idea that drug resistance in cancer therapy is link to adaptation of cancer cell to a deregulated cellular metabolism (Zhao et al., 2013) (Fig.20). Below, we will discuss the role of enzymes and metabolic pathways in resistance to anticancer drugs, as well as the mechanisms of some small molecules able to interfere with cell metabolism (Fig. 21).

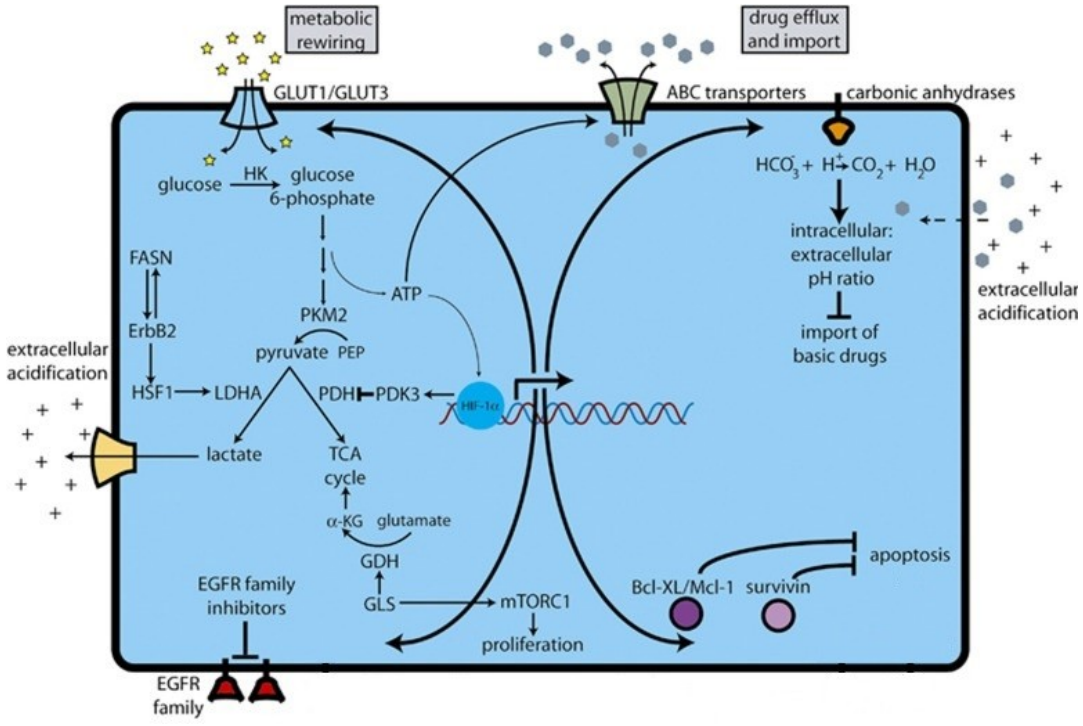

Fig. 20. Deregulated metabolism affects chemoresistance via multiple cellular pathways.

GLYCOLYSIS

Inhibiting glucose uptake may potentiate cancer therapeutics or overcome hypoxia/drug-induced resistance. The GLUT family of proteins is responsible for the transport of glucose across the plasma membrane and are often found deregulated or overexpressed in malignant cells (Macheda et al., 2005) (Fig. 20).

WZB117 is an inhibitor of GLUT1 that decreases glucose uptake, intracellular ATP levels and glycolytic enzymes leading to a lowered rate of glycolysis and cellular growth. The combination of WZB117 and cisplatin or paclitaxel displayed synergistic anticancer effects (Fig. 21) (Liu et al., 2012; Monti and Gariboldi, 2011). Under hypoxia, the GLUT1 inhibitor phloretin significantly enhances daunorubicin's anticancer effects (Fig. 21) and overcomes hypoxia-conferred drug resistance.

Multiple myeloma (MM) cells are dependent on GLUT4 activity for basal glucose consumption, maintenance of anti-apoptotic Mcl-1 protein levels, growth and viability. Ritonavir displays off-target inhibitory effects on GLUT4 and inhibits glucose consumption and proliferation by reducing Mcl-1 expression to induce apoptosis. Ritonavir also inhibits viability of primary myeloma cells and increases the sensitivity to doxorubicin (Fig.21) (McBrayer et al., 2012). Temozolomide is used with radiation and chemotherapy to treat glioblastoma, yet nearly all glioblastoma patients develop resistance. Long-term treatment of glioblastoma cells with temozolomide *in vitro* induces partial resistance *in vivo* through up-regulation of GLUT3, suggesting involvement in temozolomide resistance and that selective targeting of GLUT3 could delay the acquisition of such resistance in glioblastoma cells (Le Calve et al., 2010).

Targeted metabolism	*Targeted metabolic enzymes*	*Metabolic inhibitors*	*Cancer therapeutics/other inhibitors*	*Cancer types in vitro and/or in vivo)*
Glycolysis	GLUT1	Phloretin	Daunorubicin	Colon cancer (*in vitro*), leukemia (*in vitro*)
		WZB117	Cisplatin/paclitaxel	Lung cancer (*in vitro*), breast cancer (*in vitro*)
	GLUT4	Ritonavir	Doxorubicin	Multiple myeloma (*in vitro*)
	HK	2-DG	ABT-737/ABT-263	Leukemia (*in vitro*), cervical cancer (*in vitro*), hepatocarcinoma (*in vitro*), breast cancer (*in vitro*), small lung cancer (*in vitro*), lymphoma (*in vitro*), prostate cancer (*in vitro* and *in vivo*)
			Trastuzumab	Breast cancer (*in vitro* and *in vivo*)
			Prednisolone	Leukemia (*in vitro*)
		3-BrPA	Daunorubicin	Leukemia (*in vitro*)
			Doxorubicin	Multiple myeloma (*in vitro* and *in vivo*)
			Oxaliplatin/5-FU	Colon cancer (*in vitro*)
			Prednisolone	Leukemia (*in vitro*)
		LND	ABT-737	Leukemia (*in vitro*), lymphoma (*in vitro*)
			Prednisolone	Leukemia (*in vitro*)
	PKM2	shRNA	Cisplatin	Lung cancer (*in vivo*)
			Docetaxel	Lung cancer (*in vitro* and *in vivo*)
	LDHA	FX11	FK866	Lymphoma (*in vivo*)
		Oxamate	Paclitaxel	Breast cancer (*in vitro*)
			Trastuzumab	Breast cancer (*in vitro* and *in vivo*)
Citric acid cycle	PDK3	siRNA	Paclitaxel	Cervical cancer (*in vitro*)
			Cisplatin/paclitaxel /oxaliplatin	Colon cancer (*in vitro*)
	PDK	DCA	Omeprazole	Fibrosarcoma (*in vitro* and *in vivo*) colon cancer (*in vitro*)
			Omeprazole+tamoxifen	Fibrosarcoma (*in vitro*)
			5-FU	Colon cancer (*in vitro*)
			Sulindac	Lung cancer (*in vitro*), squamous cell carcinoma (*in vitro*)
			Irradiation	Prostate cancer (*in vitro*)
Fatty acid synthesis	FASN	Cerulenin	Docetaxel	Breast cancer (*in vitro*)
			Trastuzumab	Breast cancer (*in vitro*)
			5-FU	Breast cancer (*in vitro*)
		C75	Trastuzumab	Breast cancer (*in vitro*)
		Orlistat	Adriamycin/mitoxantrone	Breast cancer (*in vitro*)
			Gemcitabine	Pancreatic cancer (*in vitro*)

Fig. 21. Targeting cellular metabolism improves therapy in several cancer types.

HK has important roles in both glycolysis and apoptosis and inhibitors of HK, such as 2-deoxyglucose (2-DG), 3-bromopyruvate (3-BrPA) and lonidamine (LND) are in pre-clinical and early phase clinical trials.

2-DG is a glucose analog that is phosphorylated by HK to 2-DG-phosphate, which cannot be further metabolized. Accumulation of 2-DG inhibits glycolysis causing ATP depletion, cell cycle inhibition and cell death (Maher et al., 2004; Pelicano et al., 2006a). Combining 2-DG with radiation or chemotherapeutic treatments potentiates the tumor-destroying effects and enhances the clinical efficacy (Dwarakanath et al., 2009).

Trastuzumab is a humanized monoclonal antibody against ErbB2 and has shown efficacy treating ErbB2-positive breast cancer patients, yet acquired trastuzumab resistance occurs in most patients (Esteva et al., 2002; Lan et al., 2005; Hudis, 2007; Zhang et al., 2007; Slamon et al., 2001; Nagata et al., 2004; Nahta et al., 2006). Of note, overexpression of ErbB2 promotes glycolysis and increases their sensitivity to glycolytic inhibition (Zhao et al., 2009). Trastuzumab-resistant human cells also have increased glucose uptake and lactate production, indicative of increased glycolysis. 2-DG/trastuzumab combination therapy synergistically inhibits growth of both trastuzumab-sensitive and trastuzumab-resistant human breast cancers *in vitro* and *in vivo* (Fig. 21), because of more efficient glycolysis inhibition (Zhao et al., 2011). These results suggest that 2-DG can effectively enhance efficacy of trastuzumab in treating ErbB2-positive human breast cancer cells and overcome trastuzumab resistance.

3-BrPA is a glycolysis inhibitor that targets HKII and depletes cellular ATP reserves, a key determinant of chemoresistance in certain cancer types (Geschwind et al., 2004; Ko et al., 2001). In leukemia and MM cells increased glycolysis raises ATP levels, which activates ATP-binding cassette (ABC) transporters and confers drug resistance via enhanced drug efflux activity (Fig. 20). 3-BrPA causes ATP depletion, decreasing ABC transporter activity and drug efflux, therefore enhancing drug retention in cells producing preferential cell death in malignant cells. Glycolysis inhibition by 3-BrPA not only enhances the cytotoxic effects of daunorubicin and doxorubicin, but also markedly suppresses tumor growth when used with doxorubicin to treat MM-bearing mice (Fig. 21) (Nakano et al., 2011). In addition to activating ABC transporters, increased ATP levels from elevated glycolysis upregulate HIF-1α and enhance HIF-1α-mediated signaling, which can confer chemoresistance (Fig. 20). ATP depletion by 3-BrPA partially reversed the resistant phenotype and resensitized cells to chemotherapeutic agents such as oxaliplatin and 5-FU (Fig. 21) (Zhou et al., 2012).

Most treatment failure in childhood acute lymphoblastic leukemia (ALL) is ascribed to glucocorticoid (e.g., prednisolone) resistance. Increased glycolysis is directly associated to glucocorticoid resistance and inhibition of glycolysis by 2-DG, 3-BrPA or LND increases prednisolone-induced toxicity in leukemia cells (Fig. 21) (Hulleman et al., 2009). Importantly, 2-DG can reverse glucocorticoid resistance in primary leukemia cells isolated from pediatric ALL patients (Hulleman et al., 2009).

As previously reported, several data indicate that PKM2 is expressed predominantly in tumor cells (Hitosugi et al., 2009) and is important for cancer metabolism (Christofk et al., 2008). However, there are conflicting data in literature about the role of this enzyme in chemoresistance. A number of studies show a negative correlation between PKM2 expression and drug resistance. (Li et al., 2010; Martinez-Balibrea et al., 2009; Yoo et al., 2004). Decreased PKM2 protein and activity is linked to cisplatin resistance while suppression of PKM2 expression by siRNA increased cisplatin resistance (Yoo et al., 2004). Both PKM2 mRNA and protein levels are downregulated in oxaliplatin-resistant cells and PKM2 mRNA levels are inversely correlated with oxaliplatin resistance in a panel of eight colorectal cancer cell lines. Low PKM2 mRNA levels in patients are associated with high p53 protein levels and predict poor response to oxaliplatin (Martinez-Balibrea et al., 2009). In contrast, PKM2 levels are significantly up-regulated in secreted proteins of the 5-FU-resistant colon cancer cell line. Moreover, increased PKM2 is also observed in sera and tissues from colorectal cancer patients with poor response to 5-FU.

Changes in PKM2 expression are associated with drug resistance in different tumors. This indicates that PKM2 is a potential target for adjuvant cancer therapy. For example, shRNA targeting PKM2 improves the therapeutic efficacy of cisplatin by increasing apoptosis and inhibiting proliferation (Fig. 21) (Guo et al., 2011). Silencing of PKM2 enhances the efficacy of docetaxel because of increased inhibition of proliferation and apoptosis-inducing activity both *in vitro* and *in vivo* (Fig. 21) (Shi et al., 2010). A possible mechanism for the sensitization of lung cancer cells to docetaxel is that shPKM2 decreases ATP levels leading to intracellular accumulation of docetaxel (Shi et al., 2010).

LDHA catalyzes the conversion of pyruvate and NADH to lactate and NAD^+ and has a critical role in tumor maintenance. Knockdown of LDHA in tumor cells produces increased mitochondrial respiration, decreased cellular ability to proliferate under hypoxic conditions, and suppressed tumorigenicity (Fantin et al., 2006). LDHA-knockdown in the fumarate hydratase-knockdown background results in increased apoptosis via ROS production, resulting in a reduction in tumor growth and indicating that LDHA might be a promising therapeutic target.

Paclitaxel (taxol) is a widely used chemotherapeutic agent in the treatment of a variety of human cancers (Fig. 21). LDHA expression and activity is higher in taxol-resistant breast cancer cells than in taxol-sensitive cells, and down-regulation of LDHA resensitizes taxol-resistant cells to taxol. Taxol-resistant cells are more sensitive to oxamate, a pyruvate analog that inhibits glycolysis by inhibiting the conversion of pyruvate to lactate. Moreover, combination of paclitaxel with oxamate shows synergistic inhibitory effect on taxol-resistant cells (Fig. 21) by promoting cellular apoptosis (Zhou et al., 2010).

Heat shock factor-1 (HSF1) is the master regulator of the heat shock response in eukaryotes. HSF1 functions primarily to coordinate the response to heat shock, but recent studies demonstrate HSF1 exhibiting non-heat shock functions important for cancer development (Khaleque et al., 2008; Khaleque et al., 2005; Min et al., 2007). Dai et al (Dai et al., 2007) reported that HSF1 increases glucose uptake, lactate production and LDH activity. Of note, ErbB2 promotes glycolysis partially through up-regulation of HSF1 and LDHA (Fig. 20), whereas down-regulation of HSF1 leads to decreased glycolysis (Zhao et al., 2009). It has been reported that trastuzumab-resistant cells have significantly higher HSF1 protein levels than trastuzumab-sensitive cells. Thus, the inhibition of HSF1 sensitizes cells to trastuzumab and overexpression of HSF1 increased trastuzumab resistance, demonstrating that HSF1 can have an important role in resistance to this drug (Zhao et al., 2011). Finally, the combination of trastuzumab and oxamate synergistically inhibits growth of both trastuzumab-sensitive and trastuzumab-resistant cancer both *in vitro* and *in vivo* (Fig. 21), because of more efficient glycolysis inhibition (Zhao et al., 2011) Overall, high-rate glycolysis confers chemoresistance and HSF1 and LDHA may potentially act as excellent targets for overcoming this resistance in cancer patients.

PDK

PDH is responsible for the rate-limiting conversion of pyruvate to acetyl-CoA, which enters the TCA cycle to generate ATP. PDK phosphorylates PDH and inhibits its enzymatic activity. Four isotypes of PDK (PDK1-4) have been identified with PDK3 demonstrating the highest activity coupled with a lack of inhibition in response to high concentrations of pyruvate (Lu et al., 2008). Hypoxia induces PDK3 expression via up-

regulation of HIF-1α, resulting in a switch from mitochondrial respiration to glycolysis for energy production. Hypoxia-mediated PDK3 induction or forced PDK3 overexpression significantly inhibits cell apoptosis and increases resistance to cisplatin or paclitaxel (Fig. 20). Knockdown of PDK3 inhibits hypoxia-induced glycolysis and increases susceptibility of cancer cells to anticancer drugs such as cisplatin, paclitaxel or oxaliplatin (Fig. 21). Moreover, PDK3 levels are elevated and correlated with the HIF-1α level in patient colon cancer tissues and strongly correlates with the severity of the cancer while predicting poor disease-free survival outcomes (Lu et al., 2011). These findings indicate that PDK3 is potentially a novel target for improving chemotherapy or overcoming drug resistance.

Dichloroacetate (DCA) inactivates PDK leading to reactivation of PDH and a metabolic switch from glycolysis to mitochondrial respiration (Zhao et al., 2011; Kato et al., 2007). Owing to its low price, low toxicity, oral administration, long history of clinical use and ability to overcome cancer cells apoptosis resistance, DCA serves as a potential metabolic-targeting molecule for sensitizing cancer cells to chemotherapy or radiotherapy (Michelakis et al., 2008). For example, co-treatment with DCA and omeprazole exhibits synergistic antitumor activity (Fig. 21) (Ishiguro et al., 2012). DCA potentiates the anticancer effects of 5-FU (Fig. 21) via inducing more mitochondrial-mediated apoptosis. Moreover, Cao et al. (Cao et al., 2008) reported that DCA sensitizes both wild-type and Bcl-2-overexpressing cancer cells to radiation (Fig. 21) by potentiating the apoptotic machinery via interaction with Bcl-2.

FATTY ACID BIOSYNTHESIS

The fatty acid biosynthesis pathway catalyzes lipid synthesis from basic metabolites like acetyl- and malonyl-CoA. The fatty acid synthase (FASN) complex facilitates lipogenesis by synthesizing palmitate from its base components. FASN expression in normal adult tissues is generally very low or undetectable, and it is significantly up-regulated and correlates with poor prognosis in many types of cancer. The metabolic products of the FASN complex are rapidly consumed by actively dividing cells and recent data demonstrates that FASN expression is important for tumor growth and survival, suggesting that FASN is a metabolic oncogene (Flavin et al., 2010).

To date, several FASN inhibitors have shown antitumor activity including cerulenin, C75, orlistat, C93, GSK 837149A and natural plant-derived polyphenols. Cerulenin enhances the efficacy of docetaxel and trastuzumab therapy and increases 5-FU-induced growth inhibition (Fig. 21) (Vazquez-Martin et al., 2007) Similarly, C75 and trastuzumab synergistically decrease ErbB2 expression and enhance apoptotic cell death (Fig. 21) (Vazquez-Martin et al., 2007a).

FASN has an active role in ErbB2-induced breast cancer chemoresistance to docetaxel (Menendez et al., 2004) while trastuzumab-resistant breast cancer cells gain high sensitivity to FASN inhibition indicating that FASN is also important in ErbB2-induced resistance in breast cancers (Vazquez-Martin et al., 2007a). FASN is overexpressed and its activity is increased in the multidrug-resistant breast cancer cell line MCF7/AdVp3000 (Liu et al., 2008). In pancreatic cancer, there is also a positive correlation between FASN expression and resistance to chemo- or radiotherapy. FASN expression is significantly upregulated in pancreatic cancer and inhibition of FASN by siRNA or the FASN inhibitor orlistat reduces gemcitabine resistance, whereas ectopic overexpression of FASN contributes to intrinsic resistance to gemcitabine and radiation. FASN-induced radiation resistance may result from decrease in radiation-mediated ceramide production, leading to reduced caspase 8-induced apoptosis. However, the mechanism of FASN-induced gemcitabine resistance remains to be elucidated (Yang et al., 2011).

GLUTAMINOLYSIS

Metabolic flux experiments tracking ^{13}C show that cancer cells exhibiting Warburg-like metabolism do not stop utilizing the TCA cycle. These cells come to rely on glutamine as the carbon source for the TCA cycle (DeBerardinis et al., 2007). This allows the intermediates generated by the TCA cycle to feed other biosynthetic pathways as precursors (DeBerardinis et al., 2008). Therefore, cancer cells are dependent on glutamine to maintain the TCA cycle. Glutaminolysis co-induced by glutamine and leucine activates mTOR signaling, which triggers cell growth and inhibits autophagy (Duran et al., 2012). The mTOR pathway is involved in cisplatin resistance in highly malignant AFP-producing gastric cancer (AFPGC) (Kamata et al., 2007). This indicates

that elevated glutaminolysis is linked to drug resistance. Interestingly, mTOR inhibitor rapamycin enhances the antitumor effect of cisplatin in this tumor (Kamata et al., 2007).
Amino-oxyacetate is a transaminase inhibitor that targets glutamate-pyruvate transaminase, which converts glutamate to α-ketoglutarate. In melanoma cell lines, amino-oxyacetate sensitizes cells to TRAIL (tumor necrosis factor-related apoptosis inducing-ligand)-induced cell death. It inhibits proliferation of MDA-MB-231 breast cancer and SF188 glioblastoma cell lines and suppresses growth of MDA-MB-231 xenograft tumors in mice (Ramsay et al., 2011).
Bis-2-[5-phenylacetamido-1,2,4-thiadiazol-2-yl] ethyl sulfide (BPTES), an inhibitor of GLS, caused decreased aerobic cell proliferation and hypoxic cell death (Robinson et al., 2007). Inhibition of GLS by siRNA or BPTES slows the growth of glioblastoma cells with a IDH1 mutation. BPTES treatment inhibits GLS activity, lowers glutamate and α-KG levels and increases glycolytic intermediates, suggesting that simultaneous inhibition of GLS and glycolysis may be a more efficient strategy to treat mutant IDH1 patients (Seltzer et al., 2010).

ADAPTATIONS TO OXIDATIVE STRESS AND PPP

Many anticancer drugs induce oxidative stress either as a direct mechanism of cell death or as an indirect effect of exposure (Tiligada et al., 2006). It has been suggested that pathways involved in the ROS-adaptive response play a critical role in protecting cells against the damaging and cytotoxic effects of anticancer agents (Pennington et al., 2005) (Fig. 22). Thus, many redox-dependent mechanisms of adaptation to stress have been explored in terms of their potential for inducing drug resistance, with those proposed including the induction of DNA repair systems, the reprogramming of cell-cycle-regulation systems, and the up-regulation of non-enzymatic and enzymatic antioxidant defenses, and also of molecular chaperones and of stress-responsive proteins (Pelicano et al, 2004; Pennington et al., 2005).

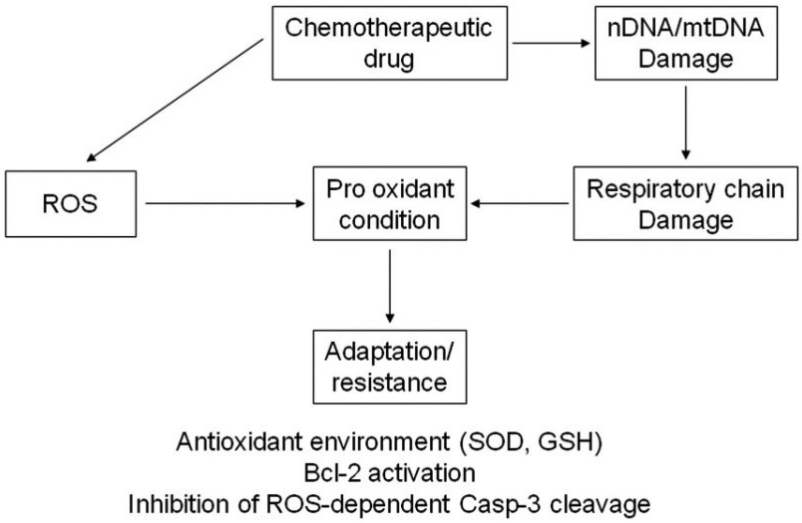

Fig. 22. Adaptation to oxidative stress and drug resistance. nDNA: nuclear DNA; mtDNA: mitochondrial DNA; SOD: superoxide dismutase.

Several studies link the up-regulation of antioxidants systems involved in cellular response to oxidative stress, to the onset of drug resistance in human malignancies (Fig. 22). Indeed, glutathione reductase/glutaredoxin (GR/GRX) system, thioredoxin/thioredoxin reductase (Trx/TrxR) system, manganese superoxide dismutase (MnSOD) and peroxiredoxins (PRXs) are associated both to resistance to chemotherapeutic drugs that induce oxidative stress and apoptosis and to high tumor grade (Landriscina et al., 2009). For example, the gene-expression profile of non-responder breast carcinomas treated with docetaxel was characterized by elevated expression of genes controlling the cellular redox environment, such as Trx, glutathione S-transferase (GST), and PRX (Iwao-Koizumi et al., 2005). Furthermore, overexpression of antioxidant enzymes appears to be present in various human tumors, thus confirming the importance for cancer cells to maintain homeostasis of intracellular redox-state, in order to avoid damages in macromolecules and to preserve genome stability (Landriscina et al., 2009).

An important redox-sensitive factor is NF-E2-related factor 2 (Nrf2) (Moi et al., 1994). In response to oxidative stress, Nrf2 controls the fate of cells through transcriptional up-regulation of antioxidant response element (ARE)-bearing genes, including those encoding for antioxidants enzymes, phase II detoxifying enzymes, and transporters. Expression of the Nrf2-dependent proteins is critical to maintain cellular redox homeostasis through elimination of toxicants/carcinogens. Nrf2 activity is regulated in

part by the association with Keap1 protein: a disruption of this association in response to stress signals causes the release of Nrf2 and its translocation into the nucleus, to effect its transcriptional activity (Fig. 23) (Itoh et al., 1999). Recent data revealed that Nrf2 and its target genes are up-regulated in resistant cancer cells (Lau et al., 2008). For example, it has been reported that the pharmacologic activation of Nfr2 induces mechanisms of cell survival in response to doxorubicin, cisplatin, and etoposide (Wang et al., 2008), whereas the downregulation of Nfr2 expression by using Nfr2-siRNA restores sensitivity to several chemotherapeutic and biologic agents (Pi et al., 2008).

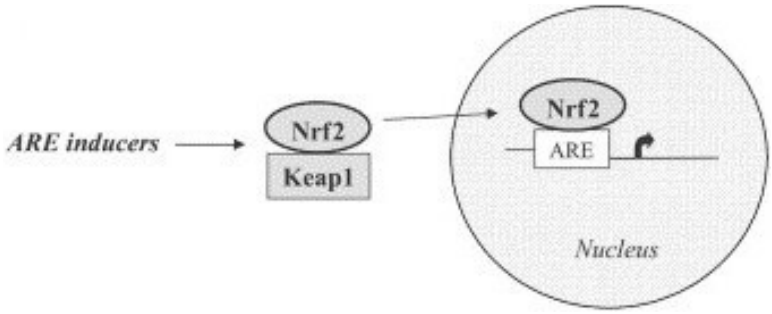

Fig. 23. Activations of Nrf2 pathway in response to stress/pro-oxidant signals.

NADPH produced from PPP is a critical antioxidant and high levels maintained through increased glycolysis in cancer cells may contribute to chemoresistance (Zhao et al., 2013). The glutathione reductase system uses NADPH reducing oxidized glutathione (GSSG) to sulfhydryl form GSH (Fig. 24). GSH removes ROS directly acting as a substrate for several peroxidases (Dringen et al., 2000; Sims et al., 2004). This tripeptide is also involved in the conjugation of foreign molecules catalyzed by GST, a target gene of Nrf2. Interestingly, GSH-depleting strategies have clearly been shown to improve the capacity of antiblastic agents to induce apoptosis in cancer cells both *in vitro* and in murine models (Esposito et al., 2002). Furthermore, several studies link the pleiotropic effects of GSH in promoting cell growth and broad resistance to therapy with the up-regulation of Bcl-2, which inhibits the activation of apoptosis and contributes to the elevation of GSH (Friesen et al., 2004; Mena et al., 2007; Osbild et al., 2006). This issue was clearly demonstrated in highly metastatic murine B16 melanoma cells that are characterized by elevated levels of both GSH and Bcl-2. Interestingly, the reduction of Bcl-2 and GSH -combined with treatment with paclitaxel,

radiation, and cytokines- eliminates melanoma cells from liver and all other systemic disease, leading to long-term survival without recurrence in melanoma-injected mice (Mena et al., 2007).

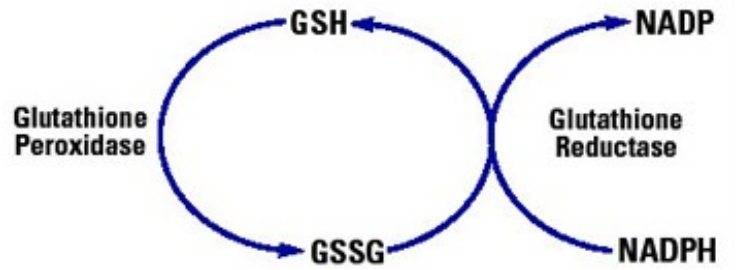

Fig. 24. The glutathione reductase system.

Finally, Polimeni et al. showed that doxorubicin-resistant human colon cancer cell line exhibites increased activity of PPP and glucose-6-phosphate dehydrogenase; these are important to keep high GSH content that, in turn, is necessary to extrude doxorubicin out from the cell through multidrug resistance-associated proteins (MRPs) (Polimeni et al., 2011).

Tumor microenvironment and resistance to cancer therapy

Besides its role as a key regulator of tumor progression, tumor microenvironment has been associated with drug delivery and drug efficacy, thus emerging as a form of *de novo* drug resistance that protects cancer cells from several therapies.

CAFs

Among the different constituents of the microenvironment, CAFs play a primary role in this phenomenon (Crawford and Ferrara, 2009; Meads et al., 2009; Roodhart et al., 2011). For example, CAFs can mediate resistance to anti-angiogenic therapy (Crawford and Ferrara, 2009): it has been shown that tumors may overcome inhibition of VEGF-mediated angiogenesis through up-regulation of PDGF-C mediated by CAFs (Crawford et al., 2009). Furthermore, pancreatic carcinoma cells co-cultured with stromal

fibroblasts become much less sensitive toward treatment with etoposide than cells cultured under standard conditions (Muerkoster et al., 2004). In keeping, in a mouse model of pancreatic adenocarcinoma, Olive et al. showed that a depletion in the stromal component leads to an in increase in chemosensitivity (Olive et al., 2009).

In addition, the achievement of EMT correlates to an increase in chemoresistance (Gal et al., 2008). Indeed, the shift in gene expression during EMT is reminiscent of the gene expression pattern present in cancer stem-like cells (Iseri et al., 2011; Kajiyama et al., 2007; Wang et al., 2009; Yang et al., 2006) and the ectopic expression of EMT genes such as Snail, ZEB1, Notch, Twist leads to a resistant phenotype *in vitro* (Cheng et al., 2007). Moreover, stromal fibroblasts can influence chemosensitivity of tumor cells by producing and activating ECM molecules. This activated ECM confers chemoresistance by integrin-mediated adhesion to fibronectin (Miyamoto et al., 2004; Van der Kuip et al., 2001). Finally, co-culture experiments and xenograft models demonstrated that the efficacy of chemotherapy-induced cell cycle arrest or senescence in stromal fibroblasts is critical for the sensitivity of the tumor compartment (Lafkas et al., 2008).

Recent data have shown that metabolic coupling between CAFs and tumor cells play an important role to confer chemoresistance. Indeed, co-culture experiments between MCF7 cells and immortalized human fibroblasts showed that tamoxifen-sensitive MCF7 become resistant by the matabolic shift from glycolytic to an oxidative state, which is dependent on mitochondrial biogenesis and oxidative phosphorilation in cancer cells. Interestingly, tamoxifen-resistance is impaired by disrupting metabolic coupling and shifting MCF7 cells back to the aerobic glycolysis (Warburg effect) (Martinez-Outschoorn et al., 2011). In keeping, also glutamine -supplied from autophagic CAFs to tumor cells- cooperates with stromal fibroblasts to confer tamoxifen-resistance in MCF7 cells, thus confirming the key role of tumor-stroma metabolic coupling and "reverse Warburg" metabolism in chemoresistance (Ko et al., 2011).

All this evidences suggests CAFs as possible targets of anti-cancer therapies in order to improve tumor chemosensitivity.

HYPOXIA

Intratumoral hypoxia and resulting HIF activation enhance chemoresistance of cancer cells in various ways. Firstly, the delivery of drugs in hypoxic area and cellular uptake

of it are affected by hypoxia or associated acidity. In this context, glycolytic metabolism leads to production of lactate, whose export results in the acidification of the extracellular environment. The resulting extracellular acidification, coupled with HIF-1α-induced expression of carbonic anhydrases (Fig. 20), causes a significant change in the pH ratio between the intracellular and extracellular environment (Ceradini et al., 2004; Greijer et al., 2005; Wykoff et al., 2000). This pH shift decreases the passive absorption of many drugs that would otherwise accumulate at a greater concentration within the cell. Secondly, some chemotherapeutic drugs require oxygen to generate free radicals that contribute to cytotoxicity. Last, hypoxia induces cellular adaptations that compromise the effectiveness of chemotherapy. In response to nutrient deprivation due to hypoxia, the rate of proliferation of cancer cells decreases but chemotherapeutic drugs are more effective against proliferating cells. On the other hand, hypoxia and HIF induce adaptation by transcriptional changes that promote cell survival and resistance to chemotherapy. Through these changes, hypoxia promotes angiogenesis, shift to glycolytic metabolism and PPP, expression of ABC transporters, cell survival and protection from apoptotic inducers (Cosse and Michiels, 2008).

A number of studies underline the relevant role of HIF-1α activation in drug resistance. For example, inhibitors of EGFR family signaling, such as gefitinib, show reduced effect under high HIF-1α expression because of an up-regulation of MET, which allows alternative signaling networks to produce similar phenotypic effects in the presence of reduced EGFR family signaling (Engelman et al., 2007; Pennacchietti et al, 2003). In addition, down-regulation of other drug targets, such as topoisomerase II or estrogen receptor α (ERα), can occur when HIF-1α expression is high and reduces the effect of drugs such as tamoxifen and etoposide (Kronblad et al., 2005; Sullivan and Graham, 2009; Wen et al., 2010). Finally, HIF-1α induces expression of genes that promote survival through anti-apoptotic signaling (survivin, Bcl-X_L, Mcl-1) or other survival mechanisms such as autophagy (BNIP3, BNIP3L).119–123. HIF-1α activation also decreases pro-apoptotic signaling by inducing the expression of decoy receptors (such as DcR2) that compete for pro-apoptotic signaling factors (Mayes et al., 2005; Pei et al., 2010). Attenuation of pro-apoptotic signaling allows cells to tolerate a higher level of chemotherapeutic insult before inducing cellular death pathways. HIF-1α signaling also works with glycolytic metabolism to trigger a variety of anti-drug mechanisms that

generate *in vitro* and clinical resistance. We have previously reported examples of how disrupting the cancer metabolism can short circuit the processes that provide protection from anticancer agents.

Furthermore, the adaptations elicited by hypoxia in cancer cells are likely responsible for the failure of anti-angiogenic therapy of metastatic tumors (De et al., 2011; Ebos and Kerbel, 2011). Recent papers have proved that anti-angiogenic drugs used in clinical settings, by reducing tumor tissue oxygenation and by activating the hypoxic transcriptional response, trigger molecular events that foster cell motility and resistance to chemotherapy (Loges et al., 2009; Paez-Ribes et al., 2009). To date, the most accepted hypotheses to explain these effects imply:

- activation of EMT by hypoxia following anti-angiogenic therapy as an escaping strategy to drive successful metastases (De et al., 2011; Maione et al., 2012; Shojaei et al., 2012);
- the metabolic shift towards PPP to fuel NADPH production and resistance to oxidative stress and to chemotherapy (De et al., 2011; Maione et al., 2012; Shojaei et al., 2012; Kruger et al., 2011; Anastasiou et al., 2011; Brahimi-Horn et al., 2011; Gruning and Ralser, 2011).

Moreover, mounting evidence indicates that hypoxic cancer cells undergo exposure to oxidative stress, thereby developing adaptive strategies to survive to the hostile milieu (Pani et al., 2010; Pani et al., 2009). Of note, hypoxic cells can enhance their antioxidant capacity and hypoxia can behave as a promoting factor for this behavior, leading cancer cells to resist to apoptosis and ultimately to chemotherapy and radiation therapy (Harris, 2002; Semenza, 2004).

OBJECTIVES

Our aim is to investigate the role of stromal fibroblasts and ROS in prostate carcinoma (PCa) progression. We focused on the metabolic reprogramming of cancer cells, clarifying the metabolic signature elicited by cancer associated fibroblasts (CAFs) in PCa cells and vice versa, and analyzing the role of reactive oxygen species (ROS) in this reciprocal interplay. Furthermore, we investigated how tumor metabolism changes in chemoresistant PCa cells, even upon contact with microenvironment, analyzing the regulation of the "Warburg effect", pentose phosphate pathway (PPP), oxidative phosphorylation and antioxidant response. Finally, considering the importance of oxidative stress in tumor-stroma interplay, we aimed at identifying natural antioxidant molecules able to disrupt the diabolic liaison between CAFs and PCa cells and to ultimately reduce CAFs-induced carcinoma progression.

As *in vitro* models, we used human prostate carcinoma cell line PC3 isolated from a bone metastasis of PCa, and human prostate fibroblasts isolated from healthy individuals affected by benign prostatic hyperplasia (HPFs, Human Prostate Fibroblasts) or from aggressive carcinoma-bearing patients (CAFs). Surgical explants were obtained thanks to the collaboration with the Urology Unit at the University of Florence.

MATERIALS AND METHODS

MATERIALS

- Unless specified, all reagents used for cell cultures were purchased from Euroclone Group, except penicillin/streptomicin from Sigma-Aldrich.
- The HIF-1 farmacological inhibitor topotecan, N-acetyl cystein (NAC), kaempferol, docetaxel and metformin were purchased from Sigma-Aldrich.
- DASA-58 was kindly provided by Prof. Cristina Nativi, Department of Chemistry, University of Florence.
- Transwells for invasion assays were from Costar (Euroclone Group). The Diff-Quik staining kit was purchased from BIOMAP SNC.
- Matrigel was purchased from BD Biosciences.
- Proteases and phosphateses inhibitors were from Sigma-Aldrich.
- Bradford reagent for protein dosage and all materials for SDS-PAGE were from Biorad.
- PVDF membrane (Polyvinylidene fluoride) used for western blotting was from Millipore.
- All the primary antibodies used were purchased from Santa Cruz Biotechnology except antibodies against HIF-1 (BD Biosciences), SIRT3 (Cell Signaling), SOD2 (Abcam), acetyl-lysine (Abcam), CA IX (Bioscience Slovakia), PKM2 (Cell Signaling), hexokinase II (Millipore). The secondary antibodies enzyme horseradish peroxidase (HRP)–coniugated were from Santa Cruz Biotechnology.
- Chemiluminescence revelation kit is from GE Health Care.
- The photographic plates were from Kodak.
- HIF-1α-siRNA (sc-35561), MCT1-siRNA (sc-37235), and SIRT3-siRNA (sc-61555), CA IX-siRNA (sc-29869), MMP-9-siRNA (sc-29400) were from Santa Cruz Biotechnology. siRNA products from Santa Cruz generally consist of pools of three to five target-specific 19–25 nucleotide siRNAs designed to knockdown gene expression. Scramble shRNA plasmid (sc-108060) and CA IX shRNA

plasmid (sc-29869-SH) from Santa Cruz was used for *in vivo* studies .TaqMan Reverse Transcription Reagents Kit, Lipofectamine 2000 and GLUT1 primers were from Invitrogen.

- Mitosox and 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) were from Molecular Probe (Invitrogen).
- The cytofluorimetric apoptosis staining kit Annexin V-IP Fluos Staining Kit was from Roche.
- The metalloproteinase catalytic activity evaluation kit Amplite^{TM} Universal Fluorimetric MMP Activity Assay Kit - Red Fluorescence was purchased from AAT Bioquest.
- The CA IX inhibitors 4-(2,4,6-trimethylpyridinium)-ethyl-benzenesulfonamide, perchlorate salt (compound 1) (FC3-148B bis), benzothiophene-3-ylmethylsulfamide (compound 2) (FC5-207A), the fluorescent CAI (compound 3) and MMP-9 inhibitors GlcNAc-SLS-HA and Lac-SLS-HA were previously reported by Alterio et al., 2009 and Calderone et al., 2006. It should be mentioned that compound 3 is a potent CA IX inhibitor, with a K_I of 24nM against this isoform. (Supuran et al. 2008). It has also been proven that it binds the enzyme only in hypoxia and not normoxia (Švastová et al., 2004).
- All radiolabelled molecules ([U-^{14}C]-glucose, [U-^{14}C]-lactate, [^{3}H]-deoxy-glucose, [1-^{14}C]-glucose and [6-^{14}C]-glucose) were purchased from PerkinElmer.

COMMON USE SOLUTIONS

- SDS−PAGE 4X Sample Buffer: 40% Glycerol, 240 mM Tris/HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% β-mercaptoethanol.
- SDS-PAGE 1X running buffer: 25 mM Tris, 192 mM glycin, 0.1% (W/V) SDS, pH 8.3.
- SDS-PAGE 1X blotting buffer: 25 mM Tris, 192 mM glycin, 10% methanol, pH 8.3,.
- RIPA lysis buffer: (50 mM Tris.HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EGTA, 1mM sodium ortovanadate, 100 mM NaF).

- Washing solution: tween 0.1 % in PBS.
- Blocking solution: non-fat dry milk 2 %, tween 0.05 % in PBS.
- PBS (Phosphate buffered saline). 0.27 g/L di KH_2PO_4, 0.2 g/L KCl, 8.01 g/L NaCl , 1.78 g/L NaH_2PO_4 pH 7.4.

METHODS

CELL CULTURES AND TREATMENTS

All cell types were cultured in DMEM high glucose (4,5 g/L) (Dulbecco's Modified Essential Medium), supplemented with 10% bovine fetal serum (FCS), 2mM glutammine, 100U/mL penicillin and 100 μg/mL streptomicin, at 37°C in a 5% CO2 humidified atmosphere.

Human PCa cells (PC3 cell line) 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 (HPFs) and prostate cancer associated fibroblasts (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 respectively. HPFs and CAFs between 4 and 10 passages were used and tested by mycoplasma presence. Activation state of CAFs was confirmed by morphology and fibroblast activation protein expression.

CAFs/PCa co-culture was performed by plating HPFs and PCa cells in different proportions (2:1, 3:1, 5:1, 10:1).

Docetaxel-resistant PCa cell line (DoceRes) was developed from PC3 sensitive cells by stepwise increased concentrations of docetaxel. As cells displayed resistance to treatments of docetaxel, the concentration was subsequently increased until the final treatment doses of 10 nM. Resistance was judged based on decreased cell death.

Conditioned media (CM) from PCa cells, HPFs and CAFs were obtained by 48h serum-starved cells (DMEM high glucose, 2mM glutammine, 100U/mL penicillin and 100

μg/mL streptomicin, at 37°C in a 5% CO2 humidified atmosphere), clarified by centrifugation and used freshly.

Treatments: 20nM NAC, 250nM topotecan, 25-50μM kaempferol, 10nM doectaxel, 5mM metformin, 40μM DASA-58, Ca IX inhibitors 1 and 2 (100 μM final concentration), MMP-9 inhibitors GlcNAc-SLS-HA and Lac-SLS-HA (54 and 76 nM final concentration, respectively) were added to cells at the beginning of experiments and maintained until the end.

ISOLATION OF PROSTATE FIBROBLASTS

HPFs and CAFs were isolated from surgical explantations. A small slice of the tissue piece was minced with sterile scalpels and pieces of <1 mm in size were plated in Petri plates and covered with covering glasses, favouring pieces compression and fragmentation. Complete DMEM supplemented with 20% FCS, 2 mM glutammine, 100 U/ml penicillin, 100 μg/ml streptomicin, 100 mg/L Kanamicyn and AMFO (Amphotericyn B).

After 3-4 days culture medium was removed and fresh medium was then added. Days required for fibroblast cells exit from bioptic fragments is highly variable and depends on the type of bioptic tissue and the number of fibroblasts composing it. Usually it takes 20-30 days and, after this period, tissutal fragments were removed with steril tweezers and trypsinyzed, thereby promoting fibroblasts isolation, as epithelial cells are not able to re-adhere to plate surface. Obtained fibroblasts were then maintained in culture with complete DMEM supplemented with 10% FCS, 2mM glutammine, 100U/ml penicillin, 100 μg/ml streptomicin.

FIBROBLASTS AND PCa CELLS ACTIVATION

HPFs were grown to subconfluence and treated for 24h with CM-PCa to obtain PCa-activated fibroblasts (PCa-AFs). Fresh serum-free medium was added PCa-AFs for an additional 24h before collection of CM-PCa-AFs. Activation state of PCa-AFs was confirmed by morphology and fibroblast activation protein expression. Note that PCa-AFs behave similarly to *in vivo* extracted CAFs (Giannoni et al., 2010). CM-HPFs, CM-CAFs and CM-PCa-AFs were used to treat PCa cells for 48h.

CELL TRANSFECTION WITH LIPOFECTAMINE

Silencing with siRNA or shRNA plasmid was performed with Lipofectamine 2000 following manufacturer's instructions (Invitrogen). Cells plated at a 90% confluence and before transfection, DMEM culture medium is removed and replaced with Optimem medium (Invitrogen), lacking serum and antibiotics that could interfere with liposomes formation. Solution containing siRNA/shRNA was added to solution with lipofectamine and incubated at room temperature for 20 minutes, in order to promote liposomes formation; then equal amounts of final solution were added to each plate. Optimem medium was removed after 4-6 h from transfection, as lipofectamine could be slightly toxic for cells. Finally, cells were maintained in complete medium for 48 h and transfection efficiency was evaluated through immunoblotting assays using specific antibodies for the silenced protein.

PROLIFERATION ASSAYS

PCa cell proliferation has been measured cytometrically by the use of carboxyfluorescein diacetate succinimidyl ester (CFSE). Cells were treated with 10 mmol/L CSFE for 15 minutes 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 coculture were grown for 5 or 7 days, in presence or absence of 1 mg/mL 2-DG, 0.5 mM DCA acid, 20 nM Antimycin A, 25-50 μM kaempferol, and PCa clones were counted under an optical microscope.

Crystal violet proliferation assay. Crystal violet (CV) is a triphenylmethane dye (4-[(4-dimethylaminophenyl)-phenyl-methyl]-N,N-dimethyl-aniline) also known as Gentian violet (or hexamethyl pararosaniline chloride). 20 x 10^3 PCa cells were plated in 24-well culture dishes and coltured in complete DMEM for 24h and 48h. Then cells was washed with PBS and incubate with crystal violet solution for 5 minutes at 37 C°. The crystal violet solution contains 0,5% crystal violet in deionized water and 20% methanol. After incubation crystal violet was removed through aspiration and three washings in PBS solution. Finally, crystal violet uptaken by cells was solubilized after

incubation in slow agitation for 1 h at 37 C° with a solution containing 0,1M sodium citrate (Sigma-Aldrich), pH 4,2. After incubation, solution containing solubilized crystal violet was collected and its absorbance was evaluated at a 595nm wavelenght. Each measurement was made in triplicate for each point of the curve of growth. Absorbance is positively correlated to crystal violet amount bound to cells.

INVASION ASSAY

Transwell system, equipped with 8μm pore polyvinylpirrolidone-free polycarbonate filters (6.5 mm diameter) were used. Cells were loaded into the upper compartment (1×10^5 PCa cells in 200μL of serum-free medium, CM-HPFs or CM-PCa-AFs). The upper sides of the porous polycarbonate filters were coated with 50 ug/cm^2 of reconstituted Matrigel and placed into 24-well culture dishes containing 500μl of complete growth medium. After 18 h of incubation at 37°C, non-invading cells were removed mechanically using cotton swabs, and the micro porous membrane was stained with Diff-Quick solutions. Chemotaxis was evaluated by counting the cells migrated to the lower surface of the filters (six randomly chosen fields).

PROSTASPHERE FORMATION

PCa cells were detached using Accutase (Sigma-Aldrich). For prostasphere formation, single cells were plated at 150 cells/cm^2 on low-attachment 100mm plate (Corning) in DMEM/F12 (Invitrogen) supplemented with B27 and N2 (Invitrogen), 5μg/mL insulin, 20ng/mL basic fibroblast growth factor (FGF) and 20ng/mL epidermal growth factor. Cells were grown under these conditions for 7 to 21 days and formed nonadherent P0 spheres termed prostaspheres.

ISOLATION OF PCa CELLS FROM CO-CULTURE

PCa cells were isolated from fibroblasts using MACS® system (Miltenyi Biotec). First, cells were detached from plates with Accutase and fibroblasts were magnetically labeled with Anti-Fibroblast MicroBeads, which bind fibroblast-specific antigen. Then, the cell suspension was loaded onto a MACS® Column, which was placed in the magnetic field of a MACS Separator. The magnetically labeled fibroblasts were retained

within the column. The unlabeled PCa cells run through; this cell fraction is thus depleted of fibroblasts.

ANNEXIN V/IODIDIUM PROPIDE CYTOFLUORIMETRIC STAINING

Cells were washed in PBS solution and detached from plates with Accutase solution. The advantages of Accutase over the traditional Trypsin/EDTA treatment are that it is less damaging to cells. In this case cells treatment with Accutase carries lower risk to disrupt Annexin V antigens, expressed on the outer side of cell membranes during apoptotic events.

After cells removal from the plates, they were resuspended in PBS solution and centrifugated at 1000 rpm for 3 minutes. Finally, pelletted cells were resuspended in 100µl containing 1µl of annexin V, 1µl of iodidium propide and 98µl of buffer solution, all provided by the kit. After 15 minutes of incubation at room temperature, cells were evaluated by flow citometry for Annexin V/iodidium propide staining.

CELL LYSIS AND PROTEIN QUANTIFICATION

In order to identify proteins of interest, cell lysates were separated through SDS-PAGE (polyacrylammide gel elettrophoresis) and revealed with Western Blotting analysis.

Cells were washed once with PBS solution and then lysated with RIPA lysis buffer supplemented with proteases inhibitors. Obtained protein lysates were collected, always kept in ice and centrifugated at 13000rpm for 10 minutes. After centrifugation, supernatant was collected and total proteins were quantified with Bradford assay.

Total protein quantification, espresse in µg/mL, is evaluated with Coomassie Brilliant Blue (Bradford protein assay), which binds to basics and aromatics aminoacidic residues (specially arginins), leading to maximum absorption at 595nm wavelegth. Thus, Coomassie Brilliant Blue intensity is positively correlated to protein concentration.

For the standard curve bovine serum albumine was used (BSA), diluting BSA 2 mg/ml concentrated in deionized water and then obtaining rising BSA concentrations from 2µg/mL to 15µg/mL.

Then Bradford reagent is prepared diluting 1/5 of starting solution with Coomassie Brilliant Blue in 4/5 of deionized water.

To run the assay, 50 µL of each sample, opportunely diluted in water in labelled eppendorfs, must be added to 950 µl of the working solution for each sample, resuspending well. After a 5 minutes incubation, the absorbance of each sample is evaluated at a wavelength of 595nm, subtracting the blank value. From values obtained from the standard curve it is possible to create a curve of absorbance in function of its concentration, thus, interpolating absorbances values to the standard curve, it is possible to calculate final protein concentration.

Correlation between absorbance and concentration is expressed by Lambert-Beer law: $A=\varepsilon dc$, where ε represents the molar extinction coefficient, d the path length and c represents sample concentration.

For each Western Blotting experiment usually 20-25 µg of total proteins are loaded on SDS-PAGE for each sample.

NUCLEAR FRACTIONATION

Cells were harvested by spinning at 1000rpm for 5 min at 4°C and then washed cells with cold PBS. After that, cells were resuspended in ice cold Buffer A (10 mM Hepes pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 1mM DTT, plus protease and phosphatase inhibitors), washed again with Buffer A (spin at 1000rpm, 5 min at 4°C) and lysated with buffer A+0.1% NP-40 (5 min. on ice). Volume of Buffer A+NP=40 should be 2-3X volume of cell pellet. You can also use a dounce homogeniser. Cell lysates were centrifuged at max speed in microcentrifuge for 10 min. at 4°C. Supernatant is cytoplasmic extract. Pellets were resuspended in Buffer C (20mM Hepes pH 7.9, 420mM KCl, 1.5mM $MgCl_2$, 1mM DTT, 25% Glycerol plus protease and phosphatase inhibitors, 1-2 volumes of pellet) and rocked for 15 min. at 4°C. After centrifugation at max speed for 15 min, supernatants (nuclear extracts) were collected and then diluted with Buffer D (20mM Hepes pH 7.9, 0.5mM DTT, 20% Glycerol plus protease and phosphatase inhibitors) to reduce the salt concentration.

SDS-PAGE ANALYSIS

Sodium dodecyl sulfate polyacrylammide gel electrophoresis (SDS-PAGE) is a technique for separating proteins based on their ability to move within an electrical current, which is a function of the length of their polypeptide chains or of their

molecular weight. This is achieved by adding SDS detergent to remove secondary and tertiary protein structures and to maintain the proteins as polypeptide chains. The anionic SDS coats the proteins (almost one SDS molecule binds every two aminoacidic residues of the polipeptidic chain), mostly proportional to their molecular weight, and confers the same negative electrical charge across all proteins in the sample.

SDS-PAGE samples are boiled for 5 minutes in a sample buffer containing SDS and β-mercaptoethanol, which leads to disolfuric bonds reduction and destabilization of eventual protein tertiary structure. In addition, sample buffer is supplemented with bromophenol blue, ionizing coloured-tracking solution for the electrophoretic run, and glycerol, which increases sample density and promotes its stratification ate the bottom of the loading well.

Once finished samples loading in the stacking gel, an electric field is applied across the gel, causing the negatively-charged proteins to migrate across the gel towards the positive electrode (anode). Stacking gel, characterized by very low acrilamide concentration (4%), is required to better stratify the samples before entering the separating gel. Separation of SDS-proteins complexes is achieved according to separating gel acrylamide concentration. Lower percentage gels are better for resolving very high molecular weight proteins, while much higher percentages are needed to resolve smaller proteins, while bromophenol blue is a very small molecule which is not affected by frictional forces, thereby representing the migration front. Proteins relative molecular mass is evaluated by comparison with protein ladder standard molecular weights, separated in the same gel. Running is carried on at 200V for almost 1 h.

WESTERN BLOTTING

After run, polyacrylamide gel is maintained for 5 minutes at room temperature in slow agitatation in the transfer blot. In order to make the proteins accessible to antibody detection they are moved from within the gel onto a membrane made of polyvinylidene difluoride (PVDF). The method for transferring the proteins is called electroblotting and uses an electric current to pull proteins from the gel into the PVDF membrane. The proteins move from within the gel onto the membrane while maintaining the organization they had within the gel. Proteins transfer is carried out at 100V for 1 h.

PVDF membrane must be previously activated through treatment with methanol for 15 seconds and left at drying for at least 15 minutes at room temperature.

After electroblotting the PVDF membrane is incubated overnight in slow agitation at 4°C with specific primary antibodies in a blocking solution containing non-fat dry milk 2% and Tween 0.05%. After incubation, the membrane is washed three times with a washing solution containing PBS 1X and Tween 0.1% and, in order to reveal the specific protein, the membrane is incubated with horseradish peroxidase conjugated secondary antibody for 1h at room temperature and then washed again for three times. In the chemiluminescence reaction horseradish peroxidase catalyzes the oxidation of luminol into a reagent which emits light when it decays. Since the oxidation of luminol is catalyzed by horseradish peroxidase, and the HRP is complexed with the protein of interest on the membrane, the amount and location of light that HRP catalyzes the emission of, is directly correlated with the location and amount of protein on the membrane. Chemiluminescent protein revelation is carried out with ECL-Amersham Pharmacia kit reagents and developing of blots is carried out in the developing room placing imaging films on top of the membrane. Exposure is repeated, varying the time as needed for optimal detection.

REAL-TIME PCR

Total RNA was extracted from cells using the RNeasy Minikit kit (Qiagen). RNA (1 µg) was reverse transcribed using TaqMan Reverse Transcription Reagents Kit following manufacturer's instructions (Invitrogen). 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.

ROS EVALUATION

For the evaluation of intracellular ROS amount, staining with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) probe was performed. H_2DCF-DA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione

and other thiols. H_2DCF-DA is solubilized in DMSO at moment of usage and avoided from light exposure.

H_2DCF-DA (5μM) probe was added to cells and incubated for 3 minutes at 37°C. After the incubation time, cells are blocked through PBS washing, quickly lisated in RIPA lysis buffer supplemented with proteases inhibitors and kept for 1 minute at 4°C. Lysates were then collected and centrifugated for 1 minute at 13000rpm. For each sample 100μl were transferred on a 96 wells multiwell and analyzed by fluorimetric quantification (excitation wavelength of 488nm, emission wavelength of 510nm). Obtained values were finally normalized on protein content of each sample.

Evaluation of mitochondrial ROS was performed adding 5μM 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.

GLUCOSE AND LACTATE UPTAKE

2-deoxy-glucose or lactate uptake was evaluated in a buffered solution (140mM NaCl, 20mM Hepes/Na, 2.5mM $MgSO_4$, 1mM $CaCl_2$, and 5mM KCl, pH 7.4) containing 0.5μCi/mL [^{3}H]-deoxyglucose or 0.2μCi/mL D-[U-^{14}C]-lactate for 15 minutes at 37°C. Cells were subsequently washed with cold PBS and lysed with 0.1M NaOH. Incorporated radioactive was assayed by liquid scintillation counting and normalized on protein content. PCa cells were treated with CM or co-cultured with HPFs for 72h (unless specified) before the analysis.

INCORPORATION OF LACTATE INTO PROTEINS

PCa cells were treated with CM or co-cultured with HPFs for 72h and then [U-^{14}C]-lactate was added for additional 24h. 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 GLUCOSE AND LACTATE

PCa cells were treated with CM or co-cultured with HPFs for 72h (unless specified) and then 0.2μCi/mL D-[U-^{14}C]-lactate or 0.5μCi/mL [U-^{14}C]-glucose were added for 15 minutes. Each dish had a taped piece of Whatman paper facing the inside of the dish wetted with 100μL of phenyl-ethylamine-methanol (1:1) to trap the CO_2. Then 200μL of 4M H_2SO_4 was added to cells. Each plate was incubated for 37°C, 5%CO_2 for 1h to permit releasing of $^{14}CO_2$. Finally, Whatman paper was removed and transferred to scintillation vials for counting.

MEASUREMENT OF PPP ACTIVITY

PPP activity was evaluated by using [1-^{14}C]-glucose and [6-^{14}C]-glucose. $^{14}CO_2$ developed from [1-^{14}C]-glucose oxidation originates by the PPP or by the TCA cycle, whereas $^{14}CO_2$ released from [6-^{14}C]-glucose originates only by TCA cycle. 2μCi [1-^{14}C]-glucose or 2μCi [6-^{14}C]-glucose were added for 1h to cells, in two different plates of same sample. Each plate had a taped piece of Whatman paper facing the inside of the dish wetted with 100μL of phenyl-ethylamine-methanol (1:1) to trap the CO_2. Then, 200μL of 4M H_2SO_4 was added to cells. Each plate was incubated for 37°C, 5%CO_2 for 1h to permit releasing of $^{14}CO_2$. Finally, Whatman paper was removed and transferred to scintillation vials for counting. The extent of PPP metabolic flux was obtained by subtracting the amount of CO_2 developed from [6-^{14}C]-glucose from the CO_2 released from [1-^{14}C]-glucose.

ANALYSIS OF CA IX ACTIVITY

CA IX activity was analyzed either by confocal microscopy or fluorescence stop-flow. For confocal microscopy analysis, cells were cultured on glass coverslips and treated with appropriate medium depending on the experiments. After the treatment, cells were washed with PBS and the CA IX fluorescent probe FITC-labeled CAI#3 (1mM, final) (compound 3) (Supuran et al., 2008) was added to living cells for 1h. Cells were then washed extensively with PBS, mounted with glycerol plastine and observed with a fluorescence microscope (Leica TCS SP5) (Švastová E. et al., 2004). An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2

hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557nm, with 20mM Hepes (pH 7.4) as buffer, and 20mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO2 hydration reaction for a period of 10–100 sec. The CO_2 concentrations ranged from 1.7 to 17mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10mM) were prepared in distilled-deionized water, and dilutions up to 0.001nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were pre-incubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, as reported earlier, and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in house as reported earlier (Supuran et al. 2008; Alterio et al., 2009).

METALLOPROTEINASE ANALYSIS

Zimography was performed using cultured media collected in our experimental conditions. Aliquots of cultured media were electrophoresed on 8% SDSPAGE co-polymerized with 0.1% (w/v) type A gelatine. Gels were washed twice in 2.5% v/v Triton X-100 for 30 min and then incubated in 50mM TRIS-HCl, pH 7.4, 200mM NaCl and 5mM $CaCl_2$ at 37°C for 24 h. After incubation, the gels were stained with 0.1% Coomassie brilliant blue in acetic acid, methanol and distilled water (1:2:3, respectively) for 60 min at room temperature. After destaining, the gels were immersed in distilled water and scanned immediately with Quantity-One Image Analysis software (Bio-Rad). Bands of gelatinase activity appeared as transparent areas against a blue background. Metalloproteinases activity was measured with Amplite™ Universal Fluorimetric MMP Activity Assay Kit according to the manifacturer's instructions.

ANALYSIS OF MMP-9 INHIBITORS BY FLUORIMETRIC ASSAYS

The values of K_i for the compounds GlcNAc-SLS-HA and Lac-SLS-HA were determined by evaluating their ability to inhibit the hydrolysis of fluorescence-quenched peptide substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Biomol, Inc). The analyses have been performed at 298K in 50mM HEPES buffer, containing 10mM $CaCl_2$, 0.05% Brij-35, at pH 7, using 1nM of MMP catalytic domains and 1μM of peptide. The experiments were performed with peptide concentration much lower than K_i. The fluorescence (excitation max 328nm; emission max 393nm) was measured for 3 min after the addition of the substrate using a Varian Eclipse fluorimeter. Fitting of rates as a function of inhibitor concentration provided the Ki values (Calderone et al., 2006).

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 6–8-wk-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 2–3 d by a caliper. Tumor volumes were determined by the length (L) and the width (W): V = (LW2)/2.

Histology. Tissues were fixed in 4% (vol/vol) phosphatebuffered formalin and paraffin embedded. Consecutive sections with 5μm thick were mounted on positively charged slides. Tissue sections were de-paraffinazed, re-hydrated and stained with hematoxylin-eosin or Azan-Mallory trichromic stainings. Metastases were counted using NIS ELEMENTS_F-2.20 software combined with a microscope Nikon Eclipse 50i.

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.

EXPERIMENTAL PART I

Aim of study

It is now well established that cancer cells undergo profound changes in their metabolism (Hanahan and Weinberg, 2011). Several studies showed that tumor cells typically use glucose by aerobic glycolysis, producing lactate and undergoing the so called Warburg effect, thereby facilitating anabolism of macromolecules needed to construct a new cell from glycolytic intermediates (Vander Heiden et al., 2009). Deregulation of oncogenes/tumor suppressor genes molecular pathways is involved in metabolic changes in cancer cells, leading to expression or down-regulation of protein and enzyme of cell metabolism (Chen and Russo, 2012).

In the last years, tumor stroma, and in particular CAFs, are emerging as key players in metabolic reprogramming of tumor mass, thus sustaining cancer cell growth and affecting tumor progression. A number of studies demonstrated the existence of metabolic coupling between stroma and tumor, but controversial results have been obtained. Indeed, colorectal carcinoma histological analyses suggest that the stroma infiltrating this cancer buffers and recycles products of anaerobic metabolism of cancer cells, in order to sustain invasive cancer growth (Koukourakis et al., 2006). On the contrary, fibroblasts undergoing activation due to deletion of Cav-1 experience oxidative stress and a HIF-1-mediated shift toward aerobic glycolysis, which is associated with elimination of mitochondrial activity through autophagy/mitophagy process (Pavlides et al., 2009; Lisanti et al., 2010). As a consequence of these profound metabolic alterations, CAFs secrete energy-rich metabolites (such as lactate, ketones, and pyruvate, derived from glycolysis) and chemical building blocks (such as amino acids nucleotides, and lipids, derived from autophagy) that can sustain and support the mitochondria-dependent growth of tumor cells (Sotgia et al., 2012). In agreement, in breast cancers, PKM2 and lactate dehydrogenase, two enzymes associated with aerobic glycolysis, are highly expressed in stromal CAFs (Pavlides et al., 2009).

In this controversial context, we analyzed if prostate fibroblasts undergo a glycolytic shift in response to PCa contact, thereby engaging a metabolic interplay with PCa cells.

Furthermore, given that CAFs have been associated by our research group to a pro-oxidant environment, deeply affecting tumor progression and metastatic spread (Giannoni et al., 2010; Giannoni et al., 2011), we also investigated the role of ROS in metabolic reprogramming of CAFs and PCa cells. We demonstrated that, following activation, CAFs shift their metabolism toward a more glycolytic one, through a HIF-1- 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.

Results

ANALYSIS OF HPFs AND CAFs FROM HUMAN PATIENTS

In order to investigate the metabolic interplay between PCa and prostatic fibroblasts, we first analyzed the metabolic signature of CAFs. We have already demonstrated that CAFs from aggressive prostate carcinoma undergo *in vivo* activation in response to factors secreted by cancer cells, achieving a "reactive" state characterized by expression of typical myfibroblasts markers, such as α-SMA and FAP (Giannoni et al., 2010). Human prostate CAFs were isolated from prostate biopsies from nine patients with PCa, whose pathological values are reported in Tab. 1. Our choice has been driven by cancer aggressiveness and PSA (Prostate-Specific Antigen) values. As healthy counterparts, we used HPFs isolated from four men with benign prostatic hyperplasia. Of note, HPFs do not express markers of activated fibroblasts (Giannoni et al., 2010). HPFs and CAFs were then used to perform *ex vivo* cultures.

	PSA (ng/ml)	Biopsy Gleason Score	Anatom.Pathol. Gleason Score	Pathological Stage
CAF 1	5.13	4+5	4+4	pT3a N0
CAF 2	9.0	4+3	3+5	pT3a N0
CAF 3	7.3	3+3	4+5	pT3a N0
CAF 4	3.4	3+4	4+5	pT3b N0
CAF 5	5.33	4+5	4+4	pT3a N0
CAF 6	6.74	3+3	3+4	pT3a N0
CAF 7	9.0	4+3	3+5	pT3a N0
CAF 8	6.32	4+4	4+3	pT3a N0
CAF 9	5.93	3+3	3+3	pT2c N0

Table 1. Pathological parameters of individuals from which CAFs have isolated; CAFs have been isolated from surgical biopsy from nine individuals with prostate carcinoma. Table reports the pathological parameters of the patients before surgery (PSA and Biopsy Gleason Score) and after surgery (Anatomy Pathologic Gleason Score and Pathological Stage).

We firstly observed that CAFs show increased basal glucose uptake compared to HPFs, consistent with increased glycolytic pathway (Fig. 1A). In keeping, the analysis of gene pathways revealed up-regulation of glycolytic enzymes in CAFs respect to HPFs, such as PKM2, ALDO-A/B/C (aldolase), ENO-1/2 (enolase), TPI-1(triose-phosphate isomerase), further confirming activation of glycolysis (Fig. 2A, 2B). We also observed

that CAFs show increased expression of monocarboxylate transporter MCT4 (Fig. 1B). MCT4 has low affinity for lactate and is consequently expressed by glycolitic cells in order to release the metabolite in extracellular compartment, thereby validating the idea that prostate CAFs increase their lactate production with respect to healthy HPFs. Finally, we observed that both cytoplasmic and mitochondrial ROS are greater in CAFs compared to HPFs, thus demonstrating that CAFs experience a state of oxidative stress compared to HPFs (Fig. 1C and 1D). This last findings are in agreement with other data in literature reporting that ROS are important players for fibroblast activation (Toullec et al., 2010). Taken together, these results indicate that prostate CAFs, under normoxic conditions, undergo a Warburg metabolism due to their activation in response to cross-talk with cancer cells.

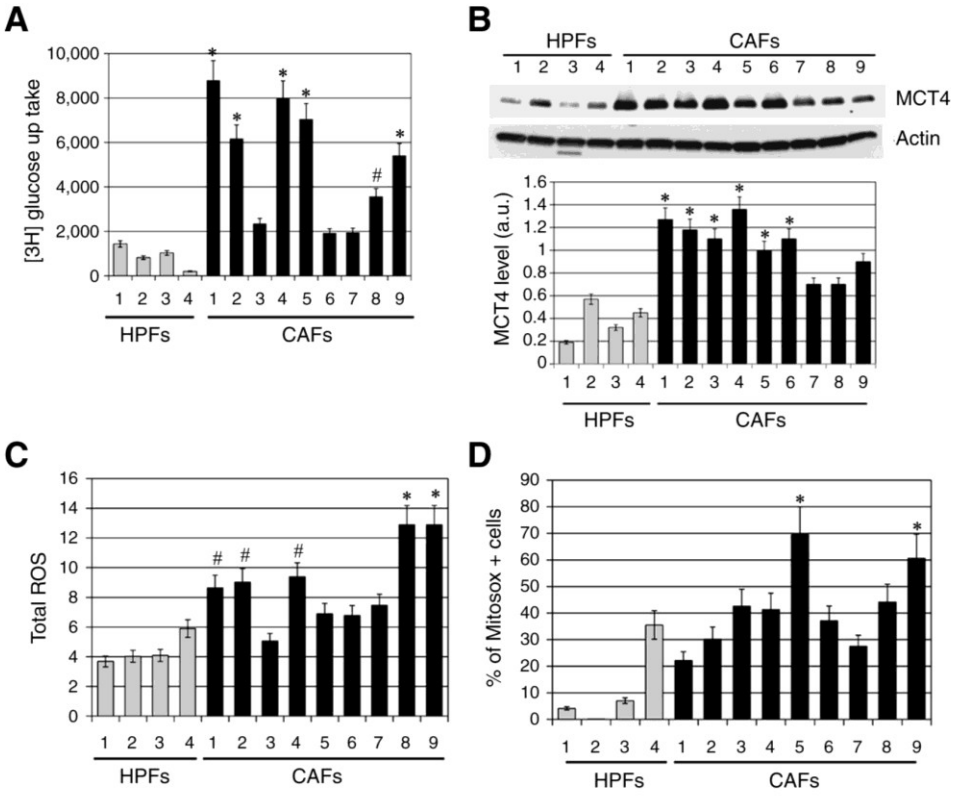

Fig. 1. Analysis of human ex vivo HPFs and CAFs. A) analysis of [^{3}H]-glucose uptake in HPFs and CAFs. **B)** MCT4 immunoblot in HPFs and CAFs. Normalization was done by actin immunoblot. The plot below reports densitometric quantitation (ratio MCT4:actin). Oxidative stress in CAFs and HPFs evaluated as level of total ROS released, analyzed using H_2DCF-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.

A

Gene set	Normalized Enrichment Score	Nominal p-value	FDR q-value
MOOTHA_GLYCOLYSIS (*C2*)	1.9957	0.0	0.0135
CARBOHYDRATE_CATABOLIC_PROCESS (*C5*)	1.7034	0.0185	0.2785

B

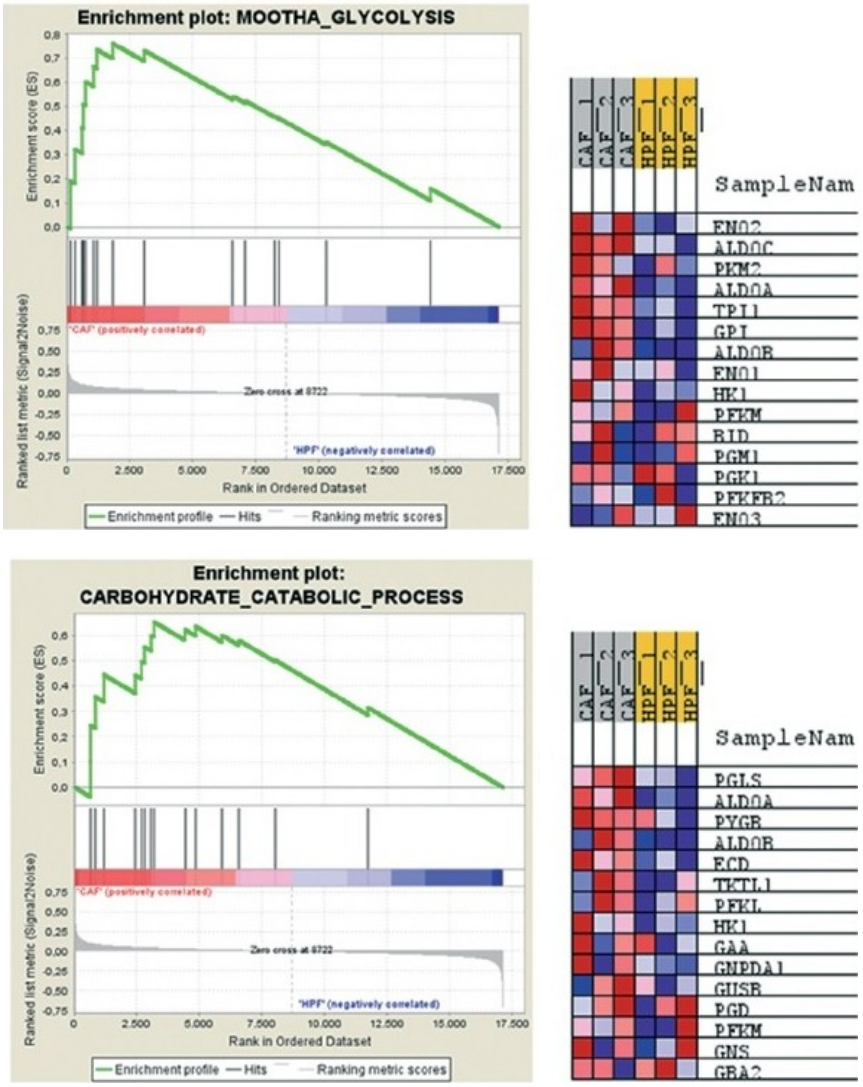

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Fig. 2 (See previous page). Enrichment of glycolitic enzymes among genes up-regulated in prostatic CAFs. Gene expression profiles were analyzed using an Illumina microarray platform on paired CAFs and HPFs isolated respectively from the tumor and the adjacent normal tissue from three prostate cancer patients subjected to radical prostatectomy. Gene Set Enrichment Analysis (GSEA) (ref1, ref2 http://broadinstitute.org/gsea) was conducted between CAFs and HPFs, and revealed, among the others, a significant enrichment for gene sets related to carbohydrate catabolism among the genes that were up-regulated in CAFs. Specifically, when the analysis was carried out searching among gene ontology gene sets (C5), enrichment was found for Carbohydrate_Catabolic_Process set, whereas and ever higher enrichment was found for a set of glycolytic enzymes (referred to as Mootha_Glycolysis) when the analysis was performed on literature based gene sets (C2). **A)** Table showing normalized enrichment scores and significance for enriched gene set. **B)** Enrichment plot (left) and heatmaps of gene intensities (left) for the gene set emerged from GEMSA. In the heatmaps, gene intensities are colored from blue (low expression) to red (high expression). (ref1) Subramanian, Tamayo et al. (2005) PNAS 102, 15545-15550 (ref2) Mootha, Lindgren et al. (2003) Nat Genet 34, 267-273.

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 conditioned medium (CM) from PCa cells. Indeed, we have already reported that PCa cells secrete soluble factors (such as IL-6) able to elicit a mesenchymal-mesenchymal transition in HPFs, leading them to an activation state similar to myofibroblasts (Giannoni et al., 2010) with expression of specific markers (e.g., FAP). We termed these *in vitro* activated HPFs as PCa-activated fibroblasts (PCa-AFs). As CAFs, PCa-AFs show a clear increase in lactate secretion compared to HPFs (Fig. 3A), likely due to up-regulation of MCT4 transporter (Fig. 3B). Activation of fibroblasts does not affect their utilization of lactate, as revealed by immunoblot analysis of MCT1 expression level (Fig. 3B). The latter has high affinity for lactate, thereby promoting its uptake in cells (Halestrap et al. 1999; Dubouchaud et al. 2000; Ullah et al. 2006). As a consequence, we found no difference in lactate up-load between HPFs and CAFs (Fig. 3B). Finally, PCa-AFs increase both expression of GLUT1 glucose transporter and glucose uptake (Fig. 3C, 3D). These data confirm that activated fibroblasts undergo a metabolic shift toward a Warburg metabolism.

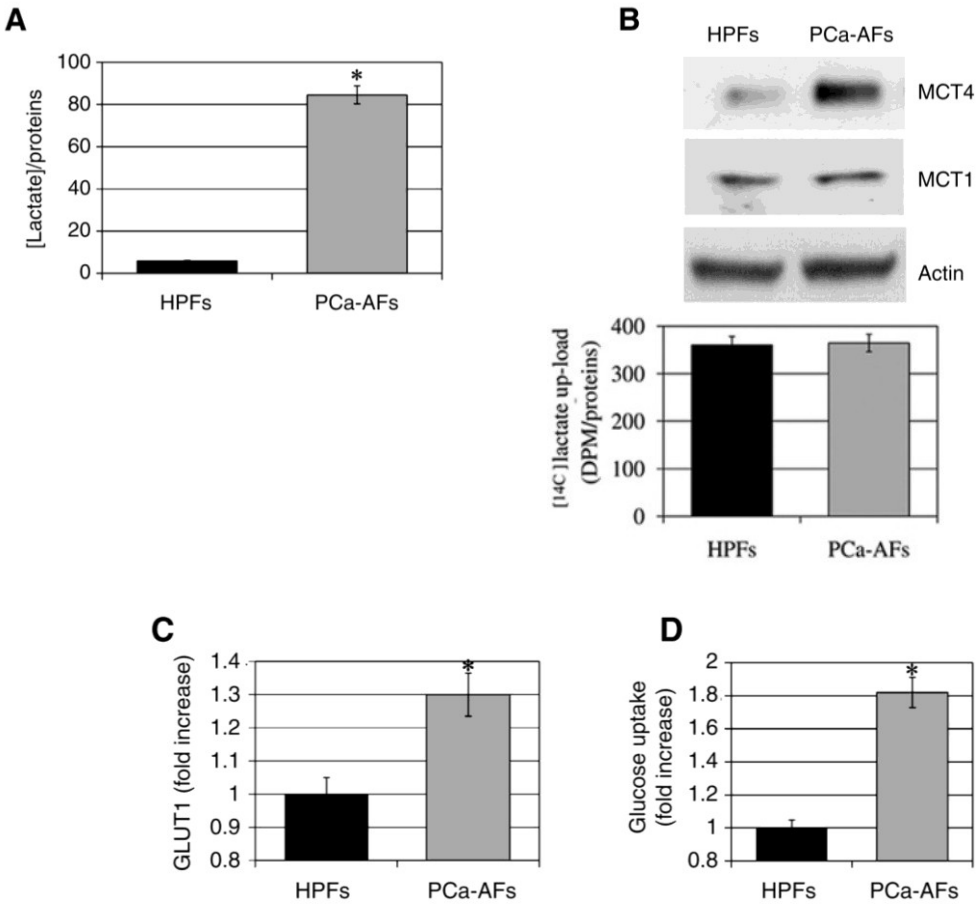

Fig. 3. Analysis of Warburg metabolism in HPFs activated *in vitro*. HPFs were treated with CM-PCa for 24 hours obtaining PCa-AFs, then serum-free medium was added for an additional 48 hours. **A)** Lactate assay in culture medium. **B)** Upper panel: MCT4 and MCT1 immunoblots in HPFs and PCa-AFs. Normalization was done by actin immunoblot. Lower panel: lactate up-load by HPFs and PCa-AFs. **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.

THE WARBURG METABOLIC SHIFT IN CAFs IS REDOX- AND HIF-1-DEPENDENT

It has been reported that differentiation of CAFs toward a myofibroblast phenotype is a redox dependent event (Toullec et al., 2012; Cat et al., 2006). HIF-1 transcription factor promotes expression of many glycolytic enzymes, thus driving anaerobic metabolism during exposure to hypoxia. Of note, even under normoxic conditions, HIF-1 can be induced by several factors, such as ROS, TCA cycle metabolites and oncogene gain of functions or tumor suppressor gene loss of function, thereby promoting glucose metabolism by aerobic glycolysis (McFate et al., 2008; Semenza, 2010b; Semenza,

2010a). We hypothesized that the conversion to a Warburg phenotype of HPFs in normoxia could be dependent on oxidative stress, which in turn could drive HIF-1 stabilization and consequent activation of its transcriptional program. To confirm this hypothesis, we firstly analyzed the intracellular ROS in HPFs and PCa-AFs. As previously reported for CAFs (Fig.1), PCa-AFs have a higher basal level of ROS with respect to HPFs (Fig. 4A). In agreement with ROS content, PCa-AFs show a clear stabilization of HIF-1 under normoxic conditions (Fig. 4C). Treatment of PCa-AFs with the ROS scavenger N-acetyl cysteine (NAC) leads to down-regulation of HIF-1 (Fig. 4C), thus confirming the ROS-mediated stabilization of this transcription factor in activated fibroblasts. Finally, the inhibition of HIF-1 either by RNA interference, by topotecan (a known HIF-1 pharmacological inhibitor; Rapisarda et al., 2004) or by NAC decreases both expression of MCT4 transporter (a target gene of HIF-1) and lactate extrusion in PCa-AFs (Fig. 4B, 4C and 4D). Taken together, these data confirm that redox-dependent stabilization of HIF-1 leads to the Warburg phenotype in activated fibroblasts.

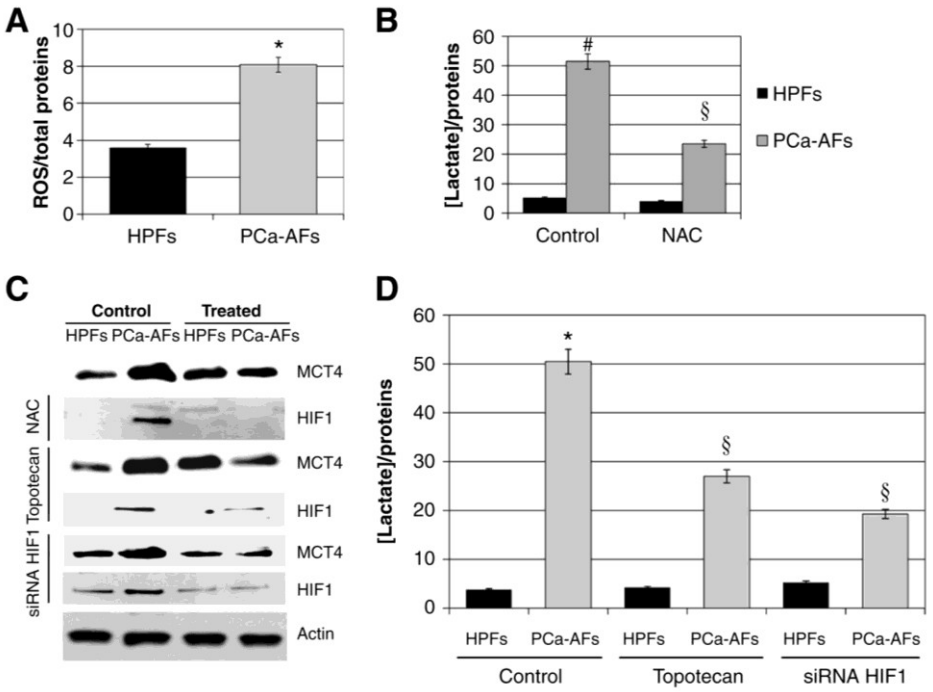

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Fig. 4 (See previous page). Warburg metabolism of PCa-AFs is ROS- and HIF1-dependent. HPFs were treated with CM-PCa for 24 hours obtaining PCa-AFs, then serum-free medium was added for an additional 48 hours. **A)** analysis of total ROS content in HPFs and PCa-AFs. **B)** Assay of lactate extruded by HPFs and PCa-AFs. A total of 20mM NAC was added to serum-free medium for 24 hours. **C)** Immunoblot analysis of redox- and HIF-1-dependence of MCT4 expression. HIF-1 was silenced in HPFs by RNA interference for 48 hours before adding CM-PCa. 20mM/L NAC and 250nM topotecan were added for 24 hours. HIF-1 silencing, topotecan, or NAC treatments are indicated on the left. Actin immunoblot was used for normalization. **D)** Assay of lactate in HPFs and PCa-AFs medium after treatment with topotecan or HIF-1 silencing as in **C**. *, $p < 0.001$ versus HPFs; #, $p < 0.001$ versus control; §, $p < 0.01$ versus HPFs.

SIRT3 IS INVOLVED IN ROS PRODUCTION AND HIF-1 STABILIZATION IN PCa-AFs

It has recently been demonstrated that the mitochondrial deacetylase SIRT3 (sirtuin-3) is involved in the control of HIF-1 expression, and redox signaling has been implicated in such control (Finley et al., 2011). Superoxide dismutase 2 (SOD2 or MnSOD) is involved to maintain ROS homeostasis in mitochondria and is a target enzyme of SIRT3. Deacetylation of SOD2 by SIRT3 induces its activations; a decrease in SIRT3 expression leads to increased SOD2 acetylation/inhibition, thereby causing ROS increase and HIF-1 stabilization (Bell et al., 2011; Chen et al., 2011). In turn, HIF-1 activation following SIRT3-loss is able to induce metabolic reprogramming toward aerobic glycolysis in cancer cells (Finley et al., 2011).

To address the possibility that the conversion to a Warburg phenotype of HPFs as a consequence of activation could be dependent on SIRT3-mediated ROS production, we firstly analyzed the expression of SIRT3 in both HPFs and PCa-AFs. Immunoblot analysis revealed a down-regulation of SIRT3 and an increase in SOD2 acetylation in PCa-AFs (Fig. 5A). As a result of SOD2 inhibition, mitochondrial ROS are strongly increased upon HPFs activation (Fig. 5B). Finally, in agreement with the idea of a redox-mediated activation of HIF-1 via SIRT3 down-regulation, we observed a clear stabilization of HIF-1 in PCa-AFs under normoxic conditions (Fig. 5A). To further confirm the role of SIRT3 in HIF-1 stabilization, we silenced SIRT3 by RNA interfering in HPFs before activation with CM-PCa. The abolish of SIRT3 expression leads to a strong overproduction of intracellular ROS associated to a dramatic increase of HIF-1 accumulation (Fig. 5C). These data demonstrate that SIRT3 acts as a key

upstream regulator of ROS production during HPFs activation, leading to HIF-1 stabilization and, ultimately, to the Warburg metabolism.

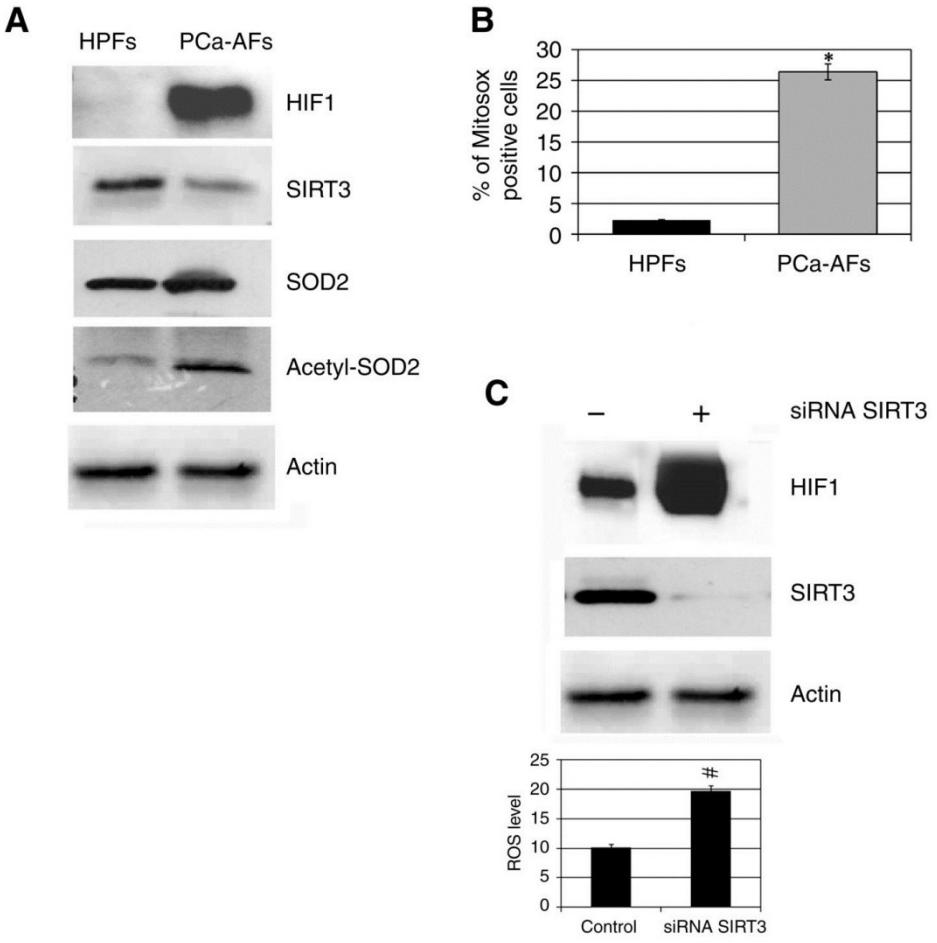

Fig. 5. HIF-1 accumulation is regulated by SIRT3 in PCa-AFs. HPFs were treated with CM-PCa for 24 hours obtaining PCa-AFs, then serum-free medium was added for an additional 48 hours. **A)** Immunoblot analysis of HIF-1, SIRT3, SOD2, and acetyl-SOD2. Actin immunoblot was used for normalization. **B)** Cytofluorimetric analysis of mitochondrial ROS using Mitosox probe. **C)** SIRT3 was silenced in HPFs by RNA interference for 48 hours before adding CM-PCa. HIF-1 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.

PCa CELLS UP-LOAD LACTATE PRODUCED BY CAFs

Several evidences in literature suggest that the glycolytic metabolism in CAFs leads to production of lactate and/or pyruvate which could be transferred to adjacent cancer cells where they enter the TCA cycle, resulting in increased oxidative phosphorylation and efficient ATP production (Pavlides et al., 2009). 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 such 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 by direct contact with PCa cells). 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 to obtain CM. The results show that lactate produced by CAFs is strongly decreased where PCa are present (both treatment with CM-CAFs or co-culture), thus suggesting that lactate has been consumed by PCa cells (Fig. 6A). Since activated fibroblasts showed unchanged MCT1 expression and unaffected lactate up-load compared to HPFs (Fig. 3B), we speculated that PCa cells 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. 6B), thereby allowing lactate up-load. In agreement, CM-CAFs or co-culture with fibroblasts do not induce MCT4 expression in PCa cells (Fig. 6B, bottom). In addition, we observed a clear up-regulation of HIF-1 under normoxic conditions when PCa cells are in contact with CAFs (both CM-CAFs and co-culture with CAFs) (Fig. 6B). Treatments with ROS scavenger NAC or topotecan lead to down-regulation both of HIF-1 and MCT1, thereby confirming that ROS-dependent stabilization of HIF-1 plays a key role even in metabolic reprogramming of PCa cells (Fig. 6B).

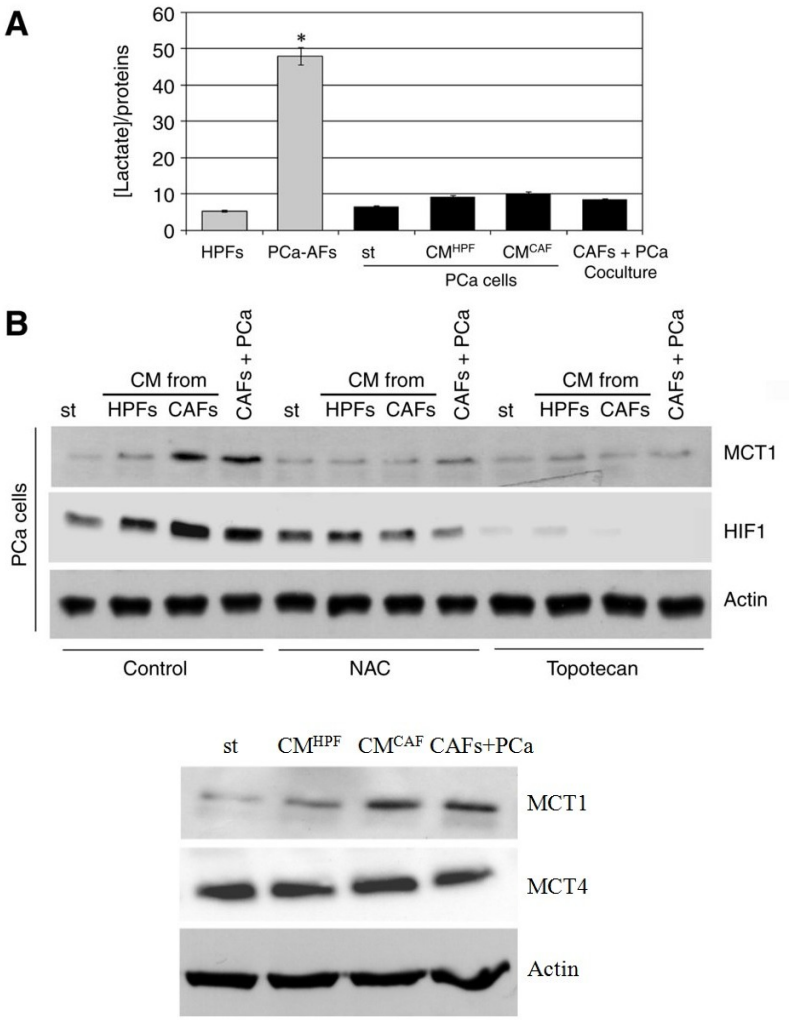

Fig. 6. PCa cells upload and use lactate produced by CAFs. PCa cells were cultured with serum-free medium (st) or with CM-HPF, CM-CAF, or co-cultured with CAFs (proportion CAFs:PCa 3:1). **A)** Assay for lactate extrusion in culture media. **B)** MCT1, MCT4 or HIF-1 immunoblots of the same samples. Actin immunoblot was used for normalization. *, $p< 0.001$ versus HPFs.

To analyze the fate 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 co-culture with CAFs (Fig. 6C, upper 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 its incorporation in [^{14}C]-proteins (Fig. 6C, middle and lower panels). We found 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 mitochondrial respiration and in anabolic reactions. Furthermore, we observed a decrease in GLUT1 expression and [^{3}H]-glucose uptake (Fig 6D). This results suggest

that PCa cells become glucose-independent and lactate-dependent upon contact with CAFs, shifting towards an aerobic metabolism and using lactate both in catabolic and anabolic processes.

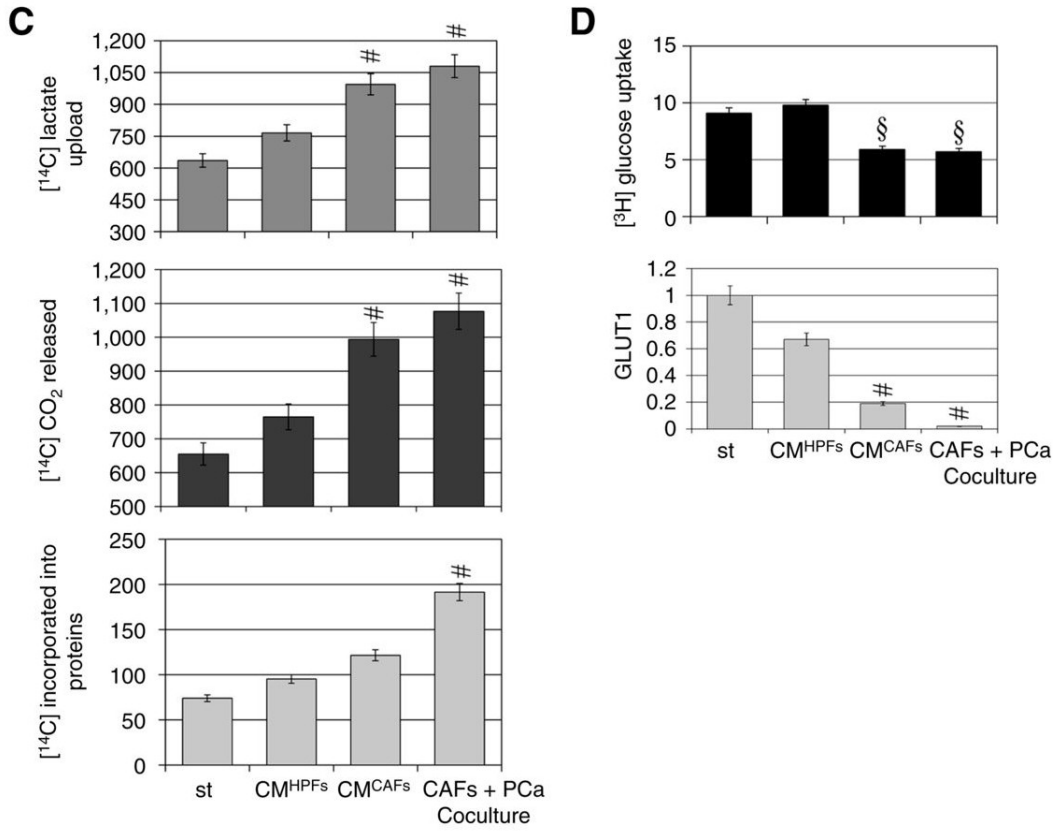

Fig. 6. PCa cells upload and use lactate produced by CAFs. PCa cells were cultured with serum-free medium (st) or with CM-HPF, CM-CAF, or co-cultured with CAFs (proportion CAFs:PCa 3:1). **C)** [^{14}C]-lactate upload (top), respiration of [^{14}C]-lactate, evaluated as [^{14}C]-CO_2 released (middle) 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 st; §, $p < 0.01$ versus st.

NATURAL ANTIOXIDANT KAEMPFEROL IMPAIRS HIF-1 STABILIZATION IN CAFs/PCa CO-CULTURE

Several studies on a wide spectrum of phenolic compounds extract from vegetables and fruits showed that these natural products can act as potent antioxidant or anticancer agents (Aravindaram et al, 2010), behaving as chemopreventive molecules (Singh et al. 2006). Kaempferol (3,4',5,7-tetrahydroxyflavone) is a flavonoid with pro- and anti-oxidant activity present in various natural sources (Marfe et al., 2009). It has been

reported that kaempferol, as well as resveratrol, is able to activate sirtuins, such as SIRT1 and SIRT3, increasing mitochondrial biogenesis and function (Rasbach et al., 2008). Furthermore, in human leukemia cell lines, kaempferol induces expression of SIRT3 and SOD2, showing anti-proliferative properties (Marfe et al., 2009).

Given the importance of ROS- and SIRT3-dependent stabilization of HIF-1 for metabolic reprogramming in our model, we analyzed the effect of kaempferol in CAFs+PCa co-culture. Treatment with kaempferol leads to a up-regulation of SIRT3, accompanied by a remarkable decrease of ROS production and a down-regulation of HIF-1 (Fig. 7). Inhibition of HIF-1 transcriptional activity was confirmed by reduced expression of its target gene carbonic anhydrase IX (CA IX) (Fig. 7). These data further confirm the mandatory role of SIRT3 and ROS in HIF-1-mediated metabolic reprogramming and represent an important example of how natural antioxidant can inhibit ROS-dependent mechanism involved in cancer progression.

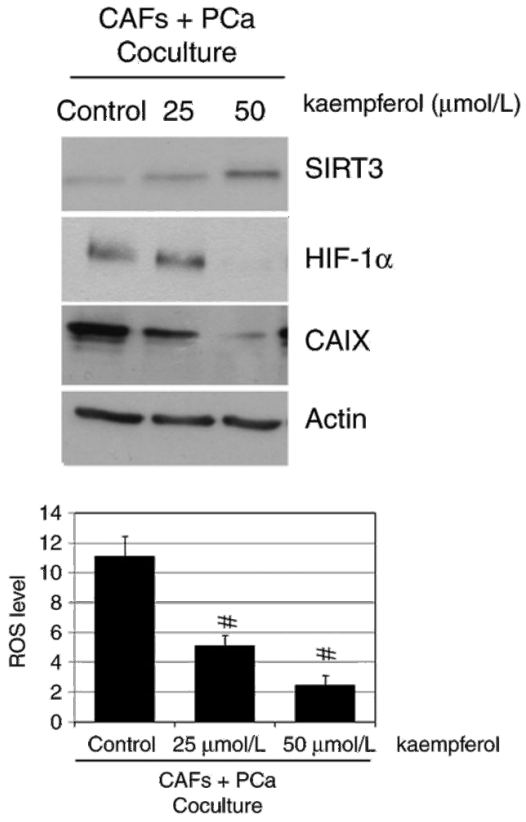

Fig. 7. Kaempferol impairs HIF-1 stabilization in CAFs+PCa co-culture. Immunoblots for SIRT3, HIF-1, and CA IX (a common target of HIF-1) were done on CAFs+PCa coculture (proportion CAFs:PCa 5:1). Note that 25μM and 50μM kaempferol was added to serum-free medium for 24 hours. Actin immunoblot was used for normalization. Total ROS production of the same samples is reported in the plot below.

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 (carboxyfluorescein succinimidyl ester), cultured alone or co-cultured with CAFs and then assayed for proliferation by flow cytometry. CFSE binds intracellular proteins and its fluorescence is halved progressively within each cell division. The results indicate that the proliferation index of PCa cells (the average number of cell divisions that a cell in the original population has undergone) increases by 30% when are in contact with CAFs, suggesting an active role of fibroblasts in sustaining PCa cells proliferation (Fig. 8A). The relevance of lactate up-load for PCa cell growth was confirmed using α-cyano-4-hydroxycinnamate (CHC), a specific MCT1 inhibitor (Sonveaux et al., 2008; Colen et al., 2011), which abolishes the trophic effect of CAFs (Fig. 8A).

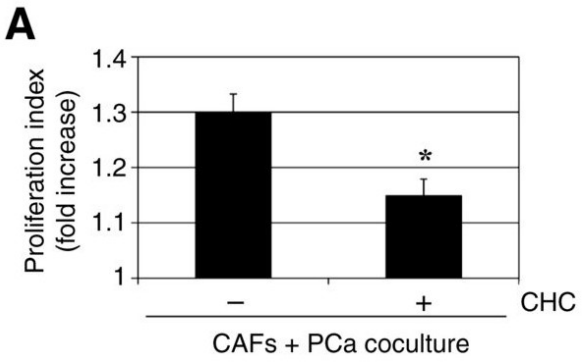

Fig. 8. Lactate produced by CAFs is useful for PCa growth. A) Analysis of PCa growth in co-culture with CAFs. PCa cells were first labeled with CFSE and then cocultured with CAFs for 48 hours, in the presence or not of 2,5mM CHC, before cytofluorimetric analysis of cell proliferation. Proliferation index has been reported in the bar graph (fold increase respect to PCa cells alone). *,$p < 0.001$ versus (−).

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) and number of PCa clones were counted. PCa islets developed after some days and we observed a clear trophic effect of CAFs for PCa cell proliferation (Fig. 8B). 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. 8B). 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. 8C). Similar results were observed after silencing of MCT1 by RNA interference (Fig. 8B) or by the use of different metabolism inhibitors (Fig. 8D). 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 Antimycin A, as well as forced re-expression of SIRT3 by kaempferol, leads to a remarkable decrease of PCa cell growth (Fig. 8D). Each of these molecules block the metabolic loop at different points and in different cell types. 2-DG (Fig. 8C) and DCA target glycolytic CAFs, being respectively an inhibitor of hexokinase and an inhibitor of pyruvate dehydrogenase kinase (PDK). Conversely, Antimycin A inhibits cytochrome *c* reductase, targeting PCa respiring cells.

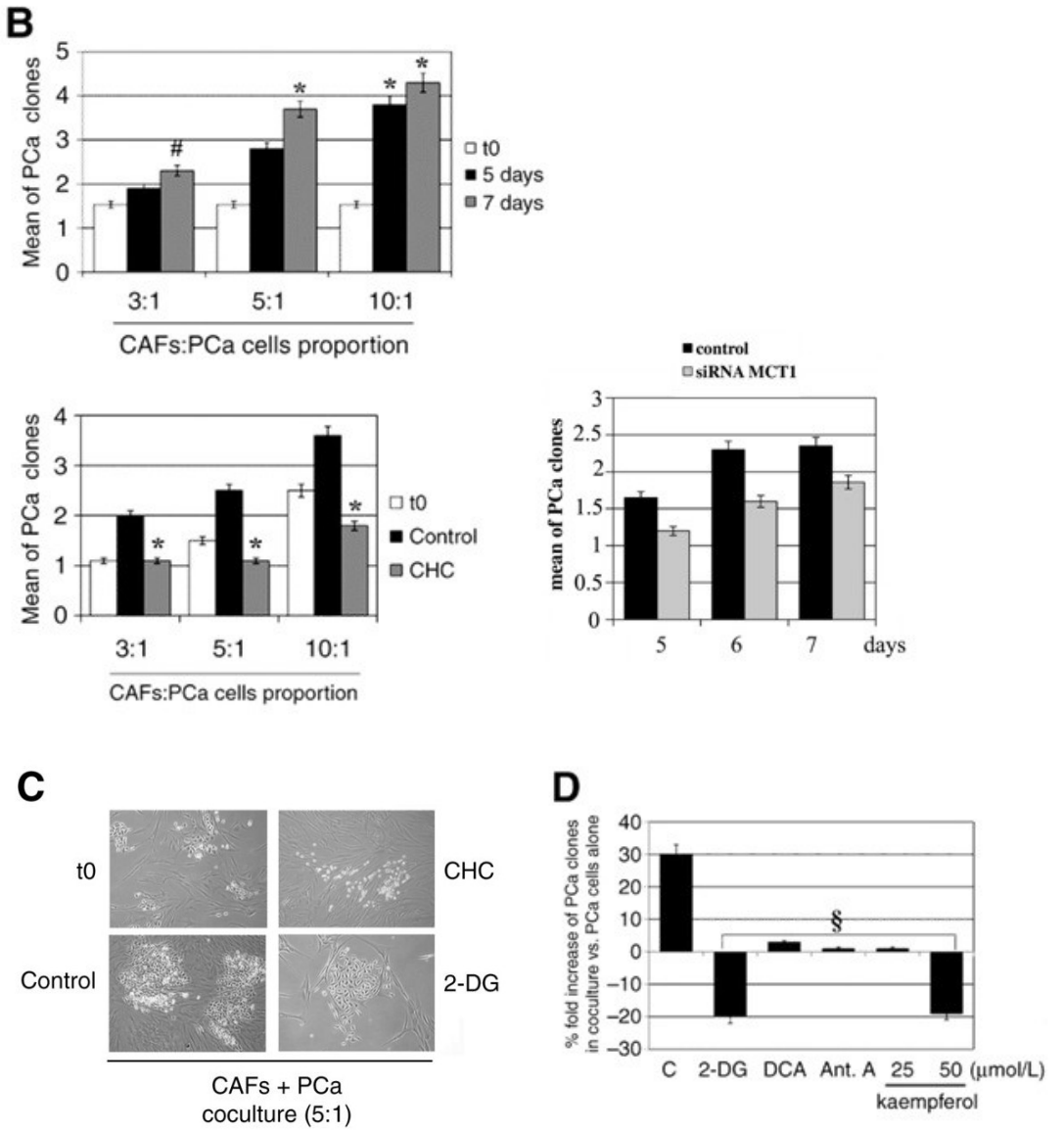

Fig. 8. Lactate produced by CAFs is useful for PCa growth. B) Number of PCa cell clones in co-culture with CAFs. A total of 3×10^3 PCa cells were seeded with increasing number of CAFs (proportion CAFs:PCa cells 3:1, 5:1, 10:1) for 5/7 days and PCa cells clones were counted under an optic microscope (top). Note that 2,5mM CHC was added to co-culture for the whole period (bottom, left panel). MCT1 silencing was obtained by RNA interference in PCa cells. 48 hours after silencing, PCa cells were used to seed a co-colture with CAFs (proportion CAFs:PCa cells 5:1). PCa clones are counted after five, six and seven days and the mean are shown in the bar graph (bottom, right panel). **C)** Representative images of PCa clones obtained in co-colture with CAFs for 7 days in the presence of 2,5 mM CHC, 1 mg/mL 2-deoxyglucose (2-DG). **D)** Increase in PCa cell clone number (% fold increase respect to PCa cells alone), in coculture with CAFs (5:1 ratio), for 7 days with 1 mg/mL 2-DG, 0.5mM DCA acid, 20nM Antimycin (Ant. A), 25 and 50 µM kaempferol. *, $p < 0.001$ versus t0; #, $p < 0.01$ versus t0; §, $p < 0.001$ versus C.

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 immunodeficient SCID bg/bg mice together with CAFs. As we already reported, co-injection of CAFs with PCa cells strongly enhances the tumor growth rate (Giannoni et al., 2010), 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. 8E).

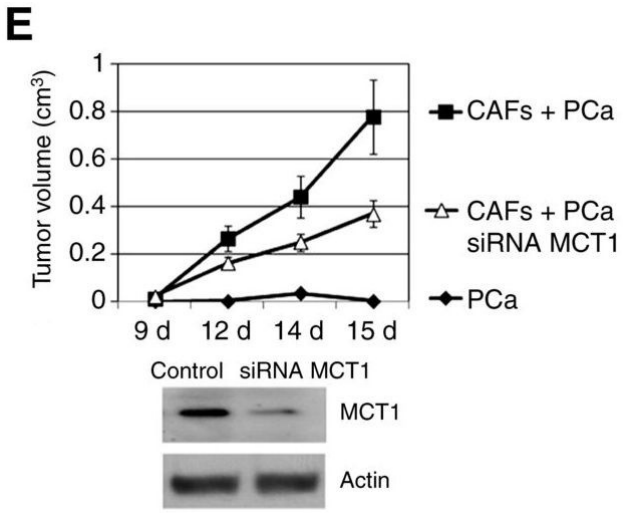

Fig. 8. Lactate produced by CAFs is useful for PCa growth. E) Xenograft growth in SCID-bg/bg mice of wild-type or MCT1-silenced PCa cells injected subcutaneously. PCa cells were injected alone or with activated fibroblasts (5:1 ratio). MCT1 silencing by RNA interference in PCa cells is shown (bottom).

EXPERIMENTAL PART II

Aim of study

Metastatic process is an hallmark of tumor malignancy and the most common cause of death for cancer patients. The key events in such metastatic pathway is EMT, an epigenetic program that leads epithelial cells to lose their cell–cell and cell-ECM interactions to undergo cytoskeleton reorganization and to gain morphological and functional characteristics of mesenchymal cells, thus generating an invasive cell able to secrete proteases to deeply change the surrounding ECM and to move away from the site of the primary tumor. Both hypoxia and CAFs have been described to elicit EMT in cancer cells. In particular, our research group demonstrated that CAFs promote EMT in PCa cells, as well as increase of stem cell markers development and spontaneous metastases (Giannoni et al., 2010). The ability of CAFs to elicit EMT and stem-like traits is due to activation of a pro-inflammatory signature involving cycloxygenase-2, NF-kB and HIF-1 which is responsible for a motogen transcriptional program (Giannoni et al., 2011).

Acidic extracellular pH has been associated with tumor progression via multiple effects including up-regulation of angiogenic factors and proteases, increased invasion and impaired immune functions. In addition, it can influence the uptake of anticancer drugs and modulate the response of tumor cells to conventional therapy. Among proteins that regulate intracellular and extracellular pH, an important role is played by carbonic anhydrases (CAs). CAs are a family of zinc metalloenzymes that rapidly catalyze the conversion of CO_2 and H_2O to H^+ and HCO_3^-.There are at least 13 human active CA isoforms expressed in different tissue and/or subcellular compartments. CA I, II, III, VII and XIII are expressed in cytosol; CA VA and VB are expressed in mitochondria; CA IV, IX, XII and XIV are expressed in plasma membrane. An hallmark feature of solid tumors is the high expression of CA IX. The latter is an hypoxia-induced enzyme that is controlled by the transcription factor HIF-1 (Parks et al., 2011). Located at the cell surface with an extracellular catalytic domain, CA IX activity contributes to acidification of tumor environment, which has been associated with acquisition of

metastatic phenotypes and chemoresistance (Kim et al., 2006; Lee et al., 2007; Robertson et al,. 2004).

The aim of this study is to address the role of CA IX in EMT regulation of PCa cells in response to CAFs. We found that CA IX is dramatically up-regulated in CAFs upon contact with cancer cells, thereby enhancing extracellular acidification and activation of MMP-2 and MMP-9-driven EMT.

Results

CA IX IS EXPRESSED IN BOTH PCa CELLS AND CAFs

We have recently demonstrated that both prostate CAFs isolated from aggressive PCa and PCa-AFs (PCa-activeted fibroblasts) are able to elicit EMT program in PCa cells (Giannoni et al., 2010). Here, we used HPFs and PCa-AFs obtained by *in vitro* activation (here termed as CAFs) to analyze their role in extracellular acidification during the EMT process induced in PCa cells. Immunoblot analysis on HPFs and CAFs revealed that only CAFs express CA IX, while HPFs do not express the enzyme (Fig. 1A). We also confirmed that CAFs isolated from patients bearing aggressive prostate carcinoma express CA IX at similar levels with respect to *in vitro* activated CAFs (Fig. 1A). Furthermore, PCa cells express CA IX and exposure to the conditioned medium (CM) from CAFs elicits an increase of expression of the enzyme (Fig. 1B).

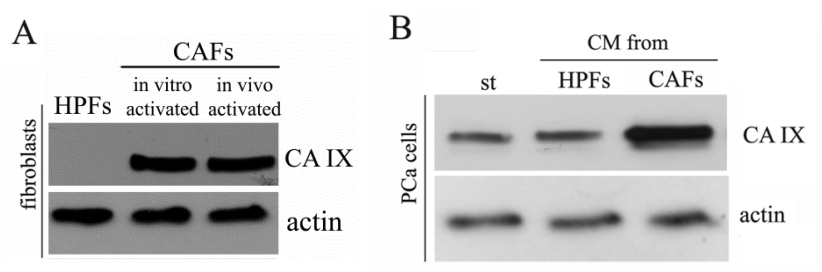

Fig. 1. CAFs show high expression of CA IX. A) Immunoblot analysis of CA IX expression in HPFs, *in vitro* activated CAFs and in CAFs isolated from human prostate carcinoma biopsy (*in vivo* activated CAFs). *In vitro* activated CAFs were obtained culturing HPFs with CM from PCa cells for 24 hours. **B)** Immunoblot analysis of CA IX expression in PCa cells. Prostate cells were treated with CM from HPFs or CAFs for 24h. Actin immunoblot was used for normalization.

The expression of CA IX in both CAFs and PCa cells upon exposure to CM-CAFs is also correlated with increase in enzymatic activity of CA IX, as revealed by fluorescence analysis after the treatment of cells with the fluorescent probe FITC-labeled CAI#3, which targets the activated state of the extracellular catalytic domain of the enzyme (Fig. 1C), as well as by stop-flow analysis (Fig. 1D). Of note, in both analyses we observed that maximum activity is reached by CA IX expressed by CAFs.

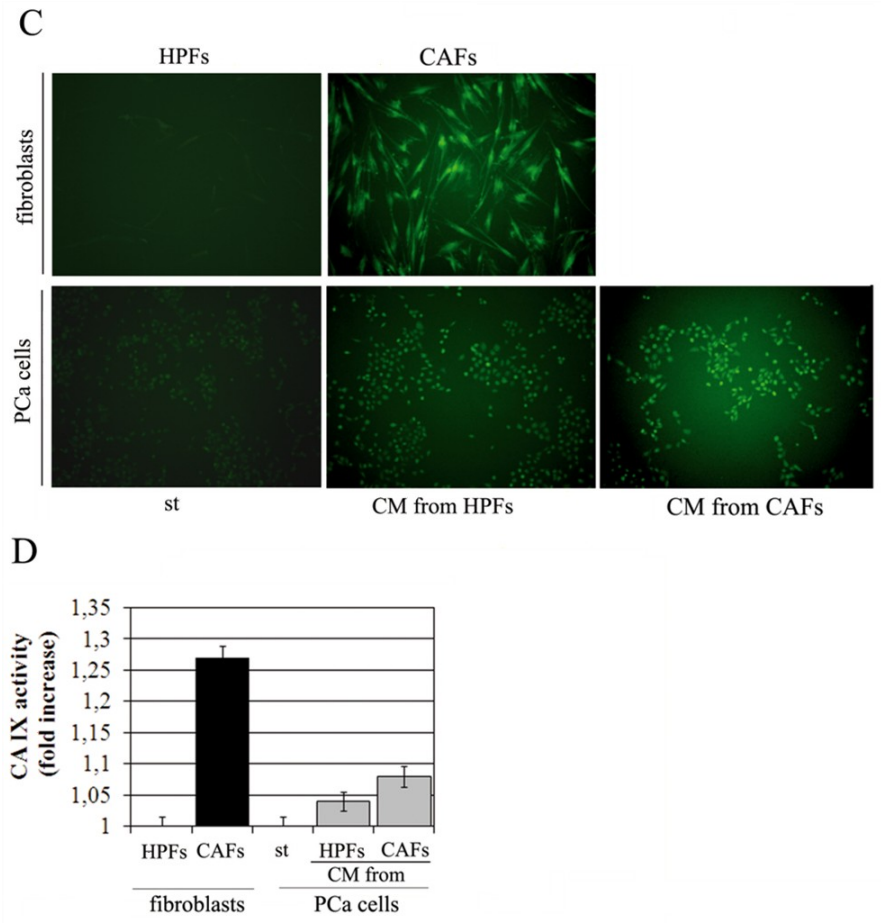

Fig. 1. CA XI activity in CAFs and PCa cells. C) Analysis of CA IX activity by fluorescence microscopy and **D)** by stop-flow analysis. *,p < 0.001 versus HPFs; §,p < 0.01 versus st.

HIF-1 ACTIVATION IS MANDATORY FOR CA IX EXPRESSION IN BOTH CAFs AND PCa CELLS

We previously demonstrated that redox-dependent stabilization of HIF-1 drive the metabolic reprogramming of CAFs toward Warburg metabolism. Furthermore, HIF-1 has already been reported as a master transcription factor involved in CA IX expression (Brahimi-Horn and Pouyssegur, 2009), although data on its involvement in the stromal counterpart are still at their infancy. We therefore analyzed CA IX expression in CAFs and PCa cells in normoxia during HIF-1 inhibition following treatment with topotecan or N-acetyl cysteine (NAC). Both treatments lead to the almost complete abolishment of CA IX expression in both cell types (Fig. 2A, 2C). In addition, silencing of HIF-1 by RNA interference in fibroblasts leads to down-regulation of CA IX, thereby confirming its mandatory role in CA IX expression in prostate carcinoma stroma (Fig. 2B).

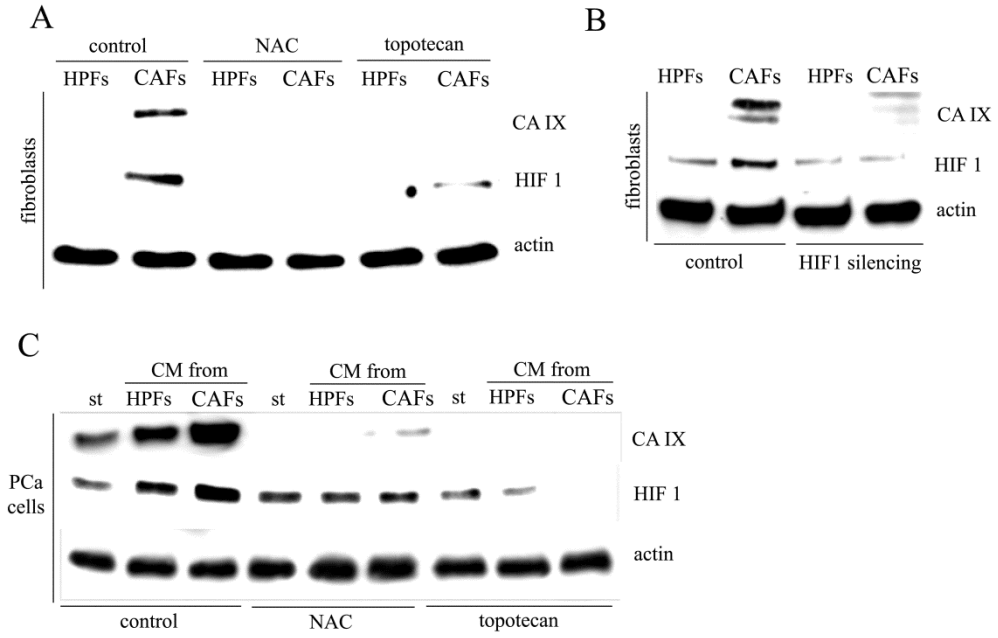

Fig. 2. CA IX expression is HIF 1-dependent both in CAFs and PCa cells. A) Immunoblot analysis of ROS- and HIF-1-dependance of CA IX expression in HPFs and CAFs. HPFs were cultured in serum-free medium or treated with CM-PCa for 24h in the presence of 20mM NAC or 250nM topotecan. **B)** HIF-1 silencing was performed in HPFs and after 48h serum-free medium (for HPFs) or CM-PCa (for CAFs) were added to the cells for 24h. **C)** Immunoblot analysis of ROS- and HIF-1-dependance of CA IX expression in PCa cells. 20mM NAC or 250nM topotecan were added to CM from HPFs or CM from CAFs and maintained throughout the experiment. In each immunoblot, actin was used for normalization.

CA IX EXPRESSION LEADS TO ACIDIFICATION OF CANCER-STROMAL ENVIRONMENT

To investigate in our model the role of CA IX in extracellular acidification, we analyze the pH of culture media of HPFs, CAFs and PCa cells after the treatment with CM from HPFs or CAFs in normoxic conditions. We observed that the activation of fibroblasts upon CM-PCa contact gives rise to an increase in extracellular acidity, thereby suggesting that the mutual interactions among stromal and cancer cells also embraces acidification of tumor microenvironment (Fig. 3A). The use of two selective inhibitors of CA IX, namely compounds 1 and 2, lead to alcalinization of extracellular milieu, confirming that CA IX plays a mandatory role in acidification of CAFs medium (Fig. 3B).

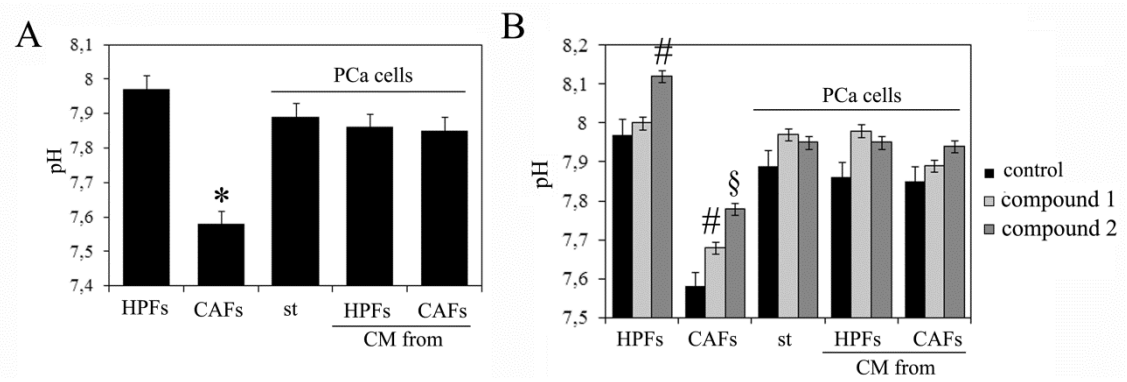

Fig. 3. CA IX induces the acidification of extracellular medium. A) Measure of extracellular pH in HPFs, CAFs and in PCa cells after treatment with different CM. **B)** Measure of extracellular pH in HPFs, CAFs and PCa cells after the treatment with CA IX inhibitors 1 (FC3-148 bis) and 2 (FC5–207A) for 48h. *,$p < 0.001$ versus HPFs; #,$p < 0.01$ versus control; §,$p < 0.001$ versus control.

CA IX ACTIVITY OF CAFs ENHANCES MMP-2 AND MMP-9 SECRETION

The ability of prostate CAFs to elicit a pro-invasive behavior in PCa cells is mainly due to a secretion of MMPs (MMP-2 and MMP-9), which drive activation of a Rac1-dependent EMT program (Giannoni et al, 2011). Owing to the acknowledged sensitivity of MMPs to acidity, we investigated if acidic environment due to CA IX expression is involved in MMPs expression and activation. Metalloproteinase analysis with zimography of fibroblasts' culture media reveal that the ability of CAFs to secrete MMP-2 and MMP-9 is severely impaired during administration of CA IX inhibitors 1 and 2 (Fig. 4A), as well as following down-regulation of CA IX by RNA interfering (Fig. 4B). Indeed, the activity of MMP-9, and to a smaller extent of MMP-2, is significantly decreased in media collected from CA IX-silenced HPFs or CAFs, thereby confirming a key role played by CA IX in MMP-2 and MMP-9 secretion by CAFs (Fig. 4C).

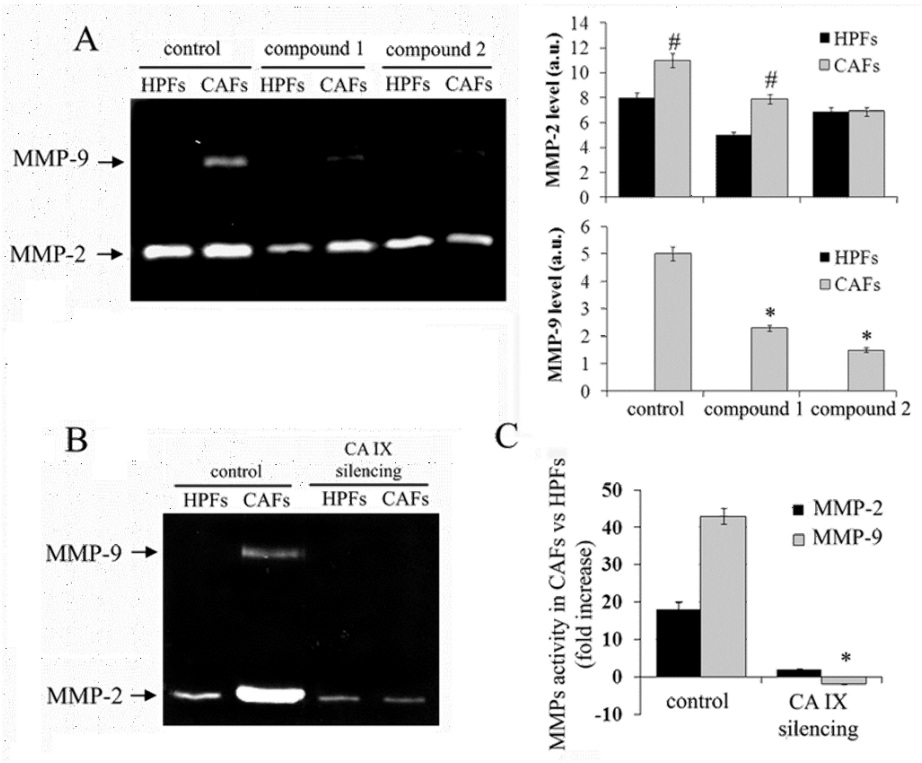

Fig. 4. Silencing of CA IX in CAFs decreases MMP-9 secretion. A) Metalloproteinase analysis with zimography of MMP-9 and MMP-2 in media of HPFs and CAFs after the treatment with the CA IX inhibitor 1 (FC3–148 bis) and 2 (FC5–207A) Bar graphs show the level of MMP-2 and MMP-9 in HPFs and CAFs after the treatment with CA IX inhibitors. **B)** CA IX silencing was performed in HPFs and CAFs, and then MMP-9 and MMP-2 were analyzed by zymography. **C)** Assay of MMP-2 and MMP-9 activity in CM from control- or CA IX-silenced CAFs. *,$p < 0.001$ versus control; #,$p < 0.01$ versus HPFs.

CA IX ACTIVITY OF CAFs IS MANDATORY TO DRIVE ACTIVATION OF EMT IN PCa CELLS

We already demonstrated that CM collected from prostate CAFs are able to elicit invasive features and EMT in PCa cells (Giannoni et al., 2010). We observed that acidification of extracellular milieu of CAFs+PCa cells co-cultures obtained omitting buffering of culture media, thereby allowing rapid acidification, greatly increased the ability of CAFs to drive the EMT program in PCa cells, as revealed by E-cadherin decrease and morphological analysis (Fig. 5A, 5B). To investigate the role of CA IX-mediated acidification and MMPs secretion, we analyzed the sensitivity of CAFs-mediated EMT to silencing of CA IX. To do that, we collected CM from HPFs and CAFs, silenced or not for CA IX, and we used them to treat PCa cells. We observed that CA IX silencing in CAFs impairs their ability to induce EMT in cancer cells, as we can

see by stable maintenance of E-cadherin expression, inhibition of invasiveness and prevention of elongation of the cells (Fig. 5D, 5E, 5C). These data suggest that, within the tumor-stroma interplay, acidification due to CA IX expression in CAFs plays a key role for activation of EMT in PCa cells.

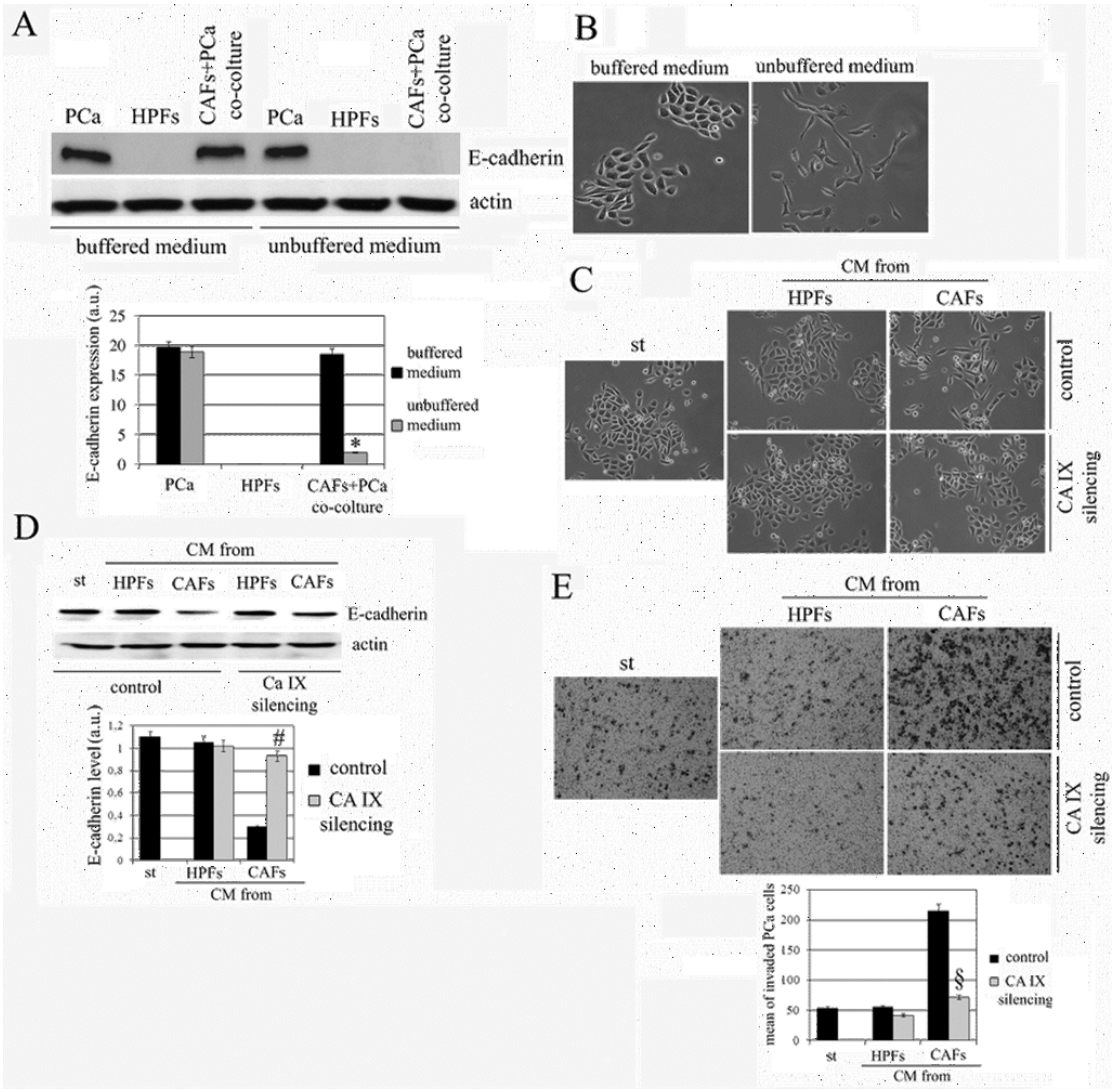

Fig. 5. Modulation of CA IX expression affects EMT in PCa cells. A) E-cadherin expression in HPFs, PCa cells and in PCa cells+CAFs co-colture cultured in DMEM buffered at pH 7 and in unbuffered medium (placed at 37°C without CO_2) for 48h. E-cadherin expression was shown in bar graph. **B)** Representative image of PCa cells treated as described in **(A)**. **C)** CA IX was silenced in HPFs and CAFs and CM were harvested. PCa cells were then treated with serum-free medium (st) or different CM for 72h, and then cells were photographed. **D)** Evaluation of E-cadherin expression in the same experimental setting described in **(C)**. In each analysis, actin immunoblot was used for normalization. a.u., arbitrary units. **E)** Boyden invasion assay of PCa cells after treatment for 18h with different CM obtained from HPFs or CAFs (control or silenced for CA IX). Bar graph represents the mean of invaded PCa cell (six fields for sample). *,$p < 0.001$ versus. buffered medium; #,$p < 0.001$ versus control CAFs; §,$p < 0.001$ versus control CAFs.

MMP-9 FROM CAFs IS A KEY PLAYER TO DRIVE ACTIVATION OF EMT IN PCa CELLS

To confirm that MMP-2 and MMP-9 are mandatory factors in EMT of PCa cells, we used nanomolar competitive inhibitors of these two matrix proteases, namely GlcNAc-SLS-HA and Lac-SLS-HA. These inhibitors belong to an unprecedented family of atoxic, water-soluble arylsulfonamides characterized by a saccharidic residue (Glc, GlcNAc or Lactose) linked, through a spacer, to the sulfonamidic nitrogen. Except for MMP-1 and -7, all compounds showed low nanomolar K_i values for the MMPs tested (MMP-2, -8, -9, -12 and -13). Peculiarly, the inhibitors here tested present a hydrophilic chain that protrudes out of the protein toward the solvent region, allowing a complete water solubility without affecting the affinity for the enzymes. GlcNAc-SLS-HA and Lac-SLS-HA inhibitors was added to CM collected from HPFs and CAFs, and we observed that inhibition of MMP-2 and MMP9 is extremely active in impairing EMT due to CAFs contact, preventing the elongation of the cells (Fig. 6A), the E-cadherin decrease (Fig. 6B) and, finally, PCa cell invasiveness (Fig. 6C).

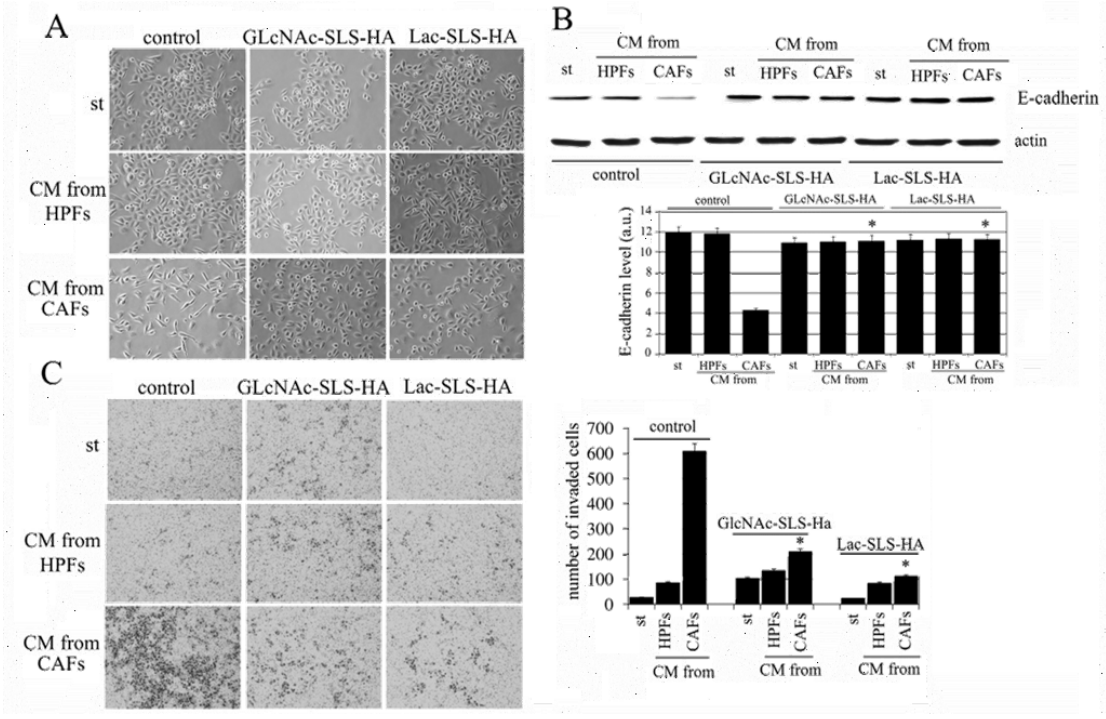

Fig. 6A, B, C. For figure legend, see next page.

Fig. 6 (See previous page). The modulation of MMP-9 expression prevents EMT in PCa cells. A) Representative images of PCa cells treated for 72h with CM from HPFs and CAFs containing MMP-9 inhibitors GlcNAc-SLS-HA or Lac-SLS-HA. **B)** Immunoblot of E-cadherin expression in the same experimental setting described in **(A)**. **C)** Invasion analysis of PCa cells in the presence of MMP-9 inhibitor GlcNAc-SLS-HA or Lac-SLS-HA. Bar graph represents the mean of invaded PCa cells (six fields for sample randomly chosen). *,$p < 0.001$ versus control CAFs.

On the basis of the acknowledged hierarchy between MMP-2 and MMP-9, we also silenced MMP-9 in CAFs by RNA interference and observed abolishment of EMT, as suggested by cell morphology (Fig. 6D), stable maintenance of E-cadherin expression (Fig. 6E) and decreased invasiveness (Fig. 6F), thereby confirming the key role played by this protease in eliciting EMT and motility in PCa cells. Furthermore, the addition of recombinant MMP-9 to CM collected from CA IX-silenced CAFs rescues the ability of PCa cells to undergo EMT, thereby confirming the hierarchy between CA IX and MMP-9 in EMT activation (Fig. 7).

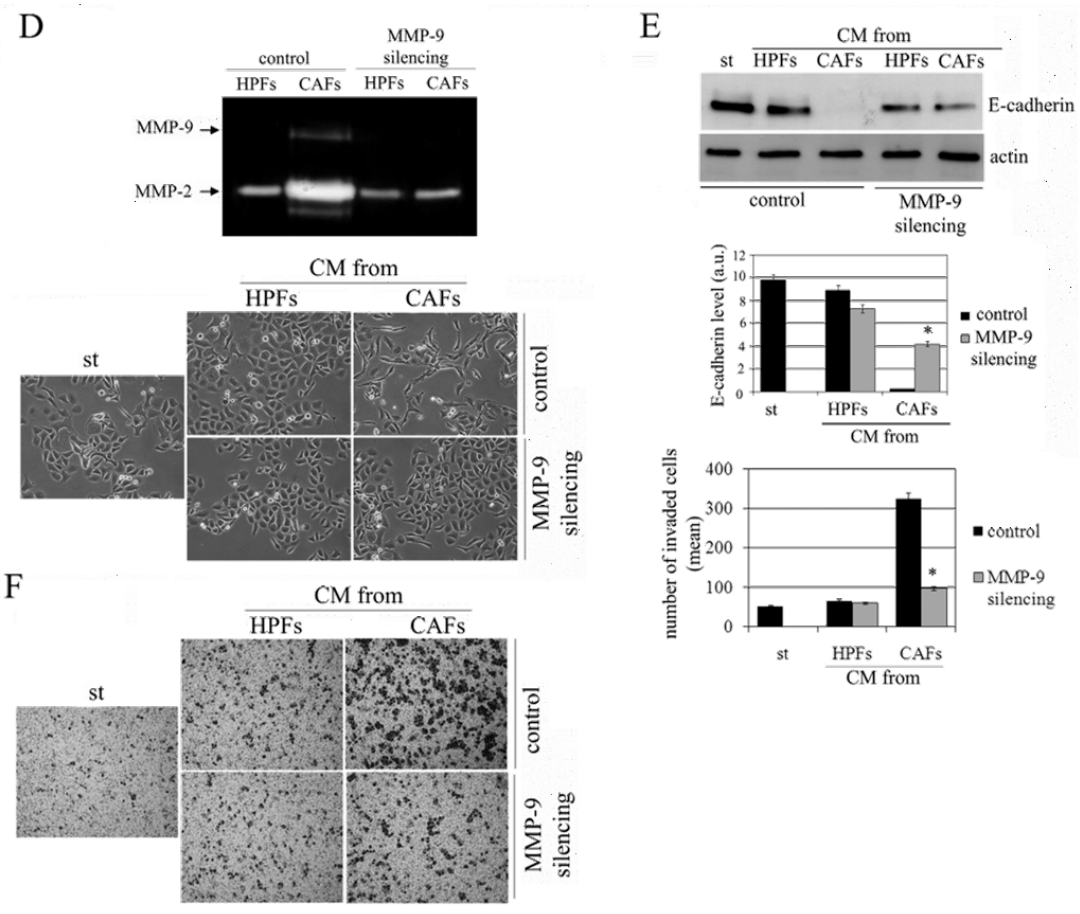

Fig. 6D, E, F. For figure legend, see next page.

Fig. 6 (See previous page). The modulation of MMP-9 expression prevents EMT in PCa cells. D) MMP-9 was silenced in HPFs and CAFs. Silencing was confirmed performing zimography of CM (upper panel). Then, CM from HPFs or from CAFs (both control and MMP-9 silenced) were added to PCa cells for 72h, and cells were photographed (lower panel). **E)** Immunoblot analysis of E-cadherin in PCa cells in the same experimental setting described in **(D)**. Actin immunoblot is used for normalization (a.u., arbitrary units). **F)** Invasion assay of PCa cells treated with CM from control HPFs or CAFs or with CM from silenced-MMP-9 HPFs or CAFs. *,$p < 0.001$ versus control CAFs.

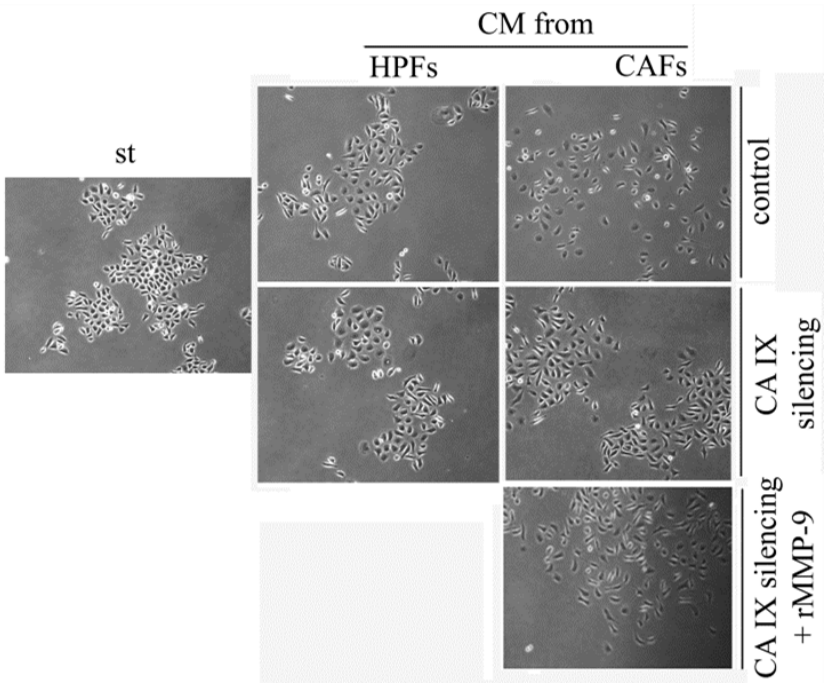

Fig.7. The addition of recombinant MMP-9 rescue EMT in PCa cells. CA IX was silenced in HPFs and CAFs. Then, CM from HPFs or from CAFs (both control and CA IX-silenced) were collected. 100µg of recombinant MMP-9 (200U/µg) were added to CM of CA IX-silenced CAFs. PCa cells were cultured for 72h with different CM and morphological analysis of EMT was performed under optical microscope.

Finally, we used a spontaneous metastasis assay in immunodeficient SCID mice to assess the role of CA IX expressed by stromal CAFs for tumor outgrowth and lung metastatic dissemination of cancer cells. We co-injected in the lateral flanks of mice PCa cells and CAFs (1:5 ratio). We already reported that in the absence of CAFs, PCa cells are unable to elicit tumors upon heterotopic injection, and that CAFs behave as synergistic bystanders eliciting survival and growth of cancer cells, allowing their escaping from primary tumors by EMT. We observed that CAFs silenced for CA IX elicit a delayed tumor onset and growth (Fig. 8A), and, more strikingly, that they are mostly unable to induce metastatic dissemination of PCa cells to lungs (Fig. 8B), likely due to their inability to drive EMT.

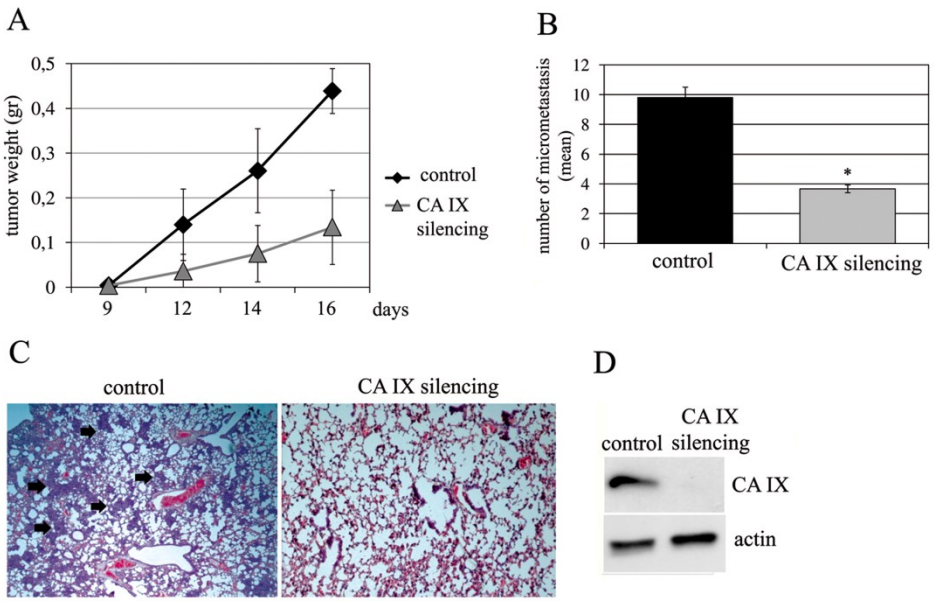

Fig. 8. CA IX silencing in CAFs decreases tumor growth and lung micrometastasis formation. A) Xenograft growth in SCID bg/bg mice of wild-type (control) or CA IX-silenced CAFs injected s.c. with PCa cells (CAFs:PCa cells ratio 1:5). **B)** Bar graph shows the mean of lung micrometastasis. **C)** Representative images of lung micrometastasis (arrows) in control and CA IX silenced treated mice. **D)** CA IX silencing by RNA interference in CAFs is shown. *,$p < 0.001$ versus control.

EXPERIMENTAL PART III

Aim of study

Drug resistance of cancer cells is recognized as the primary cause of failure of chemotherapeutic treatment in most human tumors. The ability to reduce chemoresistance would be a significant benefit for cancer patients, demonstrating the importance of research into mechanism underlying how chemoresistance arise. In this regard, mounting evidences supports the idea that deregulated cellular metabolism is linked to drug resistance in cancer therapy (Zhao et al., 2013). For example, several components of the glycolytic pathways and associated with Warburg effect, such as GLUTs transporters, HK, PKM2 and LDHA, contribute to chemoresistance in many cancer models. However, a number of data indicate that even mitochondria are involved in altered metabolism of cancer cells, and targeting mitochondrial metabolism has potential for the treatment of this disease (Ramsay et al., 2011). In cancer cells, mitochondria play a key roles, ranging from lactate respiration (Sotgia et al., 2012), to TCA cycle fueling with ketone bodies and glutamine, to citrate exportation to fuel fatty acids synthesis; of note, a few of these metabolic pathways are associated with drug resistance (Zhao et al., 2013). In this context, tumor stroma is emerging as a key player to modulate drug sensitivity in cancer cells, fueling oxidative mitochondrial metabolism with energy rich metabolites (e.g., lactate, glutamine). Indeed, MCF7 breast cancer cells become resistant to tamoxifen after co-culturing with human immortalized fibroblasts, shifting their metabolism from glycolysis to mitochondrial oxidative phosphorilation (Martinez-Outschoorn et al., 2011). Considering all of these findings, targeting cellular metabolism with small molecules interfering with protein/enzymes of metabolic pathways, can improve cancer therapy and overcome certain types of drug resistances (Zhao et al., 2013).

Here, we characterized a PCa cell line resistant to docetaxel, a promising drug for treatment of metastatic PCa. Firstly, we evaluated the phenotypic features of the resistant cells, such as proliferation, invasive properties, expression of apoptotic markers, and ability to growth in suspension. We then focused on the metabolic

adaptations induced by drug in these cells and on their role to confer advantages respect to sensitive cell line. Finally, we analyzed the role of CAFs to modulate the sensitivity of PCa cells to docetaxel.

Results

PHENOTYPIC CHARACTERIZATION OF DOCETAXEL-RESISTANT PCa CELLS

Docetaxel is a taxane used mainly for the treatment of breast, ovarian, prostate, and non-small cell lung cancer. Taxanes is a class of anti-mitotic drugs produced by the plants of the genus *Taxus*, which disrupt microtubule function stabilizing GDP-bound tubulin and thus inhibiting the process of cell division.

In our laboratory, we developed a PCa-resistant cell line (DoceRes) by treating sensitive PCa cells (Control) with increased doses of docetaxel, up to a final concentration of 10 nM. Boyden chamber assay and immunoblot analysis of sensitive and resistant cells (Fig, 1A, 1B), showed pro-invasive behavior and down-regulation of E-cadherin in DoceRes, consistent with an EMT program and a pro-metastatic phenotype. Furthermore, resistant cells show a reduced expression of the pro-apoptotic proteins Bim and Bax (Fig. 1B), and produce larger prostasphere in suspension than sensitive cells, in agreement with a decreased susceptibility to apoptosis and anoikis (Fig. 1C). Finally, DoceRes showed reduced cell growth rate compared to sensitive cells (Fig. 1D, blue and green lines). These data demonstrate that DoceRes cell line developed a more aggressive phenotype during the acquisition of chemoresistance.

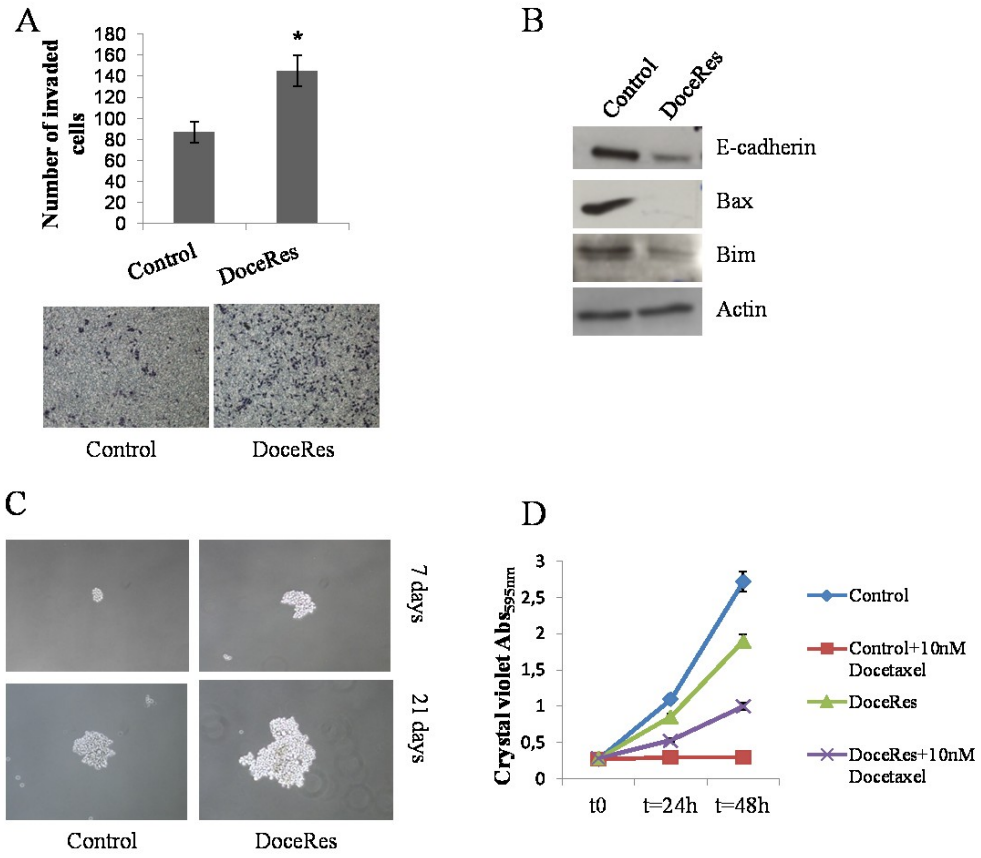

Fig.1. Docetaxel resistant PCa cells show a more aggressive phenotype compared to sensitive PCa cells. A) Boyden invasion assay of Control and DoceRes cell lines. Bar graph represents the mean of invaded cells (six fields for sample). *,p<0,01 versus Control. **B)** Immunoblot analysis of E-cadherin, Bim and Bax in Control and DoceRes. Actin immunoblot was used for normalization. **C)** Nonadheretnt P0 prostaspheres. Cells were plated in low attachement plate and grown for 7 and 21 days. Then, prostaspheres were photographed with optical microscopy. **D)** Evaluation of cell growth in complete medium by crystal violet assay. 20 x 10^3 cells were plated at the start of experiment. Cell growth was followed until 48h.

EVALUATION OF ANTIOXIDANT RESPONSE IN DoceRes CELL LINE

Anticancer drugs are able to induce oxidative stress either as a direct mechanism of cell death or as an indirect effect of exposure. For example, it has been reported that docetaxel induce ROS production through NADPH oxidase activation (Cao et al., 2005). Furthermore, it has been suggested that pathways involved in the ROS-adaptive response play a critical role in protecting cells against the damaging and cytotoxic effects of anticancer agents, thus highlighting their importance in chemoresistance (Landriscina et al., 2009). In this regard, it has been reported that Nrf2/Keap1 pathway induces mechanisms of cell survival in response to many anticancer drugs. Indeed, Nrf2

transcription factor regulates the expression of several antioxidant enzymes (such as peroxiredoxin 1, glutathione peroxidase, thioredoxin reductase), phase II detoxifying enzymes (glutathione S-transferase) and transporters in cell membrane (ABC transporters), thereby maintaining cellular redox homeostasis.

We investigated the role of Nrf2/Keap1 pathway in docetaxel resistance. Immunoblot analysis of whole cell lysate and nuclear fractions of DoceRes and Control cell lines, showed increased expression and nuclear translocation of Nrf2 transcription factor in resistant cells compared to sensitive cells, associated with a down-regulation of its repressor Keap1 and consistent with an activation of Nrf2-mediated anti-oxidant response (Fig. 2A, 2B). In agreement with these findings, we observed that resistant cells show decreased ROS production when treated with 10nM docetaxel respect to sensitive cells (Fig. 2C). These results suggest that DoceRes cell line is able to manage drug-induced oxidative stress better than sensitive cells, therefore reducing oxidative damages in cellular macromolecules through expression of Nrf2 target genes.

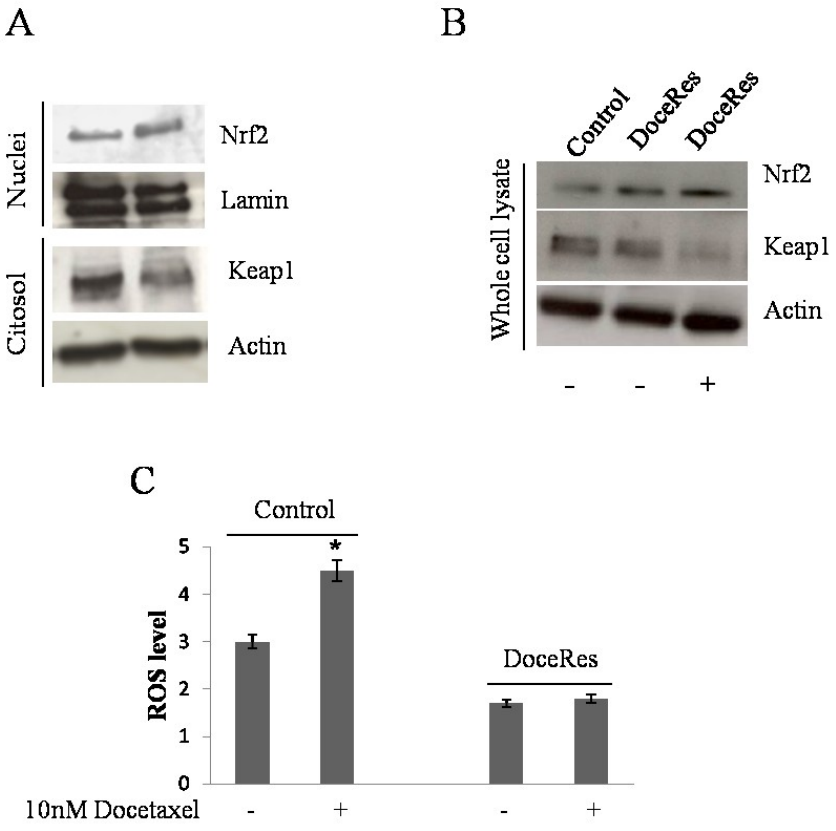

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Fig. 2. (See previous page). Resistant cells show a reduced ROS production. A) Immunoblot analysis of Nrf2 and Keap1 in sensitive and resistant cells in cellular fractions. Cells were cultured in serum-free medium for 48h before nuclear extraction. Actin and nuclear lamin immunoblot were used for normalization. **B)** Immunoblot analysis of Nrf2 and Keap1 in whole cell lysate. Cells were cultured as in **(A)**. Where indicated, 10nM docetaxel was added to medium. **C)** Evaluation of intracellular ROS with H_2DCF-DA. Cells were cultured as in **(A)** Where indicated, 10nM docetaxel was added to medium. Sample protein content was used for normalization. *,$p<0,05$ versus Control without docetaxel.

ANTIOXIDANT RESPONSE IN DoceRes IS NOT ASSOCIATED WITH INCREASED PPP ACTIVITY

NADPH is a critical source of electrons to fuel antioxidant systems in cells and PPP is an important oxidative pathway to generate this coenzyme. NADPH keeps the pool of intracellular GSH and both GSH and PPP has been associated with resistance to anticancer therapy (Zhao et al., 2013; Landriscina et al., 2009; Polimeni et al., 2011).

In order to investigate the role of PPP to fuel antioxidant response in docetaxel-resistant PCa cells, we evaluated the expression of glucose-6-phosphate dehydrogenase (G6PDH), the rate limiting enzyme of PPP, and we analyzed the metabolic flux through such pathway using two different glucose molecules radiolabelled in [1-^{14}C] or [6-^{14}C]. We found reduced expression of G6PDH and reduced activity of PPP in resistant cells compared to control (Fig. 3A, 3B). Furthermore, even the treatment of DoceRes with 10nM docetaxel, which elicited an increased Nrf2 expression, it is associated with reduced G6PDH levels and metabolic flux through PPP (Fig. 3A, 3B). Interestingly, even docetaxel treatment of sensitive cells causes a down-regulation of G6PDH expression and activity (Fig. 3C, 3D). These results confirm that PPP is not involved in counteracting oxidative stress induced by drug.

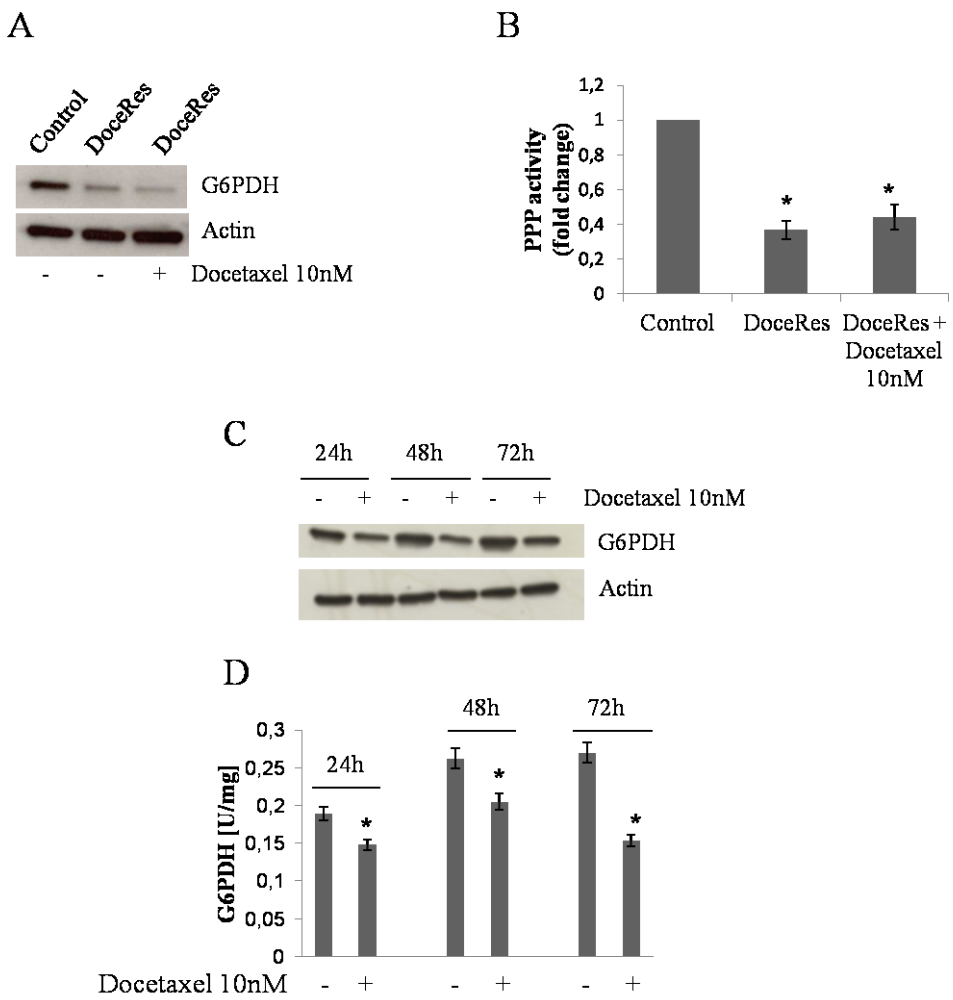

Fig. 3. No evidence of PPP engagement in docetaxel resistance. A) Immunoblot analysis of G6PDH expression in Control and DoceRes cell lines. Cells were cultured in serum-free medium for 48h before cell lysis. Where indicated, 10nM docetaxel was added to medium. Actin immunoblot was used for normalization. **B)** Analysis of PPP activity (fold change respect to Control). Cells were cultured as in **(A)**. Where indicated, 10nM docetaxel was added to medium. The PPP metabolic flux was obtained by substracting the amount of CO_2 developed from [6-^{14}C]-glucose from the CO_2 released from [1-^{14}C]-glucose. The extent of [6-^{14}C]-glucose metabolism is an index of TCA cycle alone. *,p<0,01 vs. Control. **C)** Immunoblot analysis of G6PDH in sensitive cells. Cells were cultured in serum-free medium for 24h, 48h and 72h before cell lysis. Where indicated, 10nM docetaxel was added to medium. **D)** G6PDH activity assay in sensitive cells. Cells were treated as described in **(C)**. *,p<0,05 vs. Control without docetaxel.

METABOLIC CHARACTERIZATION OF DoceRes CELL LINE

Both aerobic glycolysis and mitochondrial respiration have been associated with increased resistance to anticancer drugs (Zhao et al., 2013; Martinez-Outschoorn et al., 2011). To investigate the metabolic signature of DoceRes cell line, we analyzed both the expression of protein/enzyme involved in glycolytic pathway and the respiration of radiolabelled glucose through the release of [^{14}C]-CO_2. To better understand the metabolic adaptations of resistant cells in presence of drug, we performed these analysis even after treatment of DoceRes with 10 nM docetaxel. We firstly observed that, compared to DoceRes, Control cell line expresses basal level of HIF-1, a master regulator of glycolytic metabolism (Fig. 4A). In keeping, sensitive cells show increased expression of hexokinase II (a common target of HIF-1) and produce more lactate than resistant cells (Fig. 4A, 4D). These data suggest that Control cell line exploits a typical Warburg metabolism. Conversely, DoceRes show down-regulation of HIF-1 and hexokinase II, both in presence and in absence of drug. Moreover, docetaxel treatment leads to down-regulation of PKM2, the PK isoform involved in aerobic glycolysis (Fig. 4A). In addition, treatment with drug induces [^{14}C]-glucose oxidation to CO_2 in DoceRes cell line through mitochondrial respiration (Fig. 4B). Taken together, these results suggest that resistance to docetaxel leads to an escape from Warburg metabolism with a potential role of oxidative mitochondrial metabolism to ensure metabolic advantages during acquisition of resistant phenotype.

In order to confirm this hypothesis, we treated sensitive and resistant cells with DASA-58, which activates PKM2 inducing tetramer formation and production of pyruvate (Anastasiou et al., 2012). Sensitive and DoceRes cell lines use this metabolite differently, producing, more lactate and more CO_2 from glucose respectively (Fig. 4C, 4D). Therefore, whereas sensitive cells show a typical Warburg effect in response to DASA-58, resistant cells show an inhibition of aerobic glycolysis, consuming pyruvate in TCA cycle to oxidize it to CO_2.

In addition, compared to sensitive cells, DoceRes cell line is able to metabolize through TCA cycle other carbon source, such as lactate, further confirming an involvement of mitochondrial respiration in docetaxel resistance (Fig. 4E)

To test the importance of oxidative mitochondrial metabolism during acquisition of resistant phenotype, we used metformin, a widely used antidiabetic drug, endowed with

promising effects for cancer prevention and treatment (Sotgia et al., 2012). Metformin is reported to inhibit mitochondrial complex I activity, thereby disrupting oxidative phosphorilation (Fendt et al., 2013). We firstly observed that metformin up-regulates HIF-1 and hexokinase II both in sensitive and resistance cells, thereby confirming its ability to shift cell metabolism toward a more glycolytic one (Fig. 4F, bottom). To analyze the effect of metformin on cell growth of sensitive and resistant cells, we performed a crystal violet assay. We observed that metformin is able to inhibit cell growth selectively in DoceRes (Fig. 4F, top) and such effect is enhanced in presence of docetaxel. These data further confirm the dependence on mitochondrial metabolism of resistant cells.

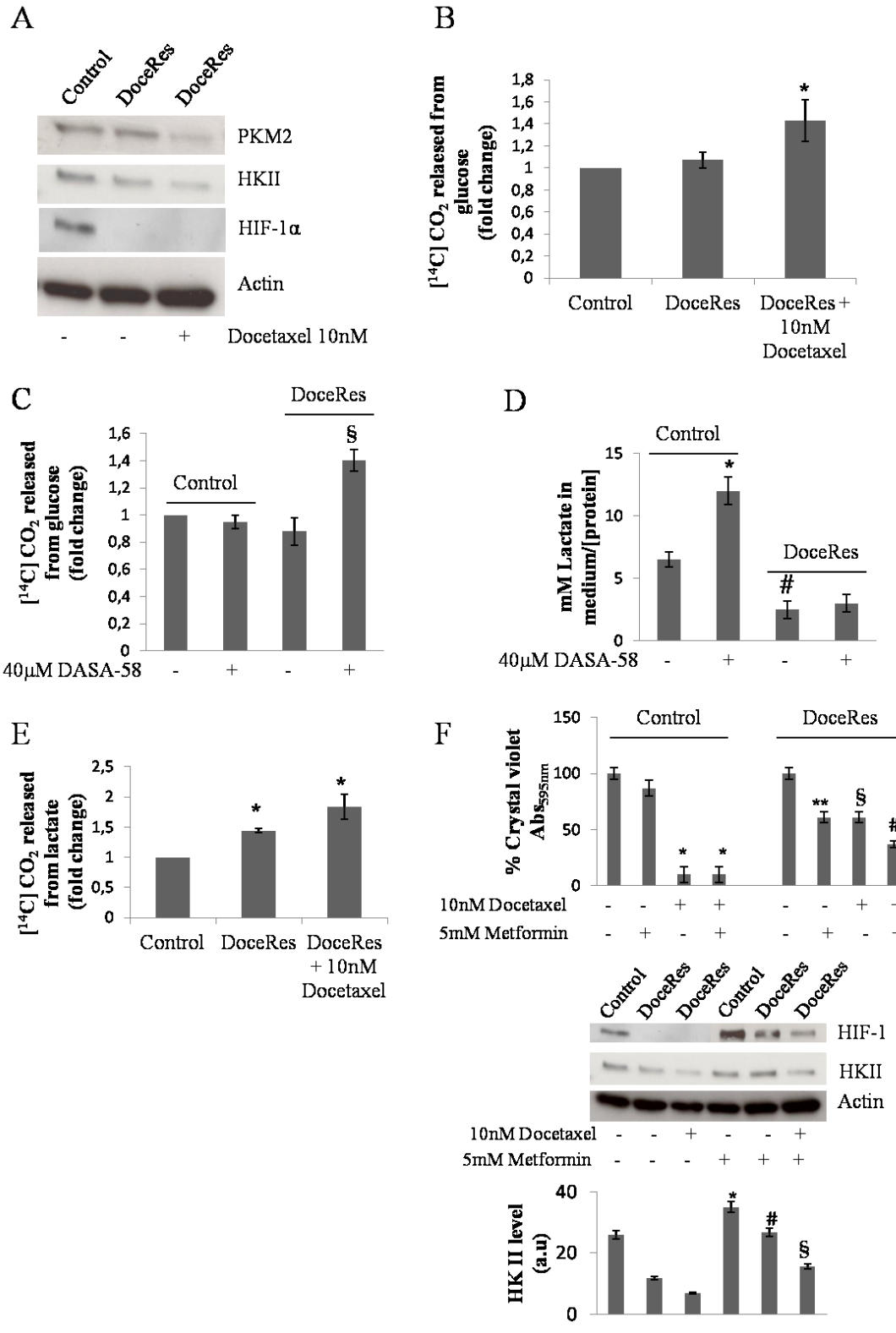

Fig. 4. For figure legend, see next page.

Fig. 4. (See previous page). Metabolic characterization of DoceRes cell line. Control and DoceRes cell lines were cultured in serum-free medium for 48h before analysis. Complete medium was used only for crystal violet assay. Where indicated, 10nM docetaxel, 40μM DASA-58, or 5mM metformin were added to medium. **A)** Immunoblot analysis of PKM2, HKII and HIF-1 in sensitive and resistant cells. Actin immunoblot was used for normalization. **B), C)** Evaluation of [^{14}C]-glucose respiration by [^{14}C]-CO_2 release (fold change respect to Control in **B**; fold change respect to Control without DASA-58 in **C**. *,p<0,05 versus Control; §,p<0,05 versus Control without DASA-58. **D)** Lactate assay in culture medium. Sample protein content was used for normalization. *,p<0,01 versus Control without DASA-58; #, p<0,01 versus Control without DASA-58. **E)** Evaluation of [^{14}C]-lactate respiration by [^{14}C]-CO_2 release (fold change respect to Control). *,p<0,01 versus Control. **F)** Upper panel: evaluation of living cells after 48h in complete medium by crystal violet assay. 20 x 10^3 cells were plated at the start of experiment. Absorbance of Control (-/-) and DoceRes (-/-) was used for normalization. *,p<0,001 versus Control (-/-); **, p<0,01 versus DoceRes (-/-); §,p<0,01 versus DoceRes (-/-); #,p<0,01 versus DoceRes with metformin. Lower panel: immunoblot analysis of HKII and HIF-1 in sensitive and resistant cells. HK II expression in the same samples was shown in bar graph. Actin immunoblot was used for normalization. a.u.; arbitrary unit: *,p<0,05 versus Control (-/-); #, p<0,01 versus DoceRes (-/-); §,p<0,01 versus DoceRes with docetaxel/without metformin.

METFORMIN IMPAIRS INVASIVENESS OF RESISTANT CELLS

Recently, it has been observed that metformin reversed EMT phenotype and decreased the invasive capacity of multidrug-resistant breast cancer cells derived from MCF7 and MDA-MB-231 cell lines (Qu et al., 2013).

To test the effect of metformin on pro-invasive phenotype of DoceRes, we evaluated the invasiveness of these cells by Boyden invasion assay. We found that metformin inhibits invasiveness only in resistant cells, with no effect in sensitive cell line (Fig. 5). These findings show that inhibition of oxidative mitochondrial metabolism in DoceRes cell line is able to revert its invasive properties, further confirming the important role of mitochondria to ensure drug resistance advantages.

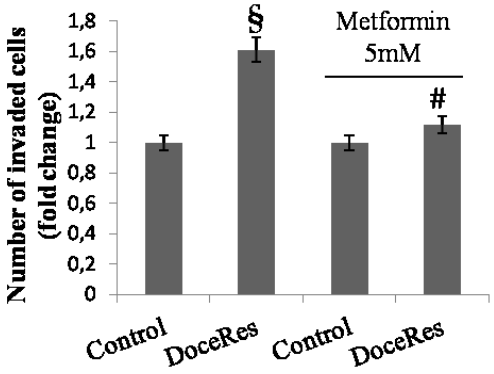

Fig. 5. Metformin impairs invasiveness of resistant cells. Boyden invasion assay of Control and DoceRes cell lines. Bar graph represents the mean of invaded cells (six fields for sample). Number of invaded cells was expressed as fold change respect to Control cells without metformin. §,p<0,05 versus Control without metformin; #,p<0,05 versus DoceRes without metformin.

CAFs REDUCE SENSITIVITY TO DOCETAXEL

CAFs have been associated with reduced chemosensitivity in a number of cancer models. Some of these studies have highlighted the importance of tumor-stroma metabolic coupling in this process. In particular, the metabolic shift from glycolysis to an oxidative mitochondrial metabolism upon contact with stromal fibroblasts (the so called "reverse Warburg" effect) plays a key role to induce tamoxifen-resistance in a breast cancer model (Martinez-Outschoorn et al., 2011).

We have previously reported that PCa cells undergo a "reverse Warburg" metabolism upon contact with CAFs. To test if CAFs are able to confer protection against docetaxel-induced apoptosis, we carried out co-cultures of sensitive PCa cells and CAFs, treating them with 10nM docetaxel. We found that CAFs are able to protect PCa cells to docetaxel toxicity, reducing the number of apoptotic cells (Fig. 6A). Same results were obtained for DoceRes+CAFs co-cultures, increasing docetaxel dose up to 20nM (Fig. 6B). Of note, lactate produced by PCa-activated fibroblasts (PCa-AFs) is uploaded by both sensitive and DoceRes cell lines, as confirmed by lactate in medium (Fig. 6C) and uptake of radioactive lactate (Fig. 6D). These findings are consistent with a “reverse Warburg” phenotype, which may be important to counteract the toxic effect of docetaxel.

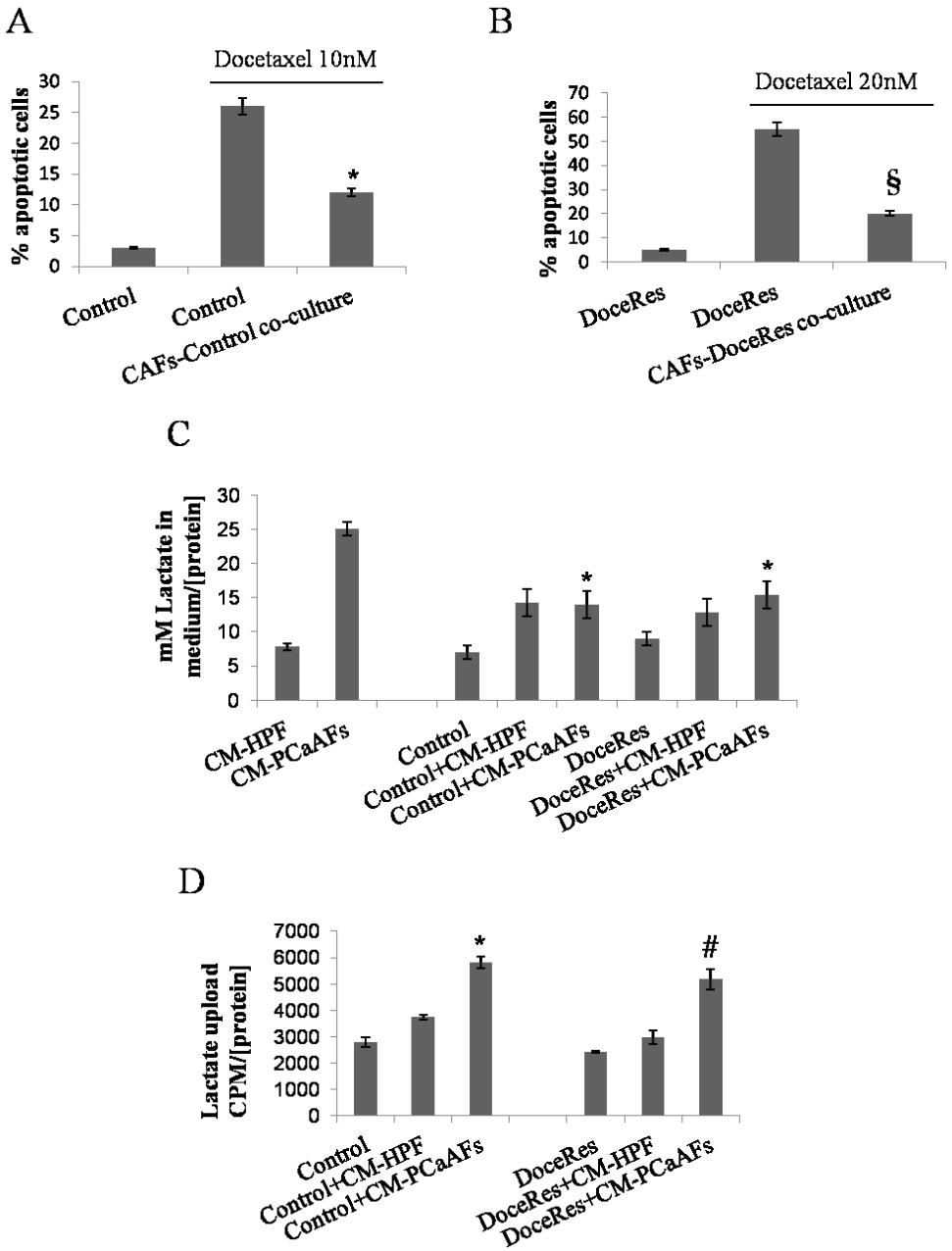

Fig. 6. CAFs reduce sensitivity to docetaxel-induced apoptosis. A), B) Analysis of docetaxel-induced apoptosis with Annexin V/Iodidium Propide cytofluorimetric staining. Control and DoceRes were cultured alone or co-cultured with CAFs (proportion CAFs:PCa 2:1) for 48h; then, docetaxel was added for 72h. After that, cells were detached from plates with Accutase. For co-culture, PCa cells were isolated using immunomagnetic beads targeting fibroblasts. Then, PCa cells were stained with Annexin V and Iodidium Propide and apoptosis was evaluated by flow citometry.*,$p<0,01$ versus Control cells alone treated with docetaxel; §,$p<0,01$ versus DoceRes cells alone treated with docetaxel. **C)** Lactate assay in culture medium. CM-HPFs and CM-PCa-AFs were obtained as previously described. Sensitive and resistant cells were treated with serum free medium (Control and DoceRes) or CM-HPFs or CM-PCa-AFs for 48h. Then, lactate in media was evaluated. *,$p<0,01$ versus CM-PCa-AFs. **D)** Analysis of [^{14}C]-lactate upload in Control and DoceRes cell lines after treatments described in **(C)**. *,$p<0,01$ versus Control+CM-HPFs; #,$p<0,01$ versus DoceRes+CM-HPFs.

DISCUSSION

Deregulation of tumor metabolism has been recently included in the so called "Hallmarks of cancers" (Hanahan and Weinberg, 2011). Several studies report the importance of metabolic reprogramming of cancer cells, which involves a number of metabolic pathway such as glycolysis, TCA cycle, glutaminolysis and fatty acids metabolism (Lunt and Vander Heiden, 2011, Chen and Russo, 2012, Cairns et al., 2011). Metabolic changes in tumor cells often result from deregulation of oncogenes/tumor suppressor genes molecular pathways, but also microenvironmental factors (e.g. hypoxia, CAFs) and mutations in metabolic enzymes affect tumor metabolism (Chen et al. 2012, Israelsen et al. 2013). These adaptations are able to provide metabolic advantages to cancer cells, from growth and survival in hostile environments to chemoresistance. A typical event associated with metabolic reprogramming of cancer cells is the so called Warburg effect (aerobic glycolysis), by which non-hypoxic tumor cells entirely rely on glycolysis in order to support the high biosynthetic and energy demands of actively proliferating cells. Interestingly, Warburg metabolism is not exclusively of tumors, as many non-transformed cells also exhibit high aerobic glycolysis during rapid proliferation (Lunt and Vander Heiden, 2011). To date, a number of studies identified molecular pathways and metabolic enzymes involved in Warburg effect in cancer cells. For example, the M2 isoform of PK is preferentially expressed in cancer, where complex regulation of its activity is important for control of aerobic glycolysis and cell metabolism (Christofk et al, 2008, Anastasiou et al., 2011).

Recently, several findings highlighted the important role of stromal fibroblast to establish a metabolic coupling with cancer cells, thus influencing their metabolic reprogramming. CAFs are a relevant component of tumor microenvironment, being the most represented cells in tumor masses. They can either be fibroblasts resident in the target organ or recruited and differentiated by circulating MSCs of bone marrow origin (Taddei et al. 2013). In any case, they respond to cancer-delivered factors able to enhance their reactivity towards a myofibroblas-like state; in turn, CAFs affect tumor

progression promoting invasive and metastatic phenotype and, ultimately, cancer malignancy.

To date, it has been reported several models of metabolic coupling between stromal and cancer cells. However, controversial data are emerged from these studies. Lisanti's group proposes that, in response to oxidative stress induced by contact with cancer cells, stromal fibroblast undergo activation due to Cav-1 down-regulation, showing a stabilization of HIF-1, a key regulator of glycolytic enzymes; the latter drives a metabolic shift towards aerobic glycolysis, which is associated with elimination of mitochondrial activity through autophagy/mitophagy process (Pavlides et al., 2009, Sotgia et al. 2012, Martinez-Outschoorn et al. 2011, Ko et al., 2011). In turn, energy-rich metabolites secreted by stromal fibroblasts (e.g. lactate/pyruvate and amino acids) can support the mitochondria-dependent growth of tumor cells, fueling oxidative mitochondrial metabolism. In agreement, immuno-histochemical analysis of sections from human breast cancer tissues reveal that PKM2 and lactate dehydrogenase, two enzymes associated with Warburg metabolism, are highly expressed in the stromal compartment (Pavlides et al., 2009). Interestingly, in this model, aerobic glycolysis is considered as a "stromal event" induced by cancer cells in order to sustain their own growth. On the contrary, histological analyses of colorectal carcinoma suggest 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, another study report 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 (Rattigan et al., 2012).

On the basis of these controversial studies, we investigated the metabolic signature of activated CAFs obtained by patients bearing aggressive prostate carcinoma. We also used healthy HPFs activated *in vitro* upon contact with PCa cells (co-culture or CM). Our *in vitro* and *in vivo* analysis revealed that CAFs and PCa cells undergo a mutual metabolic reprogramming. In particular, PCa cells induce in CAFs a Warburg-like glycolytic metabolism, thus increasing glucose uptake and its conversion into lactate. Conversely, PCa cells undergo a "reverse Warburg" phenotype, by which lactate extruded by CAFs by MCT4 transporter is uploaded by PCa cells, through their MCT1

transporter and used for fueling TCA cycle, as well as anabolic processes and cell proliferation. PCa cells gradually become glucose consumption-independent and develop a dependence on lactate upload; indeed, MCT1 expression in cancer cells is mandatory for tumor growth, as indicated by the efficiency of *in vitro* and *in vivo* targeting of MCT1 with CHC or RNA interference. In agreement with our results, it has been reported that expression of MCT4 is increased in CAFs extracted by human PCa, and such expression is correlated with poor clinical outcome both in prostate and breast cancer (Witkiewicz et al., 2012). In addition, the expression of MCT1 in cancer cells is also associated with poor prognosis (Boidot et al, 2012).

Previous studies conducted in our laboratory demonstrated that CAFs induce in PCa cells stem-like features and mesenchymal motility through EMT (Giannoni et al., 2010). These events are driven by HIF-1 and NF-kB dependent pro-inflammatory signature (Giannoni et al., 2011). In particular, we showed that CAFs are able to mimic a hypoxic environment, eliciting ROS production in PCa cells and thereby inducing HIF-1 stabilization under normoxic conditions. Here, we demonstrate that even PCa cells can elicit oxidative stress in stromal fibroblasts, as demonstrated by increased ROS production in *in vivo* and *in vitro* activated CAFs. ROS-mediated stabilization of HIF-1 drives metabolic reprogramming both in CAFs and tumor cells, leading to glycolytic metabolism in CAFs and "reverse Warburg" phenotype in PCa cells. The importance of oxidative stress and ROS-mediated activation of HIF-1 in reciprocal interplay between tumor and stomal cells, it has been demonstrated by other research group. In particular, Toullec et al. and Lisanti's group reported that ROS are key regulators during activation of fibroblasts in response to tumor, leading to stabilization of HIF-1 (Toullec et al. 2010; Sotgia et al. 2012; Lisanti et al. 2011; Pavlides et al, 2012). In addition, Toullec et al. showed that HER2-human breast adenocarcinoma exhibit a high proportion of myofibroblasts, which was significantly correlated to nodal metastases. Interestingly, this subset of tumors display a molecular signature characteristic of oxidative stress–response.

In our model, redox-dependent activation of HIF-1 in CAFs results from down-regulation of mitochondrial deacetylase SIRT3. Sirtuins are a conserved family of NAD-dependent ADP-ribosyltransferases and/or protein deaceytlases involved in metabolism, stress response and longevity. Mammals express seven sirtuins (SIRT1-7),

three of which (SIRT3-5) are localized to the mitochondrion. SIRT3 is a major mitochondrial deacetylase that targets many enzymes involved in central metabolism, resulting in the activation of many oxidative pathways. For example, SIRT3 deacetylates complex I and complex II to activate electron transport. Interestingly, SIRT3 opposes to Warburg phenotype of cancer cells, mainly acting via destabilization of HIF-1, leading to inhibition of glycolysis and activation of oxidative metabolism. As a consequence, the genetic loss of the SIRT3 in breast cancers favors aerobic glycolysis, causing oxidative stress that culminates in HIF-1 up-regulation (Finley et al., 2011). Our data show that, in activated fibroblasts, SOD2 acetylation/inactivation due to SIRT3 down-regulation causes an increase of ROS production and consequent HIF-1 stabilization, leading to glycolytic switch. Therefore, we can include CAFs as cells undergoing a SIRT3-mediated activation of HIF-1 and Warburg metabolism, enlarging the role played by SIRT3-loss to stromal cells.

Given the importance of ROS in metabolic reprogramming of CAFs and PCa cells, we aimed at identifying natural antioxidant molecules able to disrupt the diabolic liaison between tumor and stroma. To date, several studies on a wide spectrum of phenolic compounds extract from vegetables and fruits showed that these natural products can act as potent antioxidant or anticancer agents (Aravindaram et al, 2010), behaving as chemopreventive molecules (Singh et al. 2006). The molecular targets of these agents include cell signaling, cell-cycle regulators, survival/apoptotic molecules and antioxidant enzyme systems, but can also act directly as ROS scavenger (Singh et al. 2006, Aravindaram et al, 2010). For example, curcumin and green tea catechins (GTCs) show chemopreventive effect in prostate carcinoma (Singh et al. 2006). In particular, GTCs prevent PCa onset in transgenic adenocarcinoma mouse prostate (TRAMP) mice that spontaneously develop prostate cancer (Caporali et al., 2004). In addition, a randomized controlled trial showed that daily administration of GTCs in volunteers with high-grade prostate intraepithelial neoplasia prevent aggressive PCa development (Bettuzzi et al., 2006). In our study, considering the key role played by SIRT3 down-regulation and ROS in metabolic reprogramming of CAFs, we used the flavonoid kaempferol, a known SIRT3-activator, in order to inhibit such process. Kaempferol is able to increase SIRT3 expression, thus reducing ROS production and HIF-1 stabilization. As a consequence, kaempferol block the metabolic loop between CAFs

and PCa cells, leading to decrease of PCa cell growth. These results clearly demonstrate that kaempferol could be a useful molecule in the management of PCa and represents an important example of how natural antioxidant can inhibit ROS-dependent mechanism involved in cancer progression, thereby reducing tumor aggressiveness.

Our model of metabolic coupling between stroma and tumor highlight the important role of mitochondrial respiration in cancer metabolism and tumor progression, and it is in agreement with other models described in literature where energy rich metabolites derived from corrupted stoma fuel TCA cycle. The ability of tumor cells to exploit oxidative phosphorylation indicates that the traditional view of cancer metabolism requires careful revision. In particular, recent data highlight the inconsistencies of the Warburg effect in tumor cells. First of all, not all tumors are glycolytic. A systematic review of the literature in which the relative contributions to ATP production were measured from both glycolysis and oxidative metabolism indicates that cancer cells display a wide range of metabolism and that only ~30% of the tumors are glycolytic (Sotgia et al., 2012). Some tumors (for example, glioma), show an increase in both glycolysis and oxidative phosphorylation, thus displaying metabolic heterogeneity. Furthermore, some oncogenes commonly associated to aerobic glycolysis, such as c-Myc and Ras, also stimulate mitochondrial biogenesis and function. For example, c-myc induces cancer cells to utilize both oxidative phosphorylation and glycolysis and promotes glutamine oxidation by TCA cycle. In addition, several studies indicate that mitochondrial respiration is necessary to sustain aggressive tumor growth. For example, the mitochondrial protein p32 was recently shown to promote oxidative phosphorylation and to sustain tumor growth and increased levels of respiratory electron transport chain subunits predict poor clinical outcome in human breast cancer (Sotgia et al., 2012). Finally, drugs targeting mitochondrial respiration are being evaluated as anti-neoplastic agents in clinical trials (Sotgia et al., 2012; Ramsay et al., 2011). In particular, metformin, a widely used oral anti-diabetic drug, exhibits a strong anti-proliferative action in numerous cancer cell lines *in vitro* and *in vivo*. Metformin is reported to inhibit mitochondrial complex I activity, thereby disrupting oxidative mitochondrial metabolism and promoting glycolysis and lactate accumulation. Diabetic patients treated with metformin experience a lower risk of pancreatic cancer compared with those who had not taken metformin. In addition, in a large retrospective study with

more than 2,500 breast cancer patients, metformin treatment increased the complete pathological response rates following neoadjuvant chemotherapy, which points to its potential role as a new anticancer drug (Sotgia et al., 2012).

Mounting evidence supports the idea that deregulated cellular metabolism is linked to drug resistance in cancer therapy. To address this point, we developed a PCa cell line resistant to docetaxel, a promising drug for treatment of metastatic PCa and we evaluated phenotypic and metabolic features of these cells.
Resistance to docetaxel confers to PCa cells a pro-invasive behavior and a down-regulation of E-cadherin, consistent with an EMT program and a pro-metastatic phenotype. The acquisition of EMT in docetaxel-resistant PCa cells it has been reported also by Puhr and colleagues (Puhr et al., 2012), who demonstrated that EMT results from down-regulation of miR-205 and is at least in part responsible for chemotherapy failure. Moreover, we found that resistant cells show up-regulation of Nrf2, a master regulator of antioxidant response, able to maintain cellular redox homeostasis through elimination of toxicants/carcinogens. Interestingly, a link between Nrf2 and EMT it has been demonstrated, implying that chemoresistance of cancer cells upon the loss of E-cadherin might be associated with Nrf2 (Kim et al., 2012). In particular, the authors report that E-cadherin recruits Nrf2 through β-catenin, and assists the function of Keap1 for the inhibition of nuclear localization and transcriptional activity of Nrf2. In our model, up-regulation and nuclear localization of Nrf2 is associated with down-regulation both of E-cadherin and Keap1. In agreement, we observed that resistant cells show decreased ROS production when treated with docetaxel respect to sensitive cells. Given that docetaxel induce cell death in a ROS-dependent manner (Cao et al., 2005), we speculate that activation of Nrf2 transcriptional program play a key role in management of drug-induced oxidative stress in resistant cells, resulting, together with reduced expression of pro-apoptotic proteins, in cell survival. Interestingly, the docetaxel transporter MDR1 (multidrug transporter proteins-1 or P-glycoprotein), which regulates its efflux from cell, is included among Nrf2 target gene (Klaassen and Aleksunes, 2010; Hardwick et al, 2011; Ehrlichova et al., 2005), further demonstrating the important role of Nrf2 in docetaxel resistance.

Antioxidant response requires NADPH in order to fuel antioxidant systems. PPP is an important pathway to generate this coenzyme and it has been already associated with chemoresistance (Polimeni et al, 2011). The analysis of PPP in docetaxel resistant cells revealed that such pathway is down-regulated compared to sensitive cells and it is not involved in counteracting oxidative stress induced by drug. However, other metabolic enzymes produce NADPH in cells, such as malic enzyme and cytosolic IDH1; hence, further analyses are needed in order to identify a source of NADPH that sustains antioxidant response in resistant cells.

Metabolic analysis of resistant and sensitive cells suggest that chemoresistance to docetaxel induces an escape from Warburg metabolism with a potential involvement of mitochondrial respiration to confer a metabolic advantage to these cells. Indeed, docetaxel-resistant cell line shows a grater utilization of glucose by mitochondrial respiration, a down-regulation of HIF-1 actvity and PKM2 and a decreased lactate production compared to sensitive cell line. Reduced cell growth rate and down-regulation of PPP, the latter commonly associated with Warburg effect and cell growth, being a source of NADPH and nucleotides, further confirm the metabolic shift from aerobic glycolysis to oxidative mitochondrial metabolism in resistant cells. In addition, the PKM2-activator DASA-58, a well known molecule that allows the escape from Warburg metabolism (Anastasiou et al., 2011, 2012), is able to induce glucose oxidation to CO_2 only in resistant cells, whereas sensitive counterpart produce lactate. A possible explanation of this result is that down-regulation of HIF-1 in resistant cells leads to decreased expression of its target gene PDK. Therefore, the re-activation of PDH could be responsible of pyruvate oxidation in TCA cycle. Finally, resistance to docetaxel confers the ability to metabolize extracellular lactate by mitochondrial respiration. This feature could be considered as a further advantage for resistant cells, which could exploit energy rich metabolites in microenvironment derived from corrupted stroma. Taken together, these findings suggest that the shift towards oxidative mitochondrial metabolism could be an important feature useful to overcome docetaxel toxicity. In particular, we hypothesize that the recovery of mitochondrial respiration could subtract glycolytic intermediates from anabolic processes and from PPP, to drive ATP production. In turn, ATP may be used to fuel MDR transporters in order to extrude the drug.

Given the importance of oxidative mitochondrial metabolism in resistant cells, we used the mitochondrial complex I inhibitor metformin in order to impairs metabolic advantages of these cells. In agreement with other studies (Fendt et al. 2013, Sotgia et al, 2012), we found that metformin causes a shift towards glycolytic metabolism (up-regulation of HIF-1 and hexokinase II); however, such drug inhibits cell growth selectively in resistant cells, further confirming their dependence on mitochondrial respiration. Interestingly, metformin is able also to impair invasiveness of resistant cells. This result is in agreement with data obtained by Qu and colleagues (Qu et al., 2013), who demonstrated that metformin reverses EMT phenotype and decreases the invasive capacity of multidrug-resistant breast cancer cells.

Finally, our data demonstrate the protective effect of CAFs against docetaxel toxicity, both in sensitive and resistant cells, likely by inducing the "reverse Warburg" phenotype in cancer cells. These results are in agreement with Lisanti and colleagues (Martinez-Outshoorn et al., 2011), who proposes that the metabolic shift from glycolysis to an oxidative mitochondrial metabolism, upon contact with stromal fibroblasts, plays a key role during acquisition of chemoresistance in breast cancer. However, further experiments will be necessary to elucidate the role of prostate fibroblasts and lactate shuttle in resistance to docetaxel. In conclusion, our results are consistent with the idea that chemoresistance may be both a metabolic and stromal phenomenon, that can be overcome by reducing mitochondrial function.

Besides the role in metabolic reprogramming, we report that redox-based HIF-1 activation is involved in CA IX *de novo* expression in CAFs upon contact with PCa cells. In particular, we observed that CA IX up-regulation in activated fibroblasts is mandatory to elicit the secretion of MMP-2 and MMP-9 through acidification of extracellular milieu, thus driving EMT program in PCa cells.

CA IX is a common target gene of HIF-1 and it has been widely correlated to intratumoral hypoxia. Indeed, CA IX over-expression has been demonstrated in several cancer models as a master regulator of pH homeostasis, which is crucial for cancer cells in order to avoid potentially harmful effects of an highly glycolytic and therefore pro-acidic metabolism (Robertson et al., 2004; Neri and Supuran 2011). However, CA IX participation in the generation of an increasingly acidic extracellular environment and

the consequential promotion of tumor cells invasiveness, is still poorly understood. In literature, few data indicate a CA IX contribution in motility of cancer cells. Svastova et collegues pointed out the ability of CA IX to modulate E-cadherin-dependent cell adhesion through direct co-localization with β-catenin and to promote loss of cell-cell contact, the key initial step of cancer invasion (Svastova et al., 2003). Furthermore, in colorectal cancer cells it has been proved that COX-2-dependent expression of CA IX correlates with tumor stage and increases cancer cells invasiveness (Sansone et al., 2009). Finally, in cervical carcinoma cells, the over-expression of exogenous human CA IX leads to weakened cytoskeletal remodeling, disassembled focal adhesion, cell-cell adhesion and increased cell motility, mainly acting through inactivation of Rho small GTPase (Shin et al., 2011).

Unlike these data, we report that stromal fibroblasts infiltrating prostate carcinoma concur to extracellular acidification through CA IX over-expression, which are essential to drive MMP-2 and -9 secretion, rapidly leading to PCa cells EMT and metastatic dissemination in SCID mice. Interestingly, in our model, Warburg metabolism and acidification of extracellular environment due to lactate production, features commonly associated to cancer cells, are exploited by stromal compartment corrupted by tumor. However, although CAFs can be viewed as acidifying cells, due to their dramatic production of lactate, extruded together with H^+ using their over-expressed MCT-4, lactate and H^+ are both rapidly removed from tumor microenvironment by metabolically reprogrammed cancer cells, expressing MCT-1 and uploading lactate to sustain cell growth. In this context, extracellular acidification due to H^+ produced by CA IX of CAFs plays a key role in activation of MMPs, due to their well known sensitivity to acidity. In turn, MMPs drive EMT in PCa cells, by promoting the pro-oxidant/pro-inflammatory pathway previously described (Giannoni et al., 2011).

Taken together, our data highlight the plasticity of cancer cells, which engage adaptive strategies corrupting their stroma and reprogramming their metabolism to satisfy their requirements. Tumor cells rely on stromal fibroblasts both for a metabolic support and to engage invasive program in order to survive and escape from hostile environments. In our model, malignant cells are able to exploit byproducts of stromal cells in catabolic and anabolic processes, reprogramming their metabolism towards mitochondrial

respiration (the so called "reverse Warburg" phenotype) and becoming progressively independent from Warburg metabolism. This strategy could be important when conditions of nutrient starvation occur inside the tumor mass. Furthermore, metabolically reprogrammed fibroblasts are able to engage pro-invasive and pro-metastatic properties in cancer cells, by CA IX-mediated acidification of extracellular milieu. Finally, we demonstrated the key role played by ROS in the reciprocal interplay between tumor and stromal cells. In this context, we showed that antioxidant molecules could represent an effective strategy to target oxidative stress and reduce, ultimately, the aggressiveness of tumor.

The adaptations in the metabolism of cancer cells play an important role even during acquisition of chemoresistance, providing metabolic advantages useful to overcome the toxic effect of anticancer drugs. To date, both aerobic glycolysis and mitochondrial respiration have been associated with increased resistance to anticancer drugs (Zhao et al., 2013; Martinez-Outshoorn et al., 2011). Our model suggests that the shift from Warburg metabolism to mitochondrial respiration is an important mechanism to confer these advantages to resistant cancer cells. Furthermore, we include CAFs as active bystanders during the phenomenon of chemoresistance.

Our results highlight the importance to understand the metabolic reprogramming and the adaptive strategies of cancer cells in order to identify new therapeutic targets which can be used in combination with conventional chemotherapy. To date, several molecules targeting cellular metabolism are able to improve cancer therapeutics and overcome drug resistance (Zhao et al., 2013). Here, we point out the important role of mitochondrial respiration in cancer progression and chemoresistance and we propose the disruption of mitochondrial function as attractive therapy. Furthermore, our data include stromal fibroblasts as key regulators of adaptation strategies used by tumors, adding a further level of complexity to the system. Indeed, CAFs, as they are non-transformed genetically stable cells, could be ideal pharmacological targets with respect to cancer cells (Chometon and Jendrossek., 2009), which are endowed with intrinsic adaptive features.

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