



Hypoxia and cancer metabolism: genetic and functional analysis of H⁺/lactate symporters and their chaperone, BASIGIN

Ibtissam Marchiq

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ECOLE DOCTORALE SCIENCES DE LA VIE ET DE LA SANTE (ED85)

THESE

Présentée pour obtenir le titre de
Docteur es Sciences de l'Université de Nice Sophia-Antipolis
Spécialité : Aspects Moléculaires et Cellulaires de la Biologie

Hypoxie et métabolisme tumoral : analyse génétique et fonctionnelle des symporteurs H⁺/lactate et de leur chaperone, BASIGINE

Hypoxia and cancer metabolism: genetic and functional analysis of H⁺/lactate symporters and their chaperone, BASIGIN

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Centre Antoine Lacassagne par :*

Ibtissam MARCHIQ

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To my parents

"Never start stopping, never stop starting"

Marcus Tullius Cicero

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List of abbreviations

2-OG: 2-oxoglutarate

6PGD: 6-phosphogluconate Dehydrogenase

ABCB1: ATP-binding cassette transporter B1

Acetyl-coA: Acetyl coenzyme A

ADP: Adenosine diphosphate

AE: $\text{Cl}^-/\text{HCO}_3^-$ anion exchangers

AIF: Apoptosis-Inducing Factor

ALT: Alanine transaminase

AMPK: AMP-activated protein kinase

ASCT: Asc-type amino acid transporter

ASCT2: Asc-type amino acid transporter 2

ATP: Adenosine triphosphate

bHLH-PAS: basic-Helix-Loop-Helix Per-AHR/ARNT/Sim protein

BOLD-MRI: Blood Oxygenation Level Dependent Magnetic Resonance Imaging

BSG: BASIGIN

CA: Carbonic anhydrases

CAF: Cancer associated fibroblasts

CAIX/CAXII: Carbonic anhydrase IX/XII

CHC: α -cyano-4-hydroxycinnamate

CIE: Clathrin-independent endocytosis

CO_2 : Carbon dioxide

CREB: Cyclic AMP Response Element Binding protein

C-TAD: C-terminal trans-activation domain

DNA: Desoxyribonucleic acid

ECM: Extracellular matrix

EGF: Epidermal growth factor

EMMPRIN: Extracellular Matrix MetalloPRotease INducer

EPAS1: Endothelial PAS domain protein 1

EPO: Erythropoietin

ERK: Extracellular signal-regulated kinase

ETC: Electron Transporter Chain

F6P: Fructose 6-Phosphate

FAD: Flavin Adenine Dinucleotide
FDG-PET: ¹⁸ F-Deoxyglucose Positron Emission Tomography
FIH-1: Factor Inhibiting HIF-1
G6P: Glucose-6-Phosphate
G6PD: Glucose-6-Phosphate Dehydrogenase
GA-3P: Glyceraldehyde-3P
GBE1: Glucan (1,4- α -) branching enzyme 1
GDH: Glutamate dehydrogenase
GLS: Glutaminase
GLUD1: Glutamate dehydrogenase 1
GLUT: Glucose Transporters
GP: Glycogen phosphohrylase
GPT: Glutamic pyruvate transaminase
GS: Glycogen synthase
GSH: Glutathione
GYS1: Glycogen synthase 1
HIF: Hypoxia-inducible factor
HK2: Hexokinase 2
HNSCC: Head and neck squamous carcinoma
HRE: Hypoxia-Responsive Element
IL: Interleukin
IMM: Inner mitochondrial membrane
IPAS: Inhibitory PAS domain protein
LAT1: Large neutral amino transporter 1
LDH: Lactate dehydrogenase
LKB1: Liver kinase B1
LLC: Lewis lung carcinoma
MCF: Mitochondrial carrier family
MCT: Monocarboxylate transporter
MEF: Mouse embryonic fibroblasts
MISO – PET: Misonidazole positron emission tomography
MMP: Matrix metalloproteases
MPC: Mitochondrial pyruvate carrier
NAD⁺: Nicotinamide Adenine Dinucleotide

NDRG3: N-Myc downstream-regulated gene 3
NF-IL6: Nuclear Factor for interleukin 6
NF- κ B: Nuclear Factor kappa-B
NHE: Na^+/H^+ exchanger 1
NIRS: Near-Infrared Spectroscopy
NP 55/65: Neuroplastins 55/65
NPM: N phenylmaleimide
NRF2: Nuclear factor erythroid 2
 O_2 : Oxygen
OCT1: Organic cation transporter 1
ODD: Oxygen Dependent Domain
OMM: Outer mitochondrial membrane
OXPHOS: Mitochondrial oxidative phosphorylation
PC: Pyruvate carboxylase
PDH: Pyruvate Dehydrogenase
PFK-1: Phosphofructokinase 1
PFK2: Phosphofructo-2-kinase
PGC-1 α : Peroxisome proliferator activator receptor γ coactivator-1 α
PGM1: Phosphoglucomutase 1
PHD: Prolyl hydroxylases
pHe: Extracellular pH
pHi: Intracellular pH
PKL: Pyruvate kinase L
PKM: Pyruvate kinase M
 pO_2 : Oxygen partial pressure
PPP: Pentose Phosphate Pathway
PTEN: Phosphatase and TENsin homolog deleted on chromosome TEN
PYGL: Glycogen phosphorylase
ROS: Reactive Oxygen Species
Ru5P: Ribulose-5-Phosphate
SCO2: synthesis of cytochrome c oxidase 2
shRNA: Short hairpin RNA
SLC16A: Solute Carrier family 16
TCA: Tricarboxylic acid

TGF: Transforming growth factor
TIGAR: TP53-Induced Glycolysis and Apoptosis Regulator
TKT: Transketolase
TM: tTransmembrane domain
UGP2: UDP-glucose pyrophosphorylase 2
V-ATPase: Vacuolar-H⁺ APTase
VDAC: Voltage-dependent anion channels
VEGF: Vascular endothelial growth factor
VHL: von Hippel-Lindau protein
ZFN: Zinc finger nucleases
 α -KG: α -ketoglutarate

RÉSUMÉ/ABSTRACT

Résumé

Depuis la première mention par Otto Warburg au début du XX^{ème} siècle que les cellules cancéreuses sont dépendantes de la glycolyse fermentative, une voie métabolique nécessaire pour la production d'énergie et des intermédiaires anaboliques, des milliers d'études ont permis d'éclairer les mécanismes moléculaires impliqués dans le métabolisme aberrant des cellules cancéreuses. L'hypoxie, via les facteurs de transcription hypoxia-inducible factors (HIFs), ainsi que l'activation des oncogènes et la perte des gènes suppresseurs de tumeurs constituent des régulateurs majeurs, non seulement de «l'effet Warburg», mais également de plusieurs autres voies métaboliques comme la glutaminolyse. Le catabolisme exacerbé du glucose et de la glutamine est actuellement reconnu comme une caractéristique des cellules cancéreuses, qui leur procure un avantage prolifératif via la production et l'accumulation de plusieurs métabolites au niveau du microenvironnement. Parmi ces métabolites, l'acide lactique représente une molécule de signalisation clé, qui bien au-delà de son rôle dans l'induction du stress acide, joue un rôle crucial dans la migration des cellules cancéreuses, l'angiogenèse, l'évasion du contrôle du système immunitaire et les métastases. Mon projet de thèse s'inscrit dans le contexte d'une étude du métabolisme glycolytique associé aux cellules tumorales à division rapide pour lesquelles les cliniciens n'ont toujours pas de traitements efficaces. Durant ce projet, nous nous sommes intéressés à la caractérisation génétique et fonctionnelle des transporteurs MCT (MonoCarboxylate Transporters) 1 et 4, qui sont des symporteurs H⁺/lactate dont l'expression membranaire et la fonctionnalité requièrent la liaison avec une protéine chaperonne : CD147/BASIGINE (BSG). BSG, aussi appelée EMMPRIN (Extracellular Matrix MetalloPRotease INducer) est une glycoprotéine ubiquitaire, très fortement exprimée dans plusieurs types tumoraux, notamment dans les tumeurs les plus agressives. Etant donné la multiplicité des interactions et de fonctions de BSG, son rôle dans la promotion de la croissance tumorale est mal défini. Afin de mieux explorer la physiologie des complexes MCT/BSG, et valider le ciblage de l'export d'acide lactique comme une nouvelle approche anti-cancer, nous avons développé une stratégie visant à invalider le gène BSG et/ou MCT4, en utilisant la technologie des Zinc Finger Nucleases (ZFN), dans des lignées cellulaires cancéreuses humaines de côlon, poumon et glioblastome. D'abord, nous avons démontré, que contrairement à ce qui est largement publié dans la littérature, l'invalidation du gène *BSG* dans les cellules tumorales et les fibroblastes n'affecte pas la production des metalloprotéases, et que l'effet pro-tumoral majeur de BSG est lié à son action directe sur le métabolisme énergétique *via* la stabilisation des MCTs au niveau des tumeurs glycolytiques. Ensuite, nous avons démontré pour la première fois que l'inhibition concomitante des deux transporteurs MCT1 et MCT4 est nécessaire pour induire une baisse significative du taux de la glycolyse, de la prolifération *in vitro* et de la tumorigénécité *in vivo*. En outre, nos résultats montrent que l'invalidation de *BSG* induit une grande réduction de l'expression membranaire et de l'activité de MCT1 et MCT4, causant ainsi une accumulation du pool intracellulaire des acides lactique et pyruvique, une diminution du pH intracellulaire et une baisse du taux de la glycolyse. Au cours de cette thèse, nous avons également montré que ces cellules invalidées pour les complexes BSG/MCT, sont capables de proliférer aussi bien que les cellules parentales *via* un 'switch' métabolique de la glycolyse vers la phosphorylation oxydative. Enfin, nous avons démontré que l'inhibition des complexes MCT/BSG en combinaison avec la phenformine, un inhibiteur du complexe I de la mitochondrie, induit une baisse accrue du niveau d'ATP des cellules tumorales en normoxie et en hypoxie, provoquant une mort cellulaire par « catastrophe métabolique », ce qui fournit une stratégie anti-cancer très prometteuse.

De ce fait, notre approche génétique valide le potentiel clinique des inhibiteurs pharmacologiques de MCT1 et MCT4, actuellement en développement

Abstract

Since Otto Warburg reported the ‘addiction’ of cancer cells to fermentative glycolysis, a metabolic pathway that provides energy and building blocks, thousands of studies have shed new light on the molecular mechanisms contributing to altered cancer metabolism. Hypoxia, through hypoxia-inducible factors (HIFs), in addition to oncogenes activation and loss of tumour suppressors constitute major regulators of not only the “Warburg effect” but also many other metabolic pathways such as glutaminolysis. Enhanced glucose and glutamine catabolism has become a recognized feature of cancer cells, leading to accumulation of metabolites in the tumour microenvironment, which offers growth advantages to tumours. Among these metabolites, lactic acid, besides imposing an acidic stress, is emerging as a key signalling molecule that plays a pivotal role in cancer cell migration, angiogenesis, immune escape and metastasis. In this thesis, we focused on the genetic and functional characterization of monocarboxylate transporters (MCT) 1 and 4, which are H^+ /lactate symporters that require an interaction with an ancillary protein, CD147/BASIGIN (BSG), for their plasma membrane expression and function. BSG, also called EMMPRIN for Extracellular Matrix MetalloPRoteases INducer, is a conserved transmembrane glycoprotein that is strongly expressed in several tumour types. Considering the multiplicity of functions and interactions of BSG, its role in promotion of tumour growth has remained poorly defined. To further explore the physiology of MCT/BSG complexes and validate the blockade of lactic acid export as an anti-cancer strategy, we designed experiments using Zinc Finger Nuclease mediated *BSG* and/or *MCT4* gene knockouts in human colon adenocarcinoma, lung carcinoma and glioblastoma cell lines. First of all, we demonstrated that, in contradiction with the abundant literature, the disruption of the *BSG* gene in tumour cells and fibroblasts has no action on the production of matrix metalloproteases and that the major protumoural action of BSG is to control the energetics of glycolytic tumours *via* MCT1/4 activity. Second, we showed for the first time that combined inhibition of both MCT1 and MCT4 transporters is required to achieve a significant reduction in glycolysis, the proliferation rate *in vitro*, and the tumour growth *in vivo*. Moreover, our findings reported that disruption of the *BSG* gene dramatically reduced the plasma membrane expression and lactate transport activity of both MCT1 and MCT4, leading to increased accumulation of intracellular pools of lactic and pyruvic acids, decreased intracellular pH and reduced rate of glycolysis. Thirdly, we found that these BSG/MCT disrupted cells survived and resumed growth by redirecting part of their energy metabolism from glycolysis towards oxidative phosphorylation. Finally, we showed that the inhibition of MCT/BASIGIN complexes highly sensitises tumour cells to phenformin, an inhibitor of mitochondrial complex I. Phenformin addition to these MCT-disrupted cells in normoxic and hypoxic conditions induces a rapid and major drop in cellular ATP provoking cell death by ‘metabolic catastrophe’.

Therefore our genetic approach validates the anti-cancer potential of MCT1 and MCT4 pharmacological inhibitors in current development.

INTRODUCTION

Introduction

The discovery in the early 20th century of genes implicated in cancer and the study of mechanisms directly involved in alterations to DNA have dominated the field of cancer research for many decades (Hanahan and Weinberg, 2011). However, while this has highlighted, in part, our understanding of processes of malignant transformation it has become evident that changes in the genome are not sufficient to explain how cancer cells replenish their stock of energy and building blocks to rapidly divide (DeBerardinis et al., 2008). This realization has revived interest in understanding cancer cell metabolism and has launched the concept of “cancer metabolic reprogramming”, first described by Otto Warburg a century ago (Warburg, 1956). Several studies over the last decades have shed light on the link between oncogenes, tumour suppressors, metabolic remodelling and tumour growth. However, while these studies confirmed the increased rates of glycolysis of cancer cells, as reported by Warburg, they also show that these cells are addicted to glutaminolysis (DeBerardinis and Cheng, 2010; DeBerardinis et al., 2007; Scott et al., 2011). These two pathways, among others, cooperate to satisfy the demand for ATP, carbon skeletons, and nitrogen required to synthesis of macromolecules of the tumour cells. In addition, increasing evidence supports the role of changes in the microenvironment, including nutrient limitation and oxygen availability, in modulating cancer metabolism. Thus hypoxia, through the hypoxia-inducible factors (HIFs), is considered to be a key player in the transactivation of genes implicated in altered metabolism, leading to the accumulation of diverse metabolites in the microenvironment that promote tumour growth and metastasis (Brahimi-Horn et al., 2007; Pouyssegur et al., 2006; Semenza, 2010). Among these metabolites, lactate is drawing the attention of the cancer research community, not as a by product of fermentative glycolysis, but more as a metabolic modulator at the interconnection between different cancer hallmarks including, sustained angiogenesis, evasion of immune surveillance and reprogramming of energy metabolism (Hanahan and Weinberg, 2011; Hirschhaeuser et al., 2011; Walenta and Mueller-Klieser, 2004). Therefore, proteins regulating lactate metabolism offer promising opportunities for developing new anticancer therapies (Doherty et al., 2014; Fantin et al., 2006; Marchiq et al., 2015b). In this thesis, we will: briefly present hypoxia and its role in cancer, emphasise the metabolic pathways implicated in lactate production, summarise the role of lactate and lactate transport complexes in tumour development and highlight the recent advances, benefits and risks of future therapies based on inhibition of lactate transport.

A. Hypoxia and cancer

I. Atmospheric oxygen and evolution of biological complexity

1.1. Fluctuations in oxygen have led to the evolution of life forms

Oxygen (O₂) is the third most abundant element of the universe (Klemperer, 2006), and probably one of the critical components of air, permitting eukaryotic life on Earth (Hedges et al., 2004; Kump, 2008; Thannickal, 2009). According to geochemical studies, during the last 4 billion years, the earth's atmosphere recorded several fluctuations in the O₂ concentration, ranging from 0% to 35%, which led to the evolution of life from an anoxic to an oxygen-rich environment (Castresana and Saraste, 1995; Dismukes et al., 2001; Holland, 2006) (Fig. 1). The present level of O₂ at sea level (21.1%; 160mmHg) has remained stable for the past half billion years, and is thought to be mechanistically associated with our evolutionary history and the escalating development of complex multicellular life forms (Hedges et al., 2004; Koch and Britton, 2008; Thannickal, 2009). To face the challenge of balancing the conflicting vital versus toxic effects of O₂, multicellular aerobic organisms have developed sophisticated intracellular mechanisms that are involved in antioxidant defence and the use of O₂ for energy production (Raymond and Segre, 2006; Stamati et al., 2011).

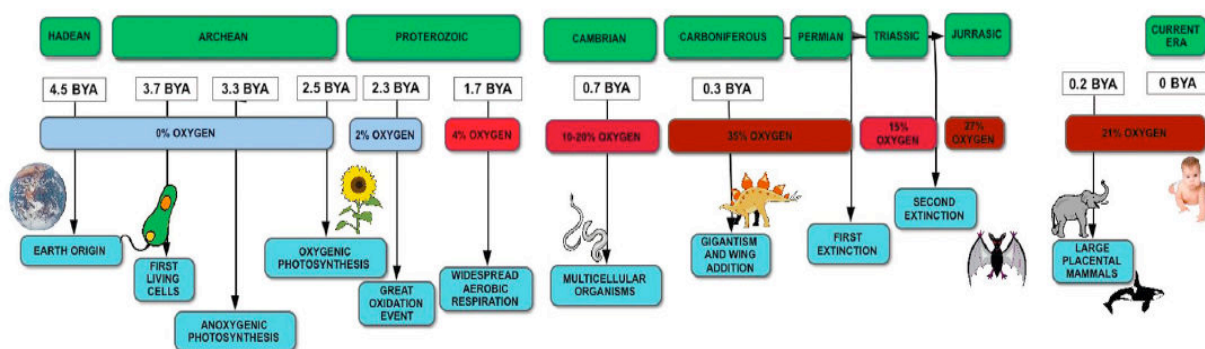


Figure 1. Changes in oxygen and the evolution of life on Earth.

The concentrations of oxygen corresponding to different eras are indicated with corresponding events in evolution. BYA: billion years ago (from (Stamati et al., 2011)).

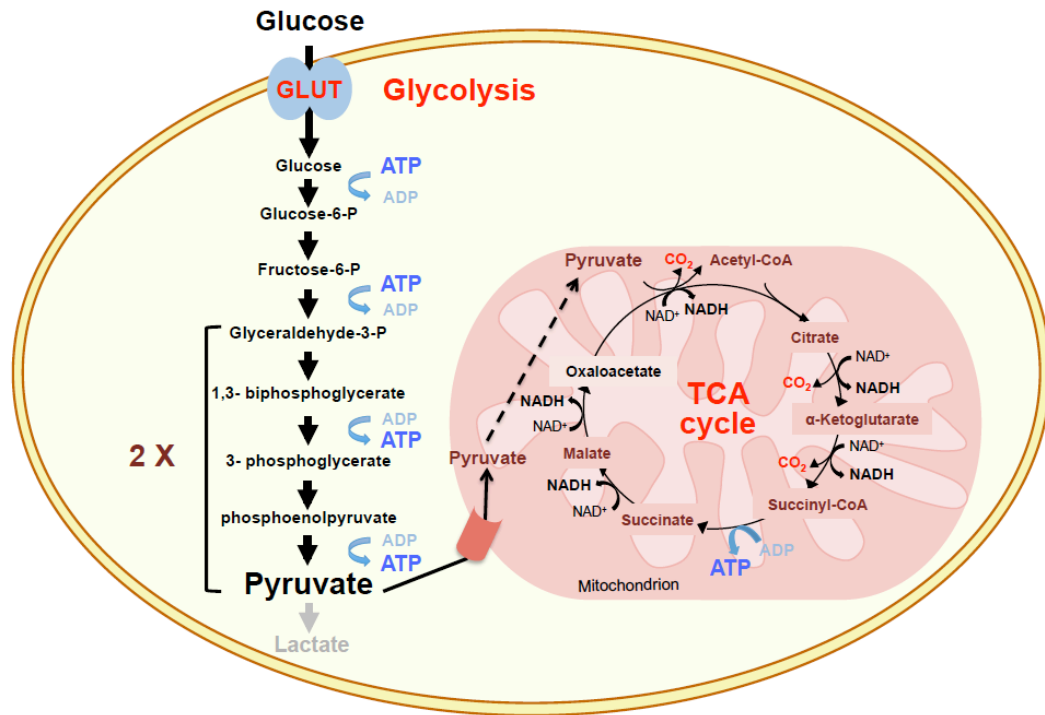
1.2. Aerobic metabolism: an efficient way to produce energy

While unicellular organisms have relied mainly on anaerobic metabolism to survive and grow during anoxic eras, the introduction of oxygen into the atmosphere has provided an extended redox potential for development of a more efficient way of producing the energy (aerobic metabolism) required for the growth of complex multicellular organisms (Raymond and Segre, 2006; Thannickal, 2009). During aerobic metabolism, consumed carbohydrates, lipids and proteins react with O_2 molecules to generate water and adenosine triphosphate (ATP) through mitochondrial oxidative phosphorylation (OXPHOS) (Babcock, 1999; Bonora et al., 2012) (Fig. 2).

This pathway comprises three different steps. First, glucose, which represents the principal energy source for cellular metabolism, is converted into pyruvate through a series of enzyme-catalysed reactions of the glycolytic pathway. Then, due to the low yield of ATP generated by glycolysis, pyruvate is converted into acetyl coenzyme A (acetyl-CoA) that enters the tricarboxylic acid (TCA) cycle (Fig. 2A). This results in the production of three molecules of carbon dioxide (CO_2) and the reduction of nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) cofactors, which provide electrons for the third step: OXPHOS.

OXPHOS consists in transfer of electrons produced from oxidation of low-redox-potential electron donors (e.g. NADH) to the high-redox-potential electron acceptor, O_2 , through the electron transport chain (ETC). The free energy released during this process is used to pump protons out, across the mitochondrial inner membrane, which creates an electrochemical gradient that is used to synthesize ATP within the mitochondrial matrix (Itoh et al., 2004; Jonckheere et al., 2012). ATP is then exported to the cytosol where it is hydrolysed to adenosine diphosphate (ADP), thus providing energy to support different processes, such as synthesis of macromolecules, active ion transport, muscle contraction, cell growth and proliferation. Thus, ATP, through the pyrophosphate that contains the high-energy phosphoanhydride bond, represents a universal energy currency at the junction between catabolism and anabolism (Bonora et al., 2012; Chi and Kemp, 2000) (Fig. 2B).

A



B

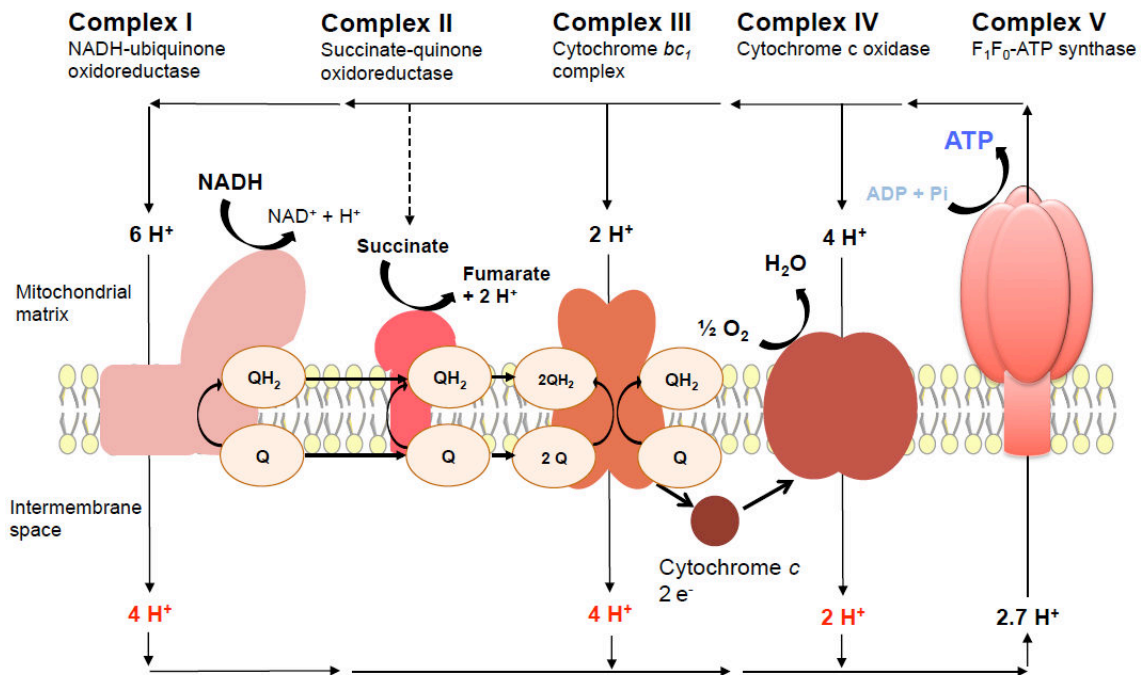


Figure 2. Schematic representation of aerobic respiration.

(A) Glycolysis produces two molecules of pyruvate and ATP from one molecules of glucose consumed. Pyruvate generated by glycolysis is converted into acetyl-CoA, which enters the TCA cycle and produces two ATP molecules and six NADH molecules. NADH is then used in mitochondrial OXPHOS for ATP production. (B) The mammalian mitochondrial electron transport chain (ETC) includes the proton-pumping enzymes complex I (NADH-ubiquinone oxidoreductase), complex III (cytochrome *bc*₁) and complex IV (cytochrome *c* oxidase), which generate proton motive force that in turn drives F₁F₀-ATP synthase. Electron transport between complexes is mediated by membrane-embedded ubiquinone (Q) and soluble cytochrome *c*. Complex I is the entry point for electrons from NADH, which are used to reduce Q to ubiquinol (QH₂). QH₂ is subsequently used by complex III to reduce cytochrome *c* in the intermembrane space (IMS), and complex IV uses cytochrome *c* to reduce molecular oxygen, which is the ultimate electron acceptor. For each NADH molecule oxidized, 10 protons are translocated across the membrane from the matrix to the IMS. Complex II (succinate-quinone oxidoreductase) provides an additional entry point for electrons into the chain (adapted from (Sazanov, 2015)).

II. Oxygen homeostasis and hypoxic stress

2.1. Physiological concentrations of O₂ in the organism

In agreement with the above, an adequate and constant O₂ supply is necessary for survival. However, in contrast to primitive multicellular organisms the small size of which has allowed simple molecular diffusion of O₂ to every cell, complex multicellular organisms with a ‘greater’ size have developed several strategies to efficiently capture (respiratory system), transport (blood) and distribute (cardiovascular system) O₂ into tissues. Moreover, a neuroendocrine system, essentially represented by erythropoietin (EPO), ensures effective regulation of O₂ concentration and delivery to whole cells of the organism (Brahimi-Horn and Pouyssegur, 2007c; Hsia et al., 2013).

Despite these vigorous systems, oxygen distribution in the human body is highly heterogeneous (Brahimi-Horn and Pouyssegur, 2007c; Kunze, 1969; Van Den Brenk and Jamieson, 1962). A cascade of O₂ occurs due to successive resistances across the pulmonary, cardiac and vascular systems. This creates a steep gradient from an atmospheric O₂ concentration at sea level (21.1%; 160mmHg at sea level), to the alveoli of the lungs (14.5%; 110mmHg), to arterial blood (13.2%; 100 mmHg) and to venous blood (5.3%; 40mmHg) (Le et al., 2006; Leach and Treacher, 1998). The oxygen level drops further in interstitial spaces of tissues (1.5-5%; 12-38mmHg) and in mitochondria (1%; 7.5mmHg) (Gnaiger et al., 1998; Gnaiger et al., 1995) (Fig. 3). This cascade is believed to protect the “energy generator factory”, mitochondria, against oxidative stress by maintaining very low levels of local O₂ concentration (Halliwell, 1999; Hsia et al., 2013). Thus, knowing the deleterious effects of oxidative stress on cell physiology, and since normal peripheral tissues are exposed to very low and variable O₂ levels, the term “normoxia”, used to describe the normal *in vitro* culture condition (21% O₂, 79% N₂ and 5% CO₂), should be revisited and possibly replaced by more physiological and reliable O₂ concentration settings, against which other experimental conditions, such as “hypoxia”, should be compared.

2.2. Hypoxia occurs in several physiological and pathological situations

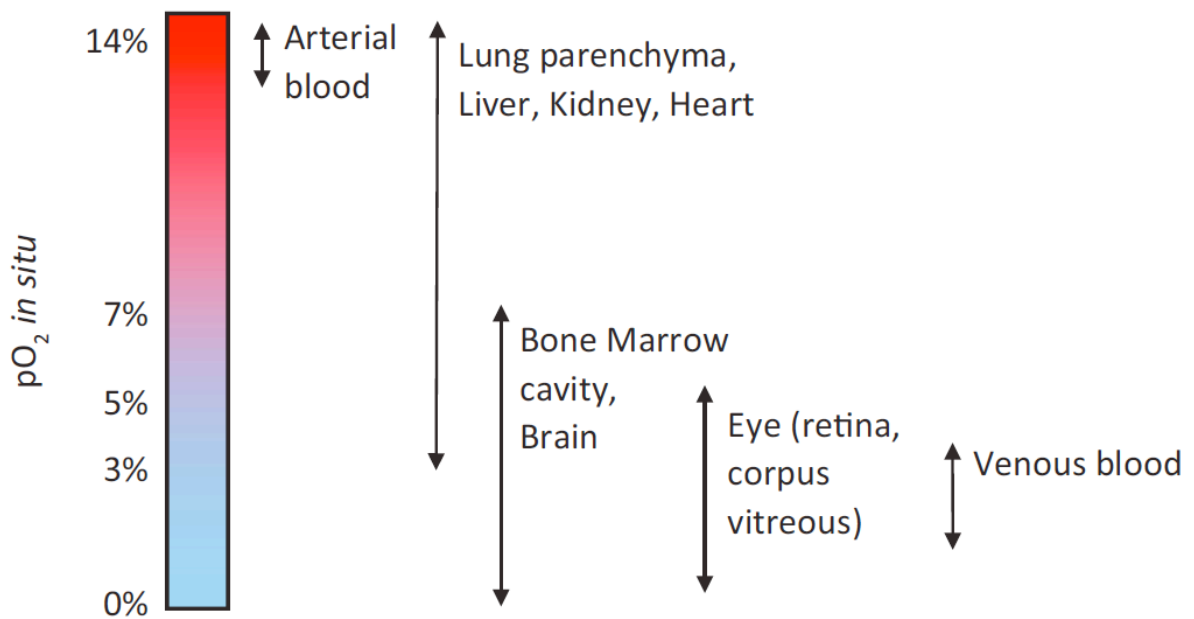
Hypoxia is defined as a state of decreased or inadequate supply of O₂ that restricts or even abolishes biological functions. This can be due to several factors, such as the low O₂ partial

pressure (pO₂) in the arterial blood, decreased tissue perfusion, decline of diffusion geometry and reduced ability of the blood to carry O₂.

Severe hypoxia generally occurs at or <1% O₂ (7.5mmHg) and frequently <0.1% O₂ (0.75mmHg). However, under normal physiological conditions, many tissues exhibit low and variable O₂ levels (3 -7%; 22.6-39.6mmHg), which suggests that the hypoxic threshold value may differ between individual tissues (Jiang et al., 1996). Moreover, measurement of pO₂ levels *in vivo* have shown the existence of physiological areas of hypoxia within tissues of different origin, such as the thymus (10mmHg; 1.3% O₂) (Braun et al., 2001), the kidney (20mmHg; 2.6% O₂) (Muller et al., 1998), the skin (8-2.3mmHg; 1.1-0.4% O₂) (Boutin et al., 2008), the bone marrow (4.5mmHg; 0.6% O₂) (Harrison et al., 2002; Parmar et al., 2007; Takubo et al., 2010) and lumen of the gastrointestinal tract (2.3mmHg; 0.3% O₂) (Colgan and Taylor, 2010; Thermann et al., 1985). This “physiological hypoxia” was described as an important cofactor in a wide range of physiological processes including, wound healing, haematopoiesis, embryogenesis and the maintenance of stem cell pluripotency (Pouyssegur et al., 2006; Semenza, 2009; Silvan et al., 2009).

Hypoxia has also been identified as a key player in a variety of pathologies, such as as type II diabetes, ischemic injury, atherosclerosis, inflammatory disorders, Alzheimer’s disease and cancer (Brahimi-Horn and Pouyssegur, 2007a; Semenza, 2011b) (Fig. 3).

A



B

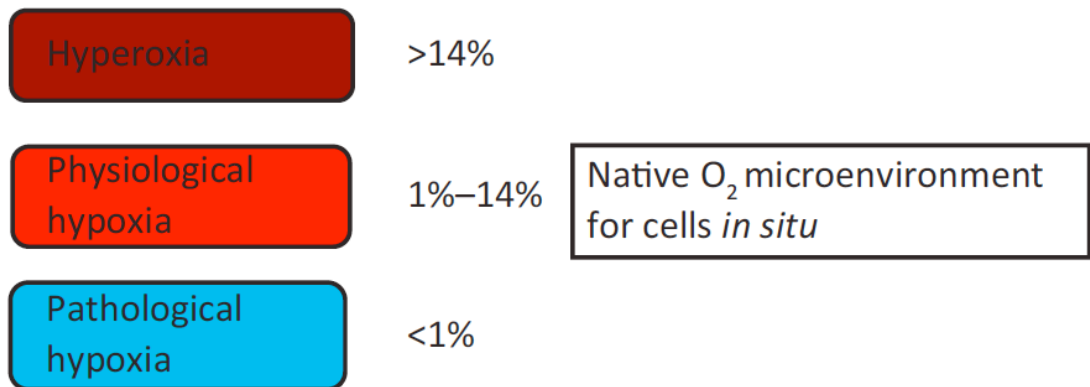


Figure 3. Physiological concentrations of oxygen in the organism and threshold of hypoxia.

(A) Values of micro-environmental O_2 found in tissues *in situ* (values taken from. (B) Native O_2 microenvironment for cells residing within different tissues in the mammalian body (Stamati et al., 2011).

III. Hypoxia: an important actor in tumour development

3.1. Defining tumour hypoxia

During tumour development, severe structural and functional abnormalities in the tumour microvasculature occur, leading to an insufficient supply of adequate amounts of nutrients and oxygen for the rapidly growing tumour mass (Carmeliet, 2005; Nussenbaum and Herman, 2010). Therefore, the expanding tumours usually grow as “cords” of well-oxygenated (normoxic), low-oxygenated (hypoxic), and oxygen-depleted (necrotic) cells that surround perfused blood vessels at a distance of up to about 150 μm (Thomlinson and Gray, 1955).

We can distinguish between three different types of tumour hypoxia (Vaupel and Harrison, 2004):

- Acute hypoxia, which is transient and affects the perfusion rate of the tumours due to abnormalities in the tumour neovasculature,
- Chronic hypoxia that results from increased diffusion distances of O_2 ($>70 \mu\text{m}$), a consequence of rapid tumour cell division and growth away from the capillaries, and
- Anaemic hypoxia that is generally linked to a treatment-related reduction of the blood's capacity to transport O_2 .

3.2. Data on tumour hypoxia has provided a better-understanding of the characteristics of tumours

The oxygenation status of solid tumours has been widely assessed over the past decades. Hypoxic regions in tissues can be detected *in vitro* using different methods, such as immunohistochemical staining of biopsies for intrinsic hypoxia markers, and histological evaluation of the microscopic appearance (Bussink et al., 2003; Williams et al., 2005). Detection of exogenous hypoxia markers, such as nitroimidazole and its derivatives (pimonidazole, EF5) by immunohistochemistry is also used to differentiate between normoxic and hypoxic tissues (Kizaka-Kondoh and Konse-Nagasawa, 2009; Lord et al., 1993; Raleigh et al., 1999). Tumour hypoxia can also be evaluated *in vivo* using several techniques including, the “gold standard” polarographic O_2 microelectrode, ^{18}F -fluoro-misonidazole positron emission tomography (^{18}F -MISO-PET), blood oxygenation level dependent magnetic resonance imaging (BOLD-MRI) and near-infrared spectroscopy (NIRS) (Baudalet and Gallez, 2002; Boushel and Piantadosi, 2000; Gambhir, 2002; Matsumoto et al., 2010; Padhani et al., 2007; Vaupel et al., 1991) (Fig. 4). In a clinical setting, it may be suitable to combine

different methods to increase the accuracy of intracellular pO₂ measurement, to distinguish the different types of tumour hypoxia and to precisely predict the sensitivity to hypoxia-dependent therapies.

Despite the differences between these methods in terms of sensitivity, accuracy and limitations to measure O₂ availability, they have highlighted several notions that will help better understanding of O₂ homeostasis in solid tumours. Thus data obtained from all the distinct studies have reported the presence of significant heterogeneity in oxygenation within individual tumours, which is less important than the inter-tumour variability. They have also shown that even if tumours could present higher median pO₂ than the limits described for hypoxia, their oxygenation levels remain lower than their respective tissue of origin. Moreover, the oxygenation status of tumours is a dynamic phenomenon that depends on the functionality and proximity of blood vessels, the initial pO₂ of the normal tissue of origin and, contrary to preconceived ideas, cannot be predicted by size, grade, stage, or histology of the tumour. Finally, although no noticeable differences in the oxygenation status between primary and metastatic tumours has been reported, studies have demonstrated the presence of more extensive hypoxic areas in recurrences when compared to their respective primary tumours (Aquino-Parsons et al., 1999; Li et al., 2007b; Vaupel and Harrison, 2004; Vaupel et al., 2007).

3.3. The importance of tumour hypoxia in malignant progression and survival

In the last two decades, accumulating evidence has shown that tumour hypoxia plays a critical role in promoting malignancy through different mechanisms including, stimulation of angiogenesis, genomic instability, stemness, immune evasion, metabolic reprogramming, and metastasis (Chan and Giaccia, 2007; Ivanovic, 2009; Rofstad, 2000; Semenza, 2010; Semenza, 2012; Subarsky and Hill, 2003). Hypoxia is also responsible for resistance to treatment, resulting in clinically aggressive phenotypes. Hypoxia induces resistance to apoptosis (Chiche et al., 2010), increases acidosis (Parks et al., 2013), decreases cell proliferation, reduces cytotoxicity of some chemotherapeutic agents and activates genes involved in drug resistance, such as ATP-binding cassette transporter B1 (ABCB1) (Comerford et al., 2002). Moreover, the chaotic tumour vasculature results in decreased drug delivery to hypoxic regions, but also in a reduced O₂ supply that limits sensitivity to radiotherapy. In fact, effectiveness of radiotherapy requires the interaction of intracellular

water with radiation to induce the formation of oxygen free radicals that cause DNA strand breaks. Therefore, a low O₂ level decreases the effects of radiation; subsequently higher radiation doses are required to achieve a therapeutic effect (Gray et al., 1953; Whillans and Hunt, 1982). To overcome this resistance, several therapeutic strategies have been developed including, the use of radiation sensitizers (nitroimidazoles), combined treatment with anti-angiogenic agents to normalize blood vessels and the use of hypoxia-activated prodrugs (anthraquinone AQ4N, SN30000) that specifically target hypoxic cells (Carmeliet and Jain, 2011; Guise et al., 2014; Hicks et al., 2010; Jain, 2005; Wilson and Hay, 2011).

Considering all the effects mentioned above, hypoxia was reported to be associated with low overall and recurrence-free survival in a wide range of tumour types, being thus an important prognostic marker for breast, lung, head and neck and cervical cancer, among others (Vaupel and Mayer, 2007; Wilson and Hay, 2011).

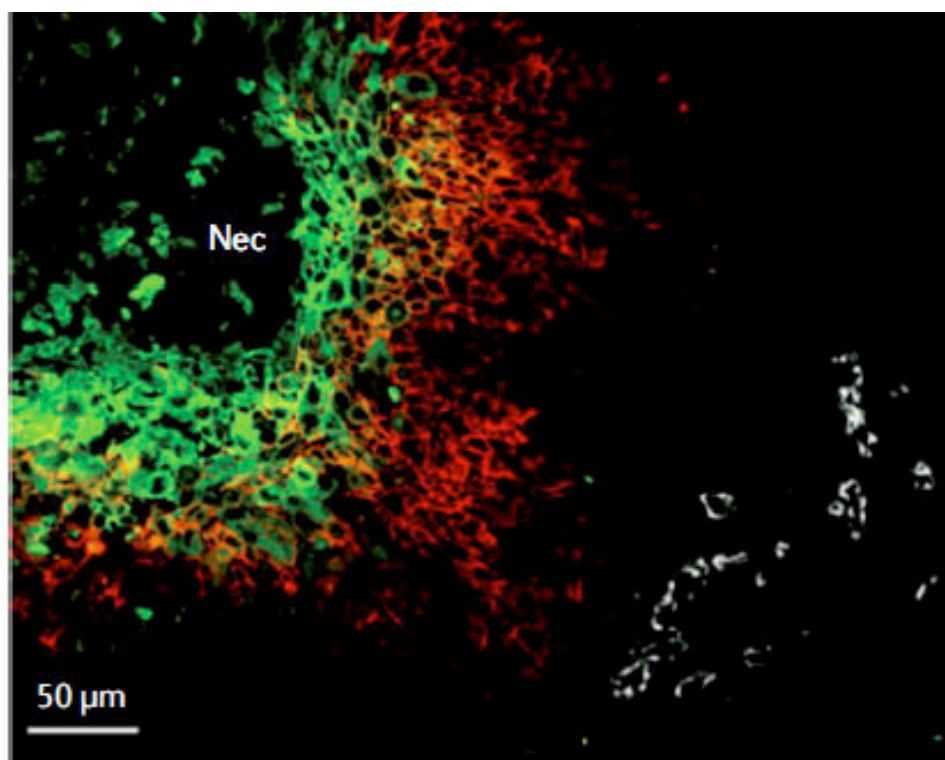


Figure 4. Oxygen dependence of hypoxia-responsive processes in tumours

Pseudocolor immunofluorescence showing the difference in distribution of covalently bound pimonidazole (green), an exogenous 2-nitroimidazole hypoxia marker, and hypoxia-inducible factor 1 (HIF1)-regulated carbonic anhydrase 9 (CA9; red), an endogenous marker of hypoxia. This distribution is shown relative to blood vessels (white) and necrosis (Nec) in a representative region of a human squamous cell carcinoma of the larynx. (from(Wilson and Hay, 2011)).

IV. Mechanisms of sensing O₂ homeostasis

Considering the importance of O₂ homeostasis in physiology, living organisms have developed several mechanisms to sense hypoxia and generate cellular adaptive responses to low pO₂. Thus during acute hypoxia (e.g. high altitude), specific O₂-sensitive ion channels, expressed on specialized chemoreceptor cells, such as glomus cells in the carotid body, are activated and induce rapid release of neurotransmitters (e.g. acetylcholine, dopamine). This leads to increased activity of both respiratory and cardiovascular systems, in order to adjust O₂ delivery to the organism. Kidney EPO-secreting cells are also stimulated by low pO₂, which results in EPO secretion and consequently increased differentiation and maturation of red blood cell precursors (Lahiri et al., 2006; Semenza, 2011a; Storz and Moriyama, 2008; Weir et al., 2005).

However, in the case of chronic hypoxia (e.g. ischemic injury, inflammatory disorders or cancer), adaptive responses rely mainly on the modulation of many transcription factors such as, Nuclear Factor kappa-B (NF-κB) (Chandel et al., 2000; Koong et al., 1994), Cyclic AMP response element binding protein (CREB) (Beitner-Johnson and Millhorn, 1998; Comerford et al., 2003) and Nuclear Factor for interleukin 6 (NF-IL6) (Yan et al., 1995), which are involved in the regulation of the expression of genes that play a role in the decrease in O₂ energy-dependent processes and the increase in the O₂ supply. Among these transcription factors, Hypoxia-Inducible Factors (HIFs) are recognized as the master O₂ sensors and regulators of hypoxia-dependent gene signalling in normal and tumour cells.

B. HIF, a key player in O₂ sensing

I. HIF subunits

To face hypoxic stress, cancer cells have developed several adaptive response mechanisms that are orchestrated by HIFs (Bertout et al., 2008; Brahimi-Horn and Pouyssegur, 2007a). Initially identified as a regulator of erythropoietin (EPO) production (Semenza and Wang, 1992), HIF is now recognized as a key promoter of several hundreds of genes involved in angiogenesis, metabolism, autophagy, migration, apoptosis, stemness, inflammation and metastasis (Majmundar et al., 2010; Ratcliffe, 2013; Semenza, 2010; Wilson and Hay, 2011).

HIF is a heterodimeric complex formed by two subunits: an oxygen-regulated HIF- α subunit (1 α , 2 α , or 3 α) and a constitutive HIF- β subunit, both of which are bHLH-PAS domain (basic-Helix-Loop-Helix Per-AHR/ARNT/Sim) proteins (Schofield and Ratcliffe, 2004; Wang et al., 1995) (Fig. 5). HIF-1 α and HIF-2 α (also known as endothelial PAS domain protein 1 (EPAS1)) are the best studied. HIF-1 α is ubiquitous, whereas the expression of HIF-2 α is restricted to the vascular endothelium, liver parenchyma, lung type II pneumocytes and kidney epithelial cells (Jain et al., 1998; Tian et al., 1997; Wiesener et al., 2003). Even if these two proteins have similar structure, their mechanisms of regulation and their target genes remain different (Carroll and Ashcroft, 2006; Patel and Simon, 2008). However, little is known about HIF-3 α . It has several splice variants, among which the inhibitory PAS domain protein (IPAS), a dominant negative inhibitor of HIF-1 α and HIF-2 α mediated transcription, is the most characterized (Makino et al., 2001; Maynard et al., 2003). IPAS is highly expressed in the thymus, cerebellar Purkinje cells and the corneal epithelium of the eye (Gu et al., 1998; Makino et al., 2001) (Fig. 5).

II. O₂-dependent regulation of HIF

2.1. Prolyl hydroxylases

Hypoxia-inducible activity is conferred by the association of the HIF- α and β -subunits. However, in the presence of O₂, HIF- α is hydroxylated by members of the Fe(II) and 2-oxoglutarate (2-OG) dependent dioxygenase superfamily, called prolyl hydroxylases (PHDs),

which regulate the stability of the protein (Berra et al., 2006; Bruick and McKnight, 2001; Kaelin and Ratcliffe, 2008). Although the three PHDs (1-3) have been shown to hydroxylate HIF- α , numerous *in vitro* studies have reported differences in the impact of these PHDs in regulating HIF- α subunits; while PHD2 is the major regulator of HIF-1 α stability, PHD-1 and PHD-3 have higher affinity for HIF-2 α (Appelhoff et al., 2004; Berra et al., 2003). Consistent with these studies, it has been shown that knock out of *Phd-2* in mice lead to lethality at early embryonic stages, whereas mice lacking *Phd-1* or *Phd-3* remain viable and do not express any apparent abnormalities (Berra et al., 2006; Takeda et al., 2006).

The mechanism of regulation consists in two steps. First, hydroxylation occurs on conserved proline residues (P402 and P564 in human HIF-1 α) situated within the ODD. Then, hydroxylated HIF- α is recognized by the β -domain of an E3 ubiquitin ligase, the von Hippel-Lindau protein (VHL) complex, which targets HIF- α for proteasomal destruction (Jaakkola et al., 2001; Ohh et al., 2000). However, in hypoxia, the hydroxylase activity of PHD is reduced since it requires O₂ as a substrate. Consequently, HIF- α proteins are stabilized and accumulate in the nucleus, where they dimerize with HIF- β and induce the transcription of a wide range of genes (Majmundar et al., 2010) (Fig. 6).

2.2. Factor inhibiting HIF

In parallel to the PHD regulatory pathway, HIF- α is subject to additional regulatory hydroxylation by factor inhibiting HIF-1 (FIH-1), an O₂-dependent asparaginyl hydroxylase, which hydroxylates HIF- α (on N803 residue in human HIF-1 α) in the C-TAD. This impairs HIF transcriptional activity by disrupting its interaction with the p300/CBP co-activators and the binding to hypoxia-responsive elements (HREs) (5'-[A/G]CGTG-3' consensus sequence) within target genes promoters or enhancers (Pugh et al., 1991; Schodel et al., 2011). FIH-1 has a lower K_m for O₂ (90 μ M) than PHD proteins (230 μ M) consequently it is able to regulate the HIF transcriptional activity even at intermediate levels of hypoxia (Koivunen et al., 2004) (Fig. 6). However, the sensitivity of HIF- α subunits to FIH-1 hydroxylation was reported to be different. Thus HIF 2- α was shown to be insensitive to FIH-1 regulation, probably due to the differences in its C-TAD (Bracken et al., 2006; Koivunen et al., 2004; Yan et al., 2007).

Moreover, unlike knock out of *Phd-2*, mice lacking *Fih-1* were recently shown to be viable and to exhibit numerous metabolic abnormalities (decreased adiposity, hyperventilation and increased insulin sensitivity), which suggests a crucial role of FIH-1 in metabolic disorders, such as obesity and diabetes (Takeda et al., 2006; Zhang et al., 2010).

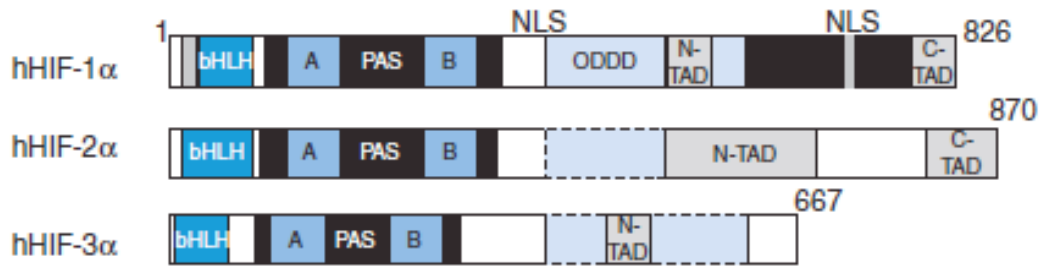


Figure 5. Domain structure of the HIF- α subunits.

HIF consists of an α and β subunit both of which belong to the bHLH family of proteins containing PAS domains. Three human isoforms of the α subunit, numbered 1–3, also termed aryl hydrocarbon receptor nuclear translocator (ARNT), are involved in the hypoxic response of cells. The labile α subunits contain an ODDD (oxygen-dependent degradation domain) that determines proteasomal degradation. Transcriptional activation domains (TADs) in the N- and C-terminal regions and a nuclear localization signal (NLS) in the N-terminal region have been identified on certain isoforms (adapted from (Brahimi-Horn and Pouyssegur, 2007b)).

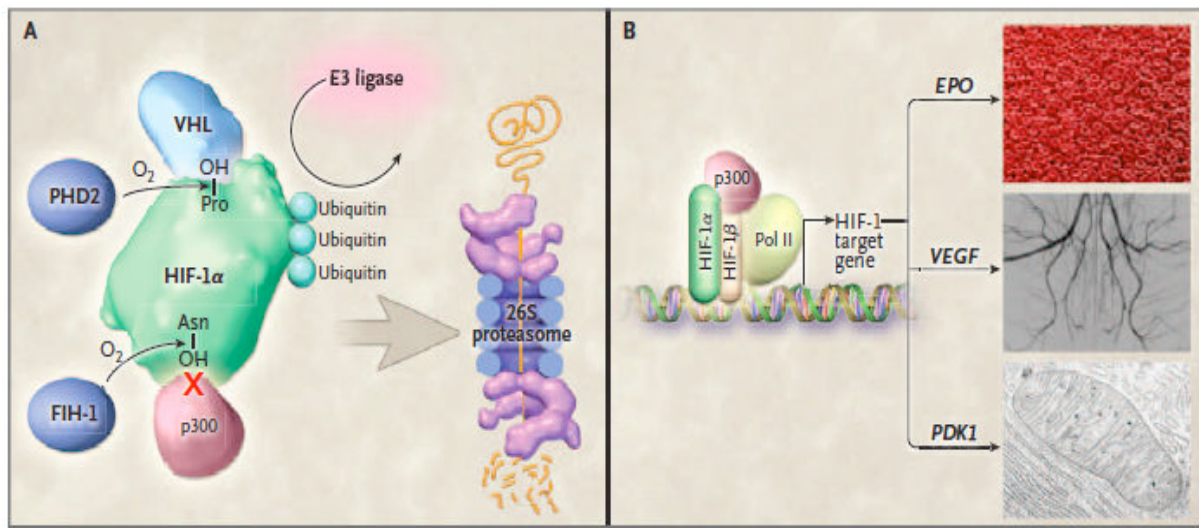


Figure 6. Oxygen Sensing, Gene Expression, and Adaptive Responses to Hypoxia.

In well-oxygenated cells (Panel A), prolyl hydroxylase domain 2 (PHD2) uses oxygen to hydroxylate hypoxia-inducible factor -1 (HIF-1 α) on proline residues (Pro–OH). The von Hippel–Lindau (VHL) protein binds to HIF-1 α containing Pro–OH and recruits a ubiquitin E3 ligase. The polyubiquitination of HIF-1 α flags the protein for degradation by the 26S proteasome. Factor inhibiting HIF-1 (FIH-1) also uses oxygen to hydroxylate HIF-1 α on an asparagine residue (Asn–OH). HIF-1 α containing Asn–OH cannot be bound by the coactivator protein p300, thereby preventing HIF-1 α from activating gene transcription. Under hypoxic conditions (Panel B), the Pro and Asn hydroxylation reactions are inhibited, and HIF- α (i.e., either HIF-1 α or HIF-2 α) rapidly accumulates, dimerizes with HIF-1 β , recruits p300, binds to hypoxia response elements, and activates the transcription by RNA polymerase II (Pol II) of hundreds of target genes, such as the following: EPO, encoding erythropoietin, which is the hormone that stimulates red-cell production (photomicrograph at top); VEGF, encoding vascular endothelial growth factor, which is the angiogenic factor that stimulates blood-vessel formation (angiogram in middle); and PDK1, encoding pyruvate dehydrogenase kinase 1, which inhibits the conversion of pyruvate to acetyl coenzyme A for oxidation in the mitochondrion (electron micrograph at bottom) (from (Semenza, 2000)).

III. O₂-independent regulation of HIF

Although hydroxylation is the major regulatory mechanism of HIF- α stability and activity, other post-translational modifications including phosphorylation, acetylation, S-nitrosylation and SUMOylation also occur and regulate HIF- α subunits (Dengler et al., 2014; Nunez-O'Mara and Berra, 2013). HIF- α was also reported to be modulated by mitochondrial reactive oxygen species (ROS) during moderate hypoxia (1.5 %), likely through inhibition of the PHD activity (Kaelin, 2005; Klimova and Chandel, 2008). Additional factors are involved in HIF- α stabilization in an O₂-independent manner including heat shock proteins (Hsp70/CHIP, Hsp90/RACK1) (Isaacs et al., 2002; Luo et al., 2010), metabolites (ascorbate, 2-OG, iron, cyclic AMP) (Kaelin and Ratcliffe, 2008), sirtuins (SIRT1, SIRT6) (Dioum et al., 2009; Zhong et al., 2010), intermittent hypoxia (Peng et al., 2006) and microRNAs (miR-17-92, miR-107, miR-210) (Taguchi et al., 2008; Yamakuchi et al., 2010). Moreover, in tumours, oncogenes and tumour suppressors are also critical activators of HIF-1 α (Agani and Jiang, 2013; Mabeesh and Amir, 2007), leading to increased translation (PI3K, PTEN) and stabilization (VHL) of HIFs. Consequently, the transcription of a wide range of genes occurs, some of which are implicated in metabolic reprogramming (Ratcliffe, 2013; Semenza, 2010).

C. Remodelling of metabolism in cancer: an efficient way to maintain cellular viability

Metabolism is roughly defined as a series of enzyme-catalysed biochemical reactions that either produce or consume energy during the transformation of a biological molecule. The metabolic networks of free-living organisms are very complex and comprise thousands of chemical reactions (pathways) that are interconnected through common enzymes, cofactors and substrates. In a simple way, metabolism can be classified into two meaningful categories:

- (1) Catabolism, which involves reactions that degrade molecules such as carbohydrates, fatty acids and amino acids in order to generate energy, redox and simple molecules.
- (2) Anabolism, which refers to chemical processes that synthesise simple molecules or assemble precursors generated by catabolic reactions to produce complex macromolecules such as proteins, nucleic acids and lipids.

During the last century, biochemists have purified, identified and described the key enzymes controlling metabolic pathways including, glycolysis, pentose phosphate pathway, the Krebs cycle, the oxidative electron chain and OXPHOS. With advances in genomics, transcriptomics, proteomics and more recently, metabolomic technologies a large scale of genes encoding metabolic enzymes, and non-genetically encoded metabolic intermediates have been widely confirmed and identified. The data generated from these technologies has established tight links between metabolism, cell signalling and common human diseases such as diabetes, obesity and cancer.

In this chapter, we will not list all the metabolic networks, but rather focus on the most important pathways that are altered in cancer namely: glucose and glutamine metabolism.

I. Glucose metabolism

1.1. Aerobic Glycolysis or the “Warburg effect”: the identity of cancer cells

In the 1920s, a German scientist Otto Warburg reported abnormalities in cancer cell metabolism, which opened the door to a new large field of cancer studies. He demonstrated that unlike the majority of normal cells, which rely primarily on mitochondrial OXPHOS to produce energy, tumour cells ardently take up glucose to perform aerobic glycolysis (Vander

Heiden et al., 2009; Warburg, 1956). This phenomenon, referred to as Warburg effect, became a distinctive metabolic characteristic of cancer cells, and proved useful for clinical detection and monitoring of tumours by [^{18}F]-deoxyglucose positron emission tomography (FDG–PET) imaging (Czernin and Phelps, 2002; Kim and Dang, 2006). Initially, the Warburg effect was proposed to be a result of impairment of mitochondrial respiration (Warburg, 1956). However, numerous recent studies have shown that mitochondrial OXPHOS in many tumours is intact (Griguer et al., 2005; Scott et al., 2011). Instead, the Warburg effect is proposed to be due to increased glycolysis that suppresses OXPHOS, which is caused by adaptation to hypoxic conditions at the early avascular stages of tumour development (Jose et al., 2011; Moreno-Sanchez et al., 2007).

Since the ATP yield of aerobic glycolysis (2 ATP per glucose molecule) is 18-fold lower than that of OXPHOS (36 ATP per glucose molecule), metabolic reprogramming implicates an increased rate of glucose uptake by tumour cells to meet the energy, macromolecular biosynthesis and redox needs required to rapid proliferation (Barthel et al., 1999; Dang and Semenza, 1999). Thus glucose transporters (GLUT1, GLUT3) and downstream glycolytic enzymes, such as hexokinase 2 (HK2), phosphofructokinase 1 (PFK-1) and lactate dehydrogenase A (LDHA), are overexpressed in more than 70% of cancers (Ayala et al., 2010; Fenske et al., 2009). This up-regulation is mainly driven by the hypoxia-induced transcriptional factor HIF-1 and by Myc, alone or in cooperation. Additional factors exacerbating growth and metabolism include oncogenes (Akt, PI3K, mTOR, Ras, Raf) and loss of tumour suppressor genes (VHL, PTEN) (Dang and Semenza, 1999; Levine and Puzio-Kuter, 2010). Elevated HIF-1 α levels in rapidly growing cells, like embryo and tumours, not only stimulates glycolysis but restricts mitochondrial respiration through the inhibition of the mitochondrial pyruvate dehydrogenase (PDH), reducing pyruvate flux into the TCA cycle (Kim et al., 2006; Papandreou et al., 2006). This HIF-1-mediated inhibition of PDH in reprogramming glucose flux is a major basis of the Warburg effect.

Besides the HIF/c-Myc axis, the tumour suppressor p53 plays a critical role in the balance between OXPHOS and glycolysis (Bensaad and Vousden, 2007; Yeung et al., 2008). In normal conditions, p53 reduces the glycolytic pathway by repressing the expression of glucose transporters GLUT1 and GLUT4 (Schwartzberg-Bar-Yoseph et al., 2004), and by inducing the expression of TP53-induced glycolysis and apoptosis regulator (TIGAR), an enzyme that decreases the levels of the glycolytic activator fructose-2, 6-bisphosphate (Bensaad et al., 2006; Bensaad and Vousden, 2007; Yeung et al., 2008). In parallel, it

promotes OXPHOS by up-regulating the synthesis of cytochrome c oxidase 2 (SCO2), which plays a role in the assembly of the cytochrome c oxidase complex (mitochondrial complex IV) and apoptosis-inducing factor (AIF), which is required to maintain the integrity of mitochondrial complex I (Vahsen et al., 2004; Zhou et al., 2003). In addition, p53 regulates glycolysis also by negatively regulating the PI3K/AKT and mTOR pathways. Thus, p53 mutations and loss in cancers results in mitochondrial reduced activity and reinforcement of their glycolytic phenotype (Zhou et al., 2003) (Fig. 7).

1.2. Pentose phosphate pathway

In most cancer cells, glucose is not only used to perform glycolysis but can also be metabolized by alternative pathways such as the pentose phosphate pathway (PPP), also known as the hexose monophosphate shunt. PPP is an anabolic cytosolic process divided into two phases. The oxidative phase converts glucose-6-phosphate (G6P) into ribulose-5-phosphate (Ru5P) and reduces NADP^+ to NADPH, through a series of irreversible reactions implicating glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD). During the non-oxidative phase, however, pentose phosphates are reversibly reconverted into glycolytic intermediates such as fructose-6-phosphate (F6P) and glyceraldehyde-3P (GA-3P) by two key enzymes, transketolase (TKT) and transaldolase (Riganti et al., 2012).

NADPH derived from PPP is crucial for cancer cell survival (Kletzien et al., 1994; Stanton, 2012). By promoting glutathione (GSH) generation, it helps cells to counteract oxidative stress, which confers them resistance to chemotherapy and radiation. NADPH is also used in reductive biosynthesis of fatty acids, sterols and other macromolecules. In parallel, Ru5P generated by PPP is necessary for nucleotides and nucleic acid synthesis and so facilitates DNA damage repair of cells (Boros et al., 2000; Tian et al., 1999). Thus to meet their constant demand of nucleotides and biosynthetic precursors, malignant and proliferative tumours frequently up-regulate the pentose phosphate pathway *via* different mechanisms. Indeed, similar to glycolysis, PPP is highly regulated by oncogenes and tumour suppressors. Many recent studies have shown that p53, PTEN and AMPK negatively regulate PPP through the inhibition of G6PD enzymatic activity, while K-Ras, mTORC1, PI3K/AKT and HIF-1 increase the level of PPP by positively regulating both its oxidative and non-oxidative branches (Bensaad and Vousden, 2007; Riganti et al., 2012; Wagle et al., 1998; Ying et al., 2012) (Fig.7).

1.3. Glycogen metabolism

In addition to the above-described glucose consuming pathways, another major metabolic adaptive pathway, namely glycogen metabolism, is attracting particular attention in cancer research. Several recent studies have shown that increased intracellular storage of glucose, in a form of glycogen, is a common feature of cancer cells (Pelletier et al., 2012; Pescador et al., 2010; Zois et al., 2014). This glycogen shunt acts as a modulator of glucose utilization under hypoxic and nutrient limiting conditions, for optimal tumour cell proliferation and growth (Ros and Schulze, 2012; Zois et al., 2014). Metabolism of glycogen involves the activity of different enzymes catalysing the synthesis (glycogen synthase (GS)) and degradation (glycogen phosphohrylase (GP)) of glycogen molecules, leading to the formation of glucose-6-phosphate (G6P), a shared intermediate of both aerobic glycolysis and the PPP. Therefore, as for these two last pathways, proteins involved in glycogen metabolism are highly regulated by hypoxia; this includes phosphoglucomutase1 (PGM1), UDP-glucose pyrophosphorylase 2 (UGP2), glycogen synthase (GYS1), glucan (1,4- α -) branching enzyme 1 (GBE1) and the liver isoform of glycogen phosphorylase (PYGL) (Favaro et al., 2012; Pelletier et al., 2012; Pescador et al., 2010). Inhibition of this last enzyme was described to noticeably affect cancer cell viability through a decreased flux into the PPP and consequently impaired NADPH levels (Favaro et al., 2012), which illustrates the tight connection between glucose metabolic pathways in supporting tumour cell survival and rapid growth under stringent conditions.

II. Addiction to glutamine: the other feature of cancer cells

Alongside glucose metabolism, many studies highlight the crucial role of glutaminolysis in tumour cell bioenergetics and metabolism. It was demonstrated that glutamine consumption is substantially increased in many cancers compared to other amino acids, and represents a feature of malignancy (DeBerardinis and Cheng, 2010; Kroemer and Pouyssegur, 2008; Wise and Thompson, 2010).

Briefly, once it enters the cells *via* membrane transporter Asc-type amino acid transporter 2 (ASCT2), glutamine is metabolized within the mitochondria, where it is first hydrolysed to glutamate and ammonium by the mitochondrial enzyme glutaminase (GLS). Generated glutamate is then converted into α -ketoglutarate (α -KG), by the glutamate dehydrogenase (GLUD1), and enters the TCA cycle, producing thus diverse metabolites (malate,

oxaloacetate, NADPH) used for fatty acids, amino acids and nucleotides biosynthesis (DeBerardinis et al., 2007; Deberardinis et al., 2008; Mates et al., 2009; Wise and Thompson, 2010). Glutamine contributes also to total ATP generation in cancer cells through TCA cycle metabolites, NADH and FADH₂, which provide electrons for the electron transport chain of mitochondria (Deberardinis et al., 2008; Guppy et al., 2002; Wise and Thompson, 2010).

As for other metabolic pathways in cancer, up-regulation of glutaminolysis is positively driven by oncogenic signals. The best-characterised regulatory mechanism implicates the transcription factor c-Myc and a variant of the Rho GTPases family, the oncogenic diffuse B-cell lymphoma protein (Dbl). c-Myc, through the transactivation of glutamine transporters on plasma membrane, and suppression of miR-23a/b, increases both the glutamine uptake and the enzymatic activity of GLS1 respectively (Dang et al., 2009; Gao et al., 2009; Wise et al., 2008); while Dbl stimulates the activity of GLS in a NF- κ B dependent manner (Wang et al., 2010). In addition, recent reports have shown that loss of the retinoblastoma tumour suppressor, as well as KRas and HIF activation, promote glutamine utilisation and metabolism in cancer cells (Gaglio et al., 2011; Gameiro et al., 2013; Reynolds et al., 2014; Wise et al., 2011) (Fig.7).

In summary, during tumour development, cells undergo metabolic reprogramming due to a combination of a poor and leaky vasculature, hypoxia and oncogenic signalling. Interaction between aberrant metabolic pathways provides cells not only with energy and macromolecules, but also a modified microenvironment. Thus through the production of diverse metabolites and enhanced glucose and glutamine metabolism a perfect nest is created for tumour cell growth and survival.

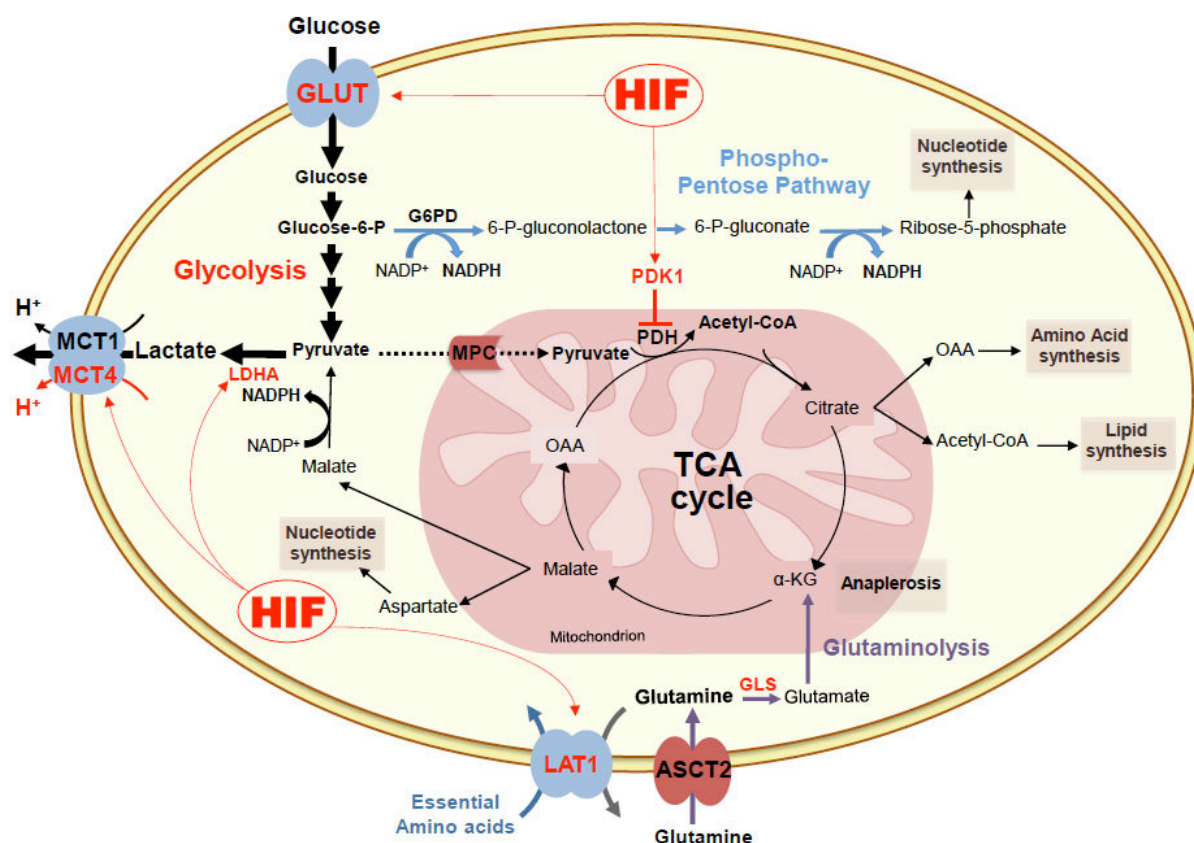


Figure 7. Schematic representation of glucose and glutamine metabolism in cancer cells.

After entering the cell through specific transporters (GLUT), glucose is metabolized to pyruvate. In cancer cells, pyruvate is mainly converted to lactate by lactate dehydrogenase A (LDHA), while its catabolism *via* the tricarboxylic acid (TCA) cycle is restricted through the inhibition of the mitochondrial pyruvate dehydrogenase (PDH) by pyruvate dehydrogenase kinase 1 (PDK1) induced by HIF-1. Glycolysis (bold arrows) generates also another important intermediate, glucose-6-phosphate (G6P) that is metabolized by the pentose phosphate pathway (blue arrows), which produces NADPH and ribose-5-phosphate for glutathione and nucleic acids synthesis. Glutaminolysis (purple arrows) is an alternative pathway for energy production for cancer cells. First converted to glutamate by glutaminase (GLS) in the cytosol, glutamine replenishes the tricarboxylic acid (TCA) cycle (anaplerosis) through the conversion of glutamate to α -ketoglutarate (α -KG). Glutaminolysis also contributes to synthesis of lipids, amino acids, nucleotides and to the generation of lactate that is transported out of the cell by the ubiquitous monocarboxylate transporter 1 (MCT1) and the hypoxia-inducible MCT4. Hypoxia-inducible factor (HIF); glucose-6-phosphate dehydrogenase (G6PD); mitochondrial pyruvate carrier (MPC); oxaloacetate (OAA); L-type amino acid transporter 1 (LAT1); Asc-type amino acid transporter 2 (ASCT2).

D. Acidosis and intracellular pH regulating systems in cancer cells

I. Tumour microenvironmental acidosis: a beneficial side effect of metabolic reprogramming?

As mentioned above, during cancer development, cells undergo substantial metabolic reprogramming due to a combination of a poor and leaky vasculature, hypoxia and oncogenic signalling. This altered metabolism, represented mainly by enhanced glycolysis, glutaminolysis and the PPP, generates large amounts of acidic by-products mainly lactic and bicarbonic acids (DeBerardinis et al., 2007; Gatenby and Gillies, 2008; Helmlinger et al., 2002; Lunt and Vander Heiden, 2011; Pouyssegur et al., 2006). This threatens the intracellular pH (pHi) homeostasis and consequently cell growth and survival, as alterations in pHi may disturb a wide range of biosynthetic and signalling processes (Gottlieb et al., 1996; Lagadic-Gossmann et al., 2004; Webb et al., 2011). To overcome this intracellular acidification, cancer cells developed adaptive strategies to extrude acid and regulate pHi. These strategies include H^+ and lactate export mechanisms through different transmembrane proteins, including Na^+/H^+ exchanger 1 (NHE1), vacuolar- H^+ APTase (V-ATPase) and monocarboxylate transporters (MCTs), but also import of bicarbonate ions via Cl^-/HCO_3^- anion exchangers (AE1-3), Na^+/HCO_3^- co-transporters (NBCe1, NBCn1) and carbonic anhydrases (mainly CAIX and CAXII) (Gatenby and Gillies, 2008; Parks et al., 2013; Reshkin et al., 2013). Collaboration of these pumps and transporters leads to the acidification of the tumour microenvironment and decreased extracellular pH (pHe), creating a reversed pH gradient (pHe (6.6-6.9) < pHi (7.2-7.5)) that promotes invasion and proliferation of tumour cells (Gillies et al., 1990; Hanahan and Weinberg, 2011; Webb et al., 2011). Indeed, an acidic pHe induces extracellular matrix (ECM) remodelling, stimulates acid-activated proteases and angiogenesis to facilitate tumour cell invasion (Gatenby and Gillies, 2008; Lagadic-Gossmann et al., 2004; Rofstad et al., 2006; Shi et al., 2001); while alkaline pHi promotes cell survival by limiting apoptotic mechanisms, which confers chemo/radioresistance to cancer cells and therefore exposes them to the selective pressure of malignant phenotypes (Gillies et al., 1990; Lagadic-Gossmann et al., 2004; Nagata et al., 2011; Rich et al., 2000; Simon et al., 1994). Several excellent reviews describe the behaviour

of cells regulated by pH dynamics in the tumour and in their environment (Gatenby and Gillies, 2008; Hanahan and Weinberg, 2011; Parks et al., 2013; Reshkin et al., 2013) (Fig. 8).

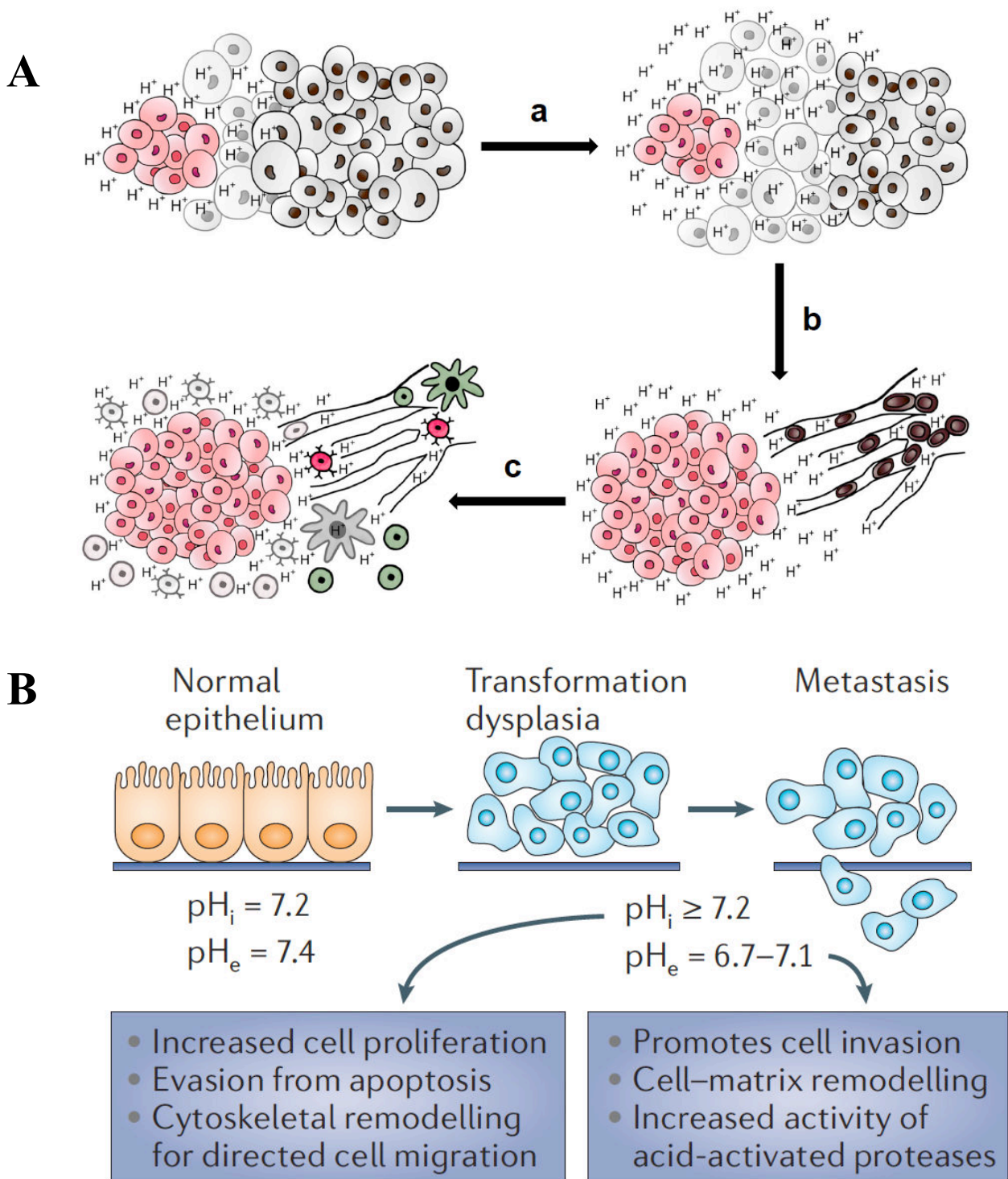


Figure 8. Dysregulated pH creates a perfect storm for cancer progression

A) Increased glycolysis by cancer cells produces an acidic microenvironment. H^+ flows along concentration gradients into adjacent normal tissue causing **a)** normal cell death, **b)** extracellular matrix degradation, and **c)** angiogenesis (adapted from (Fais et al., 2014)). **B)** Cancer cells have a reversed pH gradient compared with normal differentiated adult cells, including a constitutively higher pH_i and a lower pH_e , which facilitates the indicated adaptive behaviours (from (Webb et al., 2011)). All of these responses promote tumour growth and invasion.

II. Intracellular pH regulating systems in cancer cells

2.1. Vacuolar- H^+ ATPase

Vacuolar- H^+ ATPases (V-ATPase) are ATP-driven proton pumps, ubiquitously expressed in vacuolar membranes of eukaryotic cells play a key role in intraorganellar acidification and pH homeostasis (Forgac, 2007). V-ATPases constitute a large hetero-multimeric complex consisting of two sectors:

- The cytoplasmic ATP-hydrolytic V1-sector, which provides energy for H^+ transport through hydrolysis of ATP into ADP.
- The membranous V0-sector, which is responsible of the rotary H^+ transport across the membrane.

By pumping protons from the cytosol into the lumen of organelles, such as endosomes and lysosomes, V-ATPases are crucial for many physiological processes including, membrane trafficking, receptor-mediated endocytosis and lysosomal hydrolysis (Saw et al., 2011; Sennoune and Martinez-Zaguilan, 2007). Moreover, V-ATPases are also known to induce extracellular acidification in some normal cell lines (osteoclasts, renal cells) and cancers (pancreatic cancer, hepatocellular carcinoma) (Forgac, 2007; Ohta et al., 1996; Xu et al., 2012). Therefore, their expression level was substantially increased in several invasive cancer cell lines, such as human breast cancer cell line MDA-MB-231, and melanoma cells (Hinton et al., 2009; Horova et al., 2013; Martinez-Zaguilan et al., 1993). This plasma membrane expression that remains largely controversial could participate in extracellular acidification leading to activation of proteases, such as matrix metalloproteases, and remodelling of the ECM. Consequently, V-ATPases up-regulation could participate in cancer cell invasiveness, metastasis and positively contribute to the pathological grade of some cancers (Forgac, 2007; Parks et al., 2013) (Fig. 9).

2.2. Na^+/H^+ exchanger 1

The Na^+/H^+ exchanger isoform 1 (NHE1) is the most common isoform of the membrane-bound NHEs family (SLC9A), and is ubiquitously expressed in all mammalian cells and highly conserved in all metazoans. Through the inward-directed Na^+ gradient this plasma membrane exchanger is very efficient to export H^+ with a stoichiometry 1:1. NHE1 plays a major role in intracellular pH homeostasis, and as such its activity controls different cellular

processes including cell growth, proliferation, migration, differentiation and apoptosis (Gatenby and Gillies, 2008; Hendus-Altenburger et al., 2014; Pedersen, 2006) (Fig. 9).

In cancer cells, NHE1 has been reported to be constitutively active and positively correlated with the reversal pH gradient and metastasis (Gatenby and Gillies, 2008; Parks et al., 2013). Indeed, NHE1 possesses a pH-sensing site on its internal surface that is protonated in conditions of low pH_i, leading to increased proton extrusion and extracellular acidification. The affinity of this internal pH-sensing site is increased by all growth factors, mitogens and sperm during fertilisation, conferring to 'growth activated' cells a more alkaline pH_i (Paris and Pouyssegur, 1984). The expression of NHE1 was further described to be regulated by the epidermal growth factor (EGF), mitogens, a variety of membrane proteins and lipids, and though controversially was suggested to be a HIF-1 target gene (Chiang et al., 2008; Mo et al., 2011; Shimoda et al., 2006).

Recent studies have shown that stimulation of the NHE1 activity, in breast cancer cells and human chronic myeloid leukemia results in increased invadopodia proteolytic activity, degradation of ECM, migration and invasiveness (Bourguignon et al., 2004; Stock and Schwab, 2009; Wang et al., 2015; Wang et al., 2014). Therefore, NHE1 expression represents an advantage for cancer cells and was reported to correlate with poor clinical outcome in cervical cancer (Pedersen, 2006; Stock and Schwab, 2009; Yang et al., 2010).

2.3. HCO₃⁻ transporters

Due to the tight link between protons and pH, a majority of the research studies on pH_i regulating systems in cancer have focused on V-ATPases and NHE1. However, previous and more recent reports show that transport of HCO₃⁻ could be a promising anti-cancer target due to its role in pH homeostasis (L'Allemain et al., 1985; Parker and Boron, 2013; Parks et al., 2013; Parks and Pouyssegur, 2015; Romero et al., 2013). Bicarbonate transporters are a large family of proteins comprising cellular acid loaders, such as the electroneutral Cl⁻/HCO₃⁻ anion exchangers (AE1-3) and cellular acid extruders, mainly represented by Na⁺/HCO₃⁻ co-transporters (NBCe1, NBCn1) (Parker and Boron, 2013; Romero et al., 2013) (Fig. 9).

While transport of H⁺ is highly variable under changes in the tumour microenvironment (pH, O₂ concentration), transport of HCO₃⁻ is highly stable and active, even at mildly acidic pH_i (close to 7.2) and hypoxic conditions (Hulikova et al., 2013). Thus, numerous studies have reported a link between HCO₃⁻ transporters and cancer development. Interestingly, Na⁺/HCO₃⁻ co-transporter NBCn1 is up regulated in a wide range of human breast carcinoma

tissues, which correlates with poor prognosis (Boedtkjer et al., 2013; Han et al., 2011; Sueta et al., 2012). Furthermore, $\text{Cl}^-/\text{HCO}_3^-$ anion exchangers AE1 and AE2 are also overexpressed in colorectal and gastrointestinal cancer cells, and play a major role in tumour progression, even if this is suggested to be independent of pHi regulation (Shen et al., 2007).

In most of cancer cells HCO_3^- transporters cooperate with NHE1 to maintain pHi homeostasis. However, recent data, on 3D tumour spheroids, show that inhibition of HCO_3^- import in NHE1 expressing cells resulted in a high level of pHi acidification, which suggests that further characterization of the mechanisms underlying the expression, role and regulation of HCO_3^- transporters should be taken into consideration for future drug development (Hulikova et al., 2013; Parker and Boron, 2013; Xu et al., 2013).

2.4. Carbonic anhydrases

Carbonic anhydrases (CAs) are a large family of zinc-binding enzymes comprising 16 isoforms that catalyse the reversible hydration of CO_2 into HCO_3^- and H^+ [$\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+$]. Thus they play an important role in many physiological and pathological processes that involve pH regulation. Indeed, by generating H^+ and HCO_3^- CAs contribute to the extracellular acidification and intracellular alkalisation of cells, respectively (Parks et al., 2013; Pastorek et al., 1994; Supuran, 2008) (Fig. 9). CAs isoforms are ubiquitously expressed in several subcellular compartments of differentiated tissues, excepting CA isoform 9 (CAIX), which is abundant only in the gastrointestinal tract and is highly expressed in a wide range of cancers (Pastorekova et al., 1997; Supuran, 2008). Other isoforms are also expressed in some tumours including CAXII in kidney and breast cancers, and CAII in glial tumours (Korhonen et al., 2009; Parkkila et al., 2010; Parkkila et al., 2000).

Among all CAs isoforms, CAIX and CAXII are the only transmembrane ones that are significantly induced by hypoxia, as they have been shown to be transcriptionally regulated by HIF-1 and interestingly their catalytic site faces the extracellular compartment (Chiche et al., 2009; Wykoff et al., 2000). CAIX remains the most inducible enzyme by hypoxia and possesses a higher catalytic activity than CAXII.

Therefore, positive staining for CAIX is considered as a marker of tumour hypoxia, and an indicator of poor prognosis in several aggressive cancers (Chia et al., 2001; Giatromanolaki et al., 2001; Hussain et al., 2007; Ilie et al., 2013; Trastour et al., 2007). Indeed, through its extracellular facing catalytic site, CAIX facilitates local production of HCO_3^- ions close to HCO_3^- transporters, which results in efficient cooperation between both

systems in decreasing the pHi and in maintaining the reverse pH gradient, conferring thus chemo/radiotherapy resistance to cancer cells (Ditte et al., 2011; Morgan et al., 2007; Svastova et al., 2012). Moreover, through the generation of H^+ , and its expression in cancer associated fibroblasts (CAFs) and lamellipodial membranes, CAIX further acidifies the extracellular milieu, which promotes tumour progression and metastasis (Chiche et al., 2009; Fiaschi et al., 2013; Svastova et al., 2012).

Thus, our group and others reported that CAIX knockdown (through short hairpin RNA (shRNA)) results in significantly reduced tumour growth of colon adenocarcinoma and glioblastoma xenografts (Chiche et al., 2009; McIntyre et al., 2012), and a decreased capacity of invasion and migration through matrigel of HT-1080 fibrosarcoma cells (Radvak et al., 2013). More recent studies on a 4T1 mouse model of metastatic breast cancer showed promising effects of CAIX inhibition on tumour regression, recurrence and survival of mice (Lou et al., 2011; Parks et al., 2013). Even if these results were not as efficient on human breast cancer MDA-MB 231 tumour xenografts, clinical data and all preclinical results clearly reinforce the finding that CAIX is a master regulator of tumour survival in a hypoxic and acidic microenvironment, and support the effort made in developing specific pharmacological inhibitors for anti-cancer therapy.

MCTs and transport of lactic acid will be more detailed in the next section.

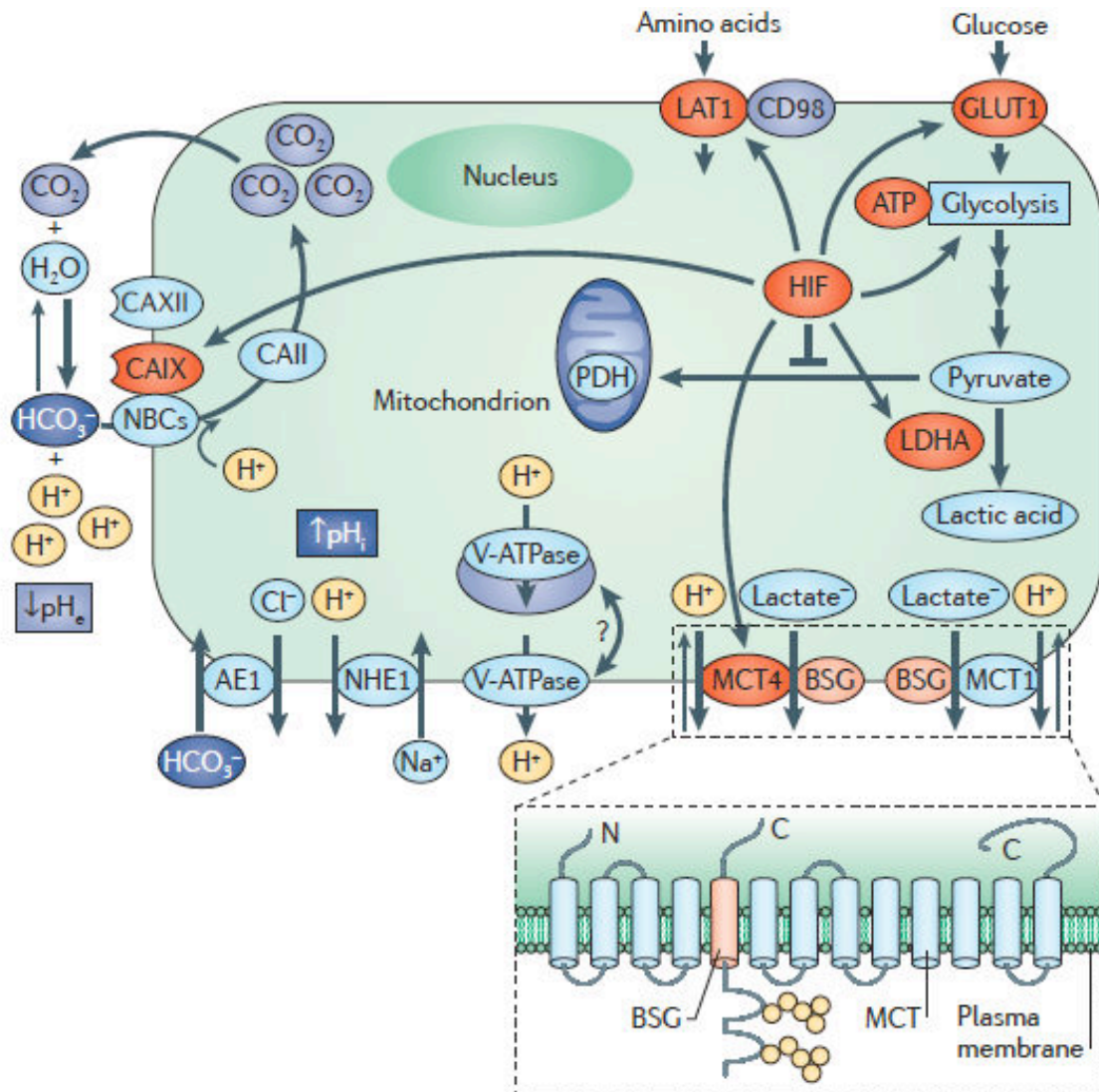


Figure 9. A cellular model of intracellular pH (pHi) regulation and metabolic interactions in the hypoxic tumour cell

Tumour cells survive their acidic surroundings by coordinating the action of pHi-regulatory proteins that include Na⁺/H⁺ exchanger 1 (NHE1), carbonic anhydrases (CAIX, CAXII and CAII), HCO₃⁻-transporters (Na⁺/HCO₃⁻-co-transporters (NBCs) and anion exchange protein 1 (AE1)), monocarboxylate transporters (MCT1 and MCT4) and potentially vacuolar H⁺-ATPases (V-ATPases). Metabolic fuel is provided to the cell by glucose and amino acid transporters (glucose transporter type 1 (GLUT1) and L-type amino acid transporter 1 (LAT1), which is chaperoned by CD98), and hypoxia promotes the expression of certain proteins that are involved in both metabolism and pHi regulation via the transcriptional activity of HIF. The inset shows MCTs in complex with basigin (BSG; also known as CD147); this complex is essential for proper protein functionality and the maintenance of glycolysis. LDHA, lactate dehydrogenase A; PDH, pyruvate dehydrogenase; pHe, extracellular pH (from (Parks et al., 2013)).

E. Lactic acidosis and lactate utilization in tumours: a new promising target for anticancer therapy development

I. Lactate: a key metabolic modulator of cancer cells and stroma

1.1. Lactate, hypoxia and acidosis

Lactate production, tumour acidosis and hypoxia are commonly thought to be linked. However, many studies have shown that high lactate concentrations are not necessarily associated with hypoxia, and that the two phenomena occur at different sites throughout tumours (Hunt et al., 2008; Yaromina et al., 2009). Aerobic glycolysis, increased glutaminolysis and low perfusion rates of blood vessels may also contribute to lactate accumulation in non-hypoxic areas (Levine and Puzio-Kuter, 2010; Vander Heiden et al., 2009; Vaupel et al., 1989). Moreover, when considered separately, the clinical significance of the two concepts is also distinct; while hypoxia without lactic acidosis is usually associated with poor prognosis, lactic acidosis in the absence of hypoxia has been recently shown to shift energy utilization of breast cancer cells from glycolysis toward OXPHOS, contributing thus to favourable clinical outcomes (Chen et al., 2008). Moreover, the high conversion rate of pyruvate into lactate, *via* the enzymatic activity of lactate dehydrogenase A (LDHA), is usually assumed to be the major mechanism responsible of tumour acidity. Nevertheless, using Ras-transfected Chinese hamster lung fibroblasts, Newell et al. (Newell et al., 1993) showed that glycolysis-deficient cells have similar extracellular pH (pHe) values than ones of parental cells, in both *in vitro* and *in vivo*, even if the former produced less lactate. They suggested, therefore, that lactic acid accumulation resulting from enhanced glycolysis is not the only process that generates tumour acidosis. Similar results were reported by two different studies using LDH-deficient cells (Yamagata et al., 1998) and glycolysis-impaired cell lines (Helmlinger et al., 2002), which found that besides lactate, increased levels of CO₂ generated by oxidative metabolism were the main cause of tumour acidity. In fact, both the TCA cycle and the PPP produce CO₂ that is hydrated by CAs to generate HCO₃⁻ and H⁺ (Levine and Puzio-Kuter, 2010; Supuran, 2008), which contributes to the acidification of the tumour microenvironment as we have previously described.

Although lactic acid is not the major player in pH_e acidification of cancer cells, an increasing number of recent studies underline its important role as a “signalling molecule” involved in different mechanisms promoting cancer cell survival, proliferation and metastasis (Gottfried et al., 2012; Hirschhaeuser et al., 2011).

1.2. Lactate a “signalling molecule” of cell migration and angiogenesis

The lactate released by cancer cells is noticeably recognized as an angiogenic promoter. Several recent studies indicate that lactate increases the production of the vascular endothelial growth factor (VEGF) and its receptor VEGFR2 by tumour cells and endothelial cells, respectively. This induction occurs through the activation of HIF-1 as the result of an indirect accumulation of pyruvate, an inhibitor of HIF-Prolyl-hydroxylases, (PHDs) (De Saedeleer et al., 2012; Lu et al., 2005; Lu et al., 2002; Marchiq et al., 2015b; Sonveaux et al., 2012). However, impact of lactate on angiogenesis is dependent not only on HIF-1 expression. Vegran et al. have also shown that lactate could induce interleukin-8 (IL-8) production by endothelial cells, through nuclear factor-kappa B (NF-κB) stimulation, which resulted in new blood vessel maturation and increased cell migration (Beckert et al., 2006; Vegran et al., 2011). Furthermore, a recent study reported a direct role of lactate in modulating angiogenesis independently of HIF. Thus, through the stabilisation of N-Myc downstream-regulated gene 3 (NDRG3) protein expression, lactate induced up-regulation of VEGF, IL-8 and CD31 levels during prolonged hypoxia, apparently *via* the ERK1/2 signalling pathway (Lee et al., 2015). Alterations in extracellular lactate levels have also been shown to increase the *in vitro* random migration of different cancer cells in a concentration dependent manner, which could facilitate metastasis (Goetze et al., 2011; Hirschhaeuser et al., 2011). Furthermore, lactate induced cancer cell motility by increasing production of other factors such as transforming growth factor-β2 (TGF-β2), hyaluronan and CD44, which play an important role in integrin activation, angiogenesis, stemness and modulation of stroma (Baumann et al., 2009; Goetze et al., 2011; Nikitovic et al., 2013; Stern et al., 2002; West et al., 1985) (Fig. 10).

1.3. Immuno-modulatory role of lactate

Besides promoting angiogenesis and migration, the metastatic potential conferred by lactic acid is linked to its emerging role in escape of immune-surveillance (Gottfried et al., 2012). Cancer-generated lactic acid was described to strongly inhibit the anticancer immune response through a decrease in the cytotoxic activity of human T-lymphocytes (Feder-Mengus

et al., 2007; Fischer et al., 2007) and natural killer cells (Husain et al., 2013; Lv et al., 2012). Lactate was also reported to inactivate cytokine release from dendritic cells and to inhibit the differentiation and activation of monocyte-derived dendritic cells (Dietl et al., 2010; Gottfried et al., 2006; Puig-Kroger et al., 2003). Enhanced immune suppression by lactate was further linked to its role in inducing the accumulation of myeloid derived suppressor cells, which further suppresses the T-lymphocytes function (Husain et al., 2013). Other studies have shown that lactate also promoted tumour-associated inflammation by increasing the production of cytokines such as IL-23 and IL-6 (Gottfried et al., 2012; Langowski et al., 2006). Moreover, recent data from syngeneic murine tumour models of Lewis lung carcinoma (LLC) and B16-F1 (B16) melanoma cancer cell lines showed that lactate-induced stabilization of HIF-1 α increased arginase 1 expression and consequently M2-like polarization of tumour-associated macrophages (Colegio et al., 2014) (Fig. 10).

1.4. Clinical significance of lactate accumulation in cancer

In view of the above, a critical role has been attributed to lactate in the development and progression of a wide range of cancers, leading to consider it as a relevant prognostic marker of poor patient survival. To confirm this, data from Mueller-Klieser's group showed for the first time that lactate accumulation was tightly linked to primary cervical cancer aggressiveness and therefore inversely correlated with patient survival (Walenta et al., 2004; Walenta et al., 2000). This negative correlation was also confirmed for patients with head and neck squamous carcinoma (HNSCC) pre-treated with lactate (Walenta and Mueller-Klieser, 2004; Ziebart et al., 2011). Another extensive and independent study have shown that lactate but not pyruvate concentration correlates significantly with tumour response to fractionated irradiation in tumour xenografts of 10 human HNSCC cell line (Sattler et al., 2010). Furthermore, high lactate levels were described, as a potential marker of human rectal adenocarcinoma (Walenta and Mueller-Klieser, 2004), glioblastoma (Park et al., 2010; Saraswathy et al., 2009) and prostate tumour aggressiveness (Keshari et al., 2013; Yaligar et al., 2012), as far as it is positively associated with resistance to radiation and probability of metastasis.

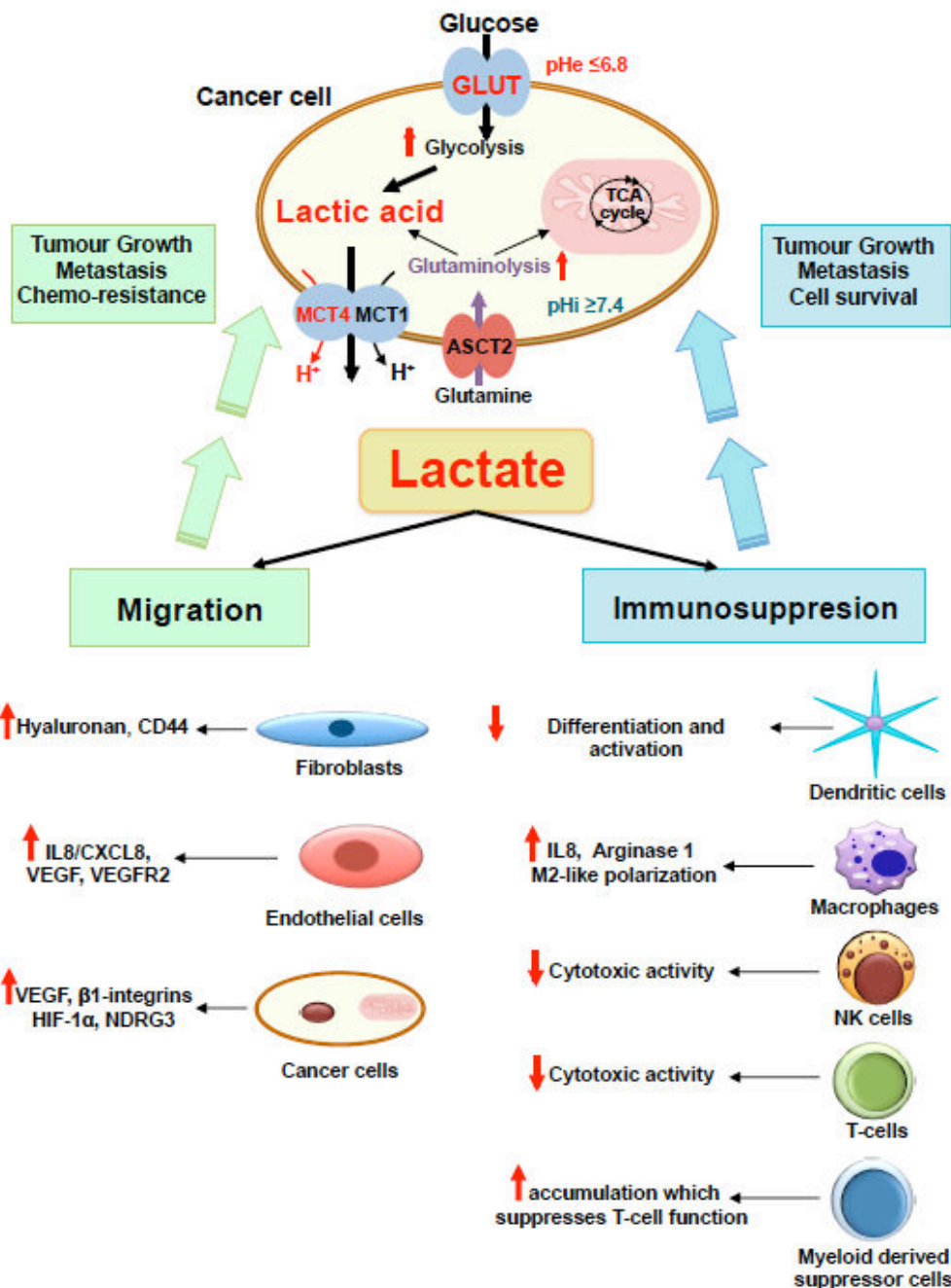


Figure 10. The different roles of cancer-generated lactic acid in promoting tumour growth and metastasis

Enhanced glycolysis and glutaminolysis generate large amounts of lactic acid that is exported by monocarboxylate transporters (MCT) 1 and 4. The accumulation of lactic acid in the extracellular milieu induces a drop in the extracellular pH (pH_e), acidification of the tumour microenvironment and promotes several cancer processes leading to cell survival, tumour growth and metastasis. Lactic acid stimulates angiogenesis by increasing the production of the vascular endothelial growth factor (VEGF) and its receptor VEGFR2 by tumour and endothelial cells. Lactate drives also angiogenesis through the activation of hypoxia-inducible factor 1 (HIF-1), the N-Myc downstream-regulated gene 3 (NDRG3) protein and the stimulation of the production of interleukin 8 (IL8). Increased extracellular lactate levels influence cancer cell motility by promoting hyaluronan production, which acts on fibroblasts and on the cancer cell cytoskeleton through interaction with CD44. More importantly, lactate generated by altered cancer metabolism plays an important role in escape of immune-surveillance, mostly through decreased cytotoxic activity of human T-lymphocytes (T-cells) and natural killer (NK) cells. Further, lactate reduces dendritic cell maturation, induces the accumulation of myeloid derived suppressor cells, and promotes M2-like polarization of tumour-associated macrophages.

II. Lactic acid transporter complexes: structure, expression and regulation

To achieve activation of cell motility and suppression of the immune system, as described above, cancer cells have to maintain a continuous flux of glycolysis that is intimately linked to the rate of lactic acid extrusion. For many years, lactate was thought to be removed from cells merely *via* transmembrane diffusion of its undissociated form, lactic acid. However, studies from Halestrap's group on human red blood cells established the presence of specific transmembrane lactate transporters, belonging to a family of monocarboxylate transporters (MCTs) (Halestrap, 2013; Halestrap and Denton, 1974). This family includes 14 members coded by the *Solute Carrier family 16 (SLC16A)* gene, which show sequence homology (Halestrap, 2012). However, only the first four isoforms (MCT1-4) have been functionally validated to transport monocarboxylates, such as L-lactate, pyruvate and ketone bodies (Halestrap, 2012; Halestrap, 2013). This transport is mainly controlled by the H^+ and monocarboxylate concentration gradient across the plasma membrane, which determines the net direction of transport (influx or efflux) (Poole and Halestrap, 1993). While recent studies showed that MCT12 is transporting creatine in patients with age-related cataract (Abplanalp et al., 2013), and that MCT7 is implicated in the transport of β -hydroxybutyrate in Zebra fish liver (Hugo et al., 2012), many studies have attributed different transport properties to other members of the family. Thus MCT6, MCT8, MCT9 and MCT10 have been reported to transport substrates such as bumetanide, thyroid hormones T3 and T4, carnitine and aromatic amino acids, respectively (Friesema et al., 2004; Friesema et al., 2008; Kim et al., 2002; Kolz et al., 2009; Murakami et al., 2005; Suhre et al., 2011). The transport substrates of the other 4 isoforms remain still unknown.

MCTs display distinct tissue distribution and play an important metabolic role in many physiological and pathological situations, extensively reviewed by Halestrap (Halestrap, 2013; Halestrap and Wilson, 2012). For concision and clarity, we will focus only on the structure and function of the well-characterized proton coupled MCT1 and MCT4.

2.1. MCT1/MCT4 structure, expression and regulation

2.1.1. Structure and expression pattern of MCT1 and MCT4

Due to the well-established role of lactate in metabolism and pH homeostasis within many tissues such as muscle, brain, kidney, liver and retina, a large number of studies concern

the lactate/H⁺ symporters MCT1-4. Although the crystal structure of MCTs has not been described, topology predictions indicated that these proteins contain 12 transmembrane domains (TMs) with intracellular N- and C-termini and a large cytoplasmic loop connecting TM6 and TM7. This prediction was later confirmed for rat MCT1 (Halestrap, 2012; Halestrap, 2013; Poole et al., 1996) (Fig. 11).

Figure 11. Proposed membrane topology of the MCT family.

The likely transport mechanism of both H⁺ and lactate in MCTs was first identified for MCT1, and suggested a translocation cycle including “outside-open” and “inside-open” conformations (De Bruijne et al., 1983; de Bruijne et al., 1985; Galic et al., 2003) implying interactions between lactate, H⁺ and MCT1 key residues (K38, D302 and R306)(Halestrap, 2012; Manoharan et al., 2006; Wilson et al., 2009) (Fig. 12). This mechanism is assumed to be shared by the other 3 MCTs (MCT2-4), as sequence alignment shows that 70% of the transmembrane amino acids are highly conserved for human MCT1-4. Thus, point mutations within the transmembrane domains of these transporters have been demonstrated to affect their substrate specificity, transport activity and inhibitor sensitivity (Garcia et al., 1994; Rahman et al., 1999). Indeed, even if MCT1-4 share common features and transport the same

substrates, they show different binding affinities for monocarboxylates. Therefore, MCT1 and MCT2 transport pyruvate ($K_m \approx 0.1\text{--}0.74\text{mmol/L}$) and stereoselectively L-lactate ($K_m \approx 1\text{--}3.5\text{mmol/L}$) with a very high affinity (Halestrap, 2012; Poole and Halestrap, 1993), compared to MCT4 which possesses a lower affinity for both pyruvate ($K_m \approx 153\text{mmol/L}$) and lactate ($K_m \approx 28\text{mmol/L}$) (Dimmer et al., 2000; Manning Fox et al., 2000). Consequently, the heterogeneous affinities correlate with different expression patterns within tissues (Fishbein et al., 2002). MCT4, due to its very high K_m for pyruvate and lactate, is mainly expressed in highly glycolytic cells such as white skeletal muscle fibers and astrocytes, while either or both MCT1 and MCT2 are expressed in red skeletal muscle, heart and neurons where they uptake lactate to fuel OXPHOS. MCT3 however is exclusively expressed on choroid plexus and the basolateral membranes of the retinal pigment epithelium (Philp et al., 2001), and was shown to transport L-lactate with a K_m of 6mmol/L .

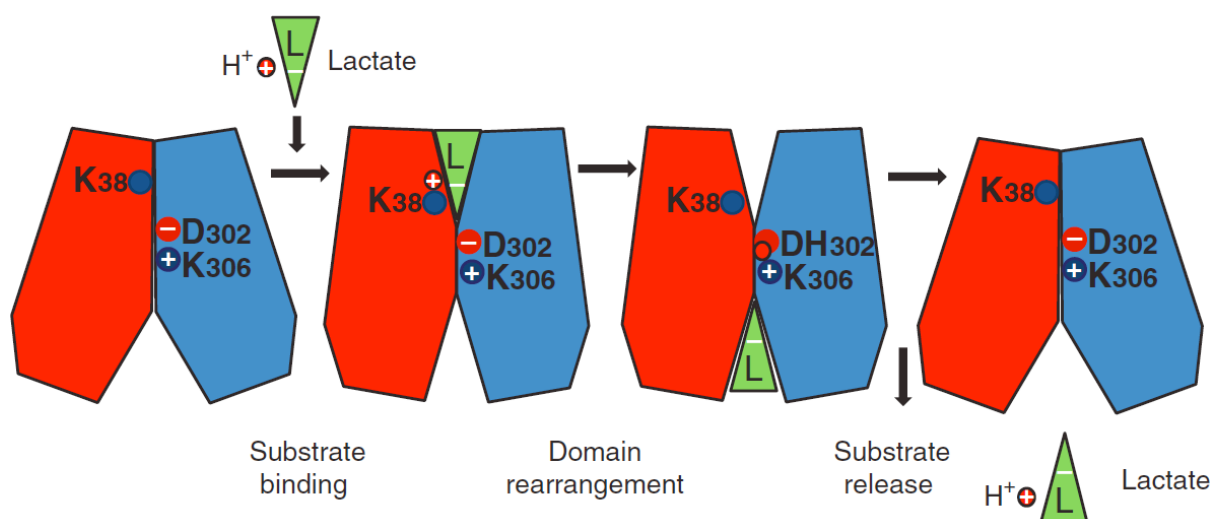


Figure 12. The proposed mechanism of lactic acid transport by MCT1.

Lactic acid protonates K38 causing the channel to open. Lactate then moves into the open extracellular side of the pore and forms an ion pair with K38. In the next step, the proton on K38 is transferred to aspartate 302 (D-) neutralizing the aspartate side chain (DH). This is followed by migration of lactate through the pore where it forms an ion pair with R306 (R+). Once K38 is deprotonated and lactate is occupying the specificity filter, the transporter relaxes back toward the closed state and releases lactic acid into the intracellular space (from (Halestrap, 2013)).

2.1.2. Mechanisms of regulation of MCT1 and MCT4

Differences in tissue distribution imply necessarily distinct regulatory mechanisms. Thus while little is known about the regulation of MCT2 and MCT3 expression, different studies highlighted the regulation of both MCT1 and MCT4 expression. Analysis of the 5'-UTR region of these two MCTs suggests that both transcripts may undergo distinct transcriptional and post-transcriptional regulatory mechanisms. Indeed, MCT4 expression is up-regulated in hypoxia through HIF-1 binding to two hypoxia response elements (HRE) upstream of the transcription start site (Ullah et al., 2006). However, while there is no evidence of a HRE on the MCT1 gene sequence, the MCT1 promoter contains potential binding sites for a number of other transcriptional factors, such as MYC, PGC-1 α , NRF-2 and CREB (Doherty et al., 2014; Hashimoto et al., 2007). Direct interaction between the p53 and MCT1 gene promoters was recently described by Ferron's group and resulted in altered MCT1 mRNA stabilization in hypoxia (Boidot et al., 2012). MCT1 expression can also be regulated in muscle cells after intense exercise through accumulation of lactate and activation of calcineurin, and AMP-activated protein kinase (AMPK) (Dubouchaud et al., 2000; Halestrap, 2012; Hashimoto et al., 2007). Further, in the pancreatic insulin secreting β cells, MCT1 is regulated by either epigenetic modification within CpG islands or microRNA-29, which target the 3'-UTR region inducing MCT1 mRNA degradation and translational repression (Pullen et al., 2011; van Arensbergen et al., 2010). Substances such as butyrate (Borthakur et al., 2008; Cuff et al., 2002), testosterone (Enoki et al., 2006) and thyroid hormone T3 (Fanelli et al., 2003) have also been described to stimulate MCT1 tissue expression.

2.2. CD147/BASIGIN structure, expression and regulation

Besides genetic regulation of MCT1-4, as described above, many studies published in the early 2000s have shown that these non-glycosylated plasma membrane transporters require a tight association with transmembrane glycoproteins for proper folding and trafficking to the cell surface. Using co-immunoprecipitation and chemical cross-linking, Kirk et al. showed that MCT1 and MCT4 specifically interacted with CD147/BASIGIN (BSG)(Kirk et al., 2000). BSG (also named EMMPRIN, gp42, HT7, neurothelin, 5A11, OX-47 and M6) is a transmembrane glycoprotein of the immunoglobulin (Ig) superfamily composed of extracellular Ig-like domains, a single membrane spanning segment and a short intracellular cytoplasmic tail (Muramatsu and Miyauchi, 2003). Alternative transcriptional initiation and

variation in splicing results in four isoforms of BSG (BSG1-4) (Belton et al., 2008) (Fig. 13A).

While little is known about the expression and functionality of both BSG3 and BSG4, studies have shown that the BSG1 isoform (three Ig-like domains) is specifically located at the retina where it closely interacts with MCT3 (Hanna et al., 2003; Ochrietor et al., 2003). However, BSG2 (referred to as BSG from now on) is the most prevalent and studied isoform. It contains two Ig-like domains and is widely expressed in tissues where it interacts with MCT1 or MCT4 (Muramatsu and Miyauchi, 2003). Cross-linking experiments together with studies using fluorescence resonance energy transfer revealed that BSG forms a homooligodimer in which a dimer of BSG binds to two monomers of MCT1 (Wilson et al., 2002) (Fig. 13B).

To reach the plasma membrane MCT2 is also assisted by two paralogs of BSG, a developmentally expressed protein named EMBIGIN (gp70, EMB) (Halestrap, 2012; Wilson et al., 2005) and synaptic glycoproteins called neuroplastins (NP 55 and NP 65) (Wilson et al., 2013).

Many *in vitro* studies have demonstrated tight collaboration of MCTs and BSG for correct plasma membrane expression. Although the first experiments showed that BSG co-localized with MCT1 on the cell surface and that co-transfection with BSG cDNA facilitated the expression of MCTs on the plasma membrane, we and others have recently shown that the surface expression and trafficking of BSG is also dependent on its association with MCTs. Thus, knockdown or gene disruption with zinc finger nucleases (ZFN) of MCT4 alone, or in combination with MCT1 knockdown, impaired the maturation of BSG, leading to its accumulation in the endoplasmic reticulum and proteasomal destruction (Gallagher et al., 2007; Le Floch et al., 2011; Marchiq et al., 2015b).

This dependency was also emphasised in *in vivo* studies of *BSG-null* mice, which showed that *BSG* gene knockout resulted in a substantial reduction in the immunohistochemical staining intensity for MCT1 and disrupted its distribution in almost all tissues (Nakai et al., 2006; Philp et al., 2003). BSG is involved in many physiological events, such as spermatogenesis, implantation, fertilization, lymphocyte responsiveness, vision, behaviour and memory (Muramatsu and Miyauchi, 2003; Weidle et al., 2010). Considering the dependence on bioenergetics of all these events the *in vitro* and *in vivo* studies mentioned above are consistent with a direct impact of a decrease in MCT expression in the phenotype of

BSG-null mice (blindness, sterility, immunodeficiency, and problems with learning and memory) (Hori et al., 2000; Igakura et al., 1998; Muramatsu and Miyauchi, 2003; Nakai et al., 2006).

However, the question whether BSG is the only ancillary protein of MCT1,3,4 remains to be answered. Indeed, MCT1 has been shown in some tissue to be properly expressed independently of BSGs (Nakai et al., 2006). We have also recently reported functional residual MCT1 and MCT4 expression in different *BSG-null* cancer cell lines (Granja et al., 2015; Marchiq et al., 2015b), suggesting the presence of unidentified proteins or mechanisms for targeting these MCTs to the cell surface. Experiments employing co-immunoprecipitation and chemical inhibition point to a role of CD44, a receptor for hyaluronan, as a co-chaperone of MCTs (Slomiany et al., 2009). Further investigation of CD44 expression in *BSG-null* mice and cancer cell lines should be performed to understand the implication of this receptor in lactate transport.

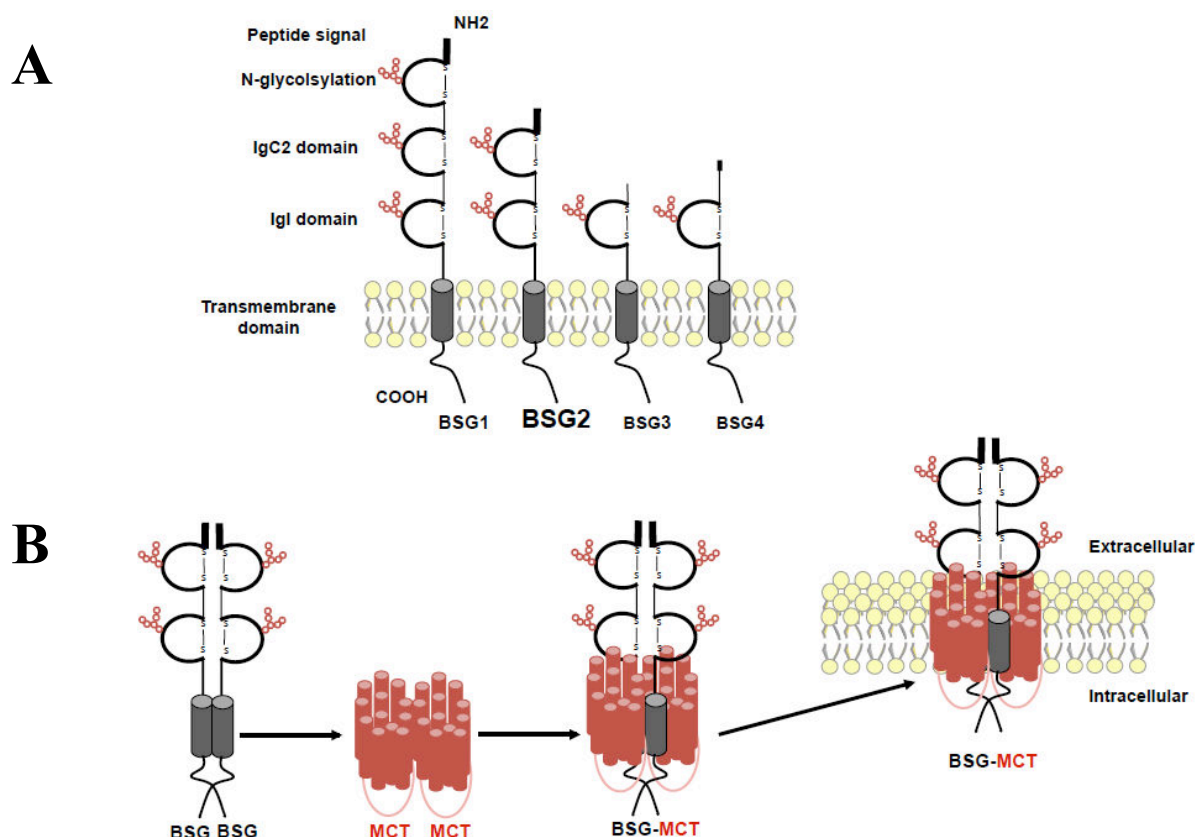


Figure 13. Schematic representation of the structure of BASIGIN (BSG) isoforms and interaction with monocarboxylate transporters (MCT).

A) Alternative transcriptional initiation and variation in splicing results in four isoforms of BSG (BSG1-4) that are composed of extracellular Ig-like domains containing glycosylation sites (red circles), a single membrane spanning segment and a short intracellular cytoplasmic tail. BSG1 is specifically located in the retina, BSG2 (in bold) is the most prevalent isoform and BSG3/BSG4 are intracellular, lacking signal peptide and much less abundant proteins. **B)** Dimers of BSG bind to two monomers of MCT, illustrated by twelve individual helices each, and form a homo-oligodimer that translocates to the plasma membrane for proper expression and functionality.

III. Clinical significance of MCTs and BSG in cancer

3.1. MCTs in cancer

Since many cancer cells rely primarily on glycolytic metabolism to support rapid proliferation, they produce increased amounts of lactic acid that should be efficiently extruded from cells to the tumour microenvironment for cell survival. Thus up-regulation of MCT1 and MCT4 has been reported in several solid tumours, such as glioblastoma, breast, colon, liver, ovarian and lung cancers (Pinheiro et al., 2012). However, the distribution pattern of these two MCTs is different, due to the disparities in the lactate content and its utilisation between tumour types, the oncogenic pathways driving each cancer and the distinct regulatory mechanisms of each MCT. MCT1/MCT4 expression was also shown to differ even within the same tumour. Sonveaux et al. (Sonveaux et al., 2008) have shown that the well-oxygenated tissues of human cervical and colon xenograft tumours express high levels of MCT1 compared to almost no detectable expression in hypoxic regions. Although it is an interesting inter-tumour coupling model, this notion has remained highly controversial and not observed in other tumour types. Other studies have also reported increased expression of MCT4, along with other glycolytic proteins in hypoxic and poorly vascularized tumour regions (Meijer et al., 2012; Rademakers et al., 2011). These differences are consistent with the notion of metabolic cooperation or “micro-ecosystem” as recently described by different groups. This implies that different tumour cell populations reprogram their metabolism, and implicate complementary pathways to meet the challenge of energy production and macromolecule synthesis in a nutrient limited environment (Bonuccelli et al., 2010; Sonveaux et al., 2008). Thus, as defined for skeletal muscle, brain and liver, a lactate shuttle is established between hypoxic and oxygenated cancer cell populations (Fig. 14). In this model, the lactate that is released as a waste product by hypoxic cells, mainly *via* MCT4, is taken up and re-used by cells expressing MCT1 in oxygenated regions. Aerobic cells will then convert lactate into pyruvate to fuel their oxidative metabolism, allowing glucose to reach the hypoxic cells at the poorly vascularized tumour core (Sonveaux et al., 2008). Thus using metabolic imaging techniques, Galie et al. (Galie et al., 2007) validated increased glucose uptake in the hypoxic tumour regions compared to the well-vascularized regions at the periphery of the tumour. Moreover, inhibition of MCT1 in different cancer cells has been reported to decrease lactate availability for oxidative cells, forcing them to take up glucose. This resulted in glucose

starvation and cell death of hypoxic cells, leading subsequently to tumour growth arrest (Sonveaux et al., 2008).

Aberrant metabolism of cancer cells is not alone in promoting the malignant phenotype, components of the tumour microenvironment, such as cancer associated fibroblasts (CAFs), endothelial cells and inflammatory cells, also play an important role. In addition to the role of lactate in inducing angiogenesis or the so-called “vascular endothelial lactate shuttle” (Fig. 14), as mentioned above, an increasing number of studies support the existence of tumour-stroma metabolic cooperation. Immunohistochemical analysis of human colorectal adenocarcinomas showed that tumour cells express high levels of GLUT1, MCT1 and HIF-1 α , indicating increased anaerobic metabolism and lactate production, whereas the expression profile of CAFs (low level of GLUT1/HIF-1 α , and high MCT1/MCT2) suggested high lactate absorption and oxidative metabolism (Koukourakis et al., 2006).

Recently, increasing evidence supports the presence of another type of tumour-stroma collaboration, referred to as the “reverse Warburg effect” (Pavlides et al., 2009; Whitaker-Menezes et al., 2011; Witkiewicz et al., 2012) (Fig. 14). In this case, CAFs first exhibit a highly glycolytic phenotype and consequently express high levels of MCT4, which is necessary to extrude lactate. This process is proposed to be induced by oxidative stress in association with loss of caveolin-1 and other metabolic alterations. Formerly, epithelial cancer cells use MCT1 to import secreted lactate, which enters the TCA cycle and drives oxidative metabolism. This phenomenon has been described for breast cancer (Witkiewicz et al., 2012), prostate cancer (Fiaschi et al., 2013), head and neck tumours (Curry et al., 2013) and osteosarcoma (Sotgia et al., 2014). Further, elevated levels of stromal MCT4 expression were reported to be a marker of poor survival in triple negative breast cancer (Witkiewicz et al., 2012). However, these studies remain highly controversial as a recent study from our group showed elevated expression levels of glycolytic markers, such as CAIX, LDHA and MCT4, in all breast cancers, with highest rates in triple negative breast cancer. More importantly staining of tumours for MCT4 but not of the stroma correlated with negative prognostic index for overall-survival (Doyen et al., 2014). Moreover, recent studies have shown decreased expression or even absence of the LDHB subunit, the enzyme converting lactate into pyruvate, in breast, prostate and gastric cancers due to hyper-methylation of the LDHB promoter (Brown et al., 2013). Thus these recent findings together with the prominent expression pattern of MCT4 in many cancers raises doubt concerning the “reverse Warburg” model proposed by Lisanti’s group (Witkiewicz et al., 2012) (Fig. 14).

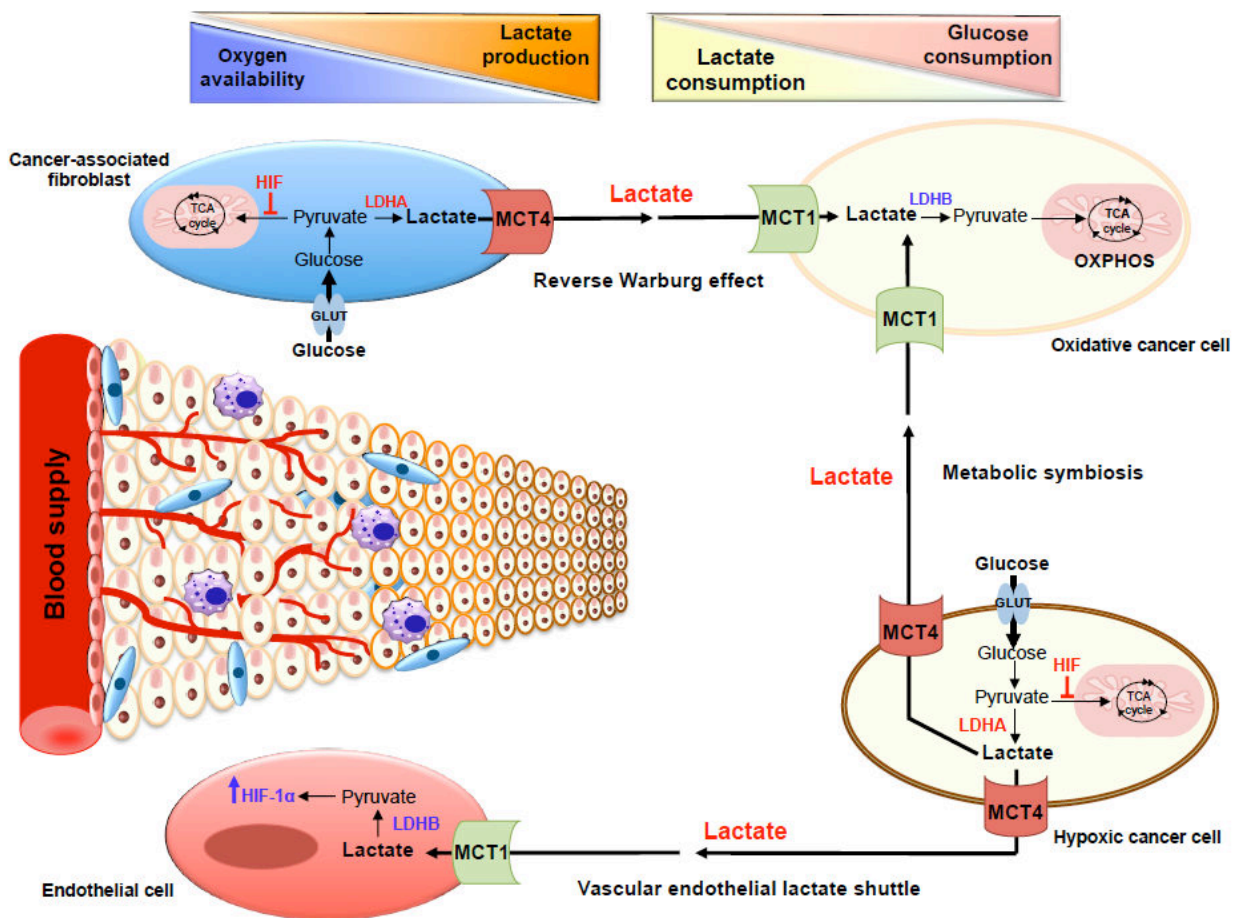


Figure 14. Model showing the tumour microenvironment and lactate shuttles in cancer.

Cells located far from the perfused blood vessels become rapidly hypoxic and rely on glycolysis for proliferation. They generate, therefore, large amount of lactic acid that is extruded by the monocarboxylate transporter 4 (MCT4). Lactate is subsequently taken up by the endothelial cells *via* the monocarboxylate transporter 1 (MCT1), and is converted into pyruvate by lactate dehydrogenase B (LDHB), a phenomenon referred to as the “vascular endothelial lactate shuttle”. Pyruvate, by stabilizing hypoxia-inducible factor-1 α (HIF-1 α), induces tumour angiogenesis. Normoxic cancer cells, that highly express MCT1, also preferentially take up lactate produced by hypoxic cancer cells to perform oxidative phosphorylation (OXPHOS). This “metabolic symbiosis” allows hypoxic regions of the tumour to acquire high levels of glucose and, subsequently, generate lactic acid. In addition, cancer-associated fibroblasts, which are highly glycolytic and express MCT4, also supply oxidative cancer cells with lactate. This tumour-stroma cooperation, termed “reverse Warburg effect” in addition to the other lactate shuttles, result in the establishment of lactate and glucose consumption gradients within tumours.

3.2. BSG in cancer

In parallel to MCT1/4 overexpression, CD147/BSG is also commonly up-regulated in cancers, and since 1990 more than 540 research articles have highlighted its pro-tumour role. Indeed, deregulation of BSG has been linked to almost every type of cancer (Li et al., 2009; Riethdorf et al., 2006). An immunohistochemical study of a large number of normal and cancer tissues has shown that BSG is overexpressed in 112 out of 129 tumour samples with a very high incidence in glioblastoma, breast cancer, pancreatic cancer, hepatocellular carcinoma, and squamous cell carcinomas, among others (Riethdorf et al., 2006). BSG expression correlated with the histological type of tumours, grade of cancers, tumour progression and recurrence, and patient survival. Moreover, BSG expression usually co-localized with MCT1/MCT4 in tumour tissues, which constitutes a prognostic marker of poor clinical outcome (Chen et al., 2010; Walters et al., 2013).

BSG was first named “EMMPRIN” for Extracellular Matrix MetalloPRotease INducer, because it was reported to be associated with increased matrix metalloproteases (MMPs) through which it promoted tumour invasiveness and metastasis. This function, based on nearly 200 studies showing a positive correlation between knockdown or ectopic expression of BSG and levels of different MMPs (1, 2, 3, 9, 11, 14 and 15), was proposed to be mediated *via* up-regulation of MMPs produced by fibroblasts neighbouring tumour cells (Weidle et al., 2010). This pro-tumoural model placed BSG at the centre of tumour invasion but so far remains only correlative with no molecular mechanisms demonstrating how BSG or its soluble form is capable of inducing MMPs. Moreover, most of these studies ignored the major role of BSG in tumour metabolism, particularly the control of the glycolytic rate, lactate transport and pH_i homeostasis through the assistance of BSG in bringing MCTs to the plasma membrane. Indeed, we and others have shown that targeting BSG with shRNA or deleting the *BSG* gene with zinc fingers nucleases (ZFNs) reduced levels of expression of MCT1/MCT4, increased the intracellular pool of lactic acid and impaired tumour growth *in vivo* (Baba et al., 2008; Granja et al., 2015; Le Floch et al., 2011; Marchiq et al., 2015b; Schneiderhan et al., 2009).

Recent studies from our group showed that BSG knockout in colon, glioma, and lung cancer cell lines promoted tumour proliferation through metabolic reprogramming (Granja et al., 2015; Marchiq et al., 2015b), but without any significant change in the expression levels of MMPs compared to parental cells. Using co-cultures of either human fibroblasts or mouse

embryonic fibroblasts (MEFs) and tumour cell lines we showed, in contrast to the published literature, that the disruption of BSG in tumour cells and in MEFs does not modify the production of MMPs. These studies concerned MMP1 and MMP13, stromelysins MMP3 and MMP11, the membrane type (MT) 1-MMP, MMP14, and finally the most described gelatinases A and B MMP2 and MMP9 (Marchiq et al., 2015a).

Besides MCTs and MMPs, BSG was reported to interact with a number of other cell surface regulatory proteins, such as β 1-integrins, cyclophilin A, ubiquitin C, caveolin-1, the CD44 glycoprotein, CD98 heavy chain (CD98hc), large neutral amino transporter 1 (LAT1), Asc-type amino acid transporter 2 (ASCT2) and VEGFR2 (Dai et al., 2009; Khayati et al., 2015; Marieb et al., 2004; Slomiany et al., 2009; Tang et al., 2008; Toole and Slomiany, 2008; Weidle et al., 2010; Xu and Hemler, 2005). Interaction with these molecules results in different roles of BSG in including angiogenesis, enhanced cell migration, invasion and chemo-resistance. Although the molecular mechanisms driving some of these interactions are described (β 1-integrins/BSG or CD44/BSG), further investigation is needed to determine whether all the putative functions attributed to BSG result from a real physical interaction with the companion molecule or to its metabolic effects.

IV. Targeting components of the MCT/BSG complexes: a new hope for anticancer therapy

4.1. Targeting BSG

Due to the interdependency of MCT1/4 and BSG for functional expression of lactate transport, and also to the key role of this glycoprotein in cancer development, it seems evident to consider BSG as a promising therapeutic target in cancer. Thus, genetic silencing studies on BSG have reported inhibition of tumour growth and increased cell death in different cancer cell lines associated with reduced, angiogenesis, MMP secretion, invasiveness and chemo-resistance (Chen et al., 2006; Li et al., 2007a; Wang et al., 2006; Weidle et al., 2010; Zou et al., 2007). On the other hand, treatment of human head and neck squamous cell carcinoma with the anti-BSG monoclonal antibody (CNTO3899) was found to reduce proliferation and induce caspase-mediated apoptosis of cells *ex-vivo*, and to impair tumour growth with increased radio-sensitivity *in vivo* (Dean et al., 2010; Dean et al., 2009). Monoclonal antibodies against BSG have also shown efficacy in treatment of hepatocellular carcinoma

and hypervascular pancreatic tumours, when administered alone or in combination with chemotherapy (Kim et al., 2014).

4.2. Targeting MCTs

The relevance of targeting lactic acid efflux to develop an anticancer strategy was initiated by the pharmacological inhibition and genetic knockdown of MCTs. Several small molecules were first described to efficiently inhibit MCT1 transport (Halestrap, 2012; Halestrap, 2013). Among these, mainly α -cyano-4-hydroxycinnamate (CHC) has been used by several groups and has demonstrated promise in inhibiting MCTs as a cancer therapy without any apparent toxicity *in vivo* (Colen et al., 2006; Colen et al., 2011; Halestrap, 2013; Morais-Santos et al., 2014; Sonveaux et al., 2008). However, the possible use of this first generation of inhibitors in the clinic faced the problem of their lack of MCT specificity. Consequently, data from all these studies did not validate MCT as an anticancer target (Halestrap, 2012).

Recently, AstraZeneca developed a new class of a highly specific and potent MCT1/MCT2 inhibitor (Ki values in the nmol/L range), named AR-C155858 (Ovens et al., 2010). Originally developed as an immunosuppressive drug that acts on T-lymphocyte activation (Bueno et al., 2007; Ekberg et al., 2007), AR-C155858 has shown a striking impairment of both *in vitro* and *in vivo* growth of HRas-transformed fibroblasts, which established for the first time the relevance of targeting MCT1 in cancer (Le Floch et al., 2011). Additionally, a second-generation of more potent MCT1 inhibitors, AZD3965 (Km=1.6 nmol/L), was recently reported to disrupt lactate efflux, glycolysis and glutathione synthesis of Burkitt lymphoma and MCF7 breast cancer cell lines, leading to cell death (Doherty et al., 2014). Other studies have also shown the anticancer effects of AZD3965 in small cell lung cancer (SCLC) and gastric cancer cells lines (Polanski et al., 2014). Treatment of tumours *in vivo* with the inhibitor induced an increase in the lactate concentration, reduced growth and enhanced radiation sensitivity (Bola et al., 2014). AZD3965 is currently undergoing phase I/II clinical trials in the United Kingdom for patients with solid tumours, prostate cancer and diffuse large cell B lymphoma.

Nevertheless, due to functional redundancy between MCT1 and MCT4, we and others have demonstrated that MCT1 inhibitors are inefficient in affecting growth and survival of highly glycolytic and hypoxic tumours. Indeed ectopic expression of MCT4 in HRas-transformed fibroblasts rendered them insensitive to MCT1 inhibition and increased their

tumour growth *in vivo* (Le Floch et al., 2011). Moreover, knockdown or knockout of MCT4 in human colon adenocarcinoma cells made them sensitive to MCT1 pharmacological inhibition, and so impaired their proliferation *in vitro* and tumour growth *in vivo* (Le Floch et al., 2011; Marchiq et al., 2015b). MCT4 silencing was also reported to decrease cancer cell migration, as MCT4 closely interacts with β 1-integrins at the leading edge of migrating cells (Gallagher et al., 2009; Gallagher et al., 2007). Considering these data and the fact that most of highly aggressive tumours predominantly express the hypoxia-induced isoform MCT4, there is an absolute need to develop MCT4-specific inhibitors as a valuable anticancer therapy.

Recently, using a molecular model of MCT4, Nancolas et al. (Nancolas et al., 2015) reported structural differences between MCT1 and MCT4, and identified N147, R306 and S364 as key residues involved in MCT1 inhibitor (AR-C155858) binding and selectivity (Fig. 15), which gives hope for development of selective small drugs inhibiting specifically MCT4. In the meantime, AstraZeneca succeeded in generating an MCT4 (AZ93) inhibitor that is likely selective and highly efficient in blocking growth of a wide range of cancer cells, but only when MCT1 gene is disrupted or MCT1 inhibited pharmacologically (Marchiq I, Critchlow S and Pouyssegur J, unpublished data).

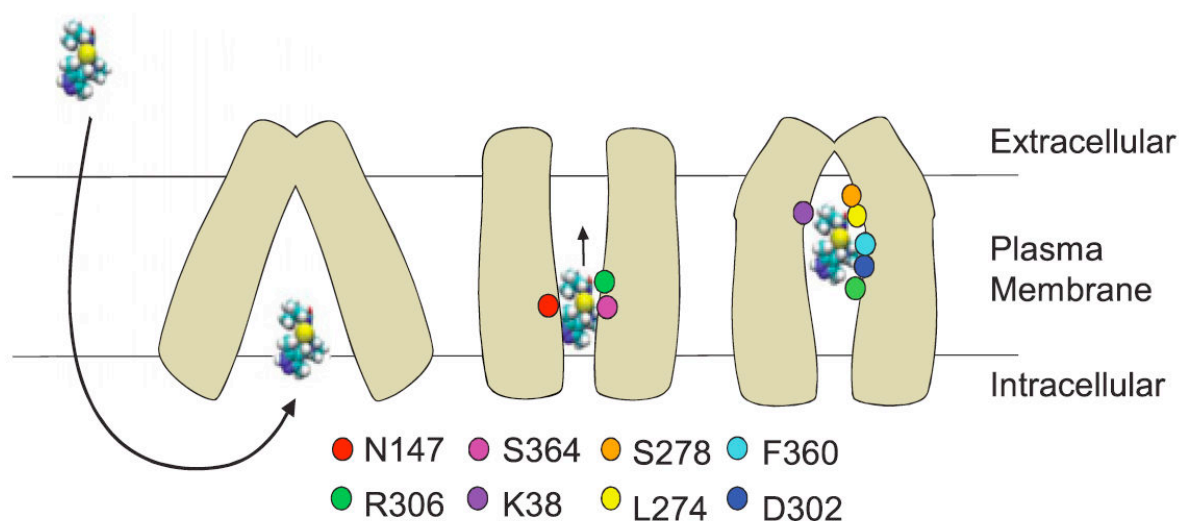


Figure 15. Schematic diagram showing the proposed mechanism of inhibition by AR-C155858

AR-C155858 crosses the plasma membrane to enter MCT1 in the inward-open conformation. An intermediate conformation is adopted, allowing binding of AR-C155858 by interaction with residues in the intracellular half including N147 (helix 5), R306 (helix 8) and S364 (helix 10). A further conformational change then allows movement of AR-C155858 further into the channel of MCT1, interacting with residues in the extracellular half including K38 (helix 1), D302 (helix 8), F360 (helix 10), L274 and S278 (helix 7) (from (Nancolas et al., 2015)).

F. Lactate into pyruvate and back: a two-way road from fermentative glycolysis to mitochondrial metabolism

I. Pyruvate metabolism

Pyruvate is a critical molecule situated at the crossroads of multiple metabolic pathways including glucose, amino acid and fatty acid metabolism. Pyruvate can be generated by several sources in the cytosol. The major one is the last irreversible step of glycolysis, where pyruvate kinase (PK) catalyses the dephosphorylation of phosphoenolpyruvate into pyruvate. The tissue-specific regulation of the PK activity is controlled by four PK isozymes: PKL, PKR, PKM1 and PKM2 encoded by two different genes, *PKLR* and *PKM* (Tsutsumi et al., 1988). Several cancers show an elevated expression level of PKM2 that, through its dynamic interconversion between a dimer (inactive) and tetramer (highly active), efficiently catalyses the formation of pyruvate and regulates glucose-derived carbon flux (Christofk et al., 2008; Wong et al., 2013).

While the main outcome of pyruvate produced by PK is reduction to L-lactate by lactate dehydrogenase (LDH), a significant amount of L-lactate is converted back to pyruvate by the reverse reaction. LDH is a tetrameric complex composed of two distinct isoforms: the LDHA, also called M isoform (for muscle), which reduces pyruvate into L-lactate and the LDHB, termed H isoform (for heart), that favours the reversible reaction leading to the production of pyruvate (Dawson et al., 1964; Quistorff and Grunnet, 2011; Stambaugh and Post, 1966). In contrast to LDHB, LDHA highly regulated by HIF-1 triggers fermentative glycolysis and represents a promising target in cancer therapy (Doherty and Cleveland, 2013). Pyruvate can also be re-formed from malate by cytosolic malic enzyme, or generated through the catabolism of several amino acids such as, serine, threonine, glycine, cysteine, tryptophan and alanine (Jeoung et al., 2014; Mendes-Mourao et al., 1975). Alanine transaminase (ALT), also referred to as glutamic pyruvate transaminase (GPT), catalyses the reversible transamination of alanine and 2-OG into glutamate and pyruvate. ALT is composed of two different isoforms: ALT1, which is localised in the cytosol and ALT2 expressed in the mitochondrial matrix (Glinghammar et al., 2009; Hopper and Segal, 1964; Rafter et al., 2012).

Once produced in the cytoplasm, pyruvate is primarily transported into the mitochondrial matrix and converted to acetyl-CoA by the PDH complex (Patel and Korotchkina, 2006). This acetyl-CoA then enters the TCA cycle leading to production of CO₂ molecules and low-redox-potential electron donors (NADH and FADH₂), which are required for OXPHOS and ATP production. Mitochondrial pyruvate is also irreversibly carboxylated into oxaloacetate by pyruvate carboxylase (PC) (Utter and Keech, 1960; Wallace, 2010), which ensures replenishment of TCA cycle components (anaplerosis) such as citrate and 2-OG, that are used in multiple anabolic pathways implicated in biosynthesis of amino acids, fatty acids and glucose (Owen et al., 2002).

However, to access the mitochondrial matrix, pyruvate must cross three layers represented by the outer mitochondrial membrane (OMM), the intermembrane space and the inner mitochondrial membrane (IMM). Similar to most other metabolites, pyruvate probably diffuses passively from the cytoplasm to the intermembrane space, through the large non-specific voltage-dependent anion channels (VDAC)/porin (McCommis and Baines, 2012). Indeed, deficiencies in VDAC1 have been reported to impair pyruvate metabolism and ATP production (Huizing et al., 1996). However, the IMM is impermeable to charged molecules, due to the importance of maintaining the proton gradient for OXPHOS and ATP production. Therefore, the movement of pyruvate through the IMM is more restrictive and requires specific transport (Fig. 16).

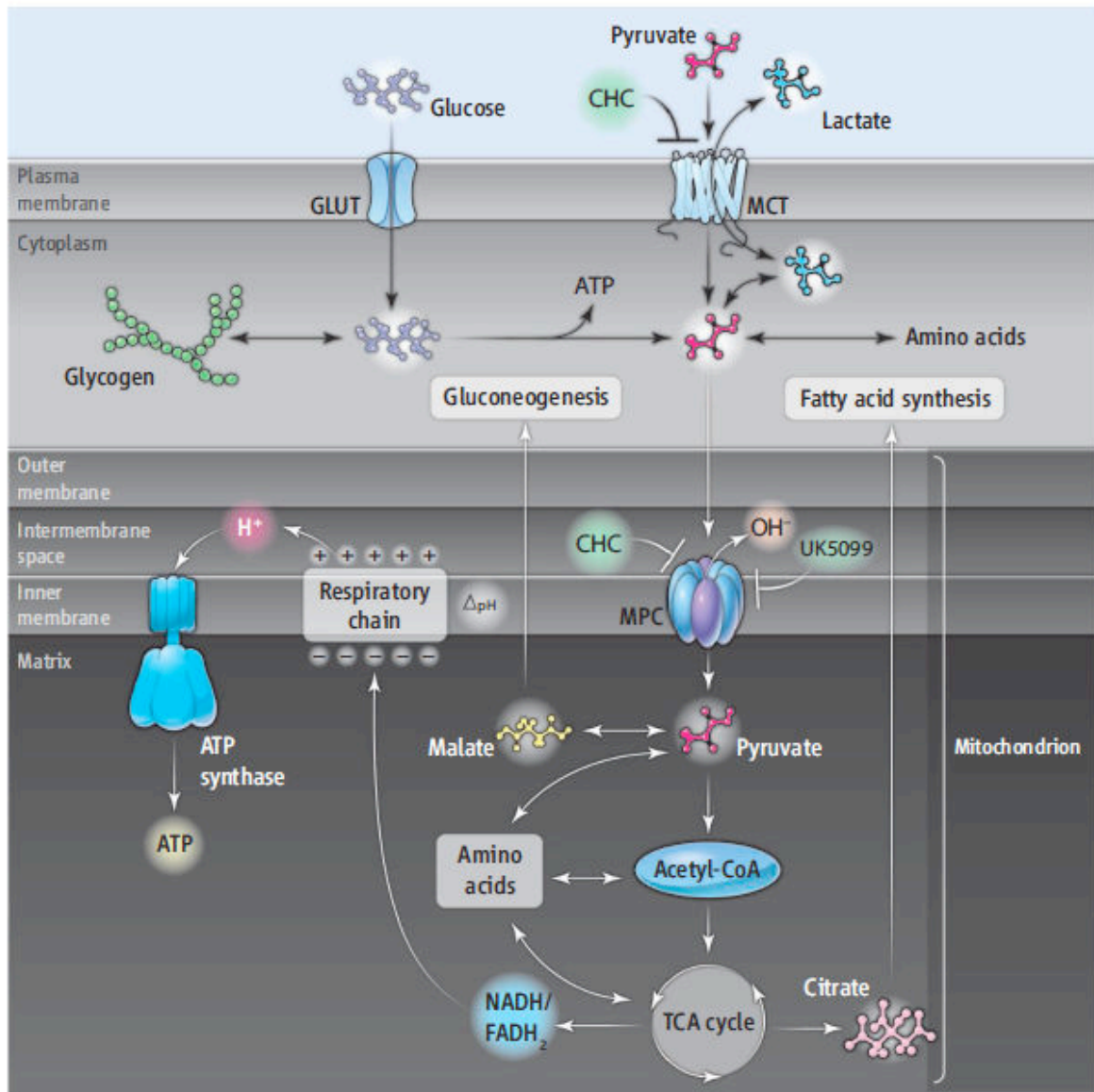


Figure 16. Pathways of pyruvate metabolism.

Pyruvate can either passively diffuse across the mitochondrial inner membrane or be transported by the mitochondrial pyruvate carrier (MPC); both processes are driven by ΔpH . MCT, monocarboxylate transporter; GLUT, glucose transporter; TCA, tricarboxylic acid; NADH, nicotinamide adenine dinucleotide, reduced; $FADH_2$, flavin adenine dinucleotide, reduced; ATP, adenosine-5'-triphosphate; CHC, cinnamic acids (Divakaruni and Murphy, 2012).

II. Transport of pyruvate into mitochondria: mitochondrial pyruvate carrier

2.1. Evidence of facilitated transport of pyruvate to the mitochondrial matrix

The mechanism of transport and the identity of pyruvate transporters in the IMM has been the subject of several investigations and controversies for several decades. It was first assumed that pyruvate could cross freely the IMM in its acidic form (Klingenberg, 1970). Thus early in the 1970s, by using purified rat liver mitochondria, inhibitors of pyruvate metabolism and ETC, mixed with radiolabelled pyruvate, Papa et al. (Papa et al., 1971) have shown that pyruvate translocation into mitochondria was saturable. They presented, therefore, evidence of specific ion-coupled transport of pyruvate across the IMM. Findings from this work were rapidly reinforced by the identification and characterization of a specific inhibitor of mitochondrial pyruvate transport by Halestrap's group (Halestrap and Denton, 1974). Indeed, α -cyano-4-hydroxycinnamate (CHC) was reported to decrease pyruvate oxidation by rat liver mitochondria (K_i of 2 to 5 $\mu\text{mol/L}$), with no effect on permeabilised mitochondria or mitochondrial enzymes. Several other cinnamate-based inhibitors of pyruvate transport were developed, however, α -cyano- β (1-phenylindol-3-yl)-acrylate (also known as UK-5099) was the most potent with K_i values in the range of nmol/L (Halestrap, 1976; Halestrap and Denton, 1975). Both CHC and UK-5099 have been reported to also inhibit MCTs at the plasma membrane and subsequently cellular uptake of pyruvate with 30- and 300-fold, respectively, less effective potency than the inhibition of mitochondrial pyruvate transport (Gray et al., 2014; Schell and Rutter, 2013; Vacanti et al., 2014) (Fig. 16).

Thiazolidinediones compounds were also described to inhibit pyruvate-stimulated respiration and some of them (GW604714X and GW450863X) are even more potent ($K_i < 1$ nmol/L) than UK-5099 (Divakaruni et al., 2013; Halestrap, 1975; Hildyard et al., 2005). Development of all these inhibitors enabled the establishment of several pyruvate transport assays that improved the understanding of the basic biochemical characteristics of mitochondrial pyruvate transport (K_m , V_{max}) prior to the purification and identification of the transporter.

Investigations into the mitochondrial pyruvate transport complex were first initiated in the beginning of the 1980s by using the thiol-blocking reagent N phenylmaleimide (NPM) (Thomas and Halestrap, 1981). Halestrap's group has shown that α -cyanocinnamate protected

the pyruvate transport from the irreversible binding of (NPM) and reduced the radiolabelling of a 15 kDa protein with [³H]-NPM. Unfortunately, purification and sequencing of this protein showed it was the subunit 4 of cytochrome c oxidase (Hildyard and Halestrap, 2003). Other studies using immobilized inhibitors as bait for purification of the mitochondrial pyruvate transporter were developed in the mid 1980s (Bolli et al., 1989; Nalecz et al., 1991). Although this method allowed the purification of several proteins from beef heart (31 and 34 kDa) (Bolli et al., 1989) and yeast (26 and 50 kDa) (Nalecz et al., 1991) isolated mitochondria, none of these proteins was identified as the mitochondrial pyruvate transporter.

When considering the mechanism of mitochondrial transport of other metabolites, it was suggested that a member of the mitochondrial carrier family (MCF), also known as the solute carrier 25 (SLC25) family, might facilitate the transport of pyruvate across the IMM. These carriers are usually 30 to 35 kDa in size and are predicted to contain six transmembrane domains with the C- and N-termini facing the intermembrane space (Gutierrez-Aguilar and Baines, 2013). Using mitochondria from *Saccharomyces cerevisiae* strains, lacking each of the 18 uncharacterized MCF proteins, a study reported that the knock out of the *YIL006w* gene resulted in decreased sensitivity to UK-5099 and reduced uptake of pyruvate. Therefore, the YIL006w protein (41.9 kDa) was proposed to be the mitochondrial pyruvate carrier (MPC) (Hildyard and Halestrap, 2003). However, work from Palmieri's group has more recently, by using experiments of reconstitution into proteoliposomes, demonstrated that this protein is a mitochondrial NAD⁺ transporter (Todisco et al., 2006).

2.2. Identification of the mitochondrial pyruvate carrier

The identity of the pyruvate carrier remained a mystery until 2012, when two independent groups published two interesting and converging stories in Science reporting the eventual identification of the MPC in the IMM (Bricker et al., 2012; Herzig et al., 2012; Schell and Rutter, 2013; Vanderperre et al., 2015). Using genetics and metabolomics Bricker et al. (Bricker et al., 2012) identified a hetero-oligomeric complex (150 kDa) of two proteins named MPC1 (12 kDa) and MPC2 (15 kDa) (also known as Brp44L and Brp44, respectively), that are highly conserved and essential for mitochondrial transport in yeast, flies and mammals. Indeed, authors have shown that genetic disruption of the MPC genes, in flies and yeast, decreased the level of acetyl CoA and TCA cycle metabolites and increased the level of glycolytic intermediates including pyruvate. Moreover, knockdown of either MPC1 or MPC2 with siRNA was reported to significantly impair pyruvate-stimulated mitochondrial

respiration (Bricker et al., 2012). Using different experimental approaches, Herzig et al. (Herzig et al., 2012) reported defective lipoic acid biosynthesis and reduced pyruvate uptake in yeast mitochondria lacking MPC proteins. Furthermore, they have shown that expression of both murine MPC proteins in *Lactococcus lactis* was sufficient to facilitate pyruvate import into the bacterium, and this transport was sensitive to MPC inhibitor UK-5099 (Herzig et al., 2012). The stoichiometry of the proteins in the hetero-oligomeric complex is currently unknown, however functional transport required both MPC1 and MPC2 (Herzig et al., 2012). Unlike the SLC25 family proteins, which contain six transmembrane domains (Palmieri, 2013), MPC paralogs are predicted to only contain two to three transmembrane domains and have recently been proposed to share structural similarity with the bacterial semiSWEET glucose transporters (SLC50 family) and the PQ-loop family of amino acid transporters (Jezegou et al., 2012; Saudek, 2012; Vanderperre et al., 2015; Yang et al., 2014). However, further investigations are needed to confirm these hypotheses and to demonstrate whether MPC proteins are effectively the carriers of pyruvate translocation across the IMM or only essential regulators or subunits of a more complex mitochondrial pyruvate carrier.

2.3. MPC in cancer

Whatever the final answer, it is evident today that MPC proteins are essential for pyruvate transport into the mitochondrial matrix. Indeed, recent work by Schell et al. (Schell et al., 2014) reported increased pyruvate flux into mitochondria after re-expression of MPCs in colon cancer cells lacking the native proteins; under-expression of MPC proteins was found in several cancer cell lines and correlated with poor survival. Moreover, the data suggest that altered pyruvate transport, due to this under-expression, might contribute to increased the “Warburg effect” (Schell et al., 2014). Consequently, enforced MPC expression was reported to impair tumour growth both *in vitro* and *vivo*. Furthermore, inhibition of MPC in mammalian cells, either genetically (siRNA) or with pharmacological inhibitors (CHC, UK-5099), was shown to suppress acetyl-CoA formation from glucose (Yang et al., 2014). However, isotopic labelling experiments demonstrated that cells survive by increasing dependency on glutamate oxidation to replenish the TCA cycle. Thus, combinatorial inhibition of MPC and glutamate dehydrogenase (GDH), the enzyme responsible for glutamine-dependent acetyl-CoA formation, was reported to considerably reduce the growth of cancer cells (Yang et al., 2014). Finally, while human mutations in the MPC1 gene were reported to be linked to hyperpyruvatemias and lactic acidosis (Bolli et al., 1989; Bricker et al.,

2012), homozygous deletion of either MPC1 or MPC2 was found to be embryonically lethal, which points out the importance of MPC and pyruvate import into mitochondria in physiology (Vanderperre et al., 2015; Vigueira et al., 2014).

III. Targeting mitochondrial metabolism: the promise of the new/old drug metformin and its analogues

As seen above, mitochondria are central metabolic organelles that act as a full-time factory producing not only ATP, but also fundamental building blocks, including fatty acids, amino acids, and nucleotides, that are required for rapid cell growth and survival. In contrast to the conventional perception of cancer metabolism, increasing evidence supports the presence of highly active mitochondrial metabolism in cancers and suggest that microenvironmental constraints (e.g. nutrient availability) dictate, in part, the metabolic phenotype of tumour cells (glycolytic vs oxidative), which is highly plastic to efficiently meet the cellular bioenergetic and biosynthetic needs. Given these observations, mitochondrial components (transporters and enzyme complexes) emerged as promising clinical targets and several pre-existing or newly synthesised drugs are being tested to validate the potential efficacy of inhibiting mitochondrial metabolism in cancer, among them, biguanides are drawing particular attention.

- Biguanides: metformin and phenformin

Biguanide is the active compound of the plant *Galega officinalis* (French lilac), which has been used for centuries without knowledge and late in the nineties for the treatment of diabetes (Witters, 2001). Biguanides are composed of two guanidine groups joined by a common nitrogen (Rena et al., 2013). Attention first focused on guanidine compounds such as synthalins (synthalin A and synthalin B) for clinical use in diabetes. However, due to their high level of toxicity these drugs were rapidly abandoned, and attention shifted to the safer derivatives, biguanides, late in the 1950s (Luft et al., 1978; Rena et al., 2013). Among these biguanides, metformin is considered today as the first-line drug treatment for type 2 diabetes due to an impressive safety record and weight neutrality, while phenformin, extensively used in the 1960s, was withdrawn from the clinic in several countries due to a high risk of lactic acidosis (about 64 cases per 100,000 patients per year) (Crofford, 1995; Gavin, 2008; Owen et al., 2000) (Fig. 17). Although metformin is widely used in the treatment of diabetes, its exact mechanism of action is still not fully elucidated. In addition, it seems to have anti-

cancer activity. Since 2005, several epidemiological studies have reported a significant reduction in cancer incidence in diabetic patients treated with metformin compared with other anti-diabetic treatments (Bowker et al., 2006; Evans et al., 2005; Zhang et al., 2013). These observations, in addition to many promising pre-clinical studies prompted over fifty prospective clinical trials to evaluate the efficacy of metformin as an anti-cancer therapy (Ben Sahra et al., 2010b; Pollak, 2013; Rena et al., 2013; Zakikhani et al., 2006).

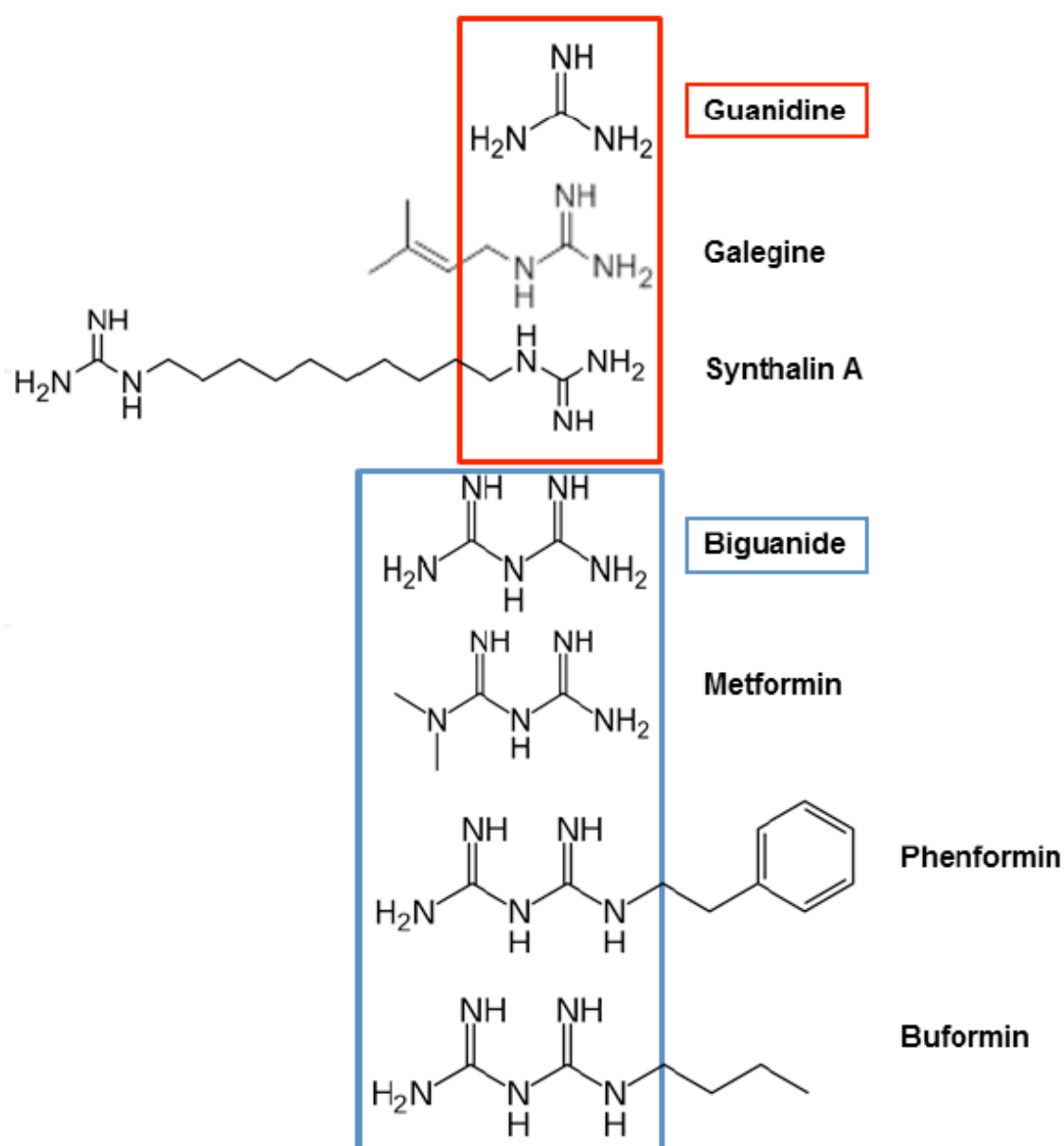


Figure 17. Structure of guanidine and biguanides (from (Cantoria et al., 2014)).

It is now believed that the anti-tumour action is mainly caused by inhibition of mitochondrial OXPHOS (Bridges et al., 2014; Dykens et al., 2008; El-Mir et al., 2000; Owen et al., 2000). Indeed, early in the 2000s, first Leverve's group (El-Mir et al., 2000) followed by Halestrap's group (Owen et al., 2000) reported that metformin reversibly inhibited the respiratory chain complex I (NADH: ubiquinone oxidoreductase) in mitochondria and sub-mitochondrial particles. Recent study has shown that metformin interacts with two distinct sites on complex I; one site perturbs the reactivity of the flavin, the other inhibits catalysis (Bridges et al., 2014). Metformin was also described to inhibit ATP hydrolysis by ATP synthase, which reduces the mitochondrial pool of ATP and subsequently increases the AMP/ATP ratio that triggers activation of the cellular energy sensor and regulator AMP-activated kinase (AMPK) by the serine/threonine kinase LKB1 (also called STK11) (Ben Sahra et al., 2010b; Hawley et al., 2010; Pollak, 2013). To restore ATP levels, AMPK induces metabolic reprogramming, mainly represented by increased glycolysis through activation of phosphofructo-2-kinase (PFK2) and down-regulation of energy consuming processes. Although this control of glycolysis by AMPK is well documented, we show recently in our group that it can even operate in AMPK-knockout cells (Pelletier et al 2015). Consequently, metformin treatment was reported to exert a noticeable anti-proliferative effect on several cancer cell lines *in vitro* and to result in tumour growth arrest in mouse models *in vivo* (Pollak, 2013; Rena et al., 2013) (Fig. 18).

Phenformin has also been shown to inhibit 50-fold more efficiently mitochondrial complex I than metformin (Dykens et al., 2008; Owen et al., 2000). Moreover, unlike metformin, the uptake of which requires a tissue-restricted cell surface organic cation transporter 1 (OCT1) and mitochondrial membrane potential, phenformin is a highly lipophilic molecule, which can more easily cross mitochondrial membranes (Owen et al., 2000; Pernicova and Korbonits, 2014; Wang et al., 2002). Consequently, phenformin exhibits broad tissue availability and was found to be more potent than metformin in reducing tumour growth (Huang et al., 2008; Owen et al., 2000). This potency in inhibiting complex I resulted in increased lactic acidosis, which limits its use in pre-clinical studies. However, it is worth noting that the treatment dose and duration in cancer therapy would be totally different from that used in diabetes. Moreover, the extensive retrospective knowledge of the side effects of phenformin, and the category of risky patients should allow establishment of safe protocols for clinical studies that take advantage of its anti-cancer potential.

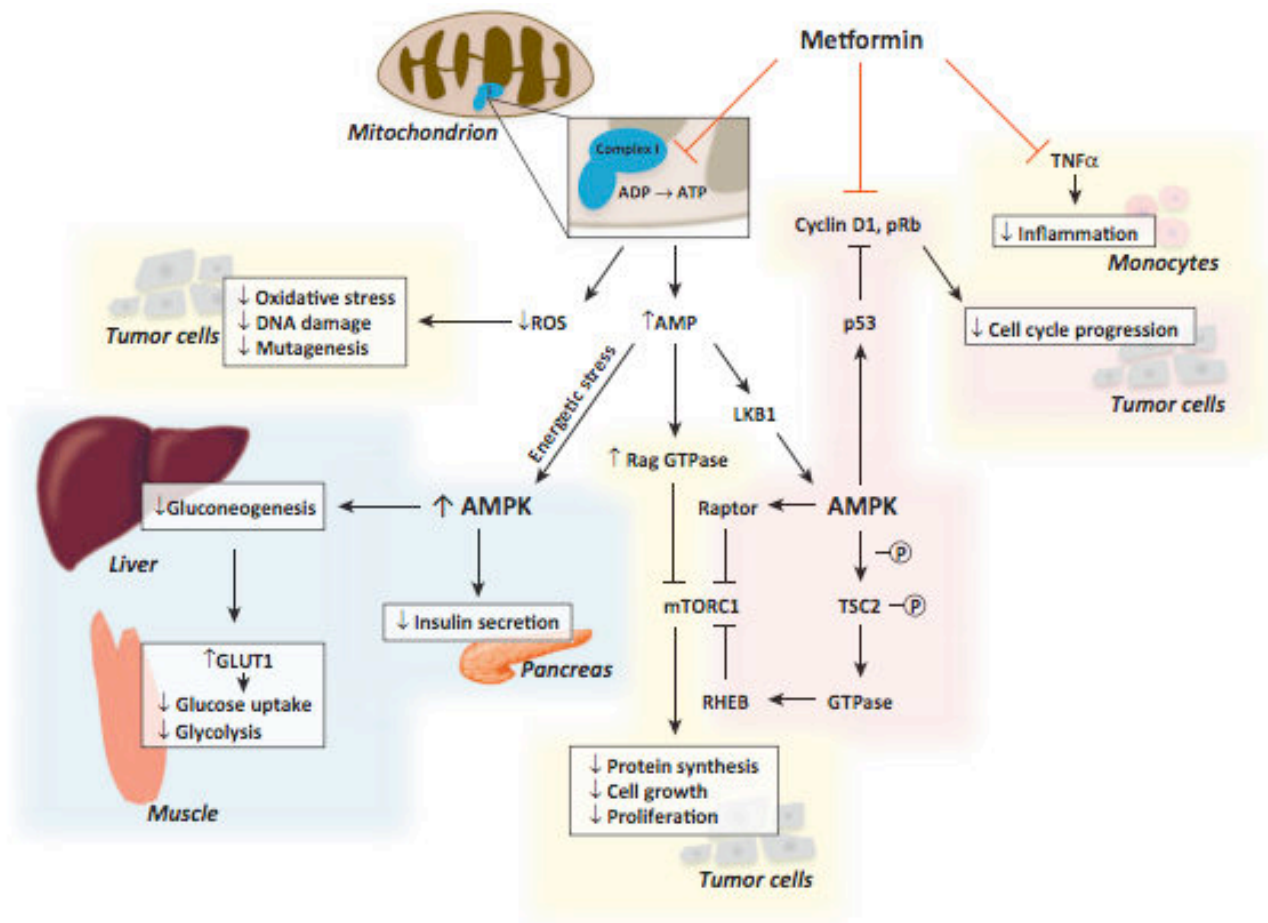


Figure 18. Schematic diagram of selected proposed mechanisms of action of metformin.

The background colour of each pathway depicts the specific mechanism that may be involved. The blue background depicts an insulin-dependent (direct) mechanism, the red background depicts an AMP-activated protein kinase (AMPK)-dependent (indirect) mechanism, and the yellow background depicts an AMPK-independent pathway. Abbreviations: pRb, retinoblastoma protein; TSC2, tuberous sclerosis protein 2; RHEB, Ras homolog enriched in brain (GTP-binding protein); GLUT1, glucose transporter protein 1 (from (Emami Riedmaier et al., 2013)).

CONTEXT AND OBJECTIVES OF THE THESIS

Context and objectives of the thesis

In response to the hypoxic tumour microenvironment, tumour cells shift their metabolism from OXPHOS towards glycolysis to satisfy their energy demands. As we have previously described, this shift leads to production of a high amount of lactic acid, which is efficiently exported by the MCTs in order to maintain a continuous glycolytic flux, ATP production and alkaline intracellular pH. The ubiquitously expressed MCT1 and the hypoxia-induced MCT4 are H⁺/lactate symporters that require an interaction with an ancillary protein, CD147/Basigin, for proper folding in the endoplasmic reticulum, for trafficking and insertion into the plasma membrane as functional transporters. BSG is a conserved transmembrane glycoprotein that is strongly expressed in several tumour types. Considering the multiplicity of its functions and interactions (MMPs, integrins, MCTs), its role in promotion of tumour growth has remained poorly defined.

Thus we aimed, during the last four years, to dissect the lactic acid transport complexes in order to better understand the role of each component (MCT1, MCT4 and BSG) in tumour development. Moreover, considering the crucial role of lactic acid in cancer, we hypothesized that blocking its extrusion will represent a promising approach to reduce tumour development, not only by inducing intracellular acidosis and ATP depletion, but also by disrupting the cell-cell interaction, as lactate is now a well-established signalling molecule.

Therefore, we addressed several questions during my PhD:

- What is the contribution of each MCT (MCT1 vs MCT4) to transport of lactate, pH_i regulation, ATP production and tumour development?
- Is HIF-1 induced MCT4 a key player in tumour cell proliferation and survival during hypoxia?
- What is the impact of deletion of the *BSG* gene on MCT1 and MCT4 expression and function?
- If residual expression and function are maintained, what is the mechanism responsible of proper folding and trafficking of these MCTs to the plasma membrane in the absence of BSG?
- Does depletion of BSG induce the expression of other MCT isoforms to maintain continuous efflux of lactate?

- Does targeting BSG have similar effects on tumour cell metabolism (pHi, glycolysis, OXPHOS), proliferation and tumour growth as concomitant inhibition of MCT1 and MCT4?
- Does the ablation of *BSG* gene reduce MMPs production by tumour cells and stroma?
- Is the pro-tumour action of BSG mainly linked to its metabolic function *via* MCT1/MCT4 activity and/or to its role in cell migration and invasion *via* enhanced production of MMPs?

Since previous studies targeting BSG/MCT complexes only used non-specific pharmacological inhibitors of MCT1 (such as CHC), or shRNA techniques, we decided to gain insight into these questions by designing experiments using specific inhibitors of MCT1 developed by Astra-Zeneca (AR-C155858, AZD3965) and ZFN-mediated *BASIGIN* and *MCT4* gene knockouts in colon adenocarcinoma, lung carcinoma and glioblastoma human cell lines.

RESULTS

Results

This section presents four publications:

A. Validation of the potential of targeting the MCT/BSG complexes for cancer therapy

PUBLICATION 1- CD147 subunit of lactate/H⁺ symporters MCT1 and hypoxia-inducible MCT4 is critical for energetics and growth of glycolytic tumors.

B. Genetic disruption of the MCT/BSG complexes causes a metabolic shift from glycolysis towards OXPHOS

PUBLICATION 2- Genetic disruption of lactate/H⁺ symporters (MCTs) and their subunit CD147/BASIGIN sensitizes glycolytic tumour cells to phenformin.

PUBLICATION 3- Disruption of BASIGIN decreases lactic acid export and sensitizes non-small cell lung cancer to biguanides independently of the LKB1 status.

C. Is BSG, also called EMMPRIN, really an inducer of MMPs?

PUBLICATION 4- Knock out of the BASIGIN/CD147 chaperone of Lactate/H⁺ symporters disproves its pro-tumour action *via* Extracellular Matrix Metalloproteases (MMPs) induction.

A. Validation of the potential of targeting the MCT/BSG complexes in cancer therapy

PUBLICATION 1- CD147 subunit of lactate/H⁺ symporters MCT1 and hypoxia-inducible MCT4 is critical for energetics and growth of glycolytic tumors.

Renaud Le Floch*, Johanna Chiche*, **Ibtissam Marchiq**, Tanesha Naïken, Karine Ilc, Clare M. Murray, Susan E. Critchlow, Danièle Roux, Marie-Pierre Simon, and Jacques Pouyssegur

(Accepted in Proceedings of the National Academy of Sciences in September 2011)

* Co-first authors

As mentioned above, hypoxia and oncogenes stimulate “fermentative glycolysis” in rapidly growing cancer cells, which generates a large amount of lactic acid (pKa = 3.86) that dissociates at physiological pH into lactate anions and protons. Consequently, lactic acid must be rapidly and efficiently extruded to maintain a permissive pH_i, which is required for several enzymatic reactions implicated in bioenergetics, metabolism, cell growth and survival. Our research group is one of the pioneering laboratories studying H⁺ dynamics and pH-regulating systems, through different studies showing the importance of NHE-1 or CAs (CAIX, CAXII) in tumour acidification and tumour development. In this publication, we aimed to explore the possibility of blocking tumour growth by inducing intracellular acidification, through Knock down/Knock out of lactate/H⁺ symporters-BSG complexes.

Before I joined Pouyssegur’s group, J Chiche and R Le Floch have established the key role of both MCT1 and MCT4 proteins in tumourigenicity. Thus by using the hamster fibroblast cell line CCL39 transformed by the oncogene Ras (CCL39-ev), and two derived cell lines defective either for glycolysis (CCL39 gly-) or for respiration (CCL39 res-), which express **only MCT1**, they showed that:

- Pharmacological inhibition of MCT1/2, with a specific inhibitor developed by AstraZeneca (AR-C155858), induced an increase in the intracellular lactate pool, reduced glycolysis and cell growth in hypoxia (1%O₂), and restricted growth of xenograft tumours *in vivo*.

- Ectopic expression of MCT4 in Ras-transformed fibroblasts (CCL39-ev) induced a total insensitivity to MCT1/2 inhibitor by restoring lactic acid extrusion, alkaline pH_i and ATP production, which resulted in increased cell growth *in vitro* and restoration of full tumourigenicity *in vivo*. The same results were obtained for the highly glycolytic cell line (CCL39 res-), which highlighted the role of MCTs in tumour growth, and pointed out the predominant role of MCT4 in highly glycolytic tumour cells.

As most human tumours were reported to over-express MCT1 and MCT4, the MCT blockade approach was extended to the human colon adenocarcinoma cell line LS174T. Thus, using specific Tet (or DOX) inducible shRNA targeting MCT1 and a constitutive shRNA targeting MCT4, it was reported that:

- Single silencing of MCT1 or MCT4 had no effect on the rate of glycolysis or on tumour growth of LS174T cells, and that combined inhibition of both transporters was required to achieve a significant reduction in glycolysis, the proliferation rate *in vitro*, and the tumour growth *in vivo*.

In the absence of a specific pharmacological inhibitor of MCT4 and considering the results showing that specific knock down of both MCT1 and MCT4 led to a strong reduction in the expression of BSG, we decided to target the chaperone with the hope to mimic the results obtained with the double silencing of MCT1/MCT4. Thus we have shown that:

- Knock down of BSG decreased expression of MCT1/MCT4, increased intracellular pools of lactate and reduced the glycolytic capacity, which resulted in a striking reduction of proliferation *in vitro* and xenograft tumour growth *in vivo*.

However, before being identified as a chaperone for MCTs, BSG was described in the literature as a pro-tumour protein, which acted on tumour cell invasion through induction of MMPs, and was subsequently named EMMPRIN for Extracellular Matrix Metalloproteinase INducer. Therefore, when I started the project, the question arises to know how BSG acted on

tumour growth: does it influence the metabolism through functional stabilization of MCT1/MCT4 at the membrane, or does it induce metalloproteases that regulate invasion? Or even a combination of these two factors?

To answer these questions, we decided to uncouple MCTs from BSG expression. Using the ZFN approach, we established *MCT4* and *BSG* “Knock out” cells in the human colon adenocarcinoma cell line (LS174T). The results showed that:

- Inhibition of MCT1 in *MCT4*-null, *BSG*^{high} cells suppressed tumour growth. In contrast, in *BSG*-null cells, in which the activity of MCTs has been maintained by EMBIGIN, a BSG paralog, no reduction of the tumoural phenotype was observed. Taken together, these findings suggested to us that the major protumoural role of BSG was the maintenance of MCT1/MCT4 activity to sustain high glycolysis.

Thus, we have shown for the first time that blocking lactic acid export by targeting BSG could be an efficient anti-cancer therapy. However, at this level, several fundamental questions remain unsolved such as, the impact of a total knock out of the *BSG* gene on MCT1/MCT4 expression and function? Is such an approach feasible, as knock down of BSG severely restricted tumour cell growth? What will be the impact of *BSG* deletion on the metabolic status of cancer cells and on the expression of MMPs by cancer cells and normal fibroblasts?

The following ‘result sections’ of my manuscript have addressed these important questions.

CD147 subunit of lactate/H⁺ symporters MCT1 and hypoxia-inducible MCT4 is critical for energetics and growth of glycolytic tumors

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Malignant tumors exhibit increased dependence on glycolysis, resulting in abundant export of lactic acid, a hypothesized key step in tumorigenesis. Lactic acid is mainly transported by two H⁺/lactate symporters, MCT1/MCT4, that require the ancillary protein CD147/Basigin for their functionality. First, we showed that blocking MCT1/2 in Ras-transformed fibroblasts with AR-C155858 suppressed lactate export, glycolysis, and tumor growth, whereas ectopic expression of MCT4 in these cells conferred resistance to MCT1/2 inhibition and reestablished tumorigenicity. A mutant-derivative, deficient in respiration (res⁻) and exclusively relying on glycolysis for energy, displayed low tumorigenicity. These res⁻ cells could develop resistance to MCT1/2 inhibition and became highly tumorigenic by reactivating their endogenous *mct4* gene, highlighting that MCT4, the hypoxia-inducible and tumor-associated lactate/H⁺ symporter, drives tumorigenicity. Second, in the human colon adenocarcinoma cell line (LS174T), we showed that combined silencing of MCT1/MCT4 via inducible shRNA, or silencing of CD147/Basigin alone, significantly reduced glycolytic flux and tumor growth. However, both silencing approaches, which reduced tumor growth, displayed a low level of CD147/Basigin, a multifunctional protumoral protein. To gain insight into CD147/Basigin function, we designed experiments, via zinc finger nuclease-mediated *mct4* and *basigin* knockouts, to uncouple MCTs from Basigin expression. Inhibition of MCT1 in MCT4-null, Basigin^{high} cells suppressed tumor growth. Conversely, in Basigin-null cells, in which MCT activity had been maintained, tumorigenicity was not affected. Collectively, these findings highlight that the major protumoral action of CD147/Basigin is to control the energetics of glycolytic tumors via MCT1/MCT4 activity and that blocking lactic acid export provides an efficient anticancer strategy.

Warburg effect | lactate transport | EMMPRIN | Embigin | cancer metabolism

Interest in the “Warburg effect” has been intensely revived in recent years (1–6). The fact that rapidly proliferating cancer cells have adopted fermentative glycolysis rather than oxidative phosphorylation (OXPHOS) to supply energy is not unique to cancer but is shared by highly proliferating normal cells. Recent work has highlighted an intimate coupling of PFKFB3, a key regulator of glycolysis, with the G₁-phase cell cycle machinery (7). It is now realized that fermentative glycolysis, although generating a low yield of ATP per glucose molecule consumed, is the pathway of choice for cell division because it generates carbon source intermediates for anabolic reactions (3). ¹⁸FDG-PET scan imaging reveals the simplest cancer signature of intense proliferation, reflecting a sort of “glucose addiction” for cell division. In consequence of rapid growth, these tumors have to face a hypoxic and acidic microenvironment linked to a restricted and often chaotic vascularization (8). Through HIF-1 activation, hypoxic tumor cells have evolved adaptive mechanisms to survive nutrient-deprived conditions such as induction of autophagy via BNIPs, induction

and mobilization of glycogen stores (9) and increased migration via EMT induction (10). Acidic burden is the price paid by intense metabolism, primarily lactic acid, as the obligatory byproduct of fermentative glycolysis. Although normal proliferating cells use glycolysis, some human tumor cells are so “addicted” to this metabolic pathway that targeting glycolysis directly has become a promising therapeutic strategy in oncology (11–15).

In our studies we have focused on exploring the role of intracellular pH (pHi) as a therapeutic strategy to target glycolytic tumors (8, 16, 17). Within an acidic microenvironment, “successful” tumors cells are those capable of actively extruding protons. Cancer cells have activated several solutions like activation of a robust Na^+/H^+ exchanger (18), the HIF-1 induction of membrane-bound carbonic anhydrases CAIX and XII (19), and the efficient export of lactic acid through the HIF-1 induction of monocarboxylate transporter 4 (MCT4) (20). Here, we have further explored a pHi-anticancer strategy by analyzing blockade of lactic acid export (21). Four MCT family members (MCT1–MCT4) have been functionally characterized as $\text{H}^+/\text{lactate}$ symporters capable of mediating bidirectional transport of lactic acid across the plasma membrane (22). MCT1 is widely expressed, whereas the expression of the hypoxia-inducible MCT4 lactate transporter tends to be restricted to “glycolytic” tissues such as muscle and increased expression has been reported in several malignancies (20). A key glycoprotein, CD147/Basigin/EMMPRIN assists MCT1 and MCT4 in folding, stability, membrane expression, and functionality (23). CD147/Basigin/EMMPRIN is an evolutionary conserved member of the Ig superfamily that has gained considerable attention before being identified as an MCT subunit (24, 25). It was first described as a potent protumoral factor capable of promoting tumor invasion (26) via the induction of matrix metalloproteinases (MMPs) and has been reported to be involved in multiple physiological functions including embryo implantation, spermatogenesis, and vision (27). Here, we addressed the following questions: Is CD147/Basigin’s protumoral action linked or independent of its role in lactic acid export? Can these two actions be uncoupled? Can we suppress tumor growth by simply inhibiting MCTs? With the use of a specific MCT inhibitor (28) and inducible MCT1/4 shRNAs, we established that growth of glycolytic tumors can be arrested through inhibition of lactate

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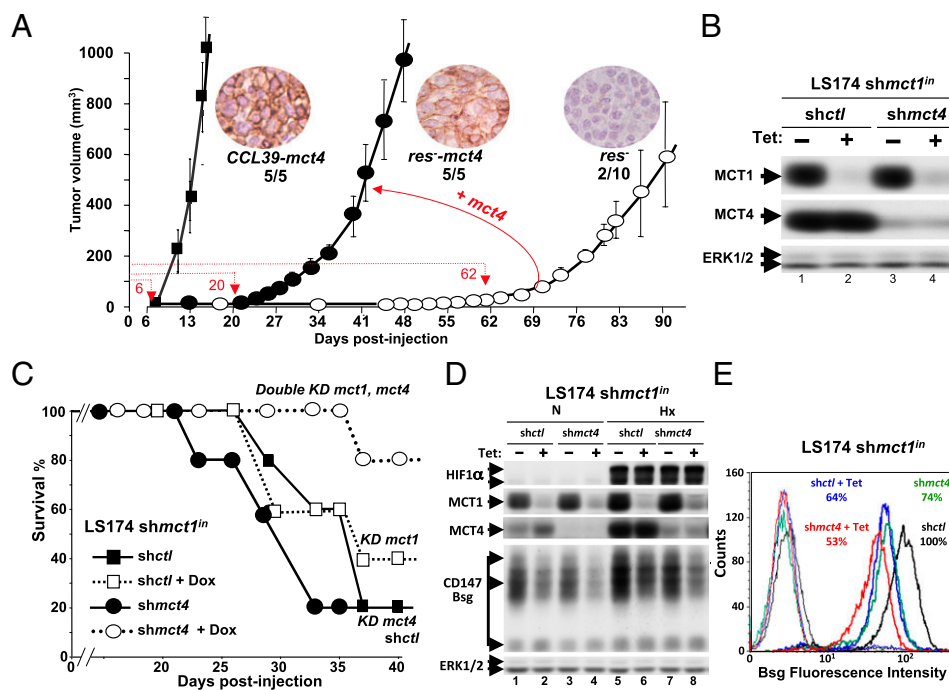


Fig. 2. Key role of hypoxia-induced MCT4 in glycolysis and tumor growth. (A) Tumorigenicity in nude mice of Ras-transformed fibroblasts CCL39-*mct4* and mutant impaired in respiration and expressing with (*res⁻-mct4*) or without (*res⁻*) MCT4. Cells (10^6) were s.c. injected into the back of nude mice. Expression of MCT4 is shown by circle insets: Immunohistological confirmation. (Magnification: 20 \times). (B) Single and double knockdown of MCT1 and MCT4 in colon carcinoma cell line LS174T. The LS174T cell line that expresses the tetracycline repressor (LS174T cell line) were stably transfected with the pTER-*mct1* plasmid that allows expression of shRNA targeting MCT1 under tetracycline treatment (LS174T *shmct1ⁱⁿ*). Second, the LS174T *shmct1ⁱⁿ* cells were infected by a lentivirus expressing a shRNA nontargeting (*shmct1*) or a shRNA targeting MCT4 (*shmct4*). The cells were cultivated in the absence (-Tet) or presence (+Tet) tetracycline for 5 d and maintained in hypoxia 1% O_2 for the last 48 h before lysis. The whole cell expression of MCT1 and MCT4 was analyzed by immunoblotting. ERK1/2 level was displayed for loading normalization. (C) Tumorigenicity in nude mice. LS174T *shmct1ⁱⁿ/shmct1* cells (1×10^6) or LS174T *shmct1ⁱⁿ/shmct4* cells where s.c. injected in nude mice drinking (+Dox) or not drinking (-Dox) doxycycline in water 4 d before cell injection and during the tumorigenicity assay. Five mice were used per condition. Mice were killed when tumor size reached the mean value of 600 mm 3 (% animal survival). This experiment was reproduced twice. (D) Cells were cultivated in the absence (-) or presence (+) tetracycline for 5 d and maintained either in normoxia or in hypoxia 1% O_2 during the last 48 h before lysis. The whole cell expression of HIF1 α , MCT1, MCT4, and CD147/Bsg was analyzed by immunoblotting. ERK1/2 was used for loading normalization. (E) The cell surface expression of CD147/Bsg was analyzed by flow cytometry on LS174T *shmct1ⁱⁿ/shmct1* cells and LS174T *shmct1ⁱⁿ/shmct4* cells cultivated in normoxia in the presence (+Tet) or absence of tetracycline (-Tet). For each condition, the CD147/Bsg fluorescence intensity was compared with the one obtained with the LS174T *shmct1ⁱⁿ/shmct1* cultivated in the absence of tetracycline (*shmct1* -Tet, dark curve, 100%); *shmct1* + Tet (blue curve, 64%); *shmct4* - Tet (green curve, 74%); and *shmct4* + Tet (red curve, 53%). Peaks on the left correspond to control incubation with the anti-mouse antibody alone.

expected from the lack of respiration in these cells. The very rare resistant clones (frequency $<10^{-3}$) that survived were analyzed for possible gain of function by point mutation or amplification of the *mct1* gene (Fig. S3). Two resistant clones (A and C) were isolated after incubation in serial increases (10–1,000-fold) in MCT1/2 inhibitor (Fig. S3A). These resistant clones did not show any mutation or amplification of the *mct1* gene (SLC16A1). In contrast, these two resistant clones demonstrated de novo expression of the hamster *mct4* gene (Fig. S3B and C). When analyzed for tumor incidence, these highly glycolytic MCT1/2 inhibitor-resistant cells (*res⁻-iMCT1/2R*) became highly tumorigenic (Fig. S3D), a result reflecting the reactivation of endogenous *mct4* gene.

These two independent findings indicate that lactate export via MCT1/2 is limiting for growth of highly glycolytic cells and points to the protumoral advantage conferred by the expression of hypoxia-inducible MCT4, an isoform highly expressed in rapidly growing human tumors.

Next, we analyzed the human colon adenocarcinoma cell line LS174T that expresses both MCT1 and MCT4 in normoxia. This cell line exhibits a three- to fivefold increase in MCT4 mRNA and protein expression in hypoxia 1% O_2 (Fig. 2D, compare lanes 1 and 5). Introduction of a tetracycline (Tet)-inducible shRNA targeting MCT1 (LS174T *shmct1ⁱⁿ*) reduced MCT1 expression by 90% in the presence of Tet (compare Fig. 2B,

lanes 1 and 2). This single MCT1 knockdown did not affect the rate of glycolysis, nor the rate of tumor growth (Fig. 2C). The infection of these LS174T *shmct1ⁱⁿ* cells with a lentivirus shRNA targeting MCT4 (*shmct4*) strongly reduced expression of MCT4 (compare *shmct1* with *shmct4* in Fig. 2B). It is only by achieving a double MCT1/4 knockdown (*shmct1ⁱⁿ/shmct4*; Fig. 2B, lane 4) that we could reduce the rate of xenograft tumor growth (double KD *mct1, mct4*; Fig. 2C). Collectively these results highlight the key role played by the two H^+ /lactate symporters and particularly the hypoxia-inducible MCT4 in optimizing the rate of glycolysis associated with rapid tumor growth.

Another interesting feature of this double MCT1/4 knockdown is the concomitant reduction in the accessory protein CD147/Basigin (Fig. 2D, compare lanes 1 and 4 in normoxia and lanes 5 and 8 in hypoxia for total expression of Basigin). Cell surface expression of Basigin was reduced by 26% (MCT4 knockdown) to 47% (MCT1/4 double knockdown; Fig. 2E, *shmct4* + Tet). These findings prompted us to evaluate whether direct targeting of CD147/basigin could act as a simple strategy to reduce expression of both MCT1 and MCT4.

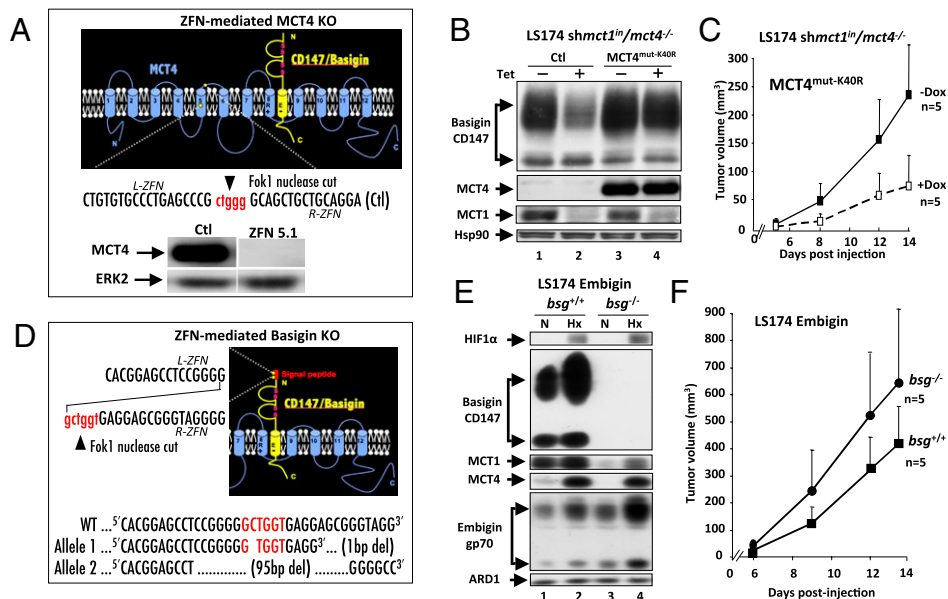
Knockdown of CD147/Basigin Reduces MCT1 and MCT4 Expression, Glycolysis, and Tumor Growth. LS174T cells were stably transfected with a Tet-inducible shRNA targeting Basigin (LS174T *shbgsⁱⁿ*)

and the total expression of Basigin, MCT1, and MCT4 in response to tetracycline (+Tet and -Tet) in either normoxic or hypoxic conditions was analyzed by immunoblotting (Fig. S44). The expression level of the upper glycosylated form of Basigin reproducibly increases in hypoxia (compare Fig. 2D, lanes 1 and 5 and compare Fig. S44, lanes 1 and 3). This hypoxia-induced Basigin is not associated with an increase in mRNA, and we propose that this Basigin increase is an indirect effect of MCT4 up-regulation. In support of this conclusion, MCT4 is induced by hypoxia, whereas MCT1 remains unchanged (Fig. 2D and Fig. S44). Knockdown of Basigin (+Tet; Fig. S44), reduced the cell surface expression of Basigin by 64% (Fig. S4B). Furthermore Basigin knockdown resulted in a pronounced reduction in MCT1 and MCT4 expression in normoxia and hypoxia (compare Fig. S44, lanes 1 and 2 or lanes 3 and 4). This observation was reproduced with three independent shRNA sequences against CD147/Basigin with a strong correlation between the level of Basigin ablation and concomitant reduction in MCT1 and MCT4 expression (Fig. S54). Knockdown of Basigin reduced the glycolytic rate by 50% (Fig. S4C, compare -Tet and +Tet); this effect was further enhanced in the presence of the MCT1/2 inhibitor (to $\approx 75\%$ control levels; Fig. S4C). Furthermore, knockdown of Basigin results in an increase in intracellular lactate (Fig. S4F), indicating that the reduced glycolytic capacity is a direct effect of loss of lactate transport. This reduced glycolytic capacity results in a significant antiproliferative effect, following Basigin knockdown (+Tet) (Fig. S4D, Fig. S4D Inset, and Fig. S5B) and a striking reduction in xenograft tumor growth in vivo as seen in Fig. S4E and Fig. S5C (compare -Dox and +Dox). Taken together, these findings reinforce the notion that blunting export of lactic acid has a profound anticancer action seen here in colon adenocarcinoma and reported in pancreatic cancer (32).

Major Protumoral Action of CD147/Basigin Is Mediated Through the Control of Lactic Acid Export. The antitumor efficacy demonstrated in Fig. S4E and Fig. S5C are mediated by direct silencing of CD147/Basigin. Is the protumoral activity of CD147/Basigin in-

timately linked to its lactic acid export function and, therefore, associated to metabolic energy? Can we uncouple the metabolic function of CD147/Basigin from its widely recognized protumoral and invasive function? To answer these questions, we designed two experiments by using the zinc finger nuclease (ZFN) targeting approach (33) (Fig. 3A and D). First, we disrupted the *mct4* gene in the LS174T *shmct1ⁱⁿ* cell line (Fig. 3A). In this cell line, referred to as *mct4^{-/-}*, the ablation of MCT4 did not modify the total expression levels of either MCT1 or CD147/Basigin in normoxia (Fig. S64), whereas in hypoxia, CD147/Basigin increased ≈ 30 –40% to accommodate the HIF1-induced expression of MCT4 in control cells only (Fig. S64, Hx). In vitro growth rates of control and *mct4^{-/-}* cells in normoxia (Fig. S6C) or in hypoxia (Fig. S6D) were identical. However, pharmacological inhibition of MCT1/2, which did not impact on cell surface expressed Basigin (71%, Fig. S6B), did not affect the in vitro growth of control cells in hypoxic conditions but dramatically reduced the growth, the rate of glycolysis, and the tumorigenicity of *mct4^{-/-}* cells (Fig. S6D–F). To further increase cell surface Basigin, we expressed in *mct4^{-/-}* cells, MCT4^{mut-L40R}, a catalytically inactive MCT4 form based on MCT1 conservation of Lysine38 (Fig. S7 and Fig. 3B), a key residue for lactate transport (34). The lactate transport experiment demonstrated that MCT4^{mut-L40R} expressed on the plasma membrane, as judged by the induced expression of Basigin, is catalytically dead (compare Fig. S7, a and c). Silencing MCT1 reduces Basigin expression in control cells (Fig. 3B, lane 2), whereas Basigin was maintained high in MCT4^{mut-L40R} cells (compare Fig. 3B, lanes 2 and 4). Interestingly the decreased tumorigenicity correlated with low MCT activity (+Dox; Fig. 3C) but remained independent of the Basigin expression level (Fig. 3B and C). In the second experiment, we knockout *basigin* in a LS174T-derivative clone expressing rat Embigin (Fig. 3D). Embigin is a paralog of Basigin that, with the exception of the conserved transmembrane segment, shares <30% homology. Furthermore, unlike Basigin, Embigin has not been reported as a protumoral protein and does not mediate cell aggregation by homophilic interactions, suggesting different modes of function for the two (35). Interestingly,

Fig. 3. ZFN-mediated *mct4* and *basigin* gene knockouts and uncoupling MCT1 and MCT4 activity from Basigin expression. (A) Knockout of *mct4* in LS174T cell line via ZFN gene targeting. Schematic representation of the membrane organization of the MCT4 lactic acid transporter with its putative association with the ancillary glycoprotein CD147/Basigin adapted from ref. 22. The fifth transmembrane targeting domain is shown with the 5'-3' coding sequence recognized by the two R-ZFN and L-ZFN plasmids. In the middle in red, the sequence where the Fok1 nuclease could cut the double DNA strands. Below is represented the immunoblot analysis of total cell extract of parental cells (Ctl) and of the corresponding ZFN5.1 clone *mct4^{-/-}* (one G deletion found) with ERK2 as loading control. (B) Immunoblot of LS174T *shmct1ⁱⁿ/mct4^{-/-}* and LS174T *shmct1ⁱⁿ/mct4^{-/-}*-MCT4^{mut-K40R} total cell extracts. The two cell lines were cultivated in normoxia for 5 d with or without tetracycline (Tet), and the expression of Basigin, MCT4, MCT1, and Hsp90 was revealed. Note that the expression of MCT4^{mut-K40R} prevents the reduction of Basigin when MCT1 is down-regulated (+Tet; compare lanes 2 and 4). (C) Tumorigenicity of the cell line LS174T *shmct1ⁱⁿ/mct4^{-/-}*-MCT4^{mut-K40R}. The cells were s.c. injected (5×10^6) in nude mice treated (+Dox) or not treated (-Dox) with doxycycline. (D) *Basigin* knockout in LS174T-derivative clone stably expressing rat Embigin. Representation of the ZFN sequences targeted *basigin* exon 2 containing the signal peptide of basigin 2 splice variant. Below are represented the sequences of the mutated alleles of one of the disrupted *basigin* mutant analyzed in the next images. (E) The expression of HIF1 α , Bsg/CD147, MCT1, MCT4, and Embigin were analyzed by immunoblot after 48 h of culture in normoxia (N) or hypoxia 1% O₂ (Hx). (F) LS174T Embigin *bsg^{+/+}* and LS174T Embigin *bsg^{-/-}* were submitted to a tumorigenicity test by injecting (3×10^6) cells s.c. in nude mice.



controlled by pH. The key role of the H⁺/Lactate symporter MCT4 in promoting pHi regulation (40) and glycolysis would suggest a strong functional link between glycolysis and migration via the Basigin/MCT/integrin complex. On the same line, we would like to propose that many of the multiples functions previously attributed to Basigin/EMMPRIN and emphasized with *bsg*^{-/-} mice (27) are tightly linked to its major role in metabolic energy.

In conclusion, CD147/Basigin, by controlling the stability and functional plasma membrane insertion of the H⁺/lactate symporters MCTs in many tissues, plays a determinant role in metabolic energy. Pharmacological agents able to disrupt these complexes Basigin/MCTs or to inhibit MCT1 and/or MCT4, which are prominently expressed in glycolytic, aggressive and rapidly proliferating malignancies, would provide a unique avenue for anticancer therapy.

Materials and Methods

Cell Culture and Hypoxic Exposure. Chinese hamster lung CCL39 fibroblasts (American Type Culture Collection) and the CCL39-derived mutants impaired

either in respiration (*res*⁻) or in glycolysis (*gly*⁻), obtained as described (29, 30), were maintained in DMEM (Sigma) supplemented with 7.5% FCS, penicillin (10 units/mL) and streptomycin (10 µg/mL) in a humidified atmosphere of 5% CO₂/95% air or 100% air at 37 °C. The colon carcinoma LS174T cells provided by Marc van de Wetering (Utrecht) expressing the tetracyclin (Tet) repressor were maintained in DMEM supplemented with 10% inactivated FCS and blasticidin (10 µg/mL; InvivoGen). Incubation in hypoxia at 1% O₂ (Hx) was carried out at 37 °C in 95% humidity and 5% CO₂/94% N₂ in a sealed anaerobic workstation (Ruskin).

See *SI Materials and Methods* for additional details.

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Supporting Information

Le Floch et al. 10.1073/pnas.1106123108

SI Materials and Methods

Plasmids, shRNA Constructs and Stable Transgenic Cells. Full-length human *mct4* cDNA in a pCI-neo vector (*mct4*) were kindly provided by A. Halestrap (1). Parental CCL39 and respiration-disrupted CCL39-*res*⁻ cells were transfected with *mct4* or control empty vector (*ev*) and selected for neomycin-resistant clones to select a stable total population of cells expressing MCT4. CCL39-*ev*, CCL39-*mct4*, *res*⁻, *res*⁻-*mct4*, and *gly*⁻ cells were then transfected with the oncogene H-*rasV12* (Addgene) for in vivo growth studies.

The LS174T cell line was stably transfected with the pTER-sh*mct1* plasmid that allows expression of shRNA targeting MCT1 upon tetracycline treatment (LS174T sh*mct1*ⁱⁿ). The targeted sequence in human *mct1* mRNA was as follows: 5'TCAT-ATGCTAGCACCTTTA3'. The transfected cells were selected with Zeocin (500 µg/mL; InvivoGen) and positive clones screened by anti-MCT1 immunoblotting. LS174TT sh*mct1*ⁱⁿ cells were infected by lentiviral particles purchased from Sigma Aldrich expressing in the pLKO.1-Puro plasmid a shRNA nontarget (Sigma Ref. SHC002V—LS174T sh*mct1*ⁱⁿ sh*ct1*) or a shRNA targeting MCT4 (Sigma Ref. TRCN0000038474—targeted sequence in human *mct4*; 5'CGTCTACATGTACGTGTTTCAT3'—LS174T sh*mct1*ⁱⁿ sh*mct4*). The LS174T sh*bsg*ⁱⁿ clones were obtained by stable transfection of pTER-*bsg* plasmid, selected with Zeocin (500 µg/mL; InvivoGen). Three independent targeted sequences in human *cd147/bsg* mRNA were used as follows:

- sequence 1 (5'GAAGTCGTCAGAACACATCAA3');
- sequence 2 (5'GCAGGTTCTTCGTGAGTTC3'); and
- sequence 3 (5'GTACAAGATCACTGACTCT3').

Immunocytochemistry. Cells at low density were lysed in SDS sample buffer. Proteins (40 µg) were separated on 8% SDS polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore). Membranes were blotted with antibodies to monoclonal antibody to Bsg/CD147 (R&D Systems; MAB972) and rabbit polyclonal antibodies to HIF-1α (2). For MCT1 and MCT4, we prepared rabbit polyclonal antibodies against the C-terminal last 15 residues for MCT1 and the last 18 residues for MCT4 with cystein coupling to KLH (NeoMPS). Specificity of antibodies was validated by an siRNA approach by immunoblotting and immunohistochemistry. Antibody to Hsp90 (Abcam) was used as a loading control. Total ERK used as a loading control was detected with mixes of rabbit serum E1B (1/3,000—epitope against the last 16 amino acids of Chinese hamster ERK1) plus rabbit anti-ERK1 from Zymed (61–7400). The rabbit polyclonal antibody to arrest-defective-1 protein (ARD1) (epitope corresponding to the last 20 amino acids of the human ARD1) also was used as loading control. Immunoreactive bands were detected with a horseradish peroxidase anti-mouse or anti-rabbit antibody (Promega) by ECL (Amersham Biosciences or Millipore).

Determination of the in Vitro Rate of Glycolysis. The lactate exported in the supernatant of cells incubated either in normoxia (N) or in hypoxia of 1% O₂ (Hx) for 1, 3, 6, 9, and 24 h in a pyruvate-free DMEM, in the presence of iMCT1/2 (100 nM) and/or oligomycin (1 µg/mL), was determined by an enzyme-based assay using 900 µM β-NAD (BioChemika), 175 µg/mL L-lactate dehydrogenase (BioChemika), and 100 µg/mL glutamate-pyruvate transaminase (Roche) diluted in a sodium carbonate (620 mM)-L-glutamate (79 mM) buffer adjusted to pH 10. Lithium lactate was used as a standard. Measurements were

done with a microplate reader after incubation for 30 min at 37 °C. The rate of glycolysis was expressed as mmole lactate/10⁶ cells per h. Determinations were done in quadruplicate, and the experiment was repeated at least three times.

Oxygen Consumption Assay. Cells (5 × 10⁶) were resuspended in 250 µL of regular media. Cellular respiration was determined by using a Clark type oxygen electrode over 10 min. Cells were incubated at room temperature with or without oligomycin (1 µg/mL) to measure the oxygen consumption that is sensitive to the inhibitor of ATP synthase and, thus, determines the respiration that is linked to ATP turnover. The rate of oxygen consumption was expressed as mmol oxygen/5 × 10⁶ cells per min.

Clonogenicity Growth Assay. Cells (2 × 10³) were seeded onto 60-mm dishes. Twenty-four hours later after cell attachment, medium was replaced with regular DMEM supplemented with 10% serum, for 10 d of growth in the presence or in the absence of iMCT1 (100 nM) or oligomycin (1 µg/mL) or both under normoxic or hypoxic (1% O₂) conditions. Dishes were then stained with Giemsa (Fluka). For LS174T stable cell lines, 900 cells were seeded onto 60-mm dishes after 5 d of culture in the presence of tetracycline (1 µg/mL). Tetracycline was maintained until the cells were fixed with Giemsa.

ZFN-Mediated Gene Knockout of *mct4* and *basigin* Genes. LS174T cells were transiently transfected twice in a 1-wk interval with the two plasmids expressing the zinc finger nuclease (ZFN) designed by Sigma-Aldrich (CompoZr Custom ZFN) and targeting the fifth transmembrane segment of MCT4 (see sequence in Fig. 3A). Transfected cells were sorted out by resistance to puromycin applied only for 30 h. Cells were then cloned by dilution in 96 wells, and only clones with double allele mutations were selected phenotypically, confirmed by DNA sequencing, and further analyzed. For *basigin* knockout, LS174T expressing rat Embigin were transiently transfected with the two plasmids expressing the ZFN designed by Sigma-Aldrich (CompoZr Custom ZFN) targeting *basigin* exon 2 encoding signal peptide of *basigin* splice variant 2 (Fig. 3D). Several clones disrupted for the two alleles were identified by DNA sequencing, and the one selected had deletions in both alleles (1 and 95 bp, respectively), inducing a frame-shift and lack of expression of the protein (Fig. 3E).

Proliferation Assay. When indicated, tetracycline (1 µg/mL) was added in the medium 4 d before the assay. Cells (6 × 10⁴) were seeded onto 6 dishes (35 mm) per condition. Twenty-four hours after seeding, cells were detached and counted with Coulter Z1 (Beckman) every 24 h during 6 d. Proliferation units were calculated by dividing the cell number obtained for each day by the one obtained 24 h after seeding.

Flow Cytometry. Cells were detached and washed once with PBS 1% serum. Cells were incubated in PBS 1% serum with the CD147/Basigin antibody (R & D Systems; MAB972) for 30 min in ice. After washing, the cells were incubated in PBS-1% serum with an R-Phycoerythrin-conjugated AffiniPure Goat Anti-Mouse IgG (Jackson ImmunoResearch; no. 115–115-164) for 30 min in ice and dark. After antibody removal, the CD147/Basigin fluorescence intensity was analyzed by FACSCalibur (Becton Dickinson). One control was made without any antibody and for each condition, one sample of cells was incubated with the anti-mouse antibody alone. All of the data were analyzed with the Cell Quest Software.

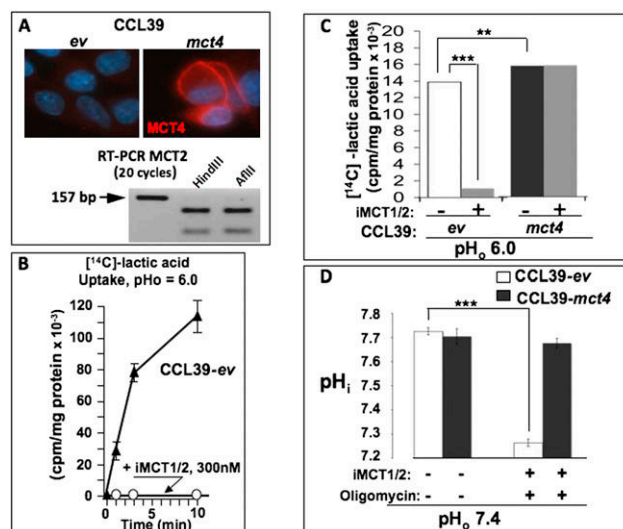


Fig. S1. Stable expression human MCT4, expression of MCT2, and sensitivity of [¹⁴C]-lactic acid uptake to iMCT1/2 in CCL39 cells. (A) Immunofluorescence to MCT4 in control empty vector plasmid transfected cells CCL39-*ev* and stable CCL39-*mct4* cells incubated in normoxia. RT-PCR of CCL39 mRNA (20 cycles) with two specific primers amplified a 157-bp fragment. The MCT2 sequence signature was validated by the size of the expected fragment (157 bp) and by restriction enzyme hydrolysis generating two fragments (105 bp and 52 bp) for HindIII and (109 bp and 48 bp) for AlfII, respectively. (B and C) [¹⁴C]-Lactic acid uptake in CCL39-*ev* and CCL39-*mct4* in the absence or presence of iMCT1/2. (B) Time course of [¹⁴C]-Lactic acid uptake in CCL39-*ev* with or without iMCT1/2. (C) Lactic acid uptake (1 min time point in triplicate) in CCL39-*ev* and CCL39-*mct4* and comparison of their sensitivity to iMCT1/2 (100 nM). (D) Resting intracellular pH (pH_i). MCT4 expression maintains the alkaline resting (pH_i) in highly glycolytic conditions (+ oligomycin) and in the absence of MCT1/2 activity (see *SI Materials and Methods* for details). Significant differences are based on the Student's *t* test (***P* > 0.01; ****P* > 0.001).

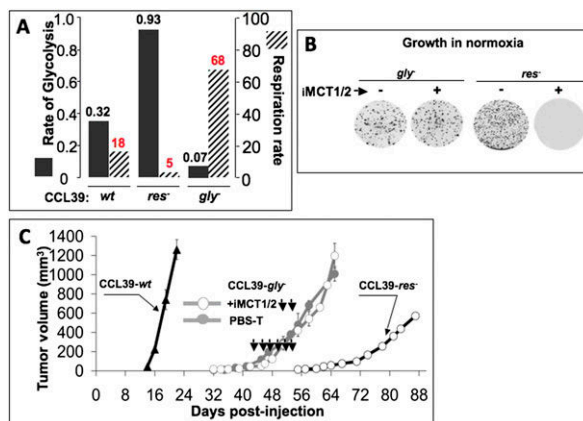


Fig. S2. Metabolic characterization of Ras-transformed CCL39 mutant fibroblasts defective in respiration (*res*⁻) or glycolysis (*gly*⁻). (A) Respective rates of respiration (hatched bars) and glycolysis (filled bars) of the three fibroblast cell lines (CCL39-wt, *res*⁻, *gly*⁻). (B) Clonogenic growth assay in response to iMCT1/2 (100 nM). Note the total insensitivity of the glycolytic-deficient cells with the respiratory-deficient cells. (C) Comparative tumorigenicity in *nude* mice of the three fibroblast cell lines (CCL39-wt, *res*⁻, *gly*⁻). Glycolytic-deficient *gly*⁻ mutant cells were treated (iMCT1/2) or not treated (PBS-T) as described in *Methods* and in Fig. 1 legend.

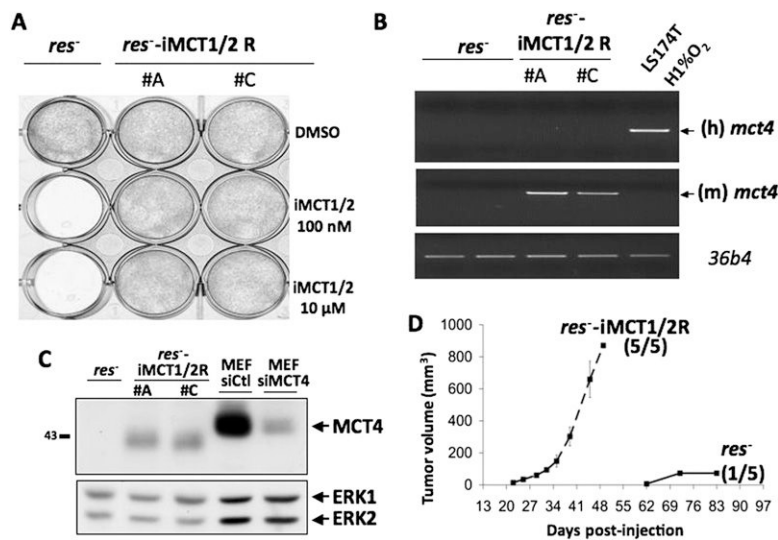


Fig. S3. (A) Viability assay on respiratory defective *res*⁻ cells and on two independent clones that were resistant to a progressive increment of iMCT1/2 concentration from 10 to 100 nM after 20 d-treatment with 100 nM iMCT1/2 (*res*⁻iMCT1/2R #A and #C). When exposed to either 100 nM or 10 μ M, a 1,000-fold higher concentration from the initial start of iMCT1/2, *res*⁻iMCT1/2R cells survive and proliferate compared with control *res*⁻ cells dying in 24 h. (B) PCR analyses of mouse (m) and human (h) *mct4* mRNA expression on *res*⁻ cells iMCT1/2 resistant cells. The two independent clones #A and #C expressed de novo the endogenous hamster MCT4 mRNA revealed by the homologous mouse (m) probe, absent in the sensitive *res*⁻ cells and distinct from the human (h) PCR product. Note that the human LS174T cells were incubated 48 h in hypoxia, and 36b4 was used as a loading control. (C) Immunoblot analysis of endogenous MCT4 in *res*⁻iMCT1/2R cells compared with *res*⁻ cells. The specificity of the MCT4 antibody was tested with Mouse Embryonic Fibroblasts (MEF) +siRNA Ctl or siRNA MCT4. Note that the hamster cells express a shorter form than the mouse MCT4. ERK1/2 was used as loading control. (D) *res*⁻ and *res*⁻iMCT1/2R cells were s.c. injected in *nude* mice. *res*⁻iMCT1/2R cells gave 100% tumors (5/5), whereas only 20% (1/5) of animals injected with *res*⁻ cells develop tumors. As observed with the *res*⁻*mct4*, *res*⁻iMCT1R tumors appeared earlier and develop faster than the *res*⁻ tumors.

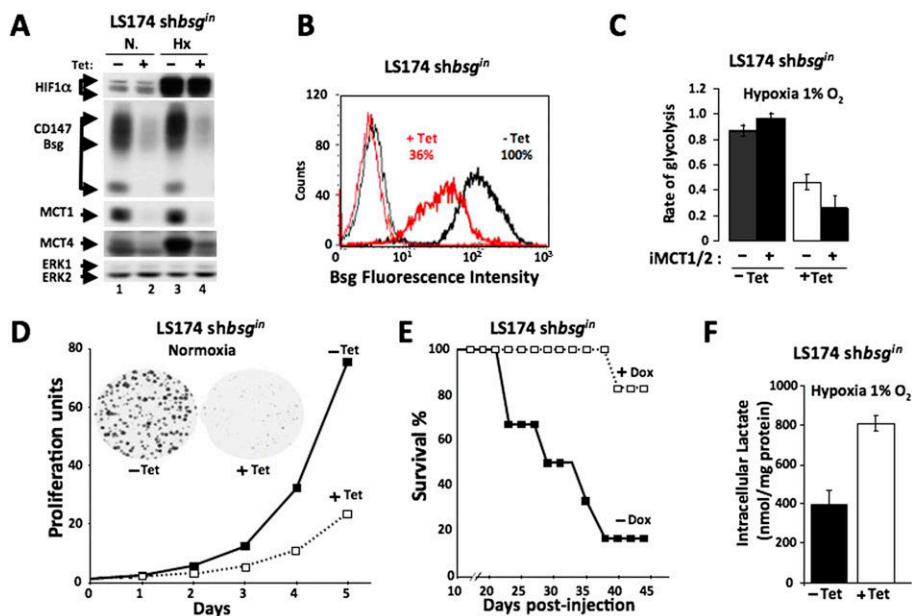
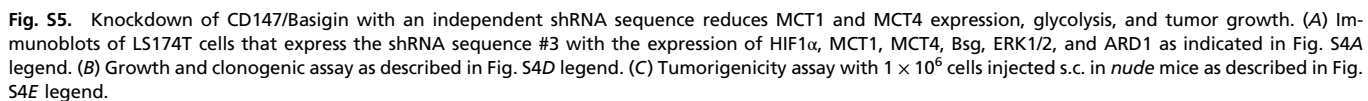


Fig. S4. Knockdown of CD147/Basigin reduces MCT1 and MCT4 expression, glycolysis, and tumor growth. (A) Knockdown of Basigin severely reduces the expression of MCT1 and MCT4. The LS174T cell line was stably transfected with the pTER-bsg plasmid that allows inducible expression of shRNA targeting CD147/Basigin (sequence 1) under tetracycline (LS174T *shbsg*ⁱⁿ). LS174T *shbsg*ⁱⁿ cells were cultivated in the absence (-Tet) or presence (+Tet) tetracycline for 5 d and maintained either in normoxia or in hypoxia 1% O₂ the last 48 h before lysis. The expression of HIF1 α , MCT1, MCT4, CD147/Bsg, and ERK1/2 was analyzed by immunoblotting as described above. (B) We report here the percentage (%) of Bsg surface expression analyzed by flow cytometry after 5 d of culture without (-Tet, black curve 100%) or with (+Tet, red curve 36%) tetracycline. Peaks on the left correspond to control incubation with the anti-mouse antibody alone. (C) The rate of glycolysis (mmol of lactate per 10⁶ cells·h⁻¹) of LS *shbsg*ⁱⁿ cells in hypoxia 1% O₂, was monitored 4-d after treatment with (+Tet) or without (-Tet) tetracycline allowing Bsg silencing. Cells were processed as described in Fig. 1C legend. (D) In vitro growth in normoxia. After 4 d of culture without (-Tet) or with tetracycline (+Tet), LS174T *shbsg*ⁱⁿ cells were submitted to a proliferation assay in normoxia with or without tetracycline. Cells were counted 24 h after seeding and daily up to 5 d. In the same experiment, a sample of LS174T *shbsg*ⁱⁿ cells (-Tet) and (+Tet) were submitted to a 10-d clonogenicity assay in normoxia with or without tetracycline (insets). (E) Tumorigenicity in *nude* mice. LS174T *shbsg*ⁱⁿ cells (1 \times 10⁵) were s.c. injected in *nude* mice drinking (+ Dox) or not drinking (-Dox) doxycycline in water 4 d before cell injection and during all of the tumorigenicity assay. Five mice were used per condition. Mice were killed when tumor size reached the mean value of 600 mm³ (% animal survival). (F) The intracellular lactate of LS174T *shmct1*ⁱⁿ was determined after 5 d of culture with or without Tet and hypoxic conditions (1% O₂) for the last 24 h.



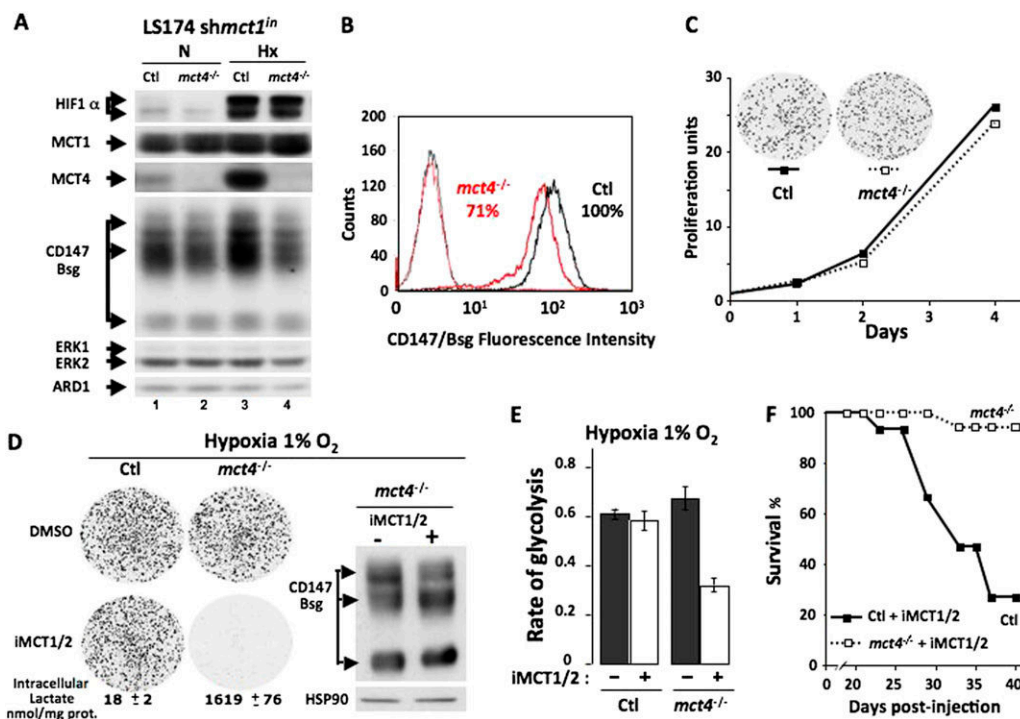


Fig. S6. Uncoupling CD147/Basigin expression and lactic acid export blunts *in vitro* and *in vivo* tumor growth. (A) Comparative expression of MCT1, MCT4, and Bsg in Ctl and *mct4*-null cells. The LS174T *shmct1ⁱⁿ/Ctl* and LS174T *shmct1ⁱⁿ/mct4^{-/-}* were cultivated in normoxia or hypoxia (1% O₂) for 48 h and lysed. The expressions of HIF1 α , MCT1, MCT4, CD147/Bsg, ERK1/2, and ARD1 were analyzed by immunoblotting. (B) Cell surface expression of CD147/Bsg analyzed by flow cytometry. Bsg surface expression of *mct4^{-/-}* cells represented 71% of the parental *mct4* proficient (Ctl). (C) Exponential *in vitro* growth in normoxia of control and *mct4*-null cells. The LS174T *shmct1ⁱⁿ/Ctl* and LS174T *shmct1ⁱⁿ/mct4^{-/-}* were submitted to a proliferation assay during 4 d and clonogenicity assay for 10 d in normal culture conditions (insets). (D) Growth in hypoxia of control and *mct4*-null cells. The LS174T *shmct1ⁱⁿ/Ctl* and LS174T *shmct1ⁱⁿ/mct4^{-/-}* cells were submitted to a 10-d clonogenicity assay in hypoxia (1% O₂) in the presence of MCT1/2 inhibitor (iMCT1/2, 100 nM) or DMSO (0.1 vol %). The intracellular lactate concentration of Ctl or *mct4^{-/-}* cells cultivated in hypoxia was determined 5 h after glucose addition in presence of iMCT1/2 (100 nM). The immunoblot on *Right* shows the level of expression of CD147/Basigin in the same cells cultivated in hypoxia 24 h with or without iMCT1/2 (100 nM). (E) The rate of glycolysis (mmol of lactate per 10⁶ cells \cdot h⁻¹) of the LS174T *shmct1ⁱⁿ/Ctl* and LS174T *shmct1ⁱⁿ/mct4^{-/-}* in hypoxia (1% O₂) is determined as described in Fig. 1. (F) LS174T *shmct1ⁱⁿ/Ctl* and LS174T *shmct1ⁱⁿ/mct4^{-/-}* cells were s.c. injected into nude mice. Six days after cell injection, mice were injected s.c. twice per day during 2 wk with MCT1/2 inhibitor (30 mg/kg) or PBS-Tween 5%. Mice were killed when tumor size reached the mean value of 600 mm³ (per animal survival).

Fig. S7. Stable expression of an inactive mutated MCT4 form in LS174T-*mct4*^{-/-} cells and lactic acid uptake. (Left) Conserved sequences of human MCT1 and MCT4. The key Lys38 residue of MCT1 corresponds to Lys40 in MCT4 that was mutated into Arg40 (MCT4-K40R). (Right) Lactic acid uptake in the three LS174T cell lines: *shmct1*ⁱⁿ (a), *shmct1*ⁱⁿ/*mct4*^{-/-} (b), and *shmct1*ⁱⁿ/*mct4*^{-/-}-MCT4^{mut-K40R} (c) in the absence (black bar) or presence of iMCT1/2 (gray bar). The uptake was conducted as a 3-min time-point in duplicate. Note that the mutated form MCT4^{mut-K40R} does not display any measurable lactic acid uptake (c, gray bar), whereas it increases the expression of Basigin (Fig. 3B, lane 4).

B. Genetic disruption of the MCT/BSG complexes causes a metabolic shift from glycolysis towards OXPHOS

PUBLICATION 2- Genetic disruption of lactate/H⁺ symporters (MCTs) and their subunit CD147/BASIGIN sensitizes glycolytic tumour cells to phenformin.

Ibtissam Marchiq, Renaud Le Floch, Daniele Roux , Marie-Pierre Simon , and Jacques Pouyssegur

(Accepted in Cancer Research in November 2014)

PUBLICATION 3- Disruption of BASIGIN decreases lactic acid export and sensitizes non-small cell lung cancer to biguanides independently of the LKB1 status.

Sara Granja *, **Ibtissam Marchiq** *, Renaud Le Floch, Conceição Souto Moura, Fátima Baltazar and Jacques Pouyssegur

(Accepted in Oncotarget in December 2014)

* Co-first authors

To further explore: i) the physiology of MCTs/BSG complexes and ii) to validate the blockade of lactic acid export as an anti-cancer strategy, we genetically disrupted, *via* ZFN, the *MCT4* and *BSG* genes in two human cell lines of colon adenocarcinoma (LS174T) and glioblastoma (U87) (publication 2). Our results showed that:

- Single ablation of the *MCT4* gene is not sufficient to alter tumour cell metabolism, pH_i and growth, confirming thus our previous findings (publication 1). However, it dramatically sensitised cells to AZD3965, a new specific pharmacological inhibitor of MCT1 that is currently undergoing phase I/II clinical trials.

- Disruption of the *BSG* gene dramatically reduced the plasma membrane expression and lactate transport activity of both MCT1 and MCT4. This leads to increased accumulation of intracellular pool of lactic and pyruvic acids, which was magnified further by the addition of MCT1 inhibitor, consequently decreasing further the pH_i and the rate of glycolysis. This led to growth arrest due to cytostasis induced by mTORC1 inhibition.
- Surprisingly, BSG-KO cells not only survived, but they exhibited normal proliferation rates.
- BSG-KO cells resumed growth by redirecting part of their energy metabolism towards OXPHOS, which sensitised them to biguanides. Unlike most of the previously published, studies in which phenformin was used as an activator of AMPK, we choose this drug more for its ability to efficiently inhibit the complex I of mitochondria.
- Treatment of MCT/BSG-disrupted cells with phenformin induced a rapid and major drop in cellular ATP provoking cell death by ‘metabolic catastrophe’ *in vitro* and restricting tumour growth *in vivo*.

These findings were further extended to three human non-small lung cancer (NSCLC) cell lines (A549, H1975 and H292) as the role of MCTs/BSG in lung cancer is controversial and poorly elucidated (publication 3). In this study, we confirmed our former results but we have also shown that:

- In contrary to what was previously published by other groups, the sensitivity of NSCLC cells to biguanides was independent of their LKB1 status and AMPK activation, but more linked to their rate of glycolysis. Thus disruption of the *BSG* gene in A549 (mutated LKB1), H1975 and H292 (wild type LKB1) cell lines, decreased their rate of glycolysis and further increased their rate of respiration, which rendered them equally sensitive to phenformin /metformin treatment in both normoxia and hypoxia.

Taken together, these findings underline that MCT/BSG complexes inhibition, combined with phenformin provided a novel promising anti-cancer strategy for targeting highly glycolytic tumours. This genetic approach supports the rational of developing specific pharmacological inhibitors of MCT1 and MCT4.

Genetic Disruption of Lactate/H⁺ Symporters (MCTs) and Their Subunit CD147/BASIGIN Sensitizes Glycolytic Tumor Cells to Phenformin

Ibtissam Marchiq¹, Renaud Le Floch¹, Danièle Roux¹, Marie-Pierre Simon¹, and Jacques Pouyssegur^{1,2}

Abstract

Rapidly growing glycolytic tumors require energy and intracellular pH (pHi) homeostasis through the activity of two major monocarboxylate transporters, MCT1 and the hypoxia-inducible MCT4, in intimate association with the glycoprotein CD147/BASIGIN (BSG). To further explore and validate the blockade of lactic acid export as an anticancer strategy, we disrupted, via zinc finger nucleases, *MCT4* and *BASIGIN* genes in colon adenocarcinoma (LS174T) and glioblastoma (U87) human cell lines. First, we showed that homozygous loss of *MCT4* dramatically sensitized cells to the MCT1 inhibitor AZD3965. Second, we demonstrated that knockout of *BSG* leads to a decrease in lactate transport activity of MCT1 and MCT4 by 10- and 6-fold, respectively. Consequently, cells accumulated an intracellular pool of lactic and pyruvic acids, magnified by the MCT1 inhibitor decreasing further pHi and glycolysis. As a result, we found that these

glycolytic/MCT-deficient cells resumed growth by redirecting their metabolism toward OXPHOS. Third, we showed that in contrast with parental cells, BSG-null cells became highly sensitive to phenformin, an inhibitor of mitochondrial complex I. Phenformin addition to these MCT-disrupted cells in normoxic and hypoxic conditions induced a rapid drop in cellular ATP-inducing cell death by "metabolic catastrophe." Finally, xenograft analysis confirmed the deleterious tumor growth effect of *MCT1/MCT4* ablation, an action enhanced by phenformin treatment. Collectively, these findings highlight that inhibition of the MCT/BSG complexes alone or in combination with phenformin provides an acute anticancer strategy to target highly glycolytic tumors. This genetic approach validates the anticancer potential of the MCT1 and MCT4 inhibitors in current development. *Cancer Res*; 75(1); 171–80. ©2014 AACR.

Introduction

Unlike in normal differentiated cells, most rapidly growing tumors rely primarily on glycolysis as a major source of energy even in the presence of oxygen, which is referred to as "fermentative glycolysis" (1). This phenomenon, confirmed through the use of ¹⁸F-fluoro-deoxyglucose PET (2), is now considered as a key metabolic hallmark of invasive cancers (3). Recent studies linked the Warburg effect to metabolic reprogramming, in which oncogenic transformation, as well as the transcriptional activity of c-Myc and the hypoxia-inducible factor1 (HIF1), induce glycolytic activity (4–6) associated to parallel inhibition of the pyruvate dehydrogenase (PDH) complex (7, 8).

Even if fermentative glycolysis generates only a few ATP molecules considering the large amounts of glucose consumed, this pathway appears to be well fitted for rapid growth of normal and cancer cells, as it provides not only energy, but also anabolic

precursors (6, 9, 10). However, the high glycolytic rate associated with a production of lactic acid rapidly creates a hostile acidic and hypoxic microenvironment that induces dissemination of metastatic variants (11). Hypoxia via HIF1 controls intracellular pH (pHi) by inducing the membrane-bound carbonic anhydrases IX and XII (12–14) and a major H⁺/lactate[−] symporter, MCT4 (15–17). Considering the key role of glycolysis in "glucose-addicted" tumors, targeting this bioenergetic and anabolic pathway represents an attractive therapeutic approach (18–22).

The regulation of pHi is a key determinant for many physiologic processes and is certainly essential in maintaining a high glycolytic rate. For this reason, we have exploited our knowledge on lactic acid transport to evaluate the impact of its inhibition as an anticancer strategy (21, 23). Transport of lactate across the plasma membrane is carried out by a family of solute carrier family 16 (SLC16), or monocarboxylate transporters (MCT) in which four (MCT1–4) catalyze bidirectional proton-coupled transport of lactate. MCT1 is induced by Myc and MCT4 by HIF1, and are upregulated in several cancers (17, 24). These two transporters require an accessory glycoprotein CD147/BASIGIN (BSG) for proper folding, stability, and trafficking to the cell surface (25, 26). BSG is a type I transmembrane glycoprotein that has been reported to be overexpressed in different metastatic tumor cells (24, 26). Thus targeting BSG/MCTs complex using a pharmacologic inhibitor or shRNA-mediated knockdown was reported to impair the *in vitro* and *in vivo* growth of pancreatic tumor cells (27), Ras-transformed fibroblasts, colon adenocarcinoma (21), and Myc-induced human malignancies (22), which suggested that blocking lactic acid export provides an efficient metabolic therapy to limit tumor cell growth (14).

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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In the present study, we explored further the mechanism by which blockade of lactic acid export exerts cytostasis and could be turned into a strategy to provoke tumor cell death. Because there is no pharmacologic inhibitor for MCT4, we designed genetic experiments to knockout, using the zinc finger nuclease (ZFN), either *MCT4* or *BSG* or both in two glycolytic tumor models, the colon adenocarcinoma (LS174T), and glioblastoma (U87) human cell lines. We demonstrated that tumor cells grown in normoxia or hypoxia survived the MCT blockade by reactivating OXPHOS. This key finding prompted us to test the sensitivity of these cells to phenformin a mitochondrial complex I inhibitor. Indeed, the two tumor cell lines that survive MCT blockade with minimal growth rapidly enter into "ATP crisis" and die *in vitro* and *in vivo* upon treatment with phenformin.

Materials and Methods

Cell culture and hypoxia

Colon adenocarcinoma LS174T cells (kindly provided by Dr. Van de Wetering, Oxford University, Oxford, United Kingdom), and glioblastoma U87 cells (kindly provided by Dr. Adrian Harris, Oxford University) were grown in DMEM (Gibco) supplemented with 10% FBS, penicillin (10 U/mL), and streptomycin (10 µg/mL). Chinese hamster lung CCL39 fibroblasts obtained from the ATCC and the CCL39-derived mutants impaired either in respiration (*res*⁻) or in glycolysis (*gly*⁻), were obtained as described (28, 29) and maintained in DMEM supplemented with 7.5% FBS. Cell lines were identified by DNA fingerprint and STR loci (DSMZ). With the exception of "Seahorse" experiments, all *in vitro* studies were performed in parallel, under normoxia or hypoxia 1% O₂ as reported (21) to fully express glycolytic enzymes, particularly MCT4.

Animal studies

The different LS174T stable cell lines (1×10^6 cells) suspended in 300 µL of serum-free DMEM supplemented with insulin-transferrin-selenium (Life Technologies) were injected subcutaneously into the back of 8-week-old male athymic mice (Harlan). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Nice-Sophia Antipolis (Nice, France; CIEPAL-azur agreement NCE/165; see Supplementary Data for details).

Proliferation assay

The different LS174T cell lines (6×10^4 cells) were seeded onto 6-well plates, three wells per cell line and per condition. We measured proliferation by detaching the cells by trypsinization, and counting them daily with a Coulter Z1 (Beckman) during 5 days. Proliferation units were calculated by dividing the cell number obtained for each day by the one obtained 24 hours after seeding.

Metabolic analysis

The rate of extracellular acidification (ECAR) and the oxygen consumption rate (OCR) were measured using the Seahorse XF24 analyzer (Seahorse Bioscience). LS174T cells (9×10^4) and U87 cells (3×10^5) were seeded on Seahorse 24-well plates, 24 and 3 hours, respectively, before analysis. Cells were maintained in cell culture medium without glucose, pyruvate, serum, or bicarbonate, and incubated for 45 minutes in a non-CO₂ incubator at 37°C. The wells were mixed for 2 minutes and the pH and oxygen

concentration measured every 22 seconds for 3 minutes. Different agents were then injected into the wells at a final concentration of 10 mmol/L of glucose (Sigma), 300 nmol/L of MCT1i, 50 µmol/L of phenformin (Sigma), and 1 µmol/L of rotenone (Sigma). After each addition, four data points of 3 minutes were taken to determine the OCR (pMoles O₂/minutes) and ECAR (mpH/minute). The protein concentration was measured to normalize the OCR and ECAR values.

Statistical analysis

Data are expressed as mean \pm SD. Each experiment was performed at least three times and the number of experiments is represented by *n*. Statistical analysis was done with the unpaired Student *t* test. Differences between groups were considered statistically significant when *P* < 0.05.

Results

Disruption of BSG/MCTs complex ablates lactic acid transport and decreases intracellular pH

We first knocked out the *MCT4* gene in LS174T cells that express high levels of BSG, MCT1, and MCT4; the mutations altering the two alleles are depicted in Supplementary Fig. S1A. We then knocked out the *BSG* gene in wild-type (WT) LS174T (Supplementary Fig. S1B) or in LS174T-*MCT4*^{-/-} generating the double knocked out *BSG*^{-/-}, *MCT4*^{-/-} cell line. The *MCT4*^{-/-} cell line shows the lack of expression of MCT4 in both normoxia and hypoxia (Supplementary Fig. S1A). Ablation of *MCT4* was accompanied by a slight decrease in the expression of BSG in normoxia and hypoxia (Fig. 1A), and by an increase in MCT1 expression that is more apparent in hypoxia (Fig. 1A). *BSG* knockout lead to the complete loss of both forms, the heavily glycosylated (around 65 kDa) and the immature low-molecular weight form of 39 kDa. *BSG* disruption, as expected from its chaperone role, induces decreased expression of both transporters MCT1 and MCT4 (Fig. 1A). Similar results were obtained for the human glioblastoma cell line. Knockout of the *BSG* gene in U87 cells induced a major decrease in the protein level of MCT1 and MCT4 in both normoxia and hypoxia (Supplementary Fig. S2A).

Next, we investigated the impact of these mutations on the *V*_{max} values of MCT1 and MCT4 by measuring initial lactic acid transport rates. The initial entry rates of total lactic acid transport in hypoxic LS174T cells was $61 \pm 5\%$ for MCT1 and $39 \pm 5\%$ for MCT4. Quantification was facilitated by the use of the specific MCT1 inhibitor (AR-C155858) referred to as MCT1i and the observation that LS174T cells do not express MCT2 and MCT3 (data not shown). Disruption of the *MCT4* gene did not modify the transport activity of MCT1 (Fig. 1B). However, addition of 300 nmol/L MCT1i to the *MCT4*^{-/-} cell line totally abolished facilitated lactic acid transport, confirming the nonexpression of other MCT isoforms (Fig. 1B). Disruption of *BSG* severely reduced the lactic acid transport activity. Thus, MCT1 and MCT4 expression was reduced by 10- and 6-fold, respectively, in LS174T (Fig. 1B), and by 3.9- and 3.3-fold, respectively, in U87 (Supplementary Fig. S2B), confirming that in the absence of BSG, MCTs, fully transcribed (Supplementary Fig. S3), were retained and degraded in the endoplasmic reticulum as nonfunctional transporters (25).

The next step was to analyze the pH_i in the LS174-mutant derivatives. The alkaline resting pH_i of the parental LS174 cell line, close to 8, was essentially not affected by the complete inhibition of MCT1 (Fig. 1C). The additional knockout of MCT4

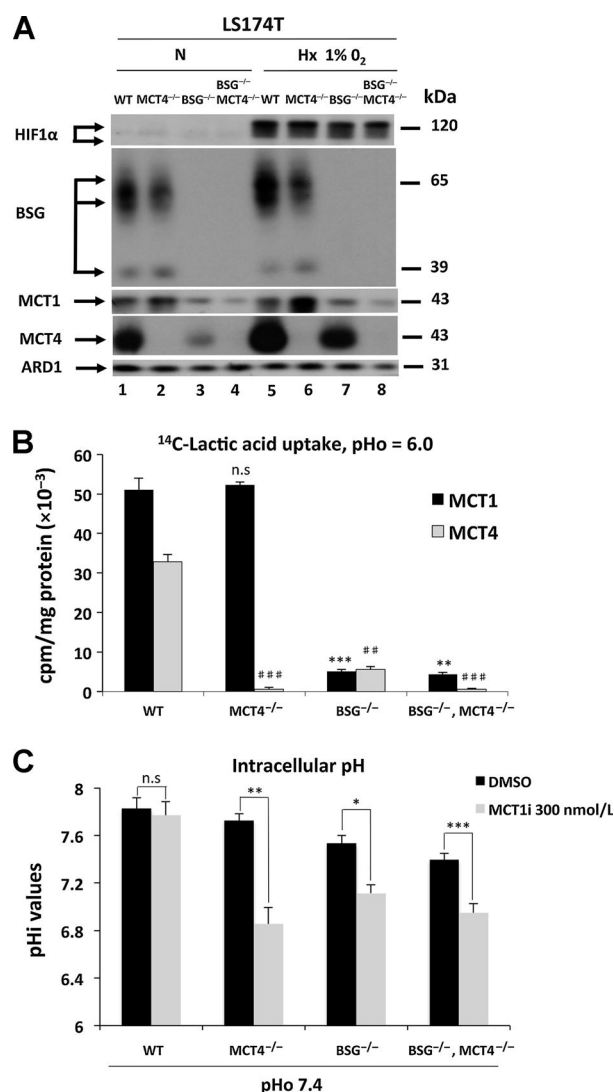


Figure 1. Disruption of BSG/MCT complexes, by ZFNs, is required to ablate lactic acid transport and decrease the pHi. A, LS174T WT and knockout cells were maintained in normoxia or hypoxia for 48 hours and lysed. The cell expression of HIF1α, MCT1, MCT4, and BSG was analyzed by immunoblotting. ARD1 was used as a loading control. B, [¹⁴C]-Lactic acid uptake in LS174T WT and MCT4/BSG-null cells. Discrimination of the total transport activity of MCT1 and MCT4 was possible by using MCT1i (300 nmol/L); **, $P < 0.001$; ***, $P < 0.0001$; n.s., not significant; and ###, $P < 0.001$; ####, $P < 0.0001$ (comparison of MCT1 and MCT4 activity, respectively, of each LS174T-mutant derived cell type vs. LS174T WT cells). C, resting pHi in the presence of MCT1i (300 nmol/L) or DMSO (0.1% v/v); *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$; n.s., not significant.

had a very modest effect on pHi. In sharp contrast, in the MCT4^{-/-} cells, MCT1i increased by 10-fold the intracellular H⁺ concentration; the resting pHi decreased from about 7.9 to 6.9 (Fig. 1C).

Inactivation of the BSG/MCT complexes decreases the rate of glycolysis and reactivates OXPHOS

When mutants derived from LS174T cells were starved for 1 hour of glucose, they rapidly accumulated intracellular lactic acid

upon glucose addition with a steady-state equilibrium that was reached within 3 to 5 hours (Fig. 2A). In agreement with the results described above, BSG^{-/-} and BSG^{-/-}, MCT4^{-/-} cells, compared with parental cells, accumulated a large intracellular pool of lactic acid ranging from 5 to 6 mmol/L lactate in parental cells to 20 mmol/L in the double knockout BSG^{-/-}, MCT4^{-/-} cells (Fig. 2A, left). The intracellular pool of lactic acid of the single mutant MCT4^{-/-} was not affected (7 mmol/L), indicating the major role played by MCT1 alone in lactic acid export. As expected, treatment of the cells with MCT1i further increased the intracellular lactate levels particularly for the MCT4^{-/-} cells reaching 38 to 40 mmol/L (Fig. 2A, right). This accumulation was less pronounced in the BSG^{-/-} cells, whereas parental cells remained less sensitive to MCT1 inhibition. With such a block in lactic acid export, retro-inhibition on lactic dehydrogenase-A (LDHA) was expected to lead to a parallel increase in pyruvic acid pool. Pyruvate concentrations were about 20-fold lower than lactate pool in parental cells but increased 4- to 6-fold in the three MCT/BSG knockout cells (Fig. 2B, right).

The intracellular glycolytic metabolite and proton accumulation mediated by MCT blockade had, not surprisingly, a strong negative impact on the rate of glycolysis. Data from the Seahorse analyzer confirmed the rapid decrease in the ECAR after BSG^{-/-} knockout (2-fold) or BSG^{-/-}, MCT4^{-/-} double knockout (3-fold), whereas the ECAR was similar in MCT4^{-/-} and parental cells (Fig. 2C). Interestingly, MCT1 inhibition did not affect the ECAR of parental cells, a result that was consistent with the relatively high expression of MCT4 in normoxic LS174T cells (Fig. 1A). However, compared with parental cells, MCT1i virtually suppressed the ECAR in the MCT4^{-/-} cells (8 and 16-fold decreases of ECAR in MCT4^{-/-} and BSG^{-/-}, MCT4^{-/-} cells, respectively; Fig. 2C).

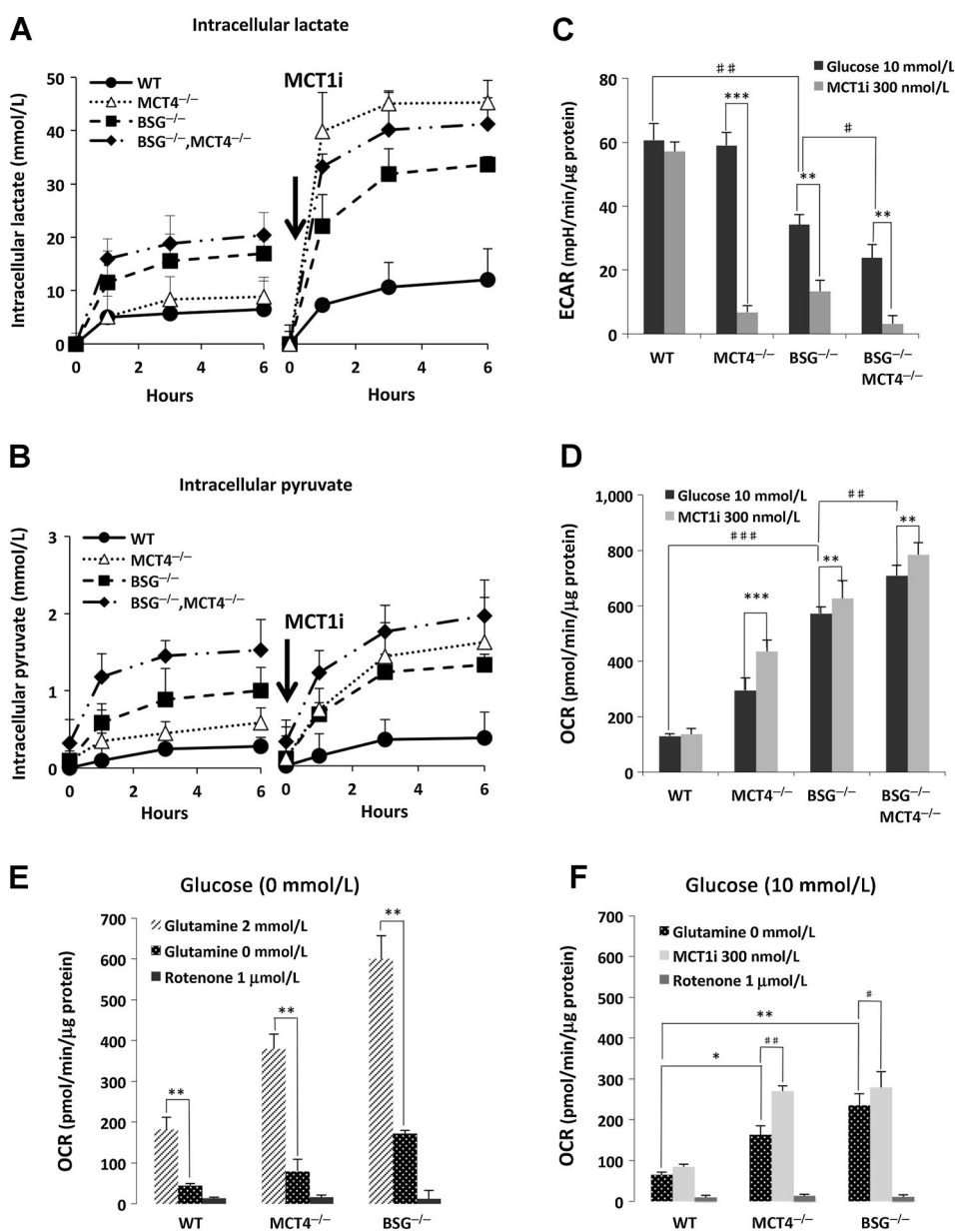
The corresponding OCR, which was low in parental cells, in contrast increased progressively with mutations affecting the intensity of the glycolytic blockade (WT < MCT4^{-/-} < BSG^{-/-} < BSG^{-/-}, MCT4^{-/-}; Fig. 2D). As expected, the OCR was increased in the disrupted cell lines upon MCT1 inhibitor. These results were confirmed in U87 cells. Indeed, both U87 BSG-null cells displayed a decreased ECAR (50%) and even an increased OCR (53%) compared with the parental U87 cells. These changes were magnified by the pharmacologic inhibition of MCT1 (Supplementary Fig. S2C and S2D).

Taken together, these findings highlight the plasticity of cancer cells and clearly demonstrate the ability of highly glycolytic tumor cells, such as LS174T and U87 cells, to rapidly shift their metabolism to OXPHOS, a mechanism of resistance to inhibition of fermentative glycolysis.

To further understand how this metabolic switch occurred and to which substrate the knockout cells relied on to increase their respiration, we first assessed the impact of glutamine deprivation on the OCR. Indeed, glutamine metabolism is well documented as a carbon and bioenergetic source for cancer cells (30).

Our results showed that the highly glycolytic parental LS174T cells displayed, in the presence of 2 mmol/L glutamine as the only exogenous carbon source (no glucose), a rather low respiratory rate (OCR = 180) that was increased when the MCT activity was partially disrupted (OCR = 380 in MCT4^{-/-} and 600 in BSG^{-/-}; Fig. 2E). Removal of glutamine from this medium sharply reduced by 3- to 4-fold the rate of respiration for the three cell lines thereby highlighting the key contribution of glutamine to respiration (Fig. 2E and Supplementary Fig. S4A

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**Figure 2.**

Inactivation of BSG/MCTs complex impairs intracellular lactate and pyruvate pools, reduces the rate of glycolysis, and reactivates OXPHOS. Time course of intracellular lactate (A) and intracellular pyruvate (B) concentrations in response to glucose (25 mmol/L) addition, in the presence of DMSO (left) or MCT1i (300 nmol/L; right). Real-time analysis of the proton production rate (ECAR; C) and OCR (D) with a Seahorse XF24 analyzer. The effect of addition of glucose (10 mmol/L) or MCT1i (300 nmol/L) is shown; **, $P < 0.001$; ***, $P < 0.0001$; and #, $P < 0.05$; ##, $P < 0.001$; ###, $P < 0.0001$ ($n = 6$). E, basal OCR of WT, MCT4^{-/-}, and BSG^{-/-} LS174T cells incubated for 1 hour with or without glutamine (2 mmol/L) in the absence of glucose. Addition of rotenone (1 μmol/L) completely abolished the OCR of these cells in either the presence or the absence of glutamine; **, $P < 0.001$. F, the OCR of the three LS174T cell lines in the absence of glutamine and with the addition of glucose (10 mmol/L), MCT1i (300 nmol/L), and rotenone (1 μmol/L); *, $P < 0.05$; **, $P < 0.001$ and #, $P < 0.05$; ##, $P < 0.001$. Data represent the average of at least three independent experiments.

and S4B). We next conducted the experiment without glutamine to analyze the contribution of glucose in OXPHOS (Fig. 2F). The rate of respiration increased in proportion to the decreased level of the MCT activity (WT < MCT4^{-/-} < BSG^{-/-}) and became maximal in the presence of MCT1i (Fig. 2F). It is interesting to note that in the presence of MCT1i, both MCT4^{-/-} or BSG^{-/-} cells respired at the same level, that is, 4-fold above the parental cells (Fig. 2F), a value that paralleled the increased intracellular pool of pyruvate in these two cell lines (5-fold increase in the first hour of MCT1i addition; Fig. 2B, right). Because this increase in OXPHOS is sensitive to rotenone (Fig. 2F) or metformin/phenformin (Supplementary Fig. S5B), mitochondrial complex I inhibitors (31–33), we propose that the accumulation of pyruvate increases, by mass action, the activity of PDH.

Disruption of the BSG/MCTs complexes arrests cell growth and sensitizes cells to phenformin

We next reported the impact of inhibition of glycolysis on the *in vitro* growth rates of the four LS174T-derived cell lines (Fig. 3A and B) and of the three U87-derived cell lines (Supplementary Fig. S2E). In normoxia, the disruption of MCT4, BSG, or both had only a modest effect on the growth rate (less than 20%, for BSG^{-/-}; Fig. 3A and Supplementary Fig. S2E, left). In hypoxia, the trend in general is that many tumor cells display a reduced growth rate (2-fold reduction for the parental LS174T cells). Again, it is the BSG^{-/-}, MCT4^{-/-} cells that display the most reduced growth rate in hypoxia compared with the parental cells (1.5-fold, Fig. 3A, right). In contrast with these modest effects on growth, the addition of the MCT1i, that does not affect the parental cell

growth, decreased the growth of U87 *BSG*^{-/-} cells by 2-fold (Supplementary Fig. S2E), and severely reduced the growth of LS174T *MCT4*^{-/-} cells by, respectively, 3-fold (Fig. 3A, left) and up to 6-fold in hypoxia (Fig. 3A, right).

Interestingly, this decrease in growth rate correlated with the decrease in pHi (Fig. 1C), which is known to restrict mTORC1 (34) as seen by inhibition of p70 S6 kinase phosphorylation (Fig. 3B). In other words, the MCT1i sensitized MCT4-null cells to growth arrest by inducing a pHi drop of 1 pH unit. The most remarkable finding is that ablation of lactic acid export induced only cytostasis with minimal loss of cell viability (data not shown). The bioenergetic plasticity reported above is certainly the basis of this acquired tumor cell viability under MCT blockade. We thus analyzed the *in vitro* growth of LS174T cells

(WT or *BSG*^{-/-}) treated with MCT1i or the lipophilic biguanide metformin analog, phenformin, or both. Figure 3C shows that the parental cells, either grown in normoxia or hypoxia, are not affected by any of the treatments, MCT1i, phenformin, or both. Disruption of *BSG* alone, however, severely restricted clonal growth in response to phenformin in normoxia but not in hypoxia (Fig. 3C). In hypoxia, although MCT1i or phenformin alone has no effect, their combination suppressed clonal growth, highlighting a clear case of synthetic lethality (Fig. 3C). Similar results were obtained by replacing 50 μ mol/L phenformin by 1 mmol/L of the hydrophilic and less permeable analog metformin.

The diverse growth phenotypes displayed in normoxia/hypoxia and in response to phenformin by the four cell lines, WT, *MCT4*^{-/-}, *BSG*^{-/-}, and *BSG*^{-/-}*MCT4*^{-/-}, are easily

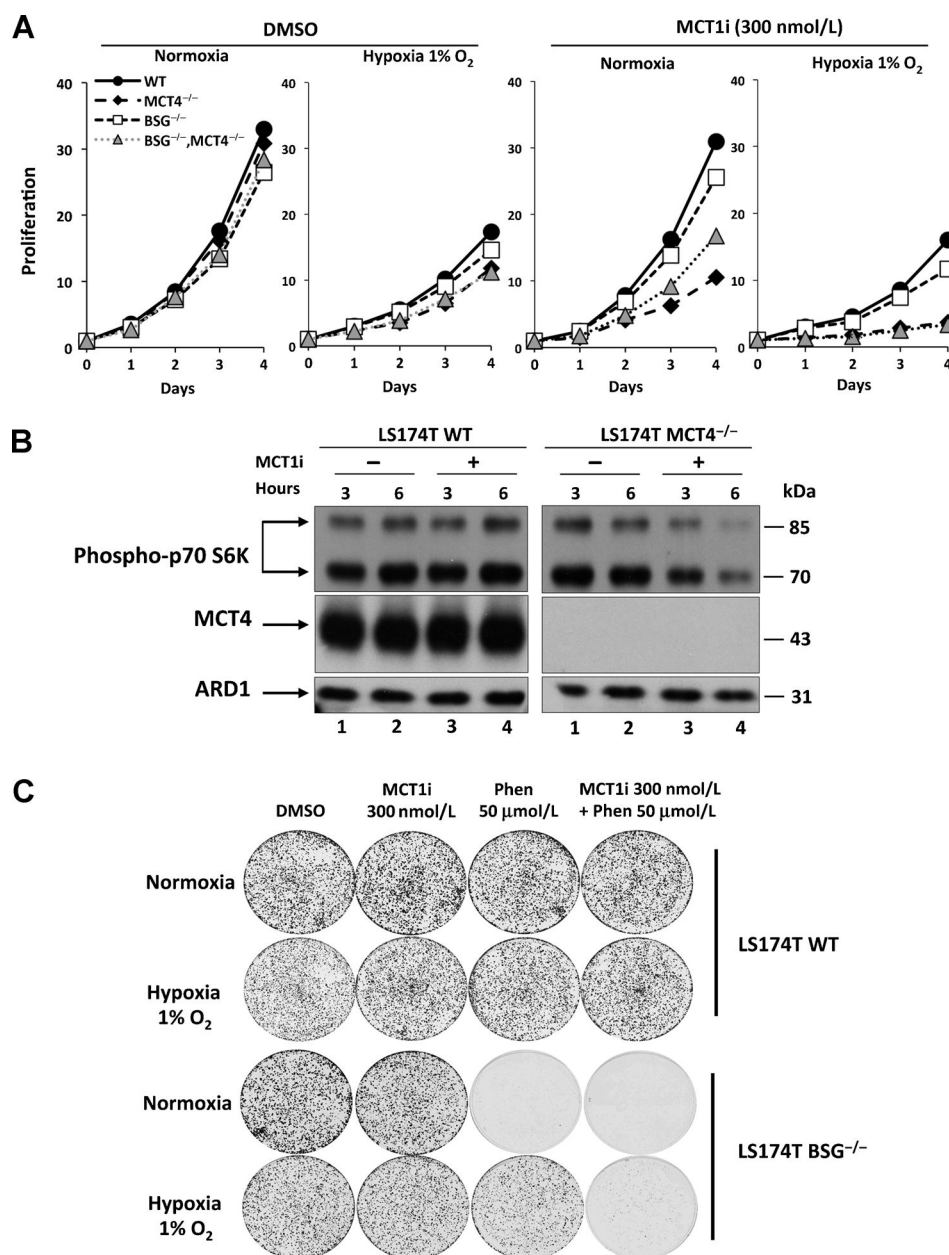


Figure 3.

Disruption of *BSG* and/or *MCT4* sensitizes tumor cells to MCT1 inhibition and/or to phenformin in both normoxia and hypoxia. A, proliferation rate of LS174T WT and mutant cells treated with DMSO or MCT1i (300 nmol/L), and incubated in either normoxia or hypoxia. B, WT and *MCT4*^{-/-} LS174T cells were maintained in normoxia, treated with DMSO or MCT1i (300 nmol/L) for 3 or 6 hours, and lysed. The expression of the phospho-p70 S6 kinase and MCT4 was analyzed by immunoblotting. ARD1 was used as a loading control. C, clonal growth of WT and *BSG*^{-/-} LS174T cells treated with MCT1 (300 nmol/L), phenformin (50 μ mol/L), or both. The results are representative of four different experiments.

explained by their energy metabolic features. In normoxia, the rates of glycolysis (ECAR), although high are further stimulated in response to phenformin for the WT and *MCT4*^{-/-} cell lines but not affected in the most MCT-disrupted cells *BSG*^{-/-} and *BSG*^{-/-}, *MCT4*^{-/-} (Supplementary Fig. S5A). The corresponding OCR showed a collapse of respiration for the four cell lines in response to phenformin (Supplementary Fig. S5B). The most dramatic phenformin-mediated growth restriction is only seen in the most restricted cell lines for MCT/glycolysis: *BSG*^{-/-} and *BSG*^{-/-}, *MCT4*^{-/-} (Supplementary Fig. S5C).

These findings received confirmation in two independent *BSG*-null tumor cell lines, glioblastoma U87 (Supplementary Fig. S2E) and lung adenoma carcinoma A549 (data not shown), pointing that targeting glycolysis (MCT inhibition) sensitized cells to phenformin. In hypoxic conditions, however, MCT4 induction induced a slight resistance to phenformin for *BSG*^{-/-} cells, whereas cells lacking both *BSG* and *MCT4* remained highly sensitive (Fig. 3C and Supplementary Fig. S5C). For *BSG*^{-/-} and *BSG*^{-/-}, *MCT4*^{-/-} cells, we postulate that the elevated intracellular lactate levels played a negative feedback on the glycolysis, thus inhibiting the increased ECAR due to phenformin (Supplementary Fig. S5A and S5B). These findings were supported by other results obtained

from the Ras-transformed fibroblast CCL39 cell line, for which we possess mutants impaired either in glycolysis (*gly*^{-/-}; Supplementary Fig. S5D; refs. 29, 35) or in respiration (*res*^{-/-}; Supplementary Fig. S5E; refs. 21, 28). As expected, only the CCL39 glycolysis-defective cells (*gly*^{-/-}) were rapidly killed by phenformin treatment (Supplementary Fig. S5F).

In conclusion, phenformin/metformin severely impairs growth and viability of tumor cells only when fermentative glycolysis is compromised.

Phenformin combined with *BSG*/*MCT*s disruption induces a major drop in cellular ATP provoking rapid cell death

As mentioned above, phenformin treatment abolishes OXPHOS (Supplementary Fig. S5B). This resulted in decreased mitochondrial ATP and led to a compensatory increase in glycolysis with increased lactate production and extracellular acidification (Supplementary Fig. S5A). Here, we showed that 24 hours of phenformin treatment had no effect on intracellular ATP levels of LS174T WT cells in both normoxia and hypoxia (Fig. 4A and B). In the case of *MCT4* knockout, phenformin only decreased the intracellular ATP by 10% showing that the compensatory glycolysis is sufficient to maintain high ATP levels.

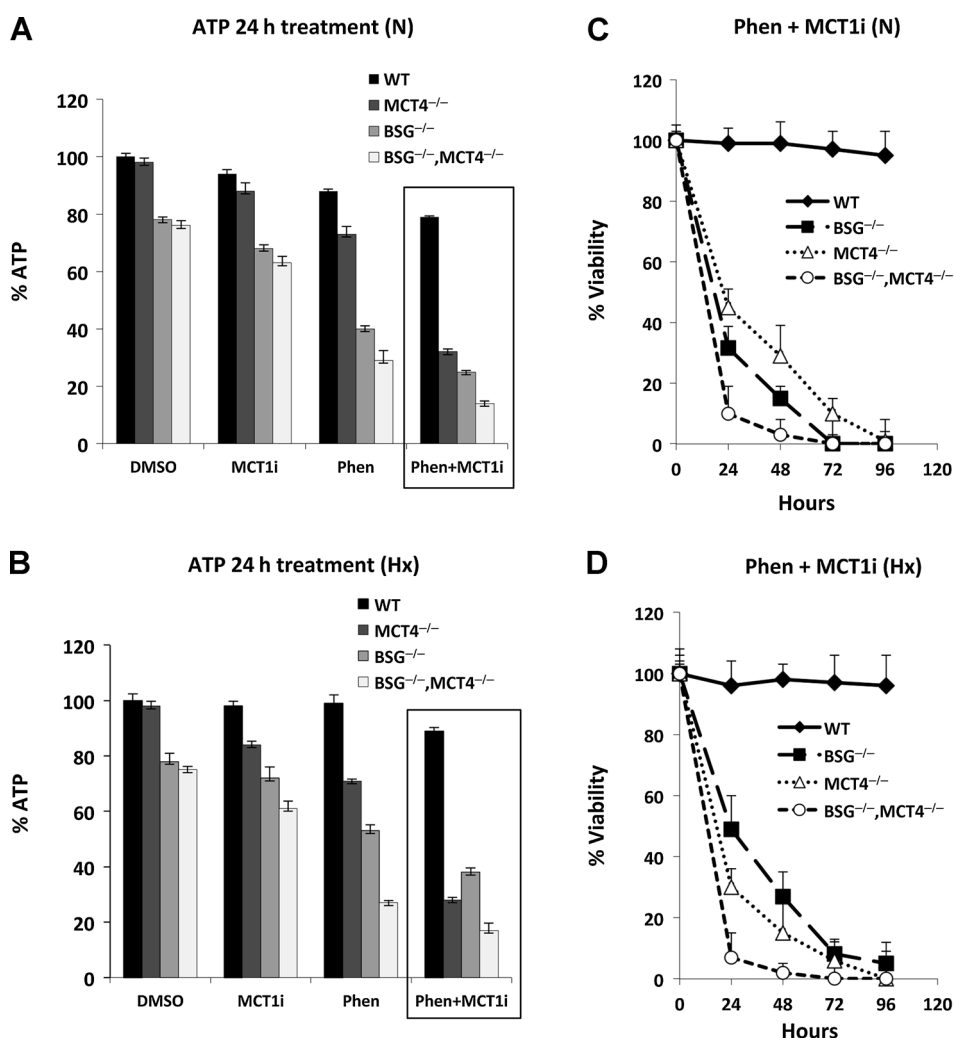


Figure 4. *BSG*/*MCT*s disruption combined with phenformin induces a major drop in cellular ATP, leading to rapid cell death. Intracellular ATP levels of LS174T WT and mutant cells after treatment with MCT1i (300 nmol/L), phenformin (50 μ mol/L), or both for 24 hours in normoxia (A) or hypoxia (B). The relative luminescence units (RLU) were normalized to the quantity of protein, and the values are given as percentages. C, cell survival of LS174T WT and mutant cells after treatment with both MCT1i (300 nmol/L) and phenformin (50 μ mol/L) in normoxia (C) or hypoxia (D).

However, *BSG*^{-/-} and *BSG*^{-/-},*MCT4*^{-/-} cells, impaired in their glycolytic flux, were not able to restore their intracellular ATP, which dropped by 60% and 80%, respectively, compared with WT cells in normoxia (Fig. 4A). MCT4 induction in hypoxia limited this decrease for *BSG*^{-/-} cells, confirming thus our findings on clonal growth.

Although MCT1 inhibition slightly decreased the intracellular ATP of *BSG*/*MCT*-disrupted cells, combination with phenformin induced an "energetic crisis" represented by a striking decrease of ATP (up to 85%; Fig. 4A and B). This ATP drop resulted in enhanced cytotoxicity and consequently rapid cell death. Indeed, 50% of either *MCT4*^{-/-} or *BSG*^{-/-} cells, and 90% of *BSG*^{-/-},*MCT4*^{-/-} cells died in both normoxia and hypoxia after 24 hours of combined treatment; and only 8% of *BSG*^{-/-} cells survived under hypoxic conditions after 96 hours (Fig. 4C and D). These

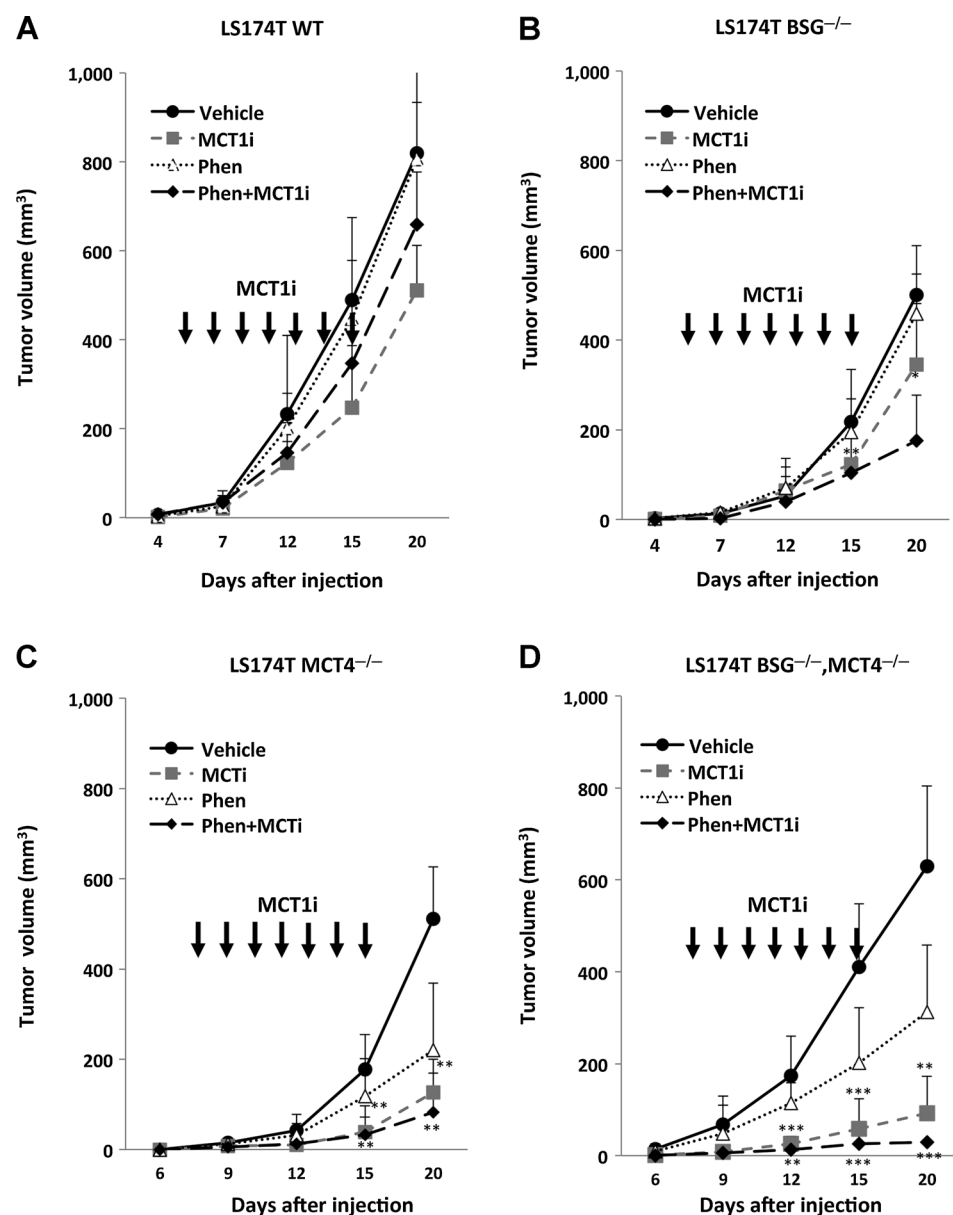
results pointed out the acute synthetic lethality achieved through the simultaneous block of lactic acid export and mitochondrial complex I.

Lactate export inhibition combined to phenformin suppresses xenograft tumor cell growth

To assess the efficacy of our combined strategy *in vivo*, LS174T-derived cell lines were injected subcutaneously to nude mice. After tumor appearance (>30 mm³), mice received 200 mg/kg/d phenformin in the drinking water, and/or, when specified, 100 mg/kg MCT1 inhibitor AZD3965 twice daily by oral gavage for 10 days. Interestingly, even if no relevant differences were seen *in vitro*, knockout of either MCT4, *BSG*, or both induced in untreated mice a modest (20%–25%) decrease in tumor growth (Fig. 5A–D). Comparing WT and *BSG*^{-/-} LS174T tumor growth, phenformin

Figure 5.

Inhibition of lactate export combined with phenformin suppresses xenograft tumor growth. A, the tumor volumes of nude mice injected subcutaneously with WT LS174T cells. B, tumor volumes of nude mice injected subcutaneously with *BSG*^{-/-} LS174T cells. At days 15 and 20, there were significant differences in tumor volume for vehicle versus phenformin/MCT1i. C, tumor volumes of nude mice injected subcutaneously with *MCT4*^{-/-} LS174T cells. At days 15 and 20, there were significant differences in the tumor volume for vehicle versus MCT1i and phenformin/MCT1i. D, tumor volumes of nude mice injected subcutaneously with *BSG*^{-/-},*MCT4*^{-/-} LS174T cells. At days 12, 15, and 20, there were significant differences in the tumor volume for vehicle versus MCT1i and phenformin/MCT1i. Statistical comparisons were performed using the Student *t* test (*, *P* < 0.05; **, *P* < 0.001; and ***, *P* < 0.0001).



treatment alone had no effect (Fig. 5A and B), suggesting, as shown in Fig. 3C, an *in vivo* hypoxic environment. In contrast and consistent with *in vitro* clonal growth (Supplementary Fig. S5C), phenformin alone reduced by 2-fold tumor growth of *MCT4*^{-/-} and *BSG*^{-/-}, *MCT4*^{-/-} tumors (Fig. 5C and D). Treatment with MCT1i alone affected tumor growth of the four tumor cell lines. The reduction in the tumor growth rate of WT and *BSG*^{-/-} tumors (2-fold) was stopped with halt in treatment (arrows, Fig. 5A and B). As expected, the strongest inhibitory effect of MCT1i alone was observed in the two *MCT4*-null cell lines: 75% and 86% reduction in tumor volume, respectively, for *MCT4*^{-/-} and *BSG*^{-/-}, *MCT4*^{-/-} tumors (Fig. 5C and D). Finally, combination of treatment (phenformin/MCT1i) reduced further the tumor volume to 84% and 96% when measured at 20 days in the two *MCT4*-null cell lines (Fig. 5C and D).

Taken together, these findings support the importance of combining blockers of lactate/H⁺ transporters (MCT1 and MCT4) with inhibitors of OXPHOS (phenformin) for a novel efficient anticancer strategy.

Discussion

In this study, we extended our previous work on the last step of glycolysis and further investigated via gene disruption the key role of MCTs/BSG complexes in controlling lactic acid export, bioenergetics, and tumor growth.

Ablation of MCTs induced inhibition of growth but not cell death

We showed that the growth of the two glycolytic cell lines, LS174 and U87, was not impaired by pharmacologic inhibition of MCT1 (MCT1i). The basal and hypoxia-induced expression of MCT4 was sufficient to ensure export of lactic acid and growth. Thus, it was clear from previous MCT knockdown studies that both MCT1/4 have to be suppressed for anticancer strategies, unless only MCT1 is expressed like in transformed fibroblasts (21) or neoplastic B cells (22). Interestingly *BSG*-knockout, as expected from Halestrap's work (26), severely reduced the MCT1/4 lactate transport activities but only minimally affected *in vitro* and *in vivo* growth of both cell lines. It is only when the full MCT activity was ablated, in *MCT4*-null cells treated with MCT1i that *in vitro* and *in vivo* growth was strongly reduced but not abolished (Figs. 3A and Fig. 5C). This strong inhibitory growth effect of MCT1i (300 nmol/L) raises the question of its specificity that we addressed previously with cells expressing only MCT1 or both MCT1/4 (21). We confirmed the specificity of MCT1i by showing that the residual growth rate seen in MCT1/MCT4 double knockout is not affected by MCT1i (data not shown). This tumor growth inhibition, however, is not due to cell death but due to cytostasis induced by mTORC1 inhibition in response to intracellular acidic stress (Fig. 3B), as we previously reported (34).

Inhibition of MCTs maintains cell survival by reactivating OXPHOS

BSG-disruption in LS174T and U87 cells impacted strongly on the plasma membrane expression of MCT1/4 thereby reducing lactate transport and inducing a 2-fold reduction in the rate of glycolysis and almost no change in the rate of growth. This minimal impact on growth was surprising until we realized reactivation of OXPHOS in *BSG*-null cells. Thus, treating *BSG*- or *MCT4*-null cells with MCT1i reduced further lactate export,

leading to substantial intracellular accumulation of lactate, pyruvate, and H⁺. This drastic metabolic and acidic stress inhibited further the glycolytic rate thereby mirroring a sharp increase in oxygen consumption. This metabolic plasticity, observed within minutes of addition of MCT1i, explains the cell survival by rapid maintenance of ATP levels. These findings contrasted with similar approaches that targeted MCT1 (22), MCT4 (36), or the upstream glycolytic step of LDHA (18, 20). In these three cases, cell death was reported to be associated with a decrease in ATP and an increase in oxidative stress. This was not the case for all the *MCT*-disrupted cell lines issued from LS174 even when associated with MCT1i treatment; ATP levels decreased no more than 20% and no reactive oxygen species (ROS) were detected (Fig. 4A and Supplementary Fig. S6A), a result apparently consistent with the antioxidant character of lactate (37). Nevertheless, this is an important issue that needs further exploration to fully understand resistance or vulnerability depending of the cancer model used.

How can we turn the cytostatic effect imposed by MCTs blockade into cell death? As ATP levels, rescued from OXPHOS, were due to activation of mitochondrial complex I (Fig. 2F and Supplementary Fig. S5B), the solution to the question came from associating phenformin with MCTs inhibition. The most remarkable point here is that only a 2-fold reduction in the glycolytic rate of *BSG*-null cells sensitized normoxic cells to death in response to phenformin or metformin. Note that these two biguanides, at their respective active concentrations (50 μmol/L, 1 mmol/L), had no detectable effect on growth and viability of WT cells (Fig. 3C, data not shown). Phenformin, alone or combined with MCT1i, reduced the ATP pool of *BSG*-null cells, respectively, by 60% and 80% in 24 hours. Loss of cell viability in either normoxia or hypoxia paralleled the rapid fall in ATP with no detectable apoptosis; this was acute necrotic cell death resulting from ATP crisis. Indeed combining phenformin with MCTs blockade induced ROS formation (Supplementary Fig. S6B), as reported by Cleveland's group (22), but cell death was not avoided by addition of N-acetyl-L-cysteine confirming cell death by "metabolic catastrophe."

Tumor lactate, a double-edged sword speaks for a MCT targeting strategy

Lactic acid generated from tumors is emerging as a major metabolite that impacts on the tumor microenvironment not only as a signaling molecule, but also as an acid stressor compromising immune surveillance (14, 38, 39). High levels of tumor lactate in cervical cancers (40) as well as high expression of MCT4, a major lactate transporter in breast cancers (17), are reported to be associated with a high frequency of metastases, tumor recurrence, and low survival. High lactate levels also signal "pseudo hypoxia" by increasing HIF1α stability (41), which promotes or enhances tumor hypoxia. We confirmed that four hypoxic markers, CA9, LDHA, PDK1 and VEGF-A, were further induced 3- to 8-fold in hypoxia by the increased pool of 40 mmol/L lactate (Supplementary Fig. S3E). Lactate as a signaling metabolite was also reported to promote M2 polarization of tumor-infiltrated macrophages (42). The second edged sword of lactate is its acidic function that together with carbonic acid (43) contributes to the extracellular acidic tumor microenvironment (14). Few reports have demonstrated the devastating impact of lactic acid on several immune cell function *in vitro* (44) and inhibition of cytolytic NK *in vivo* (45). Interestingly, the anergic state established at low pH (6–6.5) of

tumor-specific CD8(+) T lymphocytes could be reverted by restoring a physiologic pH (39).

Altogether, these protumoral features of lactate levels and acidic tumor microenvironment converge to raise optimism concerning an anticancer strategy that will reduce production and secretion of lactic acid. Here, we provided genetic evidence that targeting MCT1 and MCT4 reduced tumor growth and, if associated with the antidiabetic drugs phenformin/metformin (33), could, in a short tolerable therapeutic window, induce tumor cell death. Our optimism took off from our *in vitro* preliminary experiments on several cancer cell lines combining MCT1i and the novel specific MCT4 inhibitor in current development by AstraZeneca (46). Further experimentation with immune-competent mouse genetic cancer models and the MCT1/MCT4 inhibitors should demonstrate the benefit of this novel anticancer strategy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: I. Marchiq, J. Pouyssegur

Development of methodology: I. Marchiq, R. Le Floch, M.-P. Simon, J. Pouyssegur

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Genetic Disruption of Lactate/H⁺ Symporters (MCTs) and Their Subunit CD147/BASIGIN Sensitizes Glycolytic Tumor Cells to Phenformin

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Supplementary Data

Material & Methods:

Immunoblotting

Cells were lysed in 1.5x SDS sample buffer and the protein concentration determined using the BCA assay. 40µg of protein was separated on 8% SDS polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore). Blots were blocked in 5% non-fat milk in TN buffer (50mM Tris-HCl pH7.4, 150mM NaCl) and incubated with: mouse anti-human BSG (1:1000, MAB972 R&D Systems), mouse anti-human MCT1 (1:1000, SC-365501 Santa-Cruz Biotechnology), a rabbit anti-human/mouse MCT4 polyclonal antibody (1:1000, SC-50329 Santa-Cruz Biotechnology), rabbit anti-human phospho-p70 S6 Kinase polyclonal antibody (1:1000, Cell Signalling), and a rabbit anti-human/mouse HIF-1α polyclonal antibody (1:3000, antiserum 2087 produced in our laboratory). Detection of arrest-defective-1 protein (ARD1) (1) was used as loading control (1:30000). Immunoreactive bands were detected with horseradish peroxidase (HRP) anti-mouse or anti-rabbit antibodies (Promega) using the ECL system (Amersham Biosciences).

ZFN-Mediated Gene Knockout of the *MCT4* and *BSG* Genes

LS174T cells were transfected with the two Zing Fingers Nucleases (ZFN) designed by Sigma-Aldrich, as previously described, (2). Transfection of the ZFN (CSTZFN-1KT, CompoZr Custom ZFN) targeting the fifth transmembrane segment of MCT4 was performed with Lipofectamin 2000 (In vitrogen). Only clones (cl) with double allele mutations were selected phenotypically and confirmed by DNA sequencing (Fig. S1A).

For *BSG* knockout, LS174T-*MCT4*^{-/-} (cl199) and U87 cell lines were transfected with the ZFN designed by Sigma-Aldrich CKOZFN1227-1KT, CompoZr Custom ZFN) targeting exon 2 of *BSG* (Fig. S1B). Transfected cells were detected with a CD147 (MAB972, R&D Systems) primary antibody and with a PE-conjugated anti-mouse IgG (115-115-164, Jackson ImmunoResearch) secondary antibody as described (2). Several negative clones were detected by immunoblotting and only clones disrupted for the two alleles (DNA sequencing) were studied (cl18: *BSG*^{-/-} LS174T cells and cl12: *MCT4*^{-/-} *BSG*^{-/-} LS174T cells. Cl18 and cl39: *BSG*^{-/-} U87 cells).

[¹⁴C]-Lactate Uptake

LS174T cells (3x10⁵) seeded in 35-mm dishes were equilibrated for 5min in 20mM Mes-buffered glucose-free and HCO₃⁻-free saline solution containing 120mM NaCl, 5mM KCl, 2mM CaCl₂, 1mM MgCl₂, adjusted to pH 6.0 in the presence or in the absence of MCT1i (300nM, AR-C155858 Astra-Zeneca, UK). The transport activity of [¹⁴C]-L-lactate (NEC599050UC, Perkin Elmer) (3min time-point in triplicates) was determined as described (21). [¹⁴C]-L-lactate uptake was expressed as counts per million per mg of protein.

pHi Measurement

Cells (5x10⁵) were plated onto 35-mm dishes and incubated at 37°C, at 5% CO₂. After 24h, cells were washed with PBS and maintained in DMEM without glucose, pyruvate and serum for 1h with either DMSO (0.1% v/v) or MCT1i (300nM). 3h after addition of glucose

(25mM) and dialysed serum (10%), cells were incubated with 5 μ M of the fluorescent pH-sensitive dye BCECF-acetoxymethyl ester (BCECF/AM) for 5min at room temperature. Cells were then quickly rinsed with HEPES-buffered solution (120mM NaCl, 5mM KCl, 1mM MgCl₂, 15mM HEPES, 2mM CaCl₂, 5mM glucose, buffered at pH 7.4). The pHi was monitored using a Zeiss ICM 405 inverted microscope as described (3).

Intracellular Lactate and Pyruvate Assay

Lactate and pyruvate were measured using lactate and pyruvate assay kits (K607-100 and K609-100, Biovision) according to the manufacturer's instructions.

Cells (3x10⁵) were plated on 12-well plates and incubated in hypoxia. After 24h, cells were treated as described for pHi measurement. At the indicated times (0, 1h, 3h, and 6h) after addition of glucose (25mM) and dialysed serum (10%), cells were washed once with cold PBS, once with cold water and lysed with 100 μ L of cold water. 25 μ L of cell lysate was assayed in 96-well plates in triplicate to measure the lactate concentration.

Cells (1x10⁶ cells) were seeded in 60-mm dishes and treated as described for the determination of the intracellular lactate concentration. Cells were lysed with 350 μ L of cold water and 50 μ L of the cell lysate was used to measure the pyruvate concentration.

Clonogenicity Assay

LS174T and U87 cells, CCL39 fibroblasts and the CCL39-derived mutants (3000 cells) were plated in 60-mm dishes and incubated at 37°C, 5% CO₂. 24h after cell adherence, the medium was replaced with regular DMEM supplemented with 10% or 7.5% serum containing the MCT1i (300nM, Astra-Zeneca, UK), or phenformin (50 μ M, Sigma), or both, for 10d in normoxia or hypoxia. Dishes were stained with 5% Giemsa (Fluka) for 45min to visualize colonies.

ATP Determination & Cell Survival Assay

To assess the effect of the drugs on intracellular ATP levels, 1x10⁴ cells were seeded per well of a 96-well plate (4 wells per condition) and were treated with MCT1i (300nM) or phenformin (50 μ M) or both for 24h in normoxia or hypoxia. ATP levels were measured using a Cell-Titer-Glo kit (Promega) according to the manufacture's instructions. The relative luminescence units (RLU) were normalized to the quantity of protein, and the values are given as percentages.

For cell survival assay LS174T cells (1000 cells) were plated in 60-mm dishes and incubated at 37°C, 5% CO₂. 24h after cell adherence, cells were treated with MCT1i (300nM), and phenformin (50 μ M, Sigma). At the indicated times (24h, 48h, 72h, and 96h) after drug addition, cells were washed once with PBS and the washing buffer was centrifuged to recuperate floating cells. Fresh medium supplemented with 10% FBS was added and cells allowed to grow for 15 days in normoxia or hypoxia. Dishes were stained with 5% Giemsa (Fluka) for 45min to visualize colonies. Formed colonies were counted and the values are given as percentages.

Animal Studies

Food was given *ad libitum*. Mice were randomly divided into groups of 5 mice each.

Phenformin treated mice received 200mg/kg/day phenformin in the drinking water containing 5% sucrose (Sigma) for 10 days. Sucrose was added to make the drinking water palatable (4).

Phenformin powder was dissolved every two days in the drinking water, taking into account the mouse body weight and the volume of water consumed each day. Untreated mice (controls) received 5% sucrose in the water.

For the pharmacological inhibition of MCT1, mice were fed, twice per day, by oral gavage with 200 μ L of the vehicle (0.5% hydroxypropyl methyl cellulose/0.1% Tween 80), or with the MCT1 inhibitor AZD3965 (100mg/kg, Astra-Zeneca, UK) during 10 days after tumor apparition (>30mm³). Tumor dimensions were measured twice a week using callipers, and the tumor volume was determined by using the formula: $(4\pi/3) \times L/2 \times W/2 \times H/2$, (L, length; W, width; and H, height). When the tumor volume reached 1000mm³, mice were euthanized and the tumors collected.

RNA Extraction and Real-Time Quantitative PCR

Total RNA was extracted from the LS174T cell lines using the RNA extraction kit (Qiagen) according to the manufacturer's instructions. Total RNA (1 μ g) was added to a 20 μ L reverse transcription-PCR reaction using the Quantitect kit (Qiagen). The relative expression level of MCT1, MCT4, BSG, CA9, LDHA, PDK1 and VEGF-A was quantified by real-time quantitative PCR (qPCR), Ct values were normalized to *rplp0* Ct values and were expressed relative to the wild-type Ct values. The primers used were purchased from TaqMan and references are provided upon request.

Reactive Oxygen Species measurement and Rescue

LS174T cells (1x10⁶ cells) were treated with MCT1i (300nM), phenformin (50 μ M), or both for 24h. Cells were washed with PBS and incubated with 2',7'-dichlorofluorescein diacetate (DCFDA) (10 μ M, Sigma-Aldrich) for 30min at 37°C in the dark to measure H₂O₂. Cells were washed with PBS, trypsinized, centrifuged and resuspended in PBS 1% FBS. DCFDA was detected with a 488nm excitation laser of a flow cytometer (BD FACSCalibur) and data were analyzed with CellQuest Pro software.

For ROS rescue LS174T cells (3000 cells) were pre-treated with N-acetyl-L-cysteine (NAC) (10mM) for 1h and then treated with MCT1i (300nM) plus phenformin (50 μ M) as described (22). After 3d of treatment, cells were washed once with PBS, and the washing buffer was centrifuged to recuperate floating cells. Fresh medium supplemented with 10% FBS was added and cells allowed to grow for 15d in normoxia. Dishes were stained with 5% Giemsa (Fluka) for 45min to visualize colonies.

Supplementary Data

Supplementary Legendes Figures S1-S6:

Figure S1: ZFN-mediated *MCT4* and *BSG* gene knockout. (A) Schematic representation of the membrane organization of the lactic acid transporter MCT4 and its chaperon BSG/CD147. The two R-ZFN and L-ZFN plasmids were designed to recognize the fifth transmembrane domain of MCT4. The 5'-3' coding sequence targeted by the ZFN is shown below. The sequence where the Fok 1 nuclease cuts the double DNA strand is represented in the middle (small letters). The *MCT4*^{-/-} (cl199) and the *BSG*^{-/-} *MCT4*^{-/-} (cl12) LS174T cell lines have 14 and 31 nucleotides deleted in the two alleles, respectively. (B) The schematic represents the ZFN targeting exon 2 of *BSG* containing the signal peptide of the BSG-2 splice variant. Below are represented the sequences of the mutated alleles of LS174T *BSG*^{-/-} (cl18) (insertion of 4 nucleotides in the two alleles, capital letters), and LS174T *BSG*^{-/-} *MCT4*^{-/-} (cl12) (1 G deletion in one allele and a 4 nucleotide insertion in the other allele). Another ZFN (bis) targeting exon 7 of *BSG* containing the transmembrane domain of all BSG splice variants was used. This ZFN confirms that results obtained upon *BSG* knock out are not due to BSG-3 or BSG-4 that are lacking the exon 2 (data not shown).

Figure S2: Disruption of BSG, by zinc finger nucleases, reduces lactic acid transport, inhibits glycolysis and sensitizes U87 cells to the MCT1 inhibitor and phenformin. (A) WT and *BSG*^{-/-} U87 cells were maintained in normoxia or in hypoxia during 48h and lysed. The expression of HIF-1 α , MCT1, MCT4, and BSG was analyzed by immunoblotting. ARD1 was used as a loading control. (B) [¹⁴C]-Lactic acid uptake in the WT and the two *BSG*^{-/-} (cl8 and cl39) U87 cell lines. Discrimination of the transport activity of MCT1 and MCT4 was possible by using the MCT1i (300nM); **p<0.001, ***p<0.0001 and ###p<0.0001 (comparison of MCT1 and MCT4 activity, respectively, of each U87-mutant vs. U87 WT cells). (C) ECAR and (D) OCR of the WT and *BSG*^{-/-} U87 cell lines. The effect of addition of glucose (10mM) or MCT1i (300nM) are shown; *p<0.05, **p<0.001. (E) *In vitro* growth of U87 cells. WT and *BSG*^{-/-} U87 cells were treated with DMSO, MCT1i (300nM), phenformin (50 μ M), or MCT1i plus phenformin (from the left to the right panel). Proliferation units were calculated as described in figure 3A. The results are representative of at least three independent experiments.

Figure S3: Effect of BSG/MCTs complex disruption on BSG/MCTS mRNA expression and HIF-1 stability. Expression of the mRNA level of BSG (A), and MCT1 (B) in both WT and *MCT4*^{-/-} LS174T cell lines. Expression of the mRNA level of MCT4 (C) and MCT1 (D) in WT vs. *BSG*^{-/-} LS174T. (E) Expression of the mRNA level of CA9, LDHA, PDK1 and VEGF-A in both WT and *MCT4*^{-/-} LS174T cells treated with either vehicle (DMSO) or MCT1i (300nM) for 24h in hypoxia.

Figure S4: Glutamine-dependent and -independent rates of oxygen consumption are increased by BSG/MCTs disruption. The OCR of WT, *MCT4*^{-/-} and *BSG*^{-/-} LS174T cells incubated for 1h with 2 mM glutamine (A) or without glutamine (B). Glucose (10mM) and MCT1i (300nM) were added at the indicated times (arrows). The corresponding ECAR values for WT, *MCT4*^{-/-} and *BSG*^{-/-} LS174T cells incubated for 1h with 2mM glutamine (C) or without glutamine (D). The results are representative of three independent experiments.

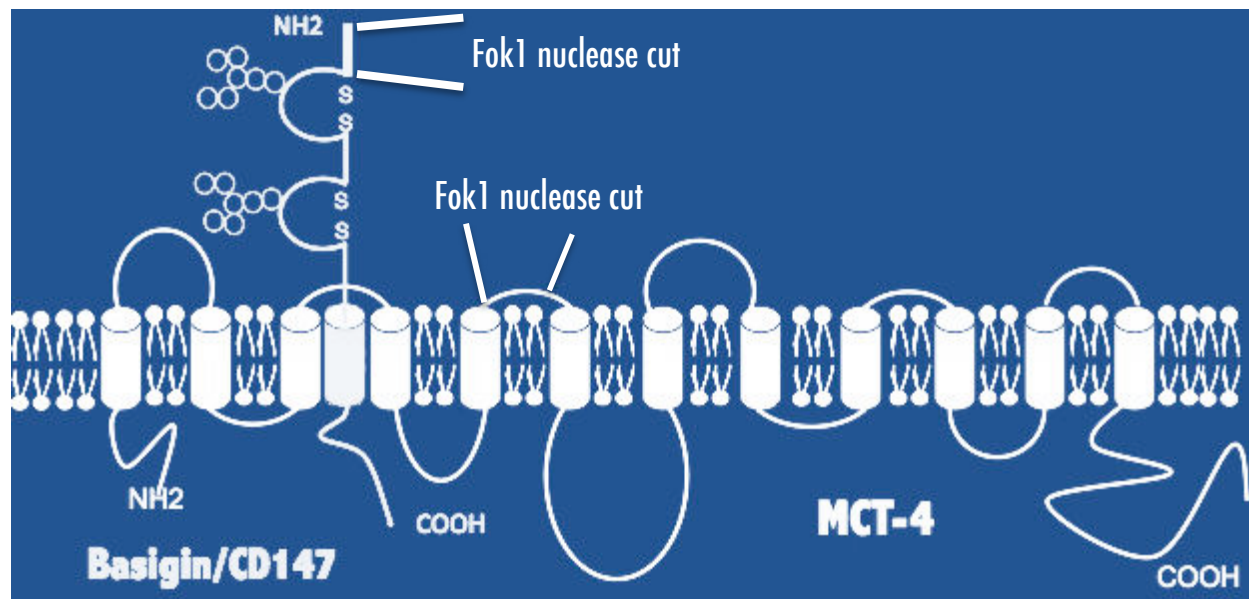
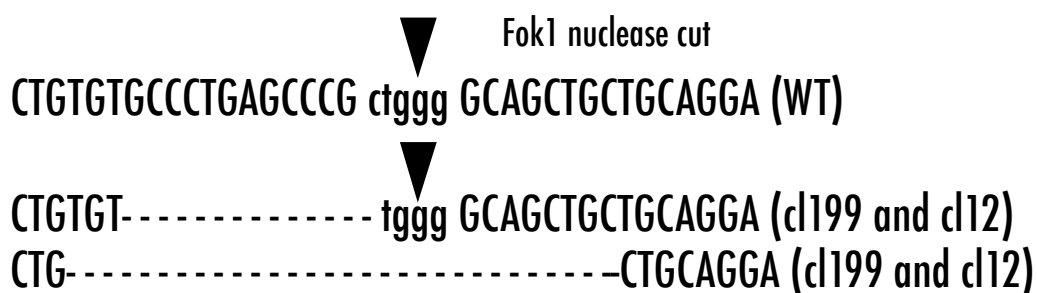
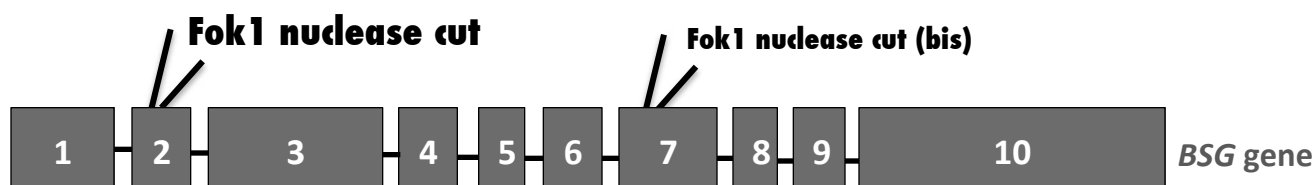
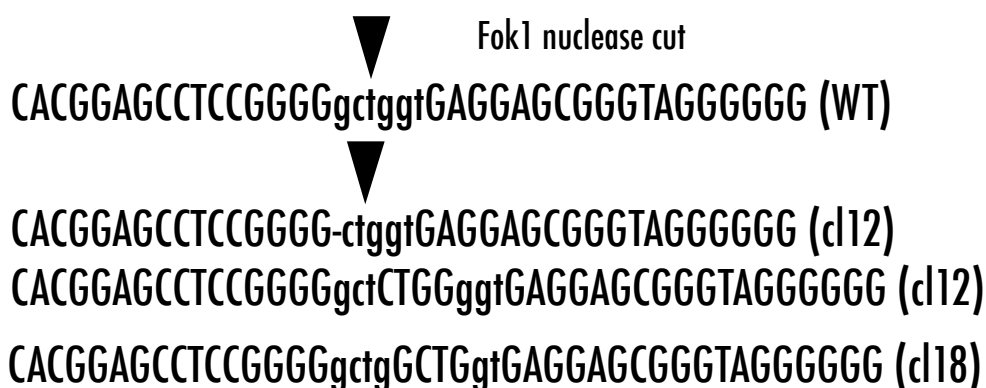
Figure S5: BSG/MCT complex knockout sensitizes cells to phenformin, a mitochondrial complex I inhibitor. (A) The ECAR and (B) the OCR of WT LS174T cells and their derived mutants in the presence of glucose (10mM) or phenformin (50μM for 2h) (n=10); *p<0.05, **p<0.001. (C) Clonal growth of the different LS174T cell lines in the presence of DMSO (0.1% v/v) or phenformin (50μM) in normoxia for 10d before staining and visualization of the colonies (results are representative of at least six different experiments). (D) the ECAR and (E) the OCR of Ras-transformed CCL39 fibroblasts (WT) and their mutants defective in either respiration (*res*−) or glycolysis (*gly*−) in response to glucose (10mM). (F) Clonogenic growth assay of the three fibroblast cell lines (CCL39-WT, *res*−, *gly*−) in response to DMSO or phenformin (50μM).

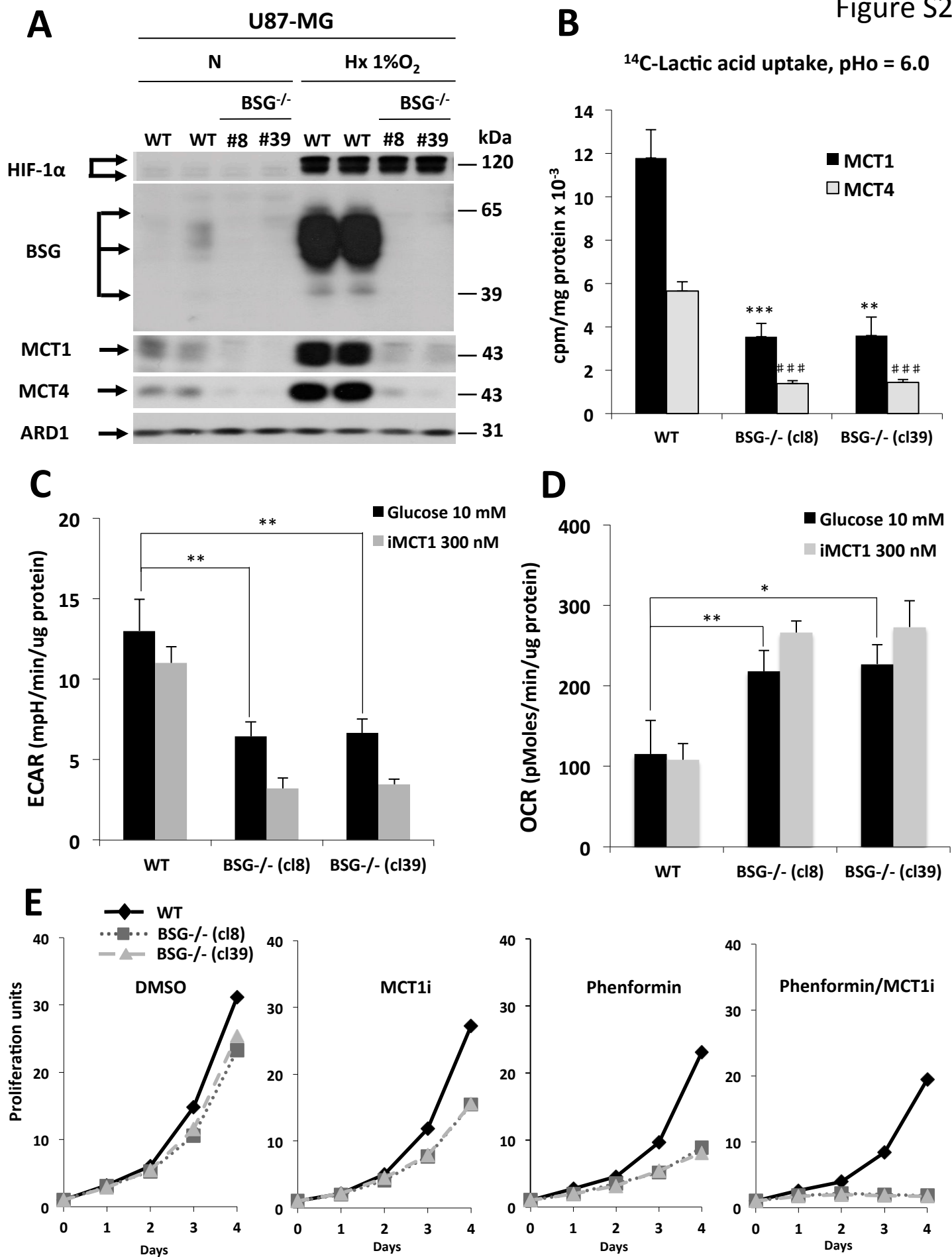
Figure S6: Phenformin/MCT1 inhibition induces an increase in the ROS content and ROS scavengers do not rescue cell death. (A) LS174T cell lines were treated with DMSO (dotted black line), or MCT1i (300nM) (gray line) for 24h and analyzed by flow cytometry for DCFDA. (B) The four LS174T cell lines were treated with vehicle (black line), or phenformin (50μM) (gray line), or both (red line) for 24h and analyzed by flow cytometry for DCFDA. (C) Cell survival by clonogenic assay. WT and mutant LS174T cell lines were pretreated with or without N-acetyl cysteine (NAC) (10mM) for 1h and then treated with DMSO or MCT1i (300nM) plus phenformin (50μM) for 3d (as described by Doherty et al. 2013). Cells were washed with PBS and fresh medium was added to allow cells to grow for 15d in normoxia. Dishes were stained with 5% Giemsa to visualize colonies.

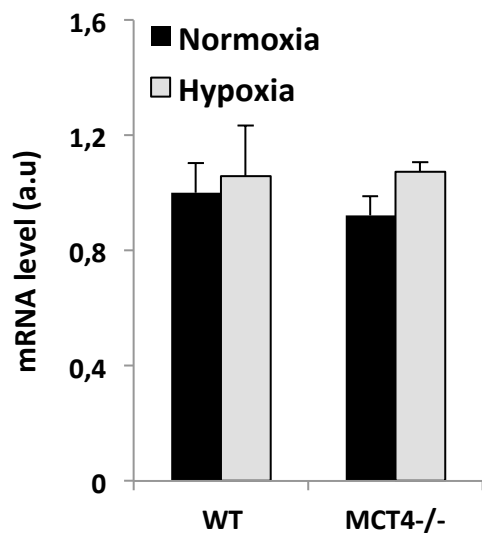
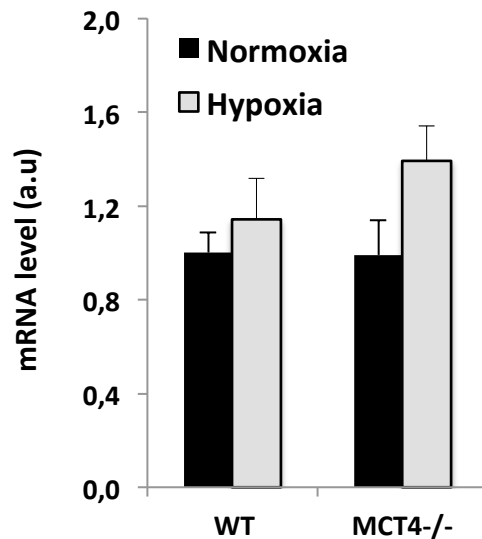
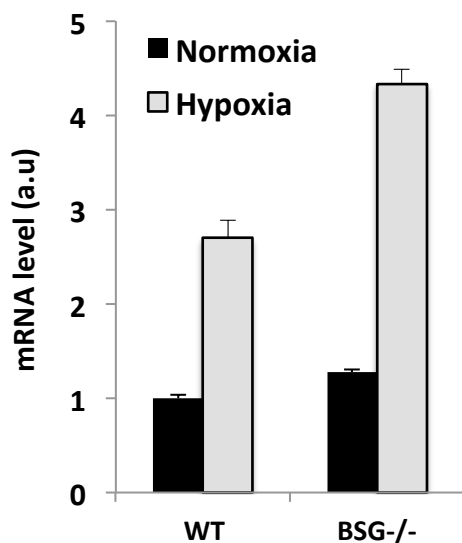
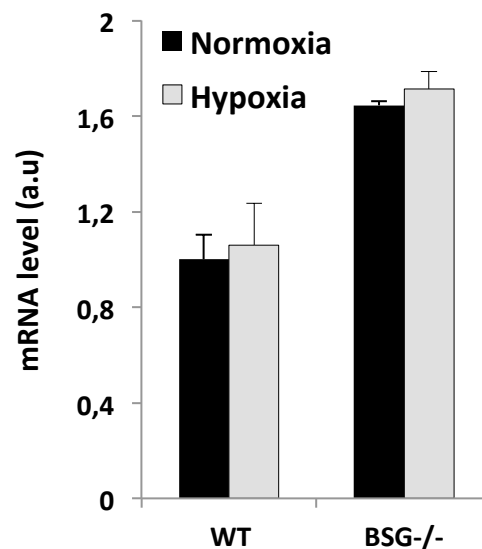
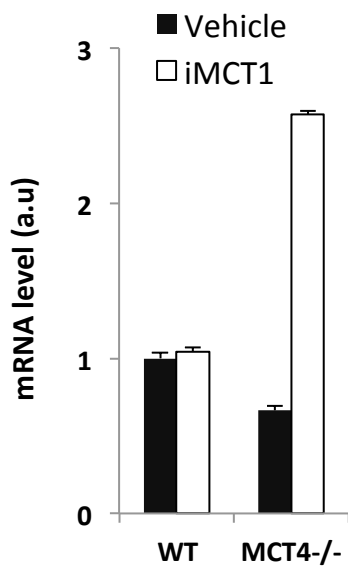
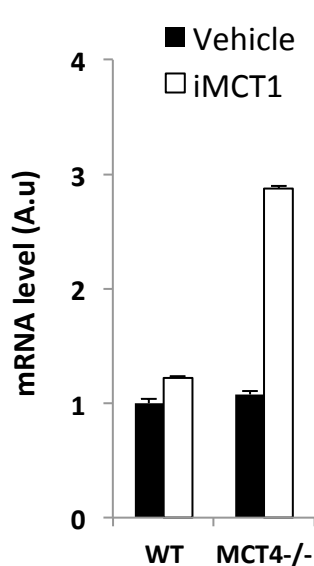
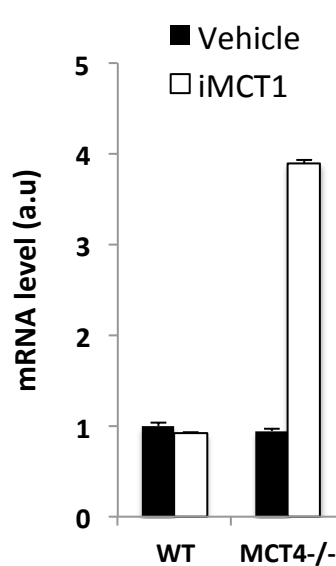
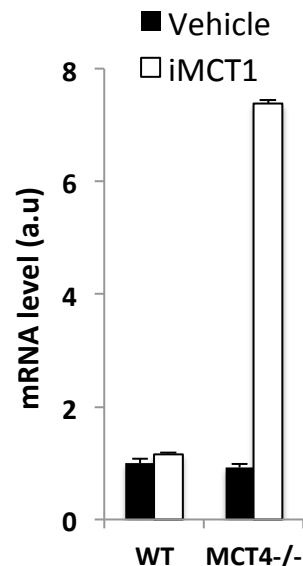
Supplementary References

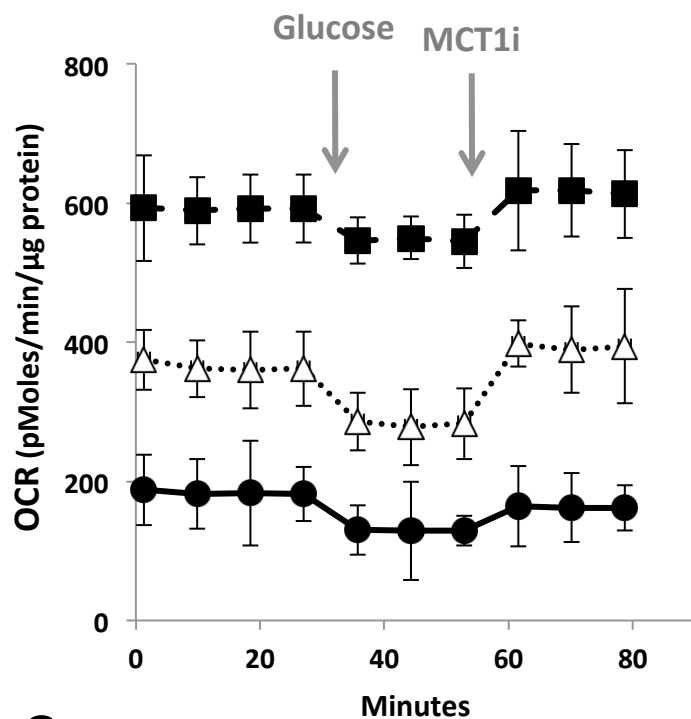
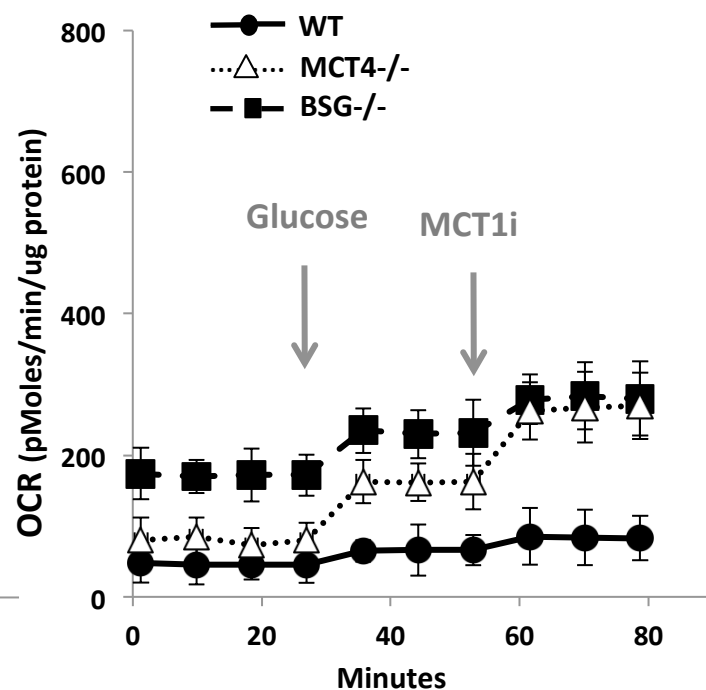
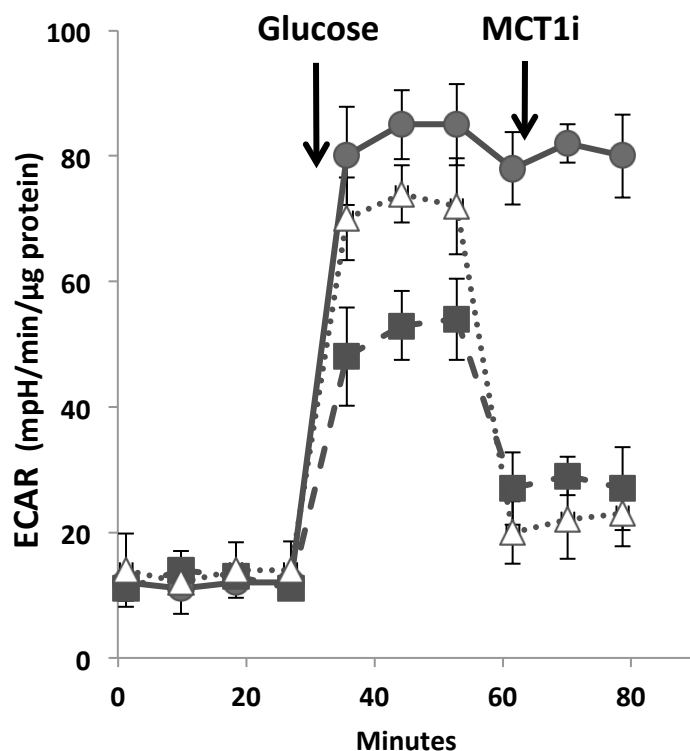
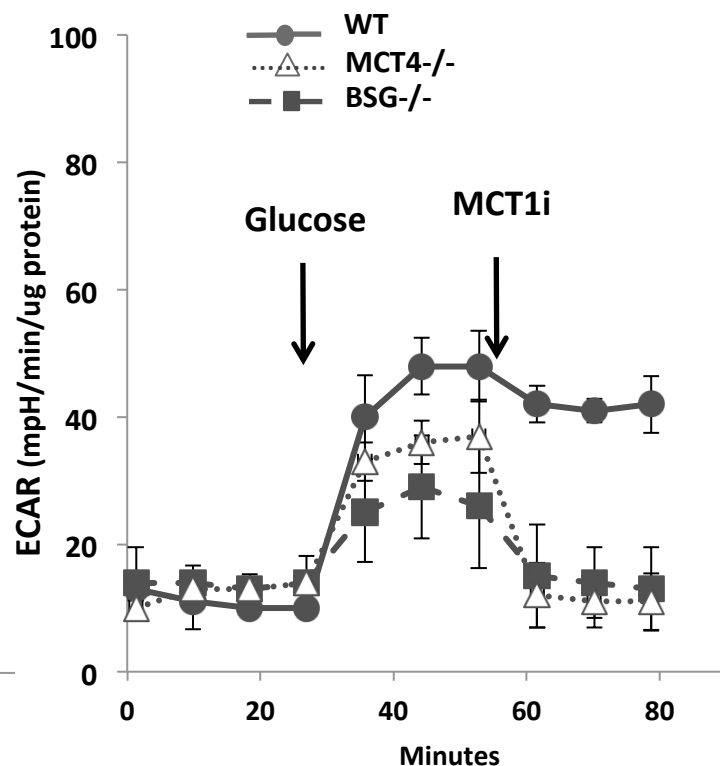
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Supplementary Figures S1-S6

A**ZFN MCT4****B****ZFN BASIGIN**

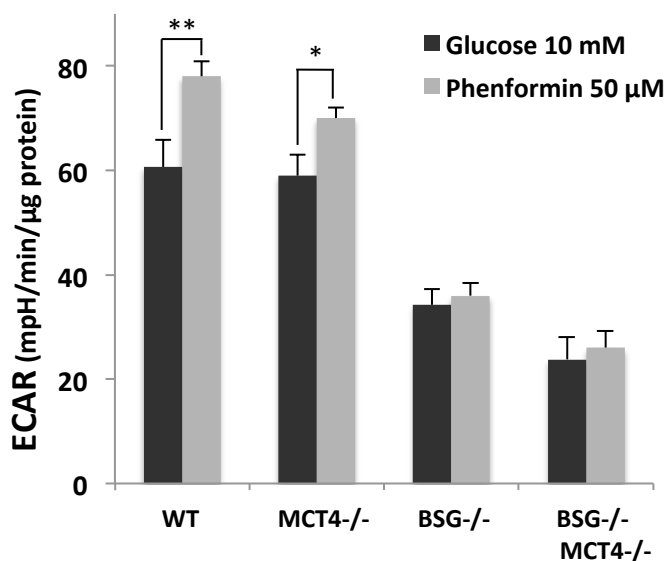


A **BASIGIN****B** **MCT1****C** **MCT4****D** **MCT1****E****CA9****LDHA****PDK1****VEGF-A**

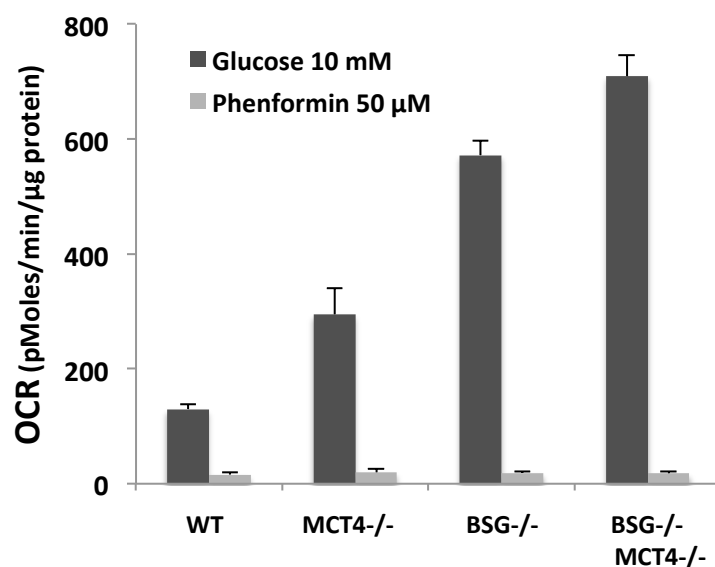
A**Glutamine (2 mM)****B****Glutamine (0 mM)****C****Glutamine (2 mM)****D****Glutamine (0 mM)**

A

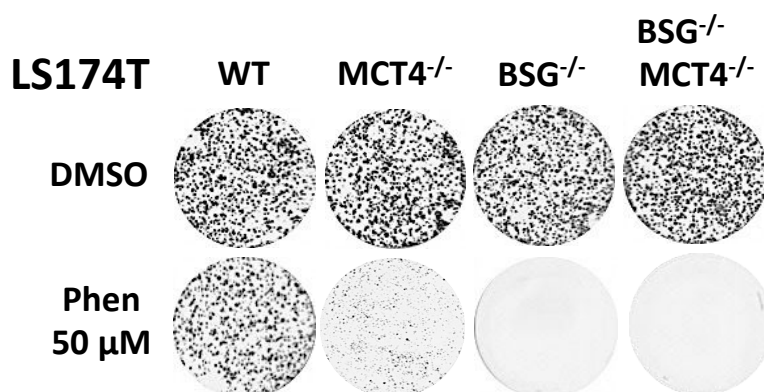
Rate of glycolysis

**B**

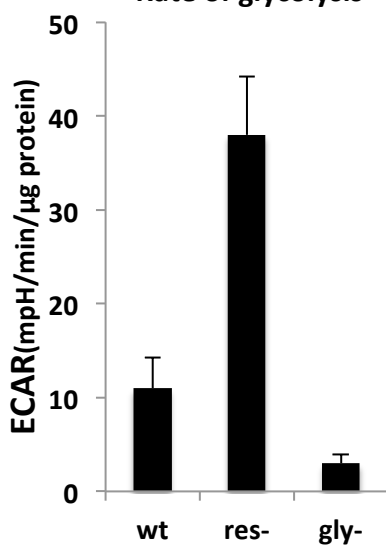
Rate of oxygen consumption

**C**

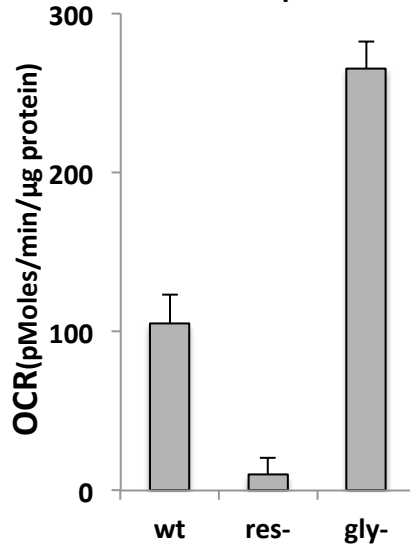
Normoxia

**D**

Rate of glycolysis

**E**

Rate of oxygen consumption

**F**

CCL39

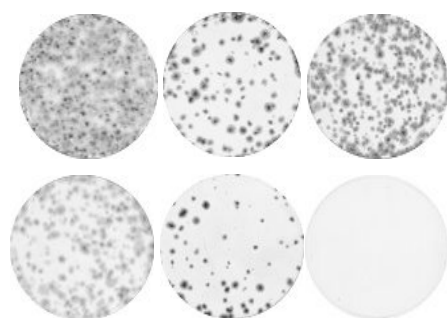
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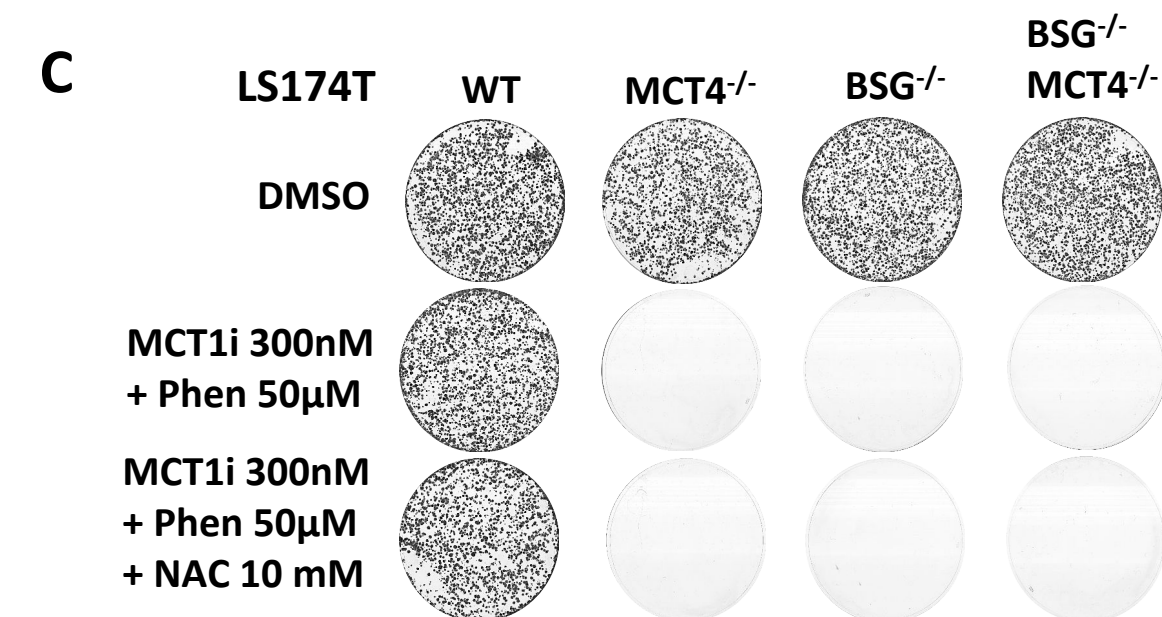
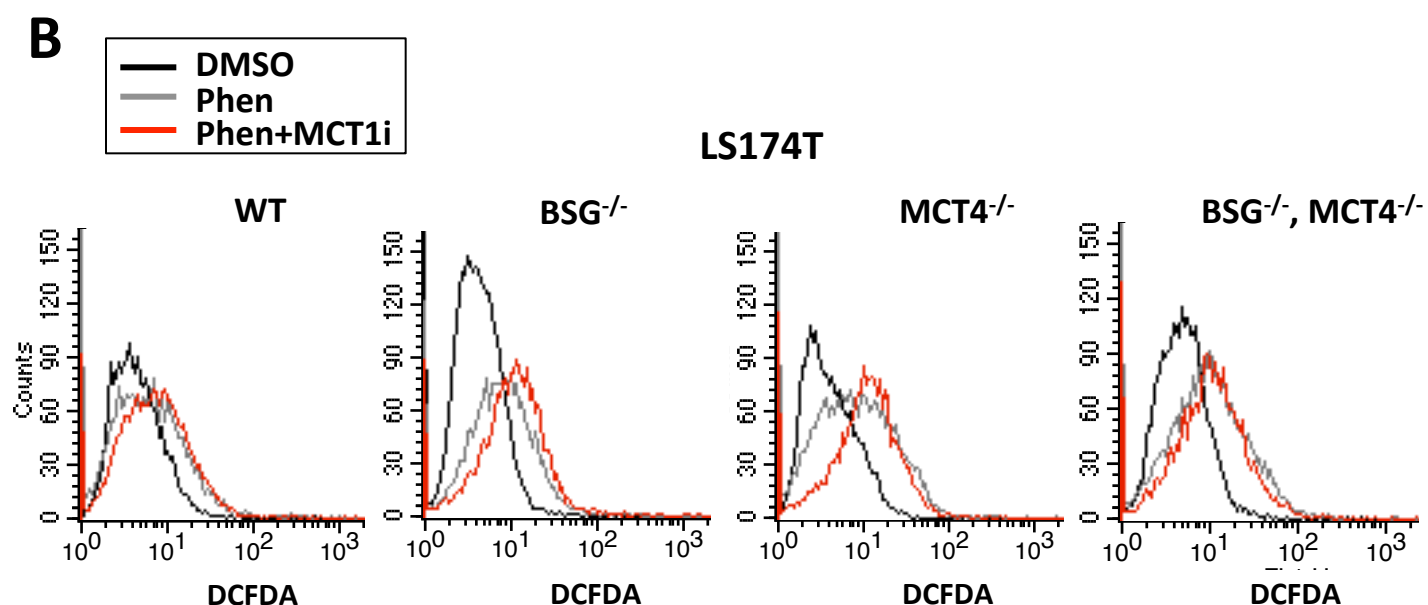
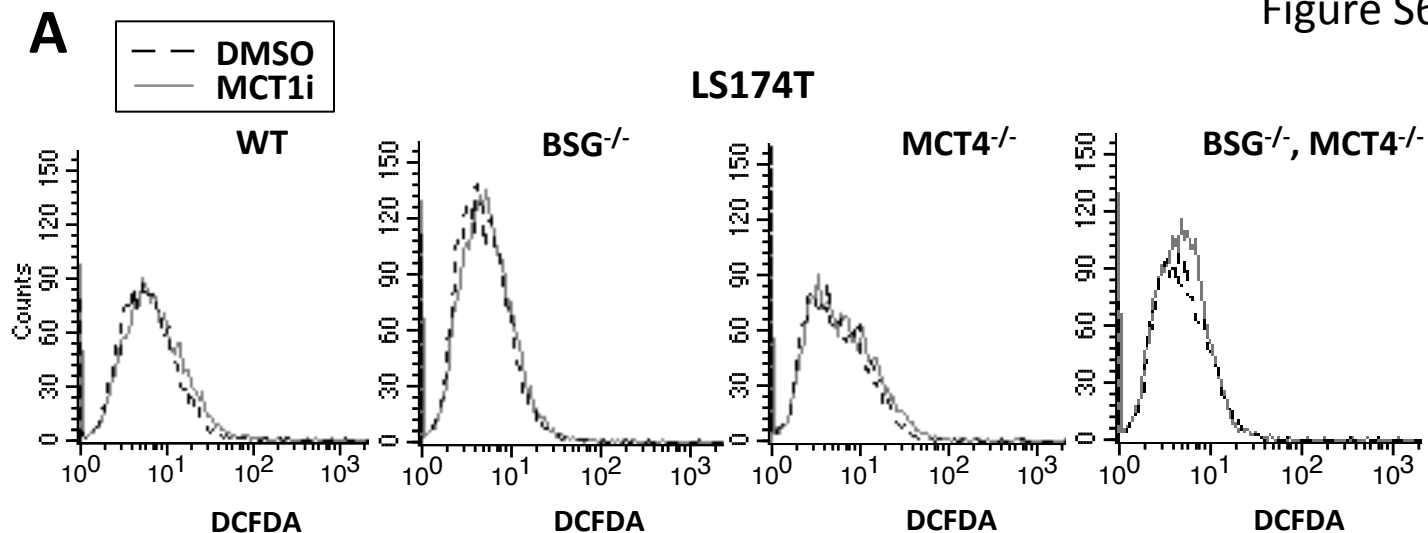
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Phen 50 μM





Disruption of BASIGIN decreases lactic acid export and sensitizes non-small cell lung cancer to biguanides independently of the LKB1 status

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ABSTRACT

Most cancers rely on aerobic glycolysis to generate energy and metabolic intermediates. To maintain a high glycolytic rate, cells must efficiently export lactic acid through the proton-coupled monocarboxylate transporters (MCT1/4). These transporters require a chaperone, CD147/BASIGIN (BSG) for trafficking to the plasma membrane and function.

To validate the key role of these transporters in lung cancer, we first analysed the expression of MCT1/4 and BSG in 50 non-small lung cancer (NSCLC) cases. These proteins were specifically upregulated in tumour tissues. We then disrupted BSG in three NSCLC cell lines (A549, H1975 and H292) via 'Zinc-Finger Nucleases'. The three homozygous *BSG*^{-/-} cell lines displayed a low MCT activity (10- to 5-fold reduction, for MCT1 and MCT4, respectively) compared to wild-type cells. Consequently, the rate of glycolysis, compared to the wild-type counterpart, was reduced by 2.0- to 3.5-fold, whereas the rate of respiration was stimulated in *BSG*^{-/-} cell lines. Both wild-type and *BSG*-null cells were extremely sensitive to the mitochondria inhibitor metformin/phenformin in normoxia. However, only *BSG*-null cells, independently of their LKB1 status, remained sensitive to biguanides in hypoxia *in vitro* and tumour growth in nude mice. Our results demonstrate that inhibiting glycolysis by targeting lactic acid export sensitizes NSCLC to phenformin.

INTRODUCTION

Non-small cell lung cancer (NSCLC) is one of the most common causes of death from cancer worldwide, accounting in 2009 for 30% and 26% of all male and female cancer deaths, respectively [34]. The overall 5-year survival rate of patients with metastatic disease remains

less than 15% [7]. Thus, a better understanding of the molecular mechanisms driving lung tumourigenesis is crucial and will provide enormous benefit in developing new pharmacological treatments.

Tumour cells are characterized by excessive aerobic glycolysis that is a hallmark of cancer cells [1, 31]. The high consumption of glucose and consequent high

production of lactate is a constant feature of most cancers, therefore high expression of glucose transporters and glycolytic enzymes is widely observed [2, 3, 8, 14, 24]. In addition, it is necessary to extrude the lactate produced to avoid lactic acidosis, which may act as a negative feedback mechanism to inhibit glycolysis [24, 35]. Monocarboxylate transporters (MCTs) constitute a family of 14 proton-linked plasma membrane transporters. Only MCT1 and MCT4 have been consistently shown to be upregulated in several human cancers [25] and to play an important role in maintaining glycolysis. For localization in the plasma membrane and for activity, these isoforms require a chaperone named CD147/BASIGIN (BSG) [15, 30, 38]

BSG, also known as EMMPRIN (Extracellular Matrix Metalloproteinase Inducer), is an evolutionary conserved protein that belongs to the immunoglobulin (Ig) superfamily. It is a widely distributed heavily glycosylated type I transmembrane glycoprotein [14, 15] and is expressed at high levels mainly in metabolically active cells such as lymphoblasts and malignant tumour cells [9, 37, 40]. BSG has been proposed to effect metalloproteinases (MMP) that degrade the extracellular matrix [37], tumour growth, chemoresistance [12, 13, 41, 42] and metastasis [22, 37]. However, the role of MCTs/BSG in lung cancer is controversial and poorly elucidated [16, 20, 27]. Therefore, we aimed to understand the role of MCTs/BSG in lactic acid extrusion, pH homeostasis and lung tumour growth. In this study we first observed that MCT1, MCT4 and BSG were overexpressed in human lung cancers when compared to adjacent non-tumour tissue. Therefore, we chose three NSCLC cell lines (A549, H1975 and H292) one of which was defective in LKB1 (A549) to analyse the impact of targeting the MCT activity on energy metabolism, on *in vitro* cell proliferation, and on *in vivo* tumour growth. We exploited RNA interference knockdown or Zinc Finger Nucleases knockout of a single gene, *BSG*, to reduce the activity of both MCT1 and MCT4. We report that the disruption of *BSG* decreased the expression and activity of MCT1 and MCT4, decreased the rate of glycolysis, increased the rate of respiration and sensitized the three *BSG*^{-/-} tumour cell lines to the inhibition of OXPHOS by metformin/phenformin (mitochondrial complex I inhibitors) *in vitro* and *in vivo*.

RESULTS

MCT expression in lung tumours

First, we analysed the expression of MCTs and BSG in a series of human lung cancer tissues. We studied 50 cases of non-small cell lung cancer (NSCLC). The series comprised squamous cell carcinoma tumours (n=8),

adenocarcinomas (n=29) and other types (n=13). All the cases had available adjacent non-tumour tissue that was also studied. We observed that MCT1, MCT4 and BSG were significantly overexpressed in tumour samples when compared to adjacent non-tumour tissue (Fig. 1, Table 1). According to the IARC, the most common histological types are squamous cell carcinomas and adenocarcinomas [36]. The levels of the three components of the lactate transport complex differed between the two histological types. MCT1 and MCT4 were expressed in all squamous cell carcinoma type cases, and BSG was expressed in 60% of the cases. MCT1, MCT4 and BSG were expressed in 20%, 30% and 40% of the cases of adenocarcinomas, respectively (Table 1).

Downregulation of BSG and MCT4 sensitizes A549 cells to oligomycin in normoxia

After confirming the expression of these biomarkers in lung tumours and taking into account the overlapping activity of both MCT1 and MCT4, we decided to evaluate the effect on growth of MCT4 and BSG silencing in A549 cells or of MCT1 pharmacological inhibition (AstraZeneca, iMCT1/2; AR-C155858). The silencing of MCT4, by shRNA, decreased MCT4 and BSG expression in hypoxia (Fig. 2A). Moreover, as expected, BSG silencing induced a parallel decrease in the expression of MCT1 and MCT4 in both normoxia and hypoxia (Fig. 2A). Silencing of MCT4 or BSG had only very modest effect on clonal growth in normoxia and hypoxia even in the presence of iMCT1/2 (Fig. 2B). Blockade of OXPHOS by oligomycin did not impact on the growth rate when the cells were cultured in hypoxia (1% O₂) (Fig. 2B, right panel). By contrast, cell growth was severely affected in normoxia by oligomycin, which was magnified in the presence of iMCT1/2 (Fig. 2B). This experiment demonstrated that hypoxic cells, in spite of appreciable silencing of MCT4 and MCT1 inhibition, remained resilient to growth inhibition by targeting glycolysis. Indeed, shRNA targeting of MCT4 did not totally abolish the activity of this transporter, and the residual expression might explain this hypoxic resistance to MCT1 and OXPHOS blockade. In the absence of a specific pharmacological inhibitor of MCT4, and taking into account the interdependency between MCTs and their chaperone, we decided to develop BSG-null cells to further explore the role of MCTs in targeting glycolysis and tumour growth.

Generation of *BSG*^{-/-} lung cancer cell lines using Zinc Finger Nucleases

First, we disrupted the *BASIGIN* (*BSG*) gene in three different non-small lung cancer cell lines (A549, H1975 and H292) by using Zinc Finger Nucleases. Knocked-out

Table 1: Expression of monocarboxylate transporters (MCT1 and MCT4) and BSG in non-tumour and lung tumour tissues.

Isoform	n	Plasma membrane expression	
		Positive (%)	<i>p</i>
MCT1			
Nontumoral	50	0 (0%)	<.001
SCC	8	8 (100%)	
AD	29	9 (31%)	
Other types	13	1 (7.7%)	
MCT4			
Nontumoral	50	0 (0%)	<.001
SCC	8	8 (100%)	
AD	29	15 (51.7%)	
Other types	13	2 (15.4%)	
BSG			
Nontumoral	50	0 (0%)	<.001
SCC	8	5 (62.5%)	
AD	29	16 (55.2%)	
Other types	13	2 (15.4%)	

SCC: Squamous Cell carcinoma, AD: Adenocarcinoma;
p association between non-tumour and tumour samples

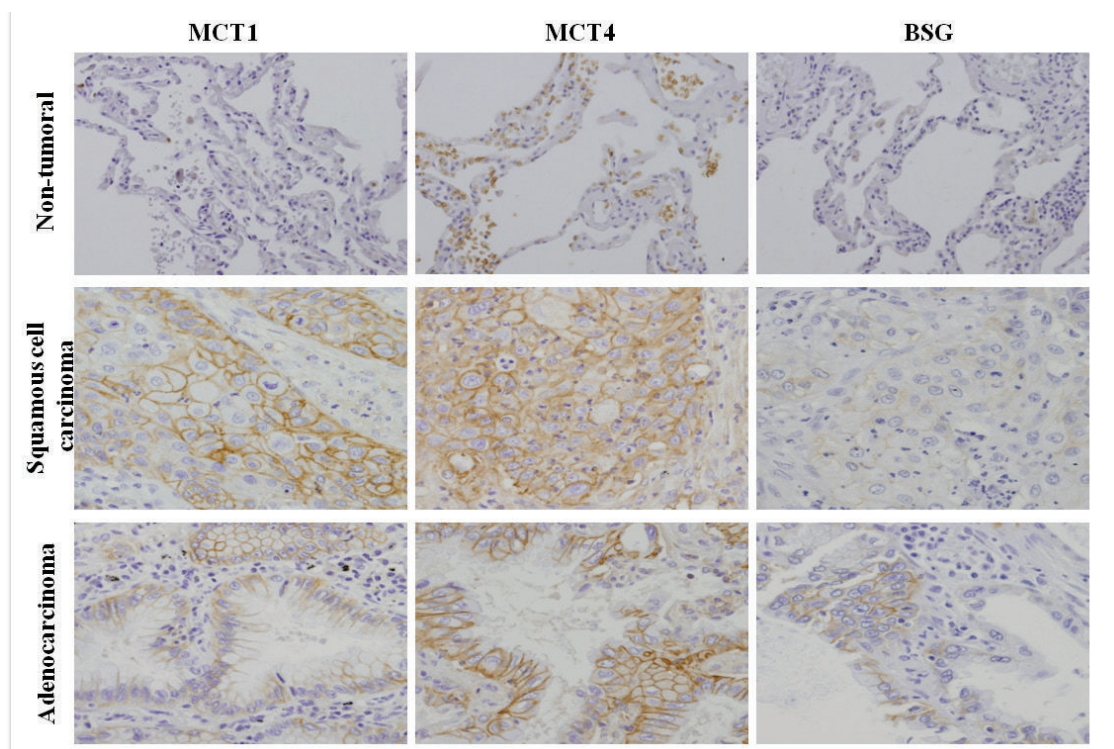


Figure 1: Immunohistochemical expression of the monocarboxylate transporters, MCT1 and MCT4, and their chaperone protein BSG in lung cancer samples. All markers were upregulated at the plasma membrane of tumour cells. Pictures were obtained using the microscope Olympus BX61, at 40 magnification.

candidates lacking expression of BSG (Figs. 3A, 4A) were identified and selected for further characterization. The site that was cut was amplified by PCR and the sequence and mutational independent status of the clones was confirmed by sequencing. Next, we analysed the expression of the MCT1 and MCT4 isoforms in normoxia and hypoxia 1% O₂ in these cells. Immunoblotting showed that the MCT1 protein was not detectable in *BSG*^{-/-} A549 cells compared to wild-type (wt) cells, while MCT4 expression was highly reduced. We also observed that the non-detectable MCT4 expression in normoxia in *BSG*^{-/-} cells remained inducible in hypoxia (Fig. 3A). Similar results were obtained for the H1975 (Fig. 4A) and H292 cell lines (data not shown). Knockout (KO) of the *BSG* gene in these cells induced a

huge decrease in the protein level of MCT1 and MCT4 in both normoxia and hypoxia.

Moreover, we demonstrated by qPCR a low level of transcript expression of BSG, but no evidence of changes in mRNA expression of MCT1, while MCT4 expression increased by 2-fold in both A549 *BSG*^{-/-} clones (Fig. 3B). This stability or even increase in MCT4 mRNA contrasted with the virtual loss of the corresponding gene product. We further assessed the impact of *BSG* disruption on the *in vitro* growth rates of the A549- (Fig. 3C, 3D) and H1975-derived cell lines (Fig. 4B) in normoxia. All *BSG*-null cells, excepting the *BSG*^{-/-} A549 clone (C153), displayed a normal growth rate compare to the parental cells (reduction of less than 5% for *BSG*^{-/-} cells).

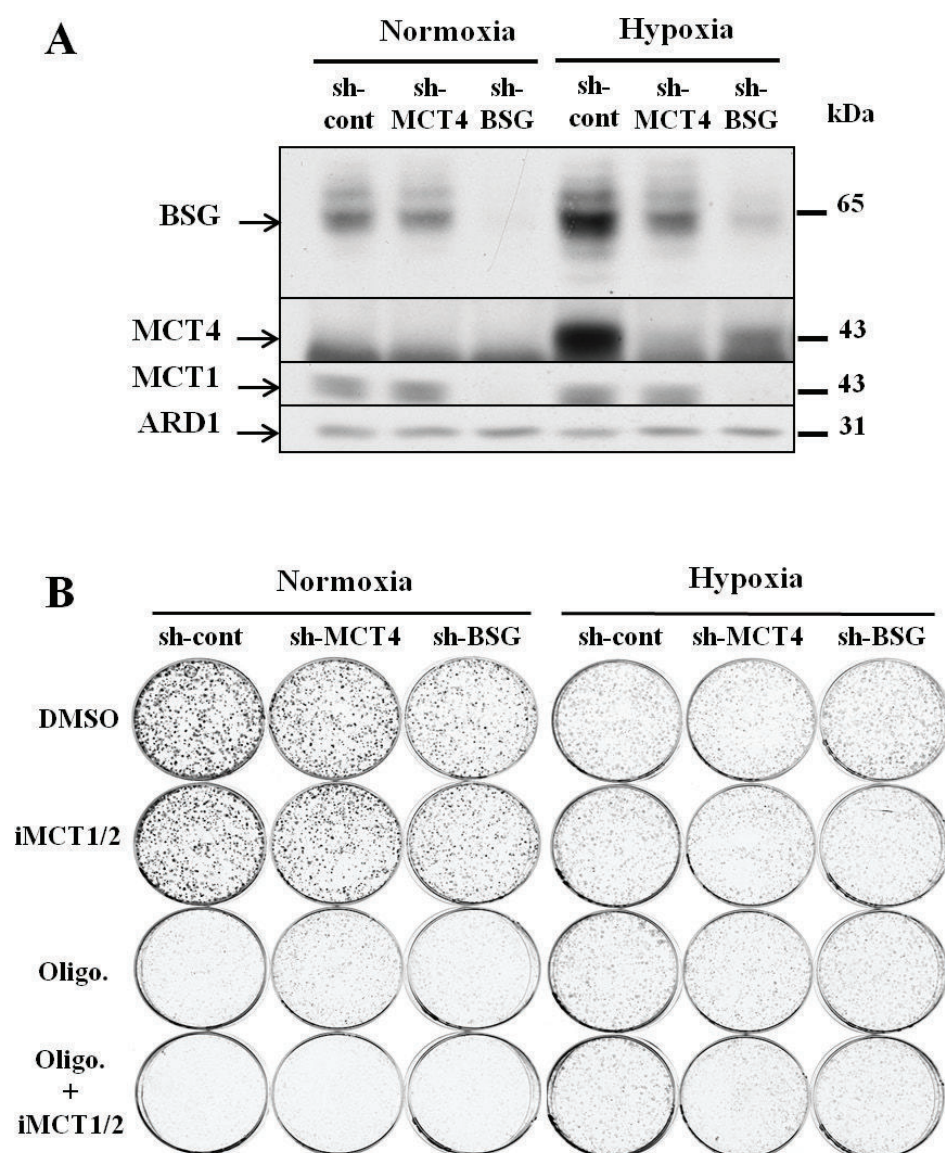


Figure 2: Downregulation of MCT4 and BSG. A: Immunoblot analysis of MCT1, MCT4 and BSG in cells transfected with either scrambled shRNA or shRNA targeting MCT4 and BSG and culture in normoxia (21% O₂) and hypoxia (1% O₂). ARD1 used as a loading control; B: Clonal growth in the absence or presence of oligomycin (1 µg/mL) or iMCT1/2 AR-C155858 (300nM) or both compounds either in normoxia and hypoxia for 8 days.

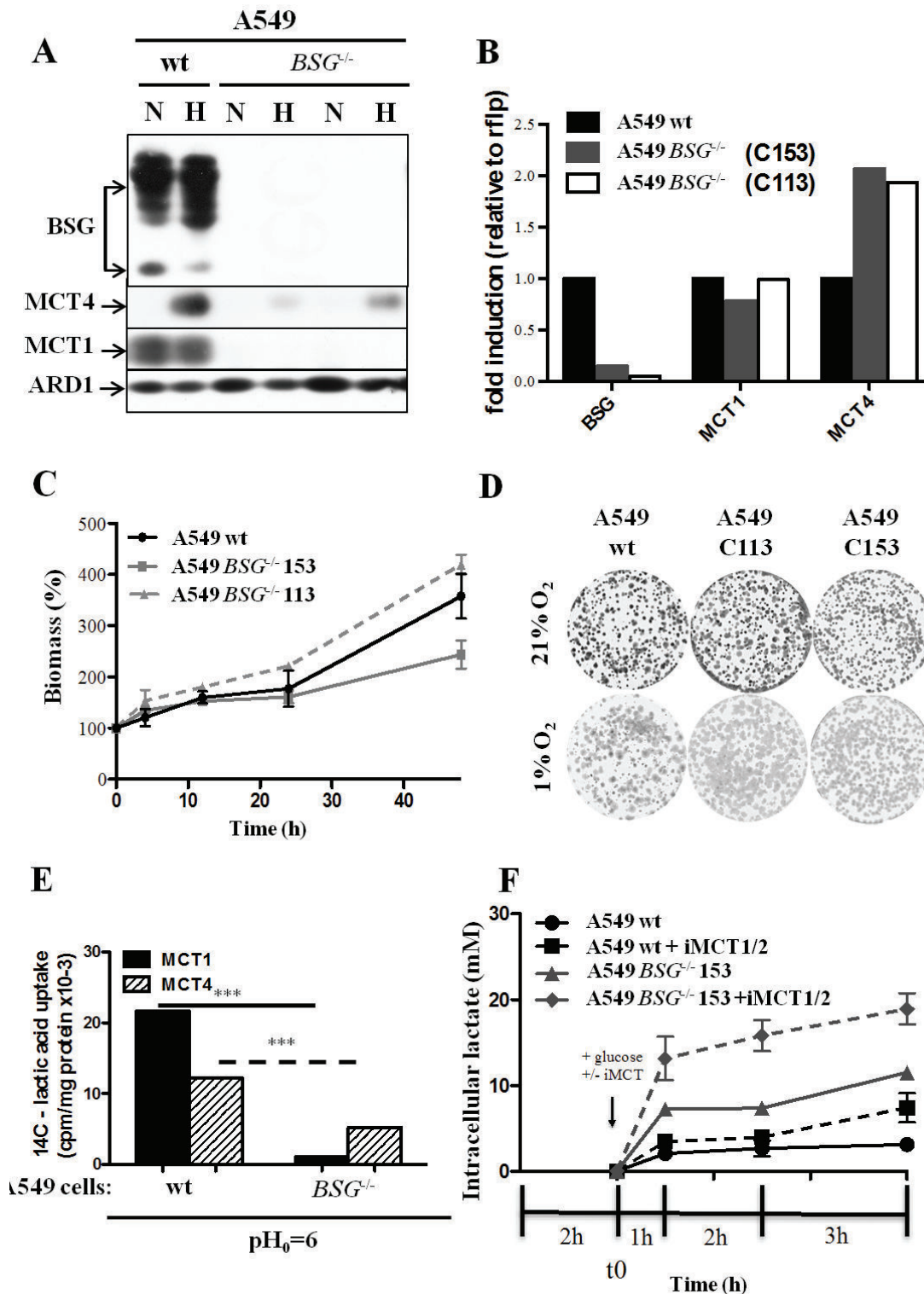


Figure 3: Effect of *BSG* disruption in lung carcinoma cell line A549. A: Immunoblot analysis of MCT1, MCT4 and BSG in wt and $BSG^{-/-}$ A549 cell lines maintained in normoxia (N) or hypoxia (1% O_2) (H) for 48h. ARD1 was used as a loading control; B: mRNA levels of MCT1, MCT4 and BSG in wt and $BSG^{-/-}$ cells (Clones C153 and C113) in hypoxia 1% O_2 ; C: Total biomass of wt and $BSG^{-/-}$ A549 (Clones C153 and C113) cells was evaluated over time using the sulphorhodamine B assay; D: Clonal growth of wt and $BSG^{-/-}$ cells (Clones C153 and C113) in normoxia (21% O_2) and hypoxia (1% O_2); E: [^{14}C]-Lactic acid uptake of wt and $BSG^{-/-}$ A549 cells in the absence or presence of iMCT1/2 (300nM) in hypoxia (1% O_2); F: Time-course of intracellular lactate concentration in response to glucose (25mM) added in the presence of either DMSO or iMCT1/2 (300nM). (***) $p < 0.0001$.

BSG gene disruption inhibits the MCT activity and increases the intracellular lactate pool

We quantified the membrane activity of both MCT1 and MCT4 by a direct measure of the initial rates of lactic acid transport in hypoxic A549 parental and *BSG*^{-/-} cells. The uptake of ¹⁴C-lactic acid was measured at a low external pH (6.0) to favour its entry in the absence or presence of the specific iMCT1/2 (Fig. 3E). Thus, we demonstrated that the ability of MCT1 to transport lactate was severely suppressed (>90%) in *BSG*^{-/-} cells, while that of the hypoxia-induced MCT4 is only decreased by 60% compared to parental cells. The transport activity of MCT1/4 was also evaluated by measuring the intracellular lactic acid pools (Fig. 3F). After 1h of glucose starvation, A549-derived cells rapidly accumulated intracellular lactic acid on addition of glucose to reach an equilibrium state within 3h. In accordance with the above experiments on

the MCT transport activity, *BSG*^{-/-} A549 cells accumulated a much higher level of intracellular lactic acid than parental cells. From 2-3mM lactate in parental cells to 11-12mM in *BSG*^{-/-} cells (Fig. 3F). Treatment of the cells with iMCT1/2 further increased the intracellular lactate pool of the *BSG*-null cells (20mM) whereas parental cells remained less sensitive.

In the highly glycolytic H1975 cell line, our results showed that the *BSG* disrupted cells accumulated higher levels of intracellular lactic acid (33mM), than the wild-type cells (3mM) (Fig. 4C) and the *BSG*^{-/-} A549 cells (Fig. 3F). However, these cells remained less sensitive to MCT1 inhibition due to the high decrease in MCT1 expression of *BSG* KO (Fig. 4A).

Taken together, these findings demonstrate that *BSG* plays a central role in controlling lactic acid export and therefore the rate of glycolysis of non-small lung cancer cells.

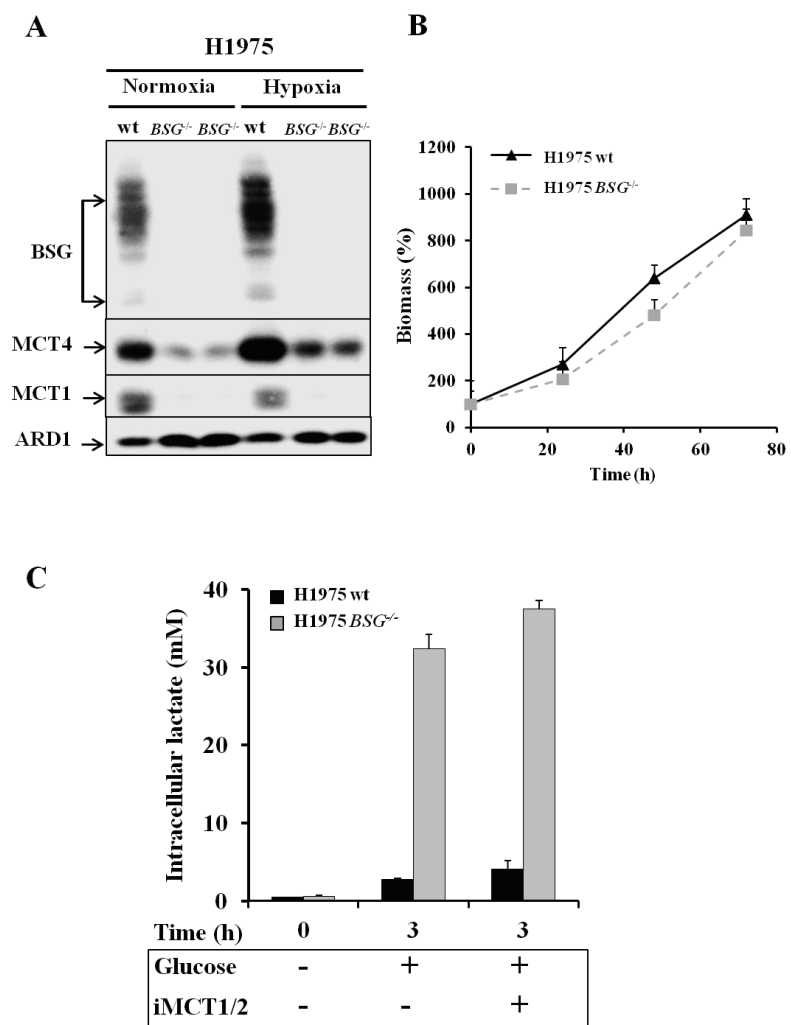


Figure 4: Effect of *BSG* disruption in lung carcinoma cell line H1975. A: Immunoblot analysis for MCT1, MCT4 and BSG in wt and *BSG*^{-/-} H1975 cell lines maintained in normoxia or hypoxia (1% O₂) for 48h. ARD1 was used as a loading control; B: Total biomass of wt and *BSG*^{-/-} H1975 cells was evaluated over time using the sulphorhodamine B assay; C: Time-course of the intracellular lactate concentration in response to glucose (25mM) added in the presence of either DMSO or iMCT1/2 (300nM).

Disruption of *BSG* decreases the rate of glycolysis and increases OXPHOS in different lung cancer cell lines

To further investigate the interplay between glycolysis and OXPHOS, under the conditions where lactic acid export was pharmacologically or genetically reduced, we used the Seahorse XF24 Analyser to measure the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of our wt and *BSG*^{-/-} cell lines. In the presence of glutamine (2mM) and glucose (10mM), *BSG*-null A549 cells showed a 2-fold reduction in ECAR compared to wt cells (Fig. 5A), whereas the more glycolytic cell line H1975 (ECAR=40 in H1975 and 10 in A549) showed a 3.4-fold reduction in ECAR upon *BSG* KO compared to parental cells (Fig. 5B). ECAR values

were further reduced (1.8-fold) in A549- and H1975-derived cell lines in the presence of iMCT1/2, reflecting inhibition of glycolysis by limiting lactic acid export (Fig. 5A, B). Addition of oligomycin or phenformin stimulated by 3- to 4-fold ECAR, respectively in wt and *BSG*^{-/-} A549 cells. However, the ECAR of wt and *BSG*^{-/-} H1975 cells was only increased by 1.3- to 1.4-fold, respectively (Fig. 5A, B). These results indicate that, in contrast to the H1975 cell line, A549 cells were moderately glycolytic as they only used one third of their glycolytic potential.

By contrast, the OCR values were high in both A549 and H1975 cell lines. These values were even increased in *BSG*-null cells compared to wt cells and further increased in response to MCT1 inhibition (Fig. 5C, D). Respiration was dramatically reduced in response to the mitochondrial complex I inhibitor phenformin (Fig. 5C, D).

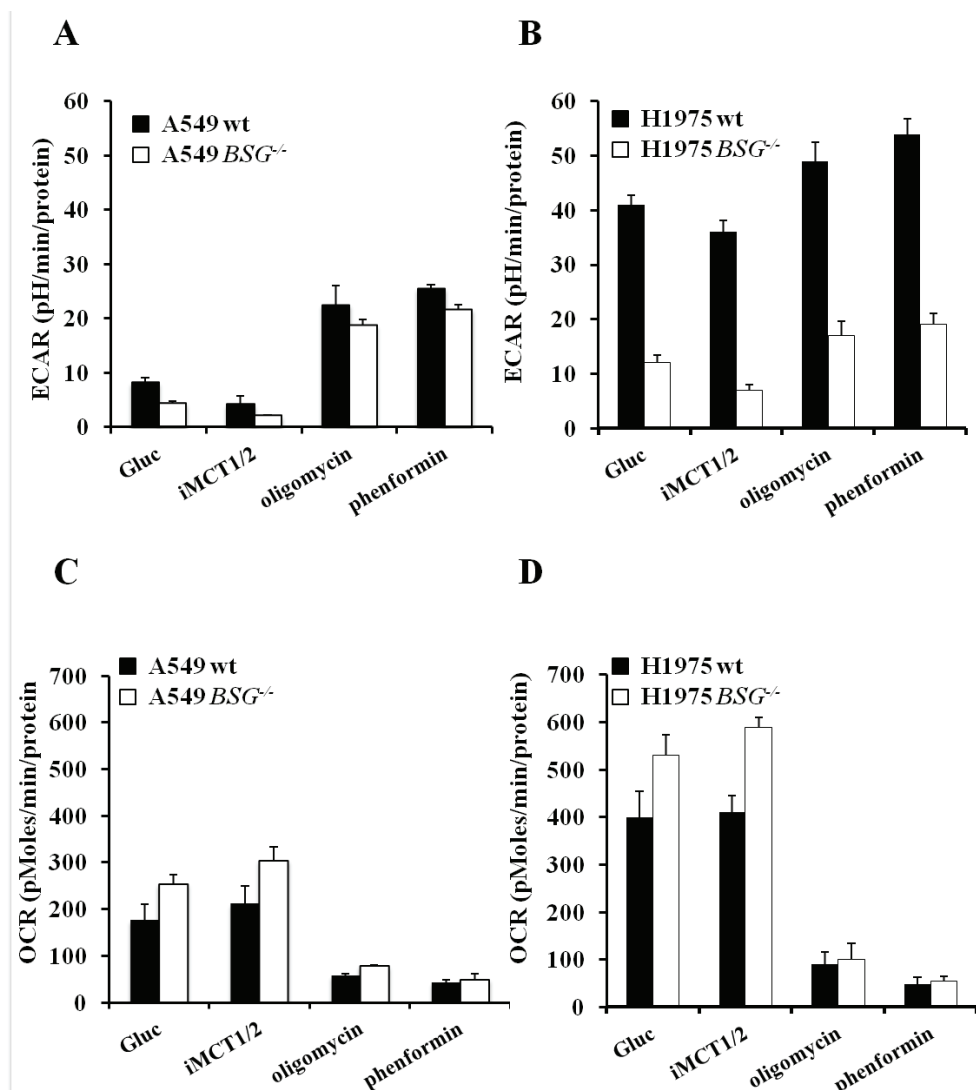


Figure 5: Analysis of bioenergetic pathways in lung carcinoma cells disrupted for *BSG*. A, B: Real time analysis of the extracellular acidification rate (ECAR) of wt and *BSG*^{-/-} A549 (A) or H1975 (B) cells using a Seahorse XF24 analyzer after injection of glucose (10mM), iMCT1/2 (1μM), oligomycin (1μg/mL) or phenformin (50μM) ; C, D: Real time analysis of the oxygen consumption rate (OCR) of wt and *BSG*^{-/-} cells A549 (C) or H1975 (D) using a Seahorse XF24 analyzer after injection of the compounds described above. Data represent the average of at least three independent experiments.

MCT inhibition sensitizes lung cancer cells to phenformin in hypoxia

Deletion of *BSG* did not affect *in vitro* clonal growth of either A549 or H1975 cells, even in the presence of iMCT1/2 (Fig. 6A, B). However, only wt A549 cells displayed an extremely high sensitivity to metformin/phenformin or oligomycin (not shown), while the more glycolytic H1975 cells remain insensitive (Fig. 6B). This is in agreement with the high oxidative/low glycolytic activity of A549 cells and perhaps also with the loss of LKB1 (see discussion) (Fig. 6A). However, in hypoxia, parental A549 cells lost sensitivity to OXPHOS inhibitors, which is consistent with optimal induction of glycolytic

enzymes through stabilization of the hypoxia-inducible factor (HIF). In sharp contrast to wt cells, *BSG*-null cells A549 and H1975 retained a high level of sensitivity to phenformin/metformin alone or in combination with iMCT1/2 (Fig. 6A, B). This striking difference between wt and *BSG*-null cells in hypoxia could easily be explained by the failure of *BSG*^{-/-} cells to express sufficient hypoxia-inducible MCT4 to sustain glycolysis (Figs. 3A, 4A).

These results were confirmed in another glycolytic non-small lung carcinoma cell line, H292, disrupted for the *BSG* gene (data not shown), highlighting the fact that targeting lactic acid export sensitizes lung carcinoma cell lines to biguanides and thus suppresses their growth in both normoxia and hypoxia.

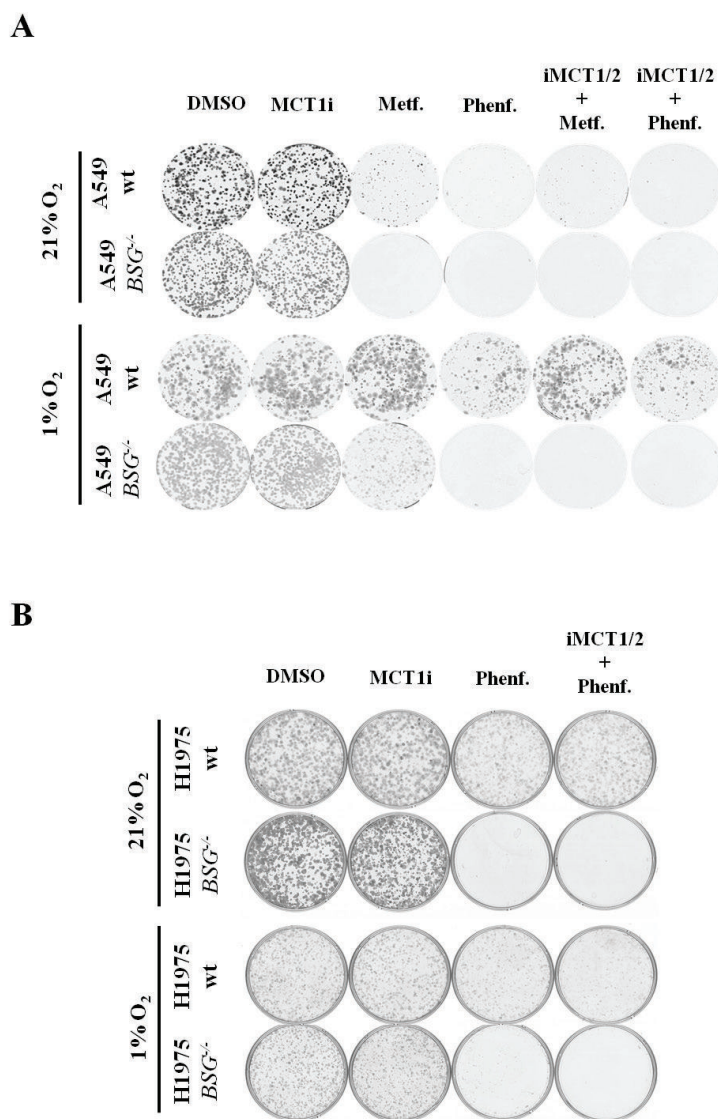


Figure 6: Disruption of *BSG* sensitizes lung carcinoma cells to phenformin in both normoxia and hypoxia. A: Clonal growth of wt and *BSG*^{-/-} A549 cells in the presence or absence of iMCT1/2 (300nM), metformin (Metf.-1mM), phenformin (Phenf. -50μM) or in combination. Cells were maintained in normoxia for 10 days (upper panel) and in hypoxia (1% O₂) for 15 days (lower panel); B: Clonal growth of wt and *BSG*^{-/-} H1975 cells in the presence or absence of iMCT1/2 (300nM), Phenformin (Phenf. -50μM) or in combination iMCT1/2. Cells were maintained in normoxia for 10 days (upper panel) and in hypoxia (1% O₂) for 12 days (lower panel).

Combination of inhibition of glycolysis and OXPHOS decreases tumour growth *in vivo*

To assess the effect of *BSG* disruption *in vivo*, we compared the tumorigenicity of wt and *BSG*^{-/-} A549 cells. Nude mice were s.c. injected with both cell populations. Surprisingly, *BSG*^{-/-} cells generated bigger tumours than wt cells (Fig. 7A, B-black lines). However, phenformin

greatly reduced the tumour burden in *BSG*^{-/-} cells than in wt cells (Fig. 7A, B). To confirm the expression levels of BSG and MCTs, tumours were collected at the end of the experiment and tested by immunohistochemistry. As expected, BSG and MCT1 expression was not detected in *BSG*^{-/-} tumors while MCT4 was present, although with a weaker expression. Moreover, treatment with phenformin did not alter the expression of BSG, MCT1 or MCT4. (Fig. 7C).

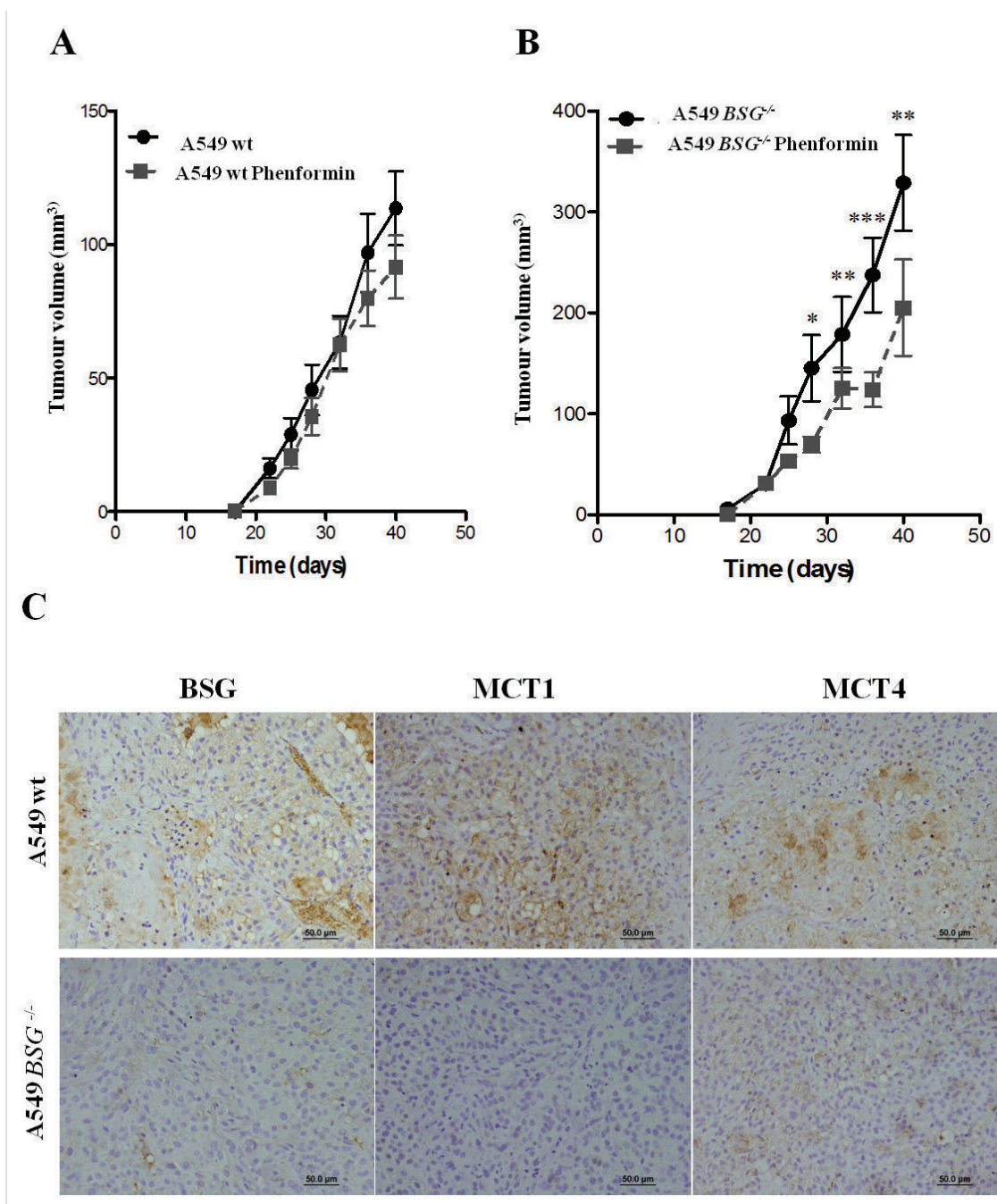


Figure 7: Xenograft tumour growth. Wt (A) and *BSG*^{-/-} cells (B) A549 cells were s.c. injected into the back of athymic nude mice. 5% sucrose with and without phenformin (200 mg/kg) was added to the drinking water for all the mice. Ten mice were studied per condition (*p<0.05, **p<0.001, ***p<0.0001); C: Immunohistochemical expression of MCT1, MCT4 and BSG in A549 wt and A549 *BSG*^{-/-} tumor samples. Pictures were obtained using the microscope Olympus.

DISCUSSION

Upregulation of glycolysis and adaptation to acidosis are key events in the transition from *in situ* to invasive cancer [11]. MCTs may play an important role through their involvement in exporting lactic acid [10]. MCTs have been described to be upregulated in several cancers [25]. However, expression in lung cancer is still controversial [16, 20, 27]. In the present study, we showed that MCTs are upregulated in lung tumour tissue when compared to adjacent non-tumour tissue. Moreover, we detected MCT1 and MCT4 in the plasma membrane of all the histological types studied. These results were in accordance with those of Koukourakis and co-workers [16]. However, they did not describe the cellular localization and histological type of the samples stained for MCT. Interestingly, we found that MCT1 and MCT4 were overexpressed in the squamous cell carcinoma subtype and MCT4 was overexpressed in all cases of adenocarcinoma subtypes, suggesting a different expression pattern of the histological types. However, due to the small number of cases, this study should be extended and replicated to obtain more solid statistically relevant data.

To understand the biological role of MCTs, we developed a strategy to inhibit their activity in a lung carcinoma cell line. For MCT1, we used the specific MCT1/2 inhibitor (AR-C155858; AstraZeneca) and found that it did not affect *in vitro* cell growth in either normoxia or hypoxia. In normoxia, A549 cells relied mainly on OXPHOS rather than on glycolysis [39], thus inhibiting MCT1, even with low expression of MCT4, did not affect cell clonogenicity. In hypoxia, in which OXPHOS was reduced and glycolysis enhanced, cells were able to survive and proliferate due to the presence of HIF-1-induced MCT4. Although, regulation of MCT expression is still not fully understood, one of the best characterized mechanisms of MCT regulation occurs through co-expression with BSG. As no pharmacological inhibitor of MCT4 is yet available and as previous published results [17] showed that the major pro-tumour action of BSG is mediated through bioenergetics, namely by controlling lactate transport, we decided to knockdown both MCT4 and BSG. As shRNA targeting of MCT4 and BSG failed to completely stop the growth of A549 cells in hypoxia, we decided to disrupt the *BSG* gene. We observed that KO of BSG resulted in a decrease in MCT1 and MCT4 expression. We observed that the mRNA level remained unchanged for MCT1 but increased 2-fold for MCT4 in the absence of BSG. As we previously reported for the LS174 colon cell line [17], BSG KO did not affect expression of MCTs at the transcriptional/translational levels but severely reduced the trafficking of MCTs from the endoplasmic reticulum (ER) to the plasma membrane [15]. In the absence of the chaperone (BSG), MCT1 and MCT4 were very unstable in the ER, which explained their

decreased expression as detected by immunoblotting (Fig. 3A) and reduced activity in lactic acid transport (Fig. 3E). Nevertheless, *BSG* gene disruption did not totally suppress the activity of MCT4 in these cells.

These results support the idea that MCTs may use an alternative chaperone for processing to the plasma membrane, as already suggested previously by our group [25, 27], or that MCTs alone could reach the plasma membrane but with a very low efficiency.

Targeting a metabolic pathway can be tricky due to the high plasticity of the metabolic networks of cancer cells. Thus, we decided to analyze the impact of disrupting BSG and OXPHOS on *in vitro* growth and tumourigenicity.

The anti-diabetic biguanide drugs metformin and phenformin used in the clinic have been shown to inhibit mitochondria complex I [6, 23], with no toxicity for metformin and little toxicity for phenformin. More recently, these biguanides have shown interesting new potential applications in oncology [5, 18, 19, 29, 32]. In this context, we report here that the three NSCLC parental cell lines (A549, H292, H1975) or their *BSG*^{-/-} derivatives were highly sensitive to metformin or phenformin for *in vitro* growth in normoxia as the cells derived their energy mainly from respiration (Fig. 6A, B and data not shown). These findings are in good agreement with the report of Wu and co-workers stating that A549 cells rely more on OXPHOS than on glycolysis [39]. However, phenformin, which induced substantially glycolysis (ECAR, Fig. 5A, B), did not rescue *in vitro* growth in normoxia (Fig. 6A). We believe that the low expression of MCT1 and MCT4, even in parental cells, precluded efficient glycolysis and therefore proliferation in normoxia. Another explanation could be related to the toxicity generated by an increase in reactive oxygen species (ROS) production as recently proposed by Shackelford and co-workers [32]. However, in hypoxia the three parental cell lines became insensitive to metformin/phenformin alone or in the presence of MCT1 inhibitor because MCT4 was highly expressed, thereby restoring glycolysis. By contrast, the *BSG*-null cells, and particularly in the presence of iMCT1, showed a high level of sensitivity to phenformin, which is consistent with ATP crisis or “metabolic catastrophe”, as discussed recently [8] and reported for colon adenocarcinoma and glioblastoma cells [19] (Fig. 6 A,B).

Only the *BSG*-null tumours were sensitive to phenformin (Fig. 7). The fact that parental cells were totally insensitive to phenformin, which contrasted with the *in vitro* data, may suggest that the tumour cells were exposed to a hypoxic microenvironment favouring glycolysis and /or production of a low level of ROS. Another explanation for the attenuated sensitivity to phenformin *in vivo* in both parental and *BSG*-null cells is that the concentration of phenformin delivered *in vivo* was lower than the concentration of 50µM used *in vitro*.

Moreover, Shackelford and co-workers showed

that inactivation of the suppressor gene *LKB1* dictated a therapeutic response of NSCLC to phenformin [6, 32]. In this context, they showed that cells with mutated *LKB1* (as in A549 cells) were more sensitive to phenformin and that this effect was dependent on AMPK activation. They hypothesized that without phosphorylation of AMPK through *LKB1*, these cells lacked the mitophagy induced by the protein *ULK1*, a downstream target of AMPK. Thus cells accumulated defective mitochondria and did not neutralize ROS that induced apoptosis. This is an interesting explanation, however our studies contradict this explanation since we found a high sensitivity to phenformin independently of the *LKB1* status. We propose instead that the level of sensitivity to phenformin is dictated by the glycolytic rate. In normoxia, the cell line with the lowest glycolytic rate (A549) was the most sensitive. A decrease in glycolysis by BSG disruption rendered the three cell lines equally sensitive to phenformin in normoxia and hypoxia.

In vitro studies of cells cultured in monolayers have given information about cell-autonomous metabolism, however they do not reflect the real physiological microenvironment in tumours. *In vivo* studies showed that BSG disruption did not reduce tumour growth as shown by other groups [4, 33]. In fact, tumour growth increased approximately 4-fold for *BSG*^{-/-} cells compared to wt cells. These results could reflect a clonal growth effect. However, *in vitro* studies showed that these KO cells had the same proliferation rate as wt cells. In addition we observed that phenformin drastically inhibited tumour growth, with a stronger impact on *BSG*^{-/-} cells compared to wt cells.

CONCLUSION

The results obtained by inhibiting the MCTs/BSG complex are promising. Nevertheless, we do not yet assume that MCTs are effective targets for cancer therapy. More effort is needed to prove that inhibition of metabolism, more specifically lactate transport, may be an alternative therapeutic strategy to use in the treatment of certain types of cancer. Therefore, the present work is an attempt to provide new data supporting the exploitation of MCTs/BSG as targets in lung cancer therapy.

MATERIALS AND METHODS

Tissue samples

Representative formalin-fixed paraffin-embedded samples from primary lung tumour tissues were retrieved from the archives of the Department of Pathology of Hospital S. João, Porto, Portugal. The tumours were classified according to the WHO criteria [36]. This

cohort included 50 cases of non-small cell lung cancer (NSCLC), 8 were squamous cell carcinomas (SCC), 29 adenocarcinomas (AC) and 13 from other types. Adjacent non-tumour tissue from each case was also selected.

Cells and culture conditions

The lung adenocarcinoma cell lines A549 (obtained from American Type Culture Collection, Manassas, VA, USA) and H1975 (kindly provided by Dr. Patrick Brest, Fr) were maintained in Dulbecco's modified eagle medium (DMEM, Gibco, Life Technologies Corporation) supplemented with 10% foetal calf serum (FCS – Gibco Life Technologies Corporation), penicillin (10units/mL) and streptomycin (10µg/mL), in a humidified atmosphere of 5% CO₂ at 37 °C. Incubation in hypoxia was carried out at 1% oxygen.

Immunohistochemistry

Sections of 3-µm were used for immunohistochemical (IHC) analysis. IHC for MCT1 and CD147/BASIGIN was performed according to the avidin-biotin-peroxidase methodology (R.T.U. Vectastin Elite ABC kit; Vector Laboratories Inc.), as previously described by our group [26]. MCT4 IHC was performed with the Ultravision Detection System Anti-polyvalent, HRP (Lab Vision Corporation, Thermo Fisher Scientific), as previously described [21, 28]. In brief, paraffin embedded sections were deparaffinized in xylene and hydrated in a graded series of ethanol solutions. For antigen retrieval, slides were incubated either with 1mM EDTA buffered solution, pH 8.0 (CD147) or citrate buffer, pH=6.0 (MCT1, MCT4) for 20 min in a water bath at 98°C. After endogenous peroxidase inactivation, incubation with the primary antibody was performed overnight for MCT1 and CD147 and for 2 h for MCT4 at room temperature. Tissues were stained with 3,3'-diaminobenzidine (DAB+ Substrate System, DakoCytomation, Dako, Agilent Technologies Company) and counterstained with haematoxylin. Colon carcinoma tissue was used as a positive control for MCT1, MCT4 and CD147. Stained slides were evaluated and then photographed under a bright field microscope. Sections were scored for plasma membrane expression following a semi-quantitative criterion. The score used was the sum of the percentage of positive cells (0, negative; 1, less than 5% positive cells; 2, 25% to 50% positive cells; and 3, more than 50% positive cells) and the staining intensity (0, negative; 1, weak; 2, moderate; 3, strong). Scores between 0 to 3 were classified as negative and 4 to 6 as positive [26].

Generation of *BSG*-null cells using Zinc Finger Nucleases (ZFNs)

To generate A549, H1975 and H292 KO cells for *BASIGIN*, two ZFN plasmids (designed by Sigma-Aldrich CKOZFN1227-1KT, CompoZr Custom ZFN) targeting *BASIGIN* exon 2 were transfected using JetPRIME® Transfection Reagent (Polyplus-transfection SA), according to the manufacturer's instructions. Transfected cells were detected by fluorescence-activated cell sorting (FACS), with a CD147 (MAB972, R&D Systems) primary antibody and with a PE-conjugated anti-mouse IgG (115-115-164, Jackson ImmunoResearch) secondary antibody. Negative cells for *BSG* were selected, sorted and cloned by dilution in 96 wells. The absence of *BSG* expression was confirmed by immunoblotting and only clones lacking the two alleles of *BSG* were analysed.

[¹⁴C]-lactate uptake

[¹⁴C]-lactate uptake was measured to determine lactate transport in A549 cells, as previously published [17]. The protein concentration was determined to normalize the radioactivity to the protein content of the cells in each dish. [¹⁴C]-L-lactate uptake was expressed as counts per million per mg of protein.

Intracellular lactate assay

A549 and H1975 cells (3x10⁵) were seeded in 12-well plates and allowed to adhere overnight. After incubation in hypoxia (1% O₂) for 24h, cells were washed twice and maintained in DMEM without glucose, pyruvate and serum for 1h with either DMSO or iMCT1/2 (300nM). DMEM 5% FCS with either DMSO or iMCT1/2 was added and cells were collected at the indicated times (0, 1, 3 and 6h). For determination of the intracellular lactate concentration, cells were washed once with cold PBS1x and cold water and lysed with 200μL cold water. 50μL of cell lysate was assayed in 96-well plates in triplicate to measure the lactate concentration using a lactate colorimetric assay kit (K607-100, Biovision Incorporated).

Immunoblotting

Cells were lysed in 1.5x SDS sample buffer and incubated for 15min at 95°C. Protein concentrations were determined using the BCA Assay. 40μg of protein was separated on 8% SDS polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore). Blots were blocked in 5% non-fat milk in TN buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl) and incubated overnight with the primary antibodies for CD147/*BSG* (1:500; MAB972, R&D Systems), MCT4 (1:1000; SC-

50329, Santa Cruz Biotechnology) and for MCT1 (1:3000; rabbit polyclonal antibodies against the C-terminal last 15 residues, prepared in the laboratory). The polyclonal antibody to arrest-defective-1 protein (ARD1) was used as loading control (1:30000). Bands were detected with the ECL system (Amersham Biosciences) after incubation of blots with secondary anti-mouse or anti-rabbit antibodies (Promega) coupled to horseradish peroxidase.

Clonogenicity assay

A549 and H1975 wt and *BSG*^{-/-} cells (2x10³) were seeded in 60-mm dishes and incubated for 24h to adhere. Medium was replaced with DMEM 10% FCS with and without iMCT1/2 AR-C155858 (300nM) or Phenformin (50M) or both for 10 days in normoxia and 15 days in hypoxia (1% O₂). Dishes were stained with 5% Giemsa (Fluka) for 45min to visualize colonies.

Metabolic analysis

The extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) were measured with a Seahorse XF analyser (Seahorse Bioscience, MA, USA). A549 cells (8x10⁴) and H1975 cells (1x10⁵) were seeded on seahorse plates and allowed to grow for 24h in normoxia. Prior to measurement, Plates were incubated for 45min in a non-CO₂ incubator at 37°C with seahorse medium without glucose, pyruvate, serum or bicarbonate.

10mM of glucose (Sigma), 300nM of iMCT1/2 (AstraZeneca, UK), 50μM of phenformin (Sigma) and 1μM of oligomycin (Sigma) were injected. After each addition, three data points of 3min were undertaken to determine the oxygen and proton concentrations in the medium. The protein concentration was determined to normalize OCR and ECAR values.

Tumour xenografts

Immunodeficient female nude mice with approximately 22g body weight were housed in groups of five, under specific pathogen-free, controlled ambient conditions. Animal studies were conducted according to Centre National de la Recherche Scientifique institutional guidelines (Ciepal n° NCE/-165). A549 wt and *BSG*^{-/-} cells (2x10⁶/mouse) suspended in 300μL of serum-free DMEM supplemented with insulin-transferrin-selenium (Gibco, Life Technologies Corporation) were sub-cutaneously injected into the backs of the mice. Water containing 5% sucrose with and without 200mg/Kg phenformin (Sigma Aldrich) was added to the drinking water, and the daily intake fluids were monitored. Sucrose was added to make the drinking water palatable (MVCL Appleyard

et al. 2012). Fresh phenformin was administered every two days. Tumours were measured every 2–3 days using a calliper and the volume was determined by using the formula: $(4\pi/3) \times L/2 \times W/2 \times H/2$, where L represents length, W the width, and H the height. Tumours were harvested at approximately 1 cm³, fixed and paraffin-embedded.

Statistics

For *in vitro* studies, the GraphPad prism 5 software was used, with the Student's *t*-test, considering significant values to be $p < 0.05$.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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C. Is BSG, also called EMMPRIN, really an inducer of MMPs?

PUBLICATION 4- Knock out of the BASIGIN/CD147 chaperone of Lactate/H⁺ symporters disproves its pro-tumour action *via* Extracellular Matrix Metalloproteases (MMPs) induction.

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As we reported before, the role of BSG in the process of cellular invasion and metastasis is extensively documented in the literature and is often linked to its ability to induce MMPs. However, all the publications are based mainly on correlations and a direct link between BSG and MMPs induction has not yet been clearly established. Moreover, with few exceptions, these studies ignore the role of BSG in controlling glycolytic metabolism by assisting MCTs at the plasma membrane. Therefore, we raised the question as to whether the pro-tumour action of BSG is really linked to MMP induction, or to its role in glucose metabolism and bioenergetics, or both.

As our previous publications (1, 2 and 3) have shown, loss of MCT function induces restricted tumour growth, independently of the BSG level. Furthermore, disruption of BSG in five different tumour cell lines did not have an impact on their growth since it redirected tumour cell bioenergetics from glycolysis to OXPHOS. These findings support a predominant role of the metabolic function of BSG in tumourigenicity. To confirm this, we aimed in this study to determine if there was any direct link between BSG expression and MMP induction. Unlike previous studies, which mainly used BSG overexpression, siRNA invalidation or forced expression with recombinant soluble BSG, we designed experiments using genetic knock out of the *BSG* gene in mouse embryonic fibroblast and three human tumour cell lines derived from glioma, colon, and lung adenocarcinoma. Using co-culture experiments of either human or mouse fibroblasts and tumour cell lines, we measured the expression or activity of several MMPs including, collagenases MMP1 and MMP13, stromelysins MMP3 and

MMP11, the membrane type (MT) 1-MMP, MMP14, and finally the most described gelatinases A and B MMP2 and MMP9. Our results showed that:

- Human tumour cell lines express different levels of MMPs in the extracellular media.
- The expression and activity of MMP2 and MMP9 was not affected either in co-cultures of tumour cells with fibroblasts or after stimulation of the later by conditioned media from tumour cells.
- In contrast to published data, genetic disruption of BSG does not affect MMPs production by cancer cells and fibroblasts.

Taken together, these findings indicate that BSG does not directly induce MMPs, as suggested extensively in the literature, and if such induction really exists, it could be indirect, possibly through its interaction with MCTs, as the key role of BSG in the control of fermentative glycolysis and tumour microenvironment acidification is well-established and seems to be important in tumour development.

Knock out of the BASIGIN/CD147 chaperone of lactate/H⁺ symporters disproves its pro-tumour action *via* extracellular matrix metalloproteases (MMPs) induction

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ABSTRACT

BASIGIN/CD147/EMMPRIN is a multifunctional transmembrane glycoprotein strongly expressed in tumours. BASIGIN controls tumour metabolism, particularly glycolysis by facilitating lactic acid export through the two monocarboxylate transporters MCT1 and hypoxia-inducible MCT4. However, before being recognized as a co-carrier of MCTs, BASIGIN was described as an inducer of extracellular matrix metalloproteases (MMPs). Early on, a model emerged in which, tumour cells use the extracellular domain of BASIGIN to recognize and stimulate neighbouring fibroblasts to produce MMPs. However, this model has remained hypothetical since a direct link between BASIGIN and MMPs production has not yet been clearly established. To validate the BASIGIN/MMP hypothesis, we developed BASIGIN knockouts in three human tumour cell lines derived from glioma, colon, and lung adenocarcinoma. By using co-culture experiments of either human or mouse fibroblasts and tumour cell lines we showed, contrary to what has been abundantly published, that the disruption of BASIGIN in tumour cells and in MEFs has no action on the production of MMPs. Our findings do not support the notion that the pro-tumoural action of BASIGIN is mediated *via* induction of MMPs. Therefore, we propose that to date, the strongest pro-tumoural action of BASIGIN is mediated through the control of fermentative glycolysis.

INTRODUCTION

BASIGIN/CD147 (BSG) is a highly glycosylated transmembrane protein belonging to the immunoglobulins (Ig) super family. The main isoform described (BSG-2) is composed of two (Ig-like) extracellular domains, a single membrane spanning segment followed by an intracellular domain. BASIGIN is described as a

multifunctional protein that could work in conjunction with several partners and therefore be involved in many cellular functions. At the physiological level, it plays a role in embryonic development (spermatogenesis, vision and smell), adhesion and the inflammatory processes [1-3]. Furthermore, BASIGIN plays an important role in regulating the energy metabolism of cells through its association with the MonoCarboxylate Transporters

(bidirectional lactate/H⁺ symporters), MCT1 and MCT4, [4-8]. Moreover, BASIGIN is involved in several diseases including inflammatory asthma, arthritis, and autoimmune pathologies such as HIV-1 infection, corona virus and hepatitis B and C [2]. Interestingly, BASIGIN was recently identified as the entry gate for *Plasmodium Falciparum* PfRh5, leading to the spread of malaria [2, 9], and for the meningococcal pilus component PilE and PilV [10]. BASIGIN is a highly conserved protein in evolution, is expressed in all cell types of metazoans, and is overexpressed in conjunction with MCTs in a variety of rapidly growing tumours. Furthermore, this BASIGIN/MCTs overexpression in cancers is directly correlated with a poor prognosis for survival [2, 3, 11, 12]. BASIGIN is involved in cancer at two main levels. First, we and others have demonstrated that BASIGIN acts in cancer progression by controlling the energy metabolism of glycolytic tumours *via* its tight association with lactic acid carriers MCT1 and MCT4 [13-16]. Nevertheless, before being recognized as a chaperone of MCTs, BASIGIN (alternatively named EMMPRIN for Extracellular Matrix MetalloProtease INducer) was reported to increase tumour growth and metastasis *via* its capacity to induce the expression of extra cellular matrix metalloproteases (MMPs) and to modify the tumour microenvironment [17-19]. This invasive capacity was also associated with the HIF1-mediated induction of VEGF and its receptor VEGFR2 [20-22]. However the mechanism by which BASIGIN mediates these actions is still unclear. A large number of observations suggest that BASIGIN works through its first extracellular Ig-like domain. Tumour cells would secrete molecules of soluble BASIGIN into the extracellular medium that are capable to induce the production of MMPs after homotypic interaction with surrounding fibroblasts [23]. Thus, BASIGIN could be directly involved in the regulation of tumour growth, invasion and metastasis by acting on both stromal and tumour cells, through the induction of angiogenic factors and proteases. During tumour progression, many interactions take place between cancer cells and the stroma, which constitutes their immediate environment. The stroma is mostly composed of capillaries, immune cells, fibroblasts and the extracellular matrix. Fibroblasts are the most abundant cells of the stroma, and a key cellular component of tumours. Around cancer cells, most of the fibroblasts acquire an activated status, and are known as carcinoma associated fibroblasts (CAF). The presence of CAFs is often associated with bad clinical prognosis, and a number of recent studies indicate that CAFs could play an important role in all steps of tumour progression, from initiation until metastasis [24-26]. Therefore, the notion that BASIGIN could serve as an inducer of MMPs is an attractive and interesting hypothesis in the context of tumour microenvironment and metastasis.

However, the model placing BASIGIN at the

centre of MMPs induction remains hypothetical. Links established between BASIGIN, MMPs and invasion are often indirect and the results obtained based on siRNA knockdown or forced expression and incubation with recombinant BASIGIN, are unconvincing. To gain insight into the role of BASIGIN in the tumour microenvironment, we genetically disrupted BASIGIN in three human tumour cell lines derived from glioma, colon, and lung adenocarcinoma using zinc finger nuclease (ZFN) technology. The effect of BASIGIN knockout on tumour microenvironment regulation, and in particular on fibroblasts activation, was tested *in vitro* on co-cultures of tumour cells lines (wild type *versus* BSG-null cells) with human or mouse embryonic fibroblasts. In these models, we focused on the expression of MMPs usually described to be associated with tumour invasion, and correlated with the expression of BASIGIN. Among these MMPs, we observed collagenases MMP1 and MMP13, stromelysins MMP3 and MMP11, the membrane type (MT) 1-MMP, MMP14, and finally the most described gelatinases A and B MMP2 and MMP9 [27, 28]. However, in contradiction with the abundant literature, our findings do not support the model of BASIGIN/EMMPRIN as a key player in tumour invasion *via* MMP induction.

RESULTS

ZFN- mediated gene knockout of BSG isoform 2

We knocked out the *BASIGIN* gene using ZFN technology in LS174T, U87 and A549 cells. Several clones were obtained for all cell lines (Table 1). Among them, we selected LS174T *BSG*^{-/-} cl.18, U87 *BSG*^{-/-} cl.8 and cl.39 and A549 *BSG*^{-/-} cl.153. BSG-2 isoform knockout (Figure 1A) caused the total loss of the two forms of the protein (highly glycosylated and low non mature molecular weight) in the three cell lines (Figure 1B). Clonogenicity and growth rate analysis (data not shown) of LS174T, U87, and A549 wt *versus* *BSG*^{-/-} cells showed that BASIGIN disruption had moderate if any effect on clonal growth [16]. This result was surprising in light of shRNA effects previously reported in the literature [13, 14]. Thus we isolated independent BSG-null clones by targeting exon 7 of the *BSG* gene (Figure 1A). This exon contains the site previously chosen for shRNA and is common to all BASIGIN spliced isoforms [29]. The growth phenotype, metabolic profile and MCT expression of these new LS174 BSG-null clones were identical to those obtained by targeting *BSG* exon 2 (data not shown). This finding confirms that in tumour cells, BSG-2 is the most abundant if not the only expressed spliced form of BASIGIN [1-3].

Table 1: Primary cell cultures and cell lines used in the study.

NHF: Normal Human Fibroblasts

MEF wt: Mouse Embryonic Fibroblast
MEF BSG^{-/-}

LS174T wt: Human colon carcinoma cell line
LS174T BSG^{-/-}: clone 18

U 87 wt: Human glioblastoma cell line
U 87 BSG^{-/-}: clone 8 and 39

A549 wt: Lung carcinoma cell line
A549 BSG^{-/-}: clone 153

Human tumour cells express varying levels of MMP2 and MMP9 in extracellular medium

As MMP2 and MMP9 are usually associated with tumour progression in a BSG-dependent way, we investigated their expression in a series of BASIGIN-positive human tumour cells. Zymogel analysis of MMP2 and MMP9 gelatinase activity showed that, while MMP9

is weakly expressed in all cells tested, MMP2 expression in extracellular medium varies between tumour cell lines. MDA-MB-231, LS174T and A549 cell lines express low levels of MMP2 (Figure 1C, lanes c, d, f and Figure 2A, lane a). In contrast, U87 cells express a significant level of MMP2, which is comparable to the expression by normal human fibroblasts (NHF) (Figure 1C lanes b, h and Figure 3A, lanes a, m) or mouse embryonic fibroblasts (MEF) (Figure 2A, lane j and Figure 3A, lane n). However the differential expression of MMP2 by tumour cells does not appear to reflect the expected metastatic properties of these cells since metastatic MDA-MB-231 cells express little or no MMP2 and MMP9 in the extracellular medium.

Co-cultures of tumour cells with fibroblasts does not significantly change MMP2 and MMP9 expression in extracellular medium

We then tested in our models if, as published by [17], co-culture of tumour cells with fibroblasts was able to stimulate metalloproteases production in the extracellular medium. For this, we analysed MMP2 and MMP9 production in conditioned medium obtained from

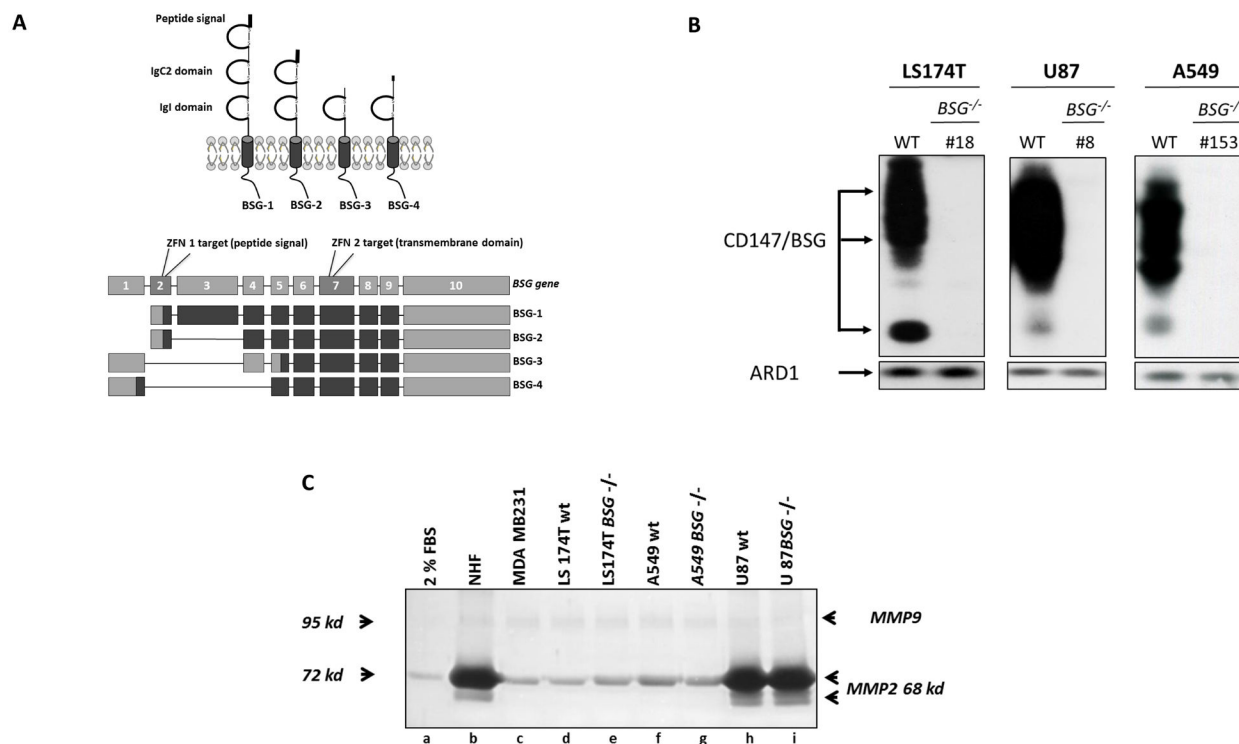


Figure 1: Zinc Finger Nucleases Knock out of BASIGIN/CD147 gene in tumour cells. **A.** Schematic representation of the four isoforms of human BASIGIN (BSG-1, BSG-2, BSG-3 and BSG-4), and mapping of the human *BASIGIN* gene sequence (exons/introns) with the four mRNA sequences for human BASIGIN spliced isoforms adapted from [29]. Two ZFN were used to generate *BSG*^{-/-} cell lines; ZFN1 targeted the exon 2 encoding peptide signal and common to BSG-1 and BSG-2, while ZFN2 was designed to target the exon 7, encoding a part of the transmembrane domain and common to all the isoforms. **B.** Western blot analysis. LS174T, U87 and A549 wt cells and *BSG*^{-/-} clones were grown in normoxia during 48h and lysed. BASIGIN expression was analysed by immunoblotting. ARD1 was used as loading control. **C.** Zymogel analysis of Conditioned Media (CM) from wt vs *BSG*^{-/-} tumour cells lines. Cells were grown for 48h in 2% FBS media (lane a). Conditioned media were harvested, and MMP2/MMP9 activities were analysed by loading ECM samples in a 10% polyacrylamide zymogel.

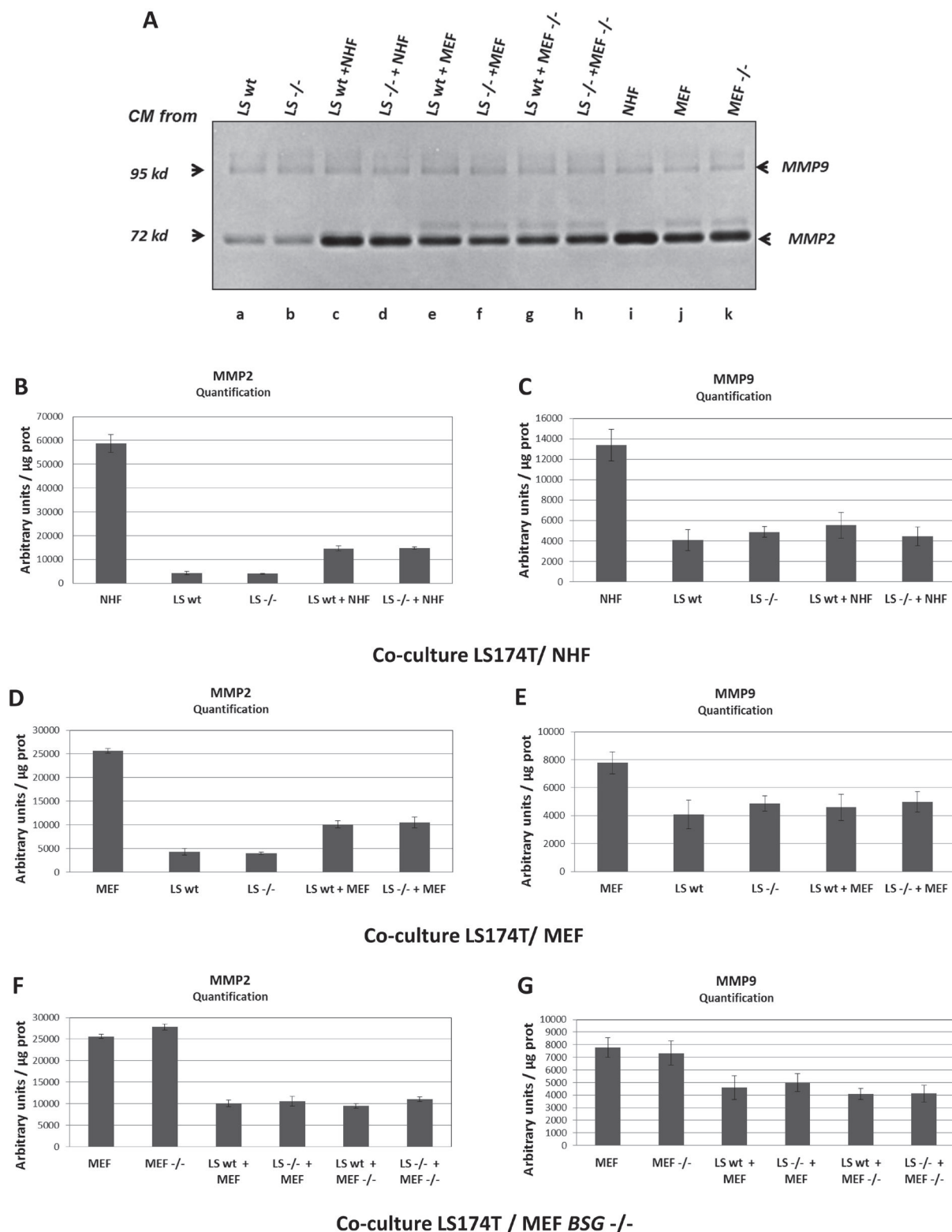


Figure 2: MMP2 and MMP9 expression in LS174T cells. A. Zymogram analysis of MMP2/MMP9 activities in CM harvested after 48h of culture. LS174T wt or *BSG*^{-/-} (referred to as LS wt and LS -/-) cells were grown in 2% FBS media alone (a, b) or in co-culture with either NHF (c, d) or with MEF (wt or *BSG*^{-/-} cells refers to as MEF and MEF -/-) (e, f g, h). CM media of NHF, MEF wt and *BSG*^{-/-} cells cultured alone was also assessed (i, j, k) B. and C. Quantification of MMP2/MMP9 activities in CM of LS174T wt vs *BSG*^{-/-} cells cultured alone, or co-cultured with NHF. D and E: Quantification of MMP2/MMP9 activities in CM of LS174T wt vs *BSG*^{-/-} cells cultured alone, or co-cultured with MEF wt. F and G: Quantification of MMP2/MMP9 activities in CM of LS174T wt vs *BSG*^{-/-} cells cultured alone, or co-cultured with MEF *BSG*^{-/-}. Results were normalized relative to protein quantity.

LS174T and U87 cultured alone, or with two different types of fibroblasts (NHF or MEF). Zymogels analysis, and quantification of MMP activities showed that co-cultures of LS174T with either NHF (Figure 2A, lane c

and Figure 2B-2C) or MEF (Figure 2A, lane e and Figure 2D-2E) did not significantly produce more MMP2 and MMP9 than tumour cells or fibroblasts cultured alone (Figure 2A, lanes a, i, j and Figure 2B-2E). The same

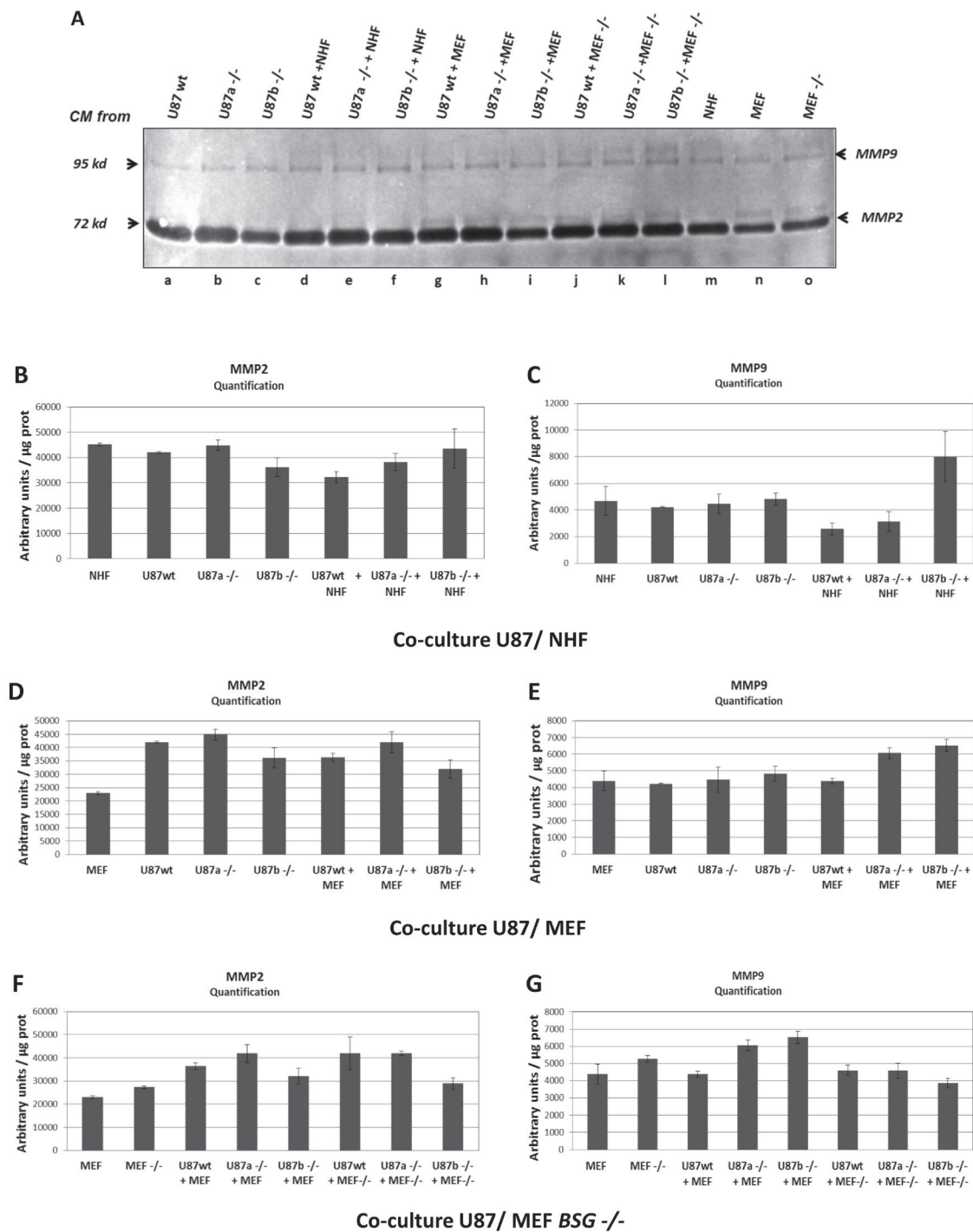


Figure 3: MMP2 and MMP9 expression in U87 cells. A. Zymogel analysis of MMP2/MMP9 activities in CM harvested after 48h of culture. U87 wt or *BSG*^{-/-} cells (clones a, and b) (referred to as U87 wt and U87^{-/-}) were grown in 2% FBS media alone (a, b, c) or in co-culture with either NHF (d, e, f) or with MEF (wt or *BSG*^{-/-} cells) (g, h, i, j, k, l). CM media of NHF, MEF wt and *BSG*^{-/-} cells cultured alone was also assessed (m, n, o). B. and C. Quantification of MMP2/MMP9 activities in CM of U87 wt vs *BSG*^{-/-} cells cultured alone, or co-cultured with NHF. D. and E. Quantification of MMP2/MMP9 activities in CM of U87 wt vs *BSG*^{-/-} cells cultured alone, or co-cultured with MEF wt. F. and G. Quantification of MMP2/MMP9 activities in CM of U87 wt vs *BSG*^{-/-} cells cultured alone, or co-cultured with MEF *BSG*^{-/-}. Results were normalized relative to protein quantity.

results were obtained with U87 cells as shown in Figure 3. Co-culture of U87 with either NHF (Figure 3A, lane d and Figure 3B-3C) or MEF (Figure 3A lane g and 3D-3E) did not produce more MMP2 and MMP9 than NHF or MEF alone (Figure 3A lanes a, m, n and 3B-3E). These results indicate that contrary to what has been previously published, fibroblasts and tumour cells do not seem to cooperate to produce more MMP in the extracellular medium *in vitro*. Conversely, it should be noted that NHF or MEF co-cultured with LS174T cells (but not with U87 cells) seem to produce less MMP2 and MMP9 than when they are cultured alone (Figure 2A lanes i, j compared to lanes c, d, e, f and Figure 2B-2E).

Activation of NHF by conditioned media (CM) from tumour cells

It is well known that when in contact with tumour cells, fibroblasts can acquire a specific CAF activated phenotype. This can result in the expression of new markers, such as alpha smooth muscle actin (α -SMA), the activation of TGF β /SMAD signalling pathways and acquisition of new properties, such as matrix remodelling [24, 25]. We thus, tested if conditioned media from LS174T and U87 tumour cells are able to

activate stimulated NHF *in vitro*. NHF stimulated by CM from LS174T cells seems to have acquired new matrix remodelling properties represented by their capacity to contract collagen gels (Figure 4A), while CM from U87 cells does not have any effect (Figure 4B). Moreover, immunoblotting analysis indicates that NHF stimulated by CM from LS174T cells express α -SMA, and that TGF β /SMAD signalling pathways is activated in these stimulated fibroblasts (Figure 4C). On the contrary, CM from U87 cells does not have any effect (Figure 4D). These data suggest that fibroblasts stimulated with CM from LS174T cells could have acquired a status similar to that of activated CAFs. Nevertheless, BSG disruption had no impact on collagen contraction or on TGF β /SMAD signalling pathway activation.

Stimulation of fibroblasts by conditioned media from tumour cells does not significantly change MMP2 and MMP9 expression in extracellular medium

It is also known that activated fibroblasts are able to secrete increased level of matrix degrading proteases such as MMP2 and MMP9 [17, 28]. Therefore, we tested if CM from LS174T and U87 tumour cells could induce MMPs

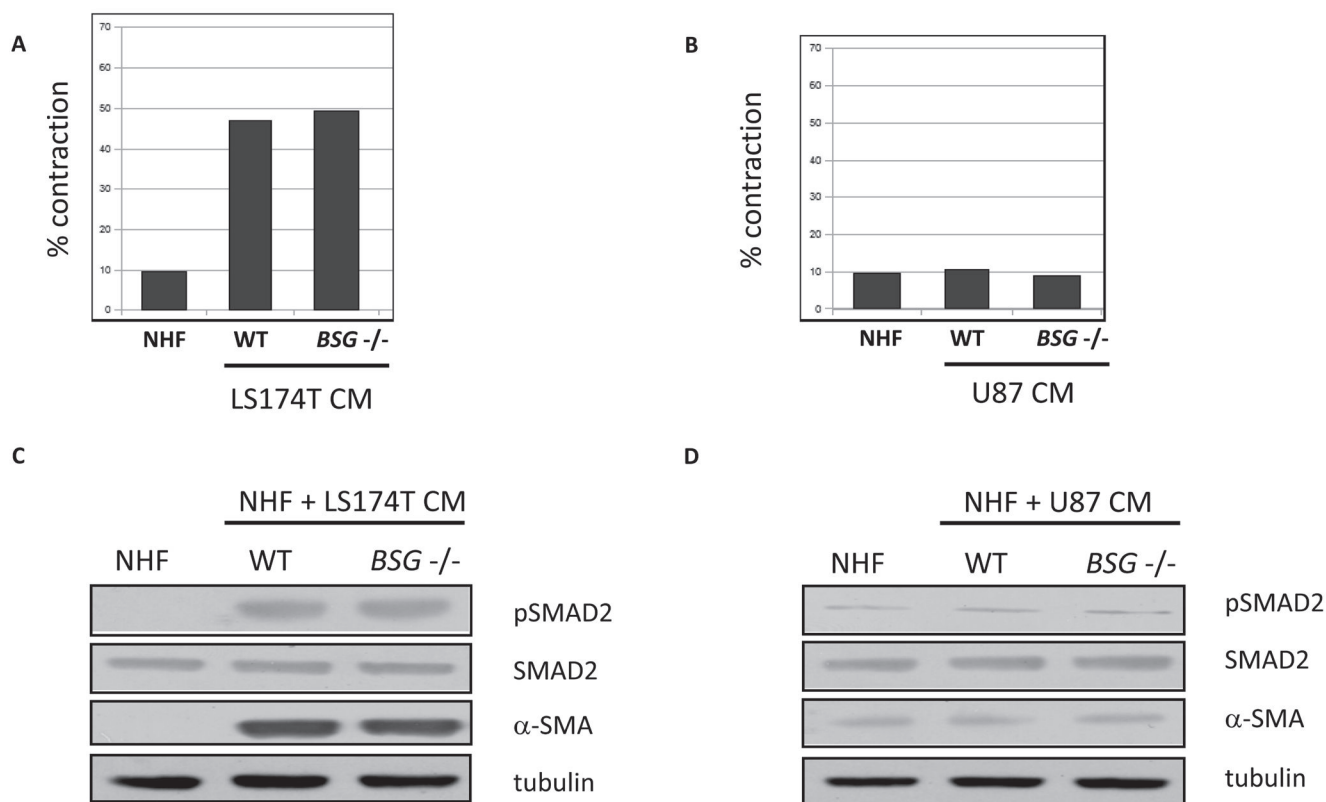


Figure 4: NHF activation by CM from tumours cells . A. and B. Quantification of matrix contraction by NHF stimulated by CM from LS174T and U87 (wt vs BSG^{-/-}) tumours cells. **C. and D.** Immuno-detection of pSMAD2 and α -SMA expressed in NHF 48h after stimulation by CM from LS174T and U87 (wt vs BSG^{-/-}) tumours cells. SMAD2 and tubulin are used as control. These figures are representative of three independent experiments.

production in stimulated fibroblasts. Zymogel analysis and quantification of MMPs activity showed that conditioned media from LS174T or U87 cells have no significant effect on MMP2 production by stimulated NHF either by direct stimulation (Figure 5A lanes a, b, d and 5B), or by delayed action (Figure 5C, lanes a, b, d and 5D). In this experiment, due to the weakness of MMP9 signal, only MMP2 activity was measured. As in co-culture experiments, it should be noted that NHF directly stimulated by CM from LS174T (but not by CM from U87 cells) produce less MMP2 than non-stimulated NHF (Figure 5A lane a compared to lanes b, c, d, e and Figure 5B).

Genetic disruption of BASIGIN does not significantly affect MMP2 and MMP9 production by tumour and fibroblastic cells

The impact of BASIGIN on MMPs production by tumour cell lines was first evaluated by analysing the MMP2 and MMP9 activities in CM from wt *versus* *BSG*^{-/-} tumour cell lines alone, or co-cultured with fibroblasts. Zymogel quantification showed that *BSG* invalidation does not significantly affect MMP2 and MMP9 production

by LS174T (Figure 1C lanes d, e, Figure 2A lanes a, b, and Figure 2B-2C), U87 (Figure 1C lanes h, i, Figure 3A lanes a, b, c and Figure 3B-3C), and A549 (Figure 1C lanes f, g) tumour cells.

MMP2 and MMP9 activities were also evaluated in MEF wt *versus* *bsg*^{-/-}. We used MEF from *BSG*-null mice invalidated for BASIGIN by an insertion of a MC1neo box into the gene [30]. In these cells, as in ZFN invalidated cells, the disruption of *BSG* gene results in the total loss of the protein (data not shown). As observed in tumour cells, the invalidation of BASIGIN in MEF does not notably modify the level of MMP2 and MMP9 in extra cellular media (Figure 2A lanes j, k, Figure 3A lanes n, o and Figure 2F-2G, 3F-3G). Moreover, the invalidation of BASIGIN does not affect MMPs production when tumour cells were co-cultured with NHF (Figure 2A, lanes c, d, Figure 3A lanes d, e, f and Figure 2B-2C, 3B-3C), with MEF wt (Figure 2A lanes e, f, Figure 3A lanes g, h, i and Figure 2D-2E, 3D-3E) or MEF *bsg*^{-/-} (Figure 2A, lanes g, h, Figure 3A lanes j, k, l and Figure 2F-2G, 3F-3G). Finally, *BASIGIN* gene disruption does not have any detectable effect on the level of MMP2 and MMP9 produced by CM stimulated fibroblasts (Figure 5A and 5C lanes b, c, d, e and Figure 5B, 5D).

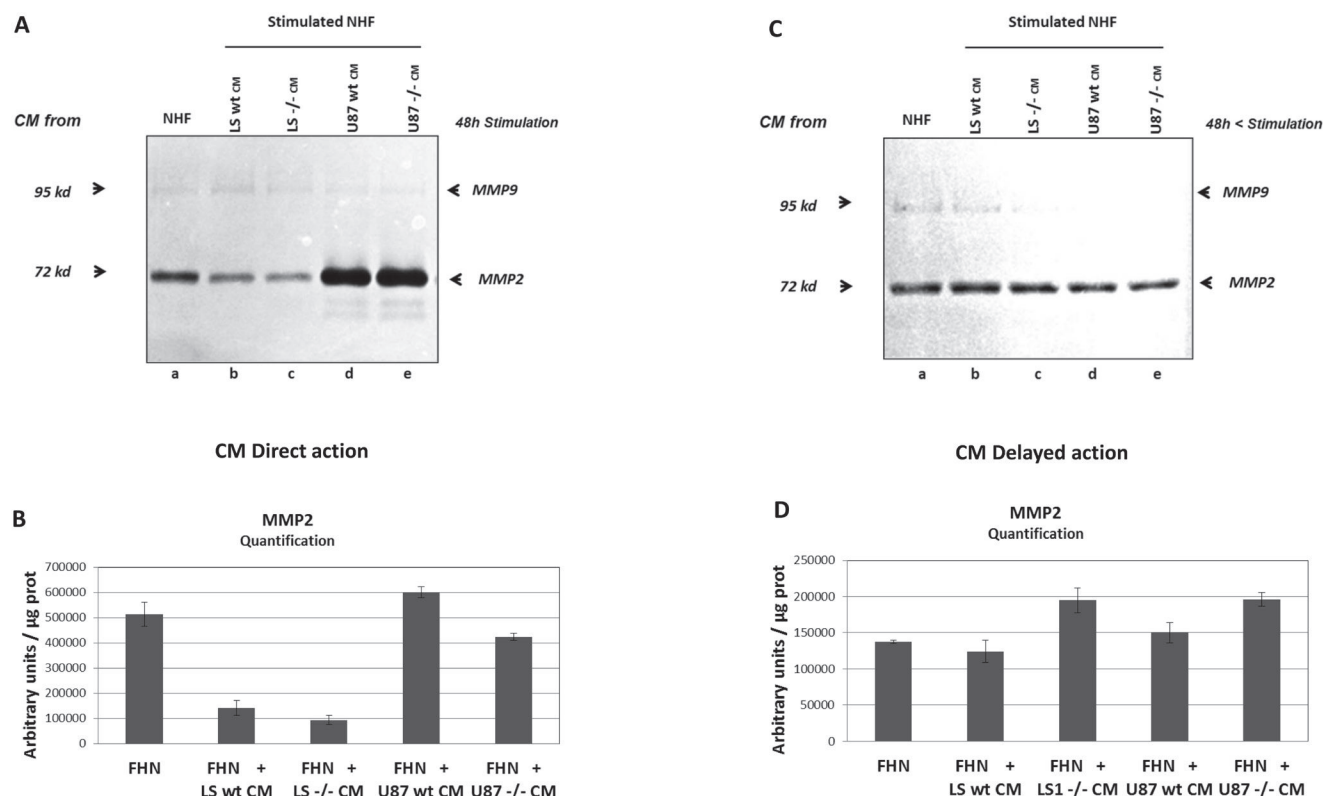


Figure 5: MMP2 and MMP9 expression by NHF stimulated by conditioned media (CM) from tumours cells. A. Zymogel analysis of MMP2/MMP9 activities in NHF CM harvested after 48h of culture (CM direct action). NHF were grown for 48h in the presence of CM from NHF as a control (a), or from tumour cells, LS174T wt (b) vs *BSG*^{-/-} (c), U87 wt (d), vs *BSG*^{-/-} (e). **B.** Quantification of MMP2 activity in CM from stimulated NHF. **C.** Zymogel analysis of MMP2/MMP9 activities in NHF CM harvested after 48h of cell culture, washed and cultured in fresh 2% FBS media during an additional 48h (CM delayed action). **D.** Quantification of MMP2 activity in CM from NHF 48h after stimulation.

Genetic disruption of BASIGIN does not significantly affect MMP2, MMP9, MMP3, MMP11 and MMP14 gene-expression in tumour cell lines

In addition to MMP2 and MMP9, we investigated the expression of MMP3, MMP11, MMP13 and MMP14 in tumour cells. These MMPs have also been reported to be associated with cancer progression in a BSG-dependent manner. As these MMPs have no gelatinase activity, we

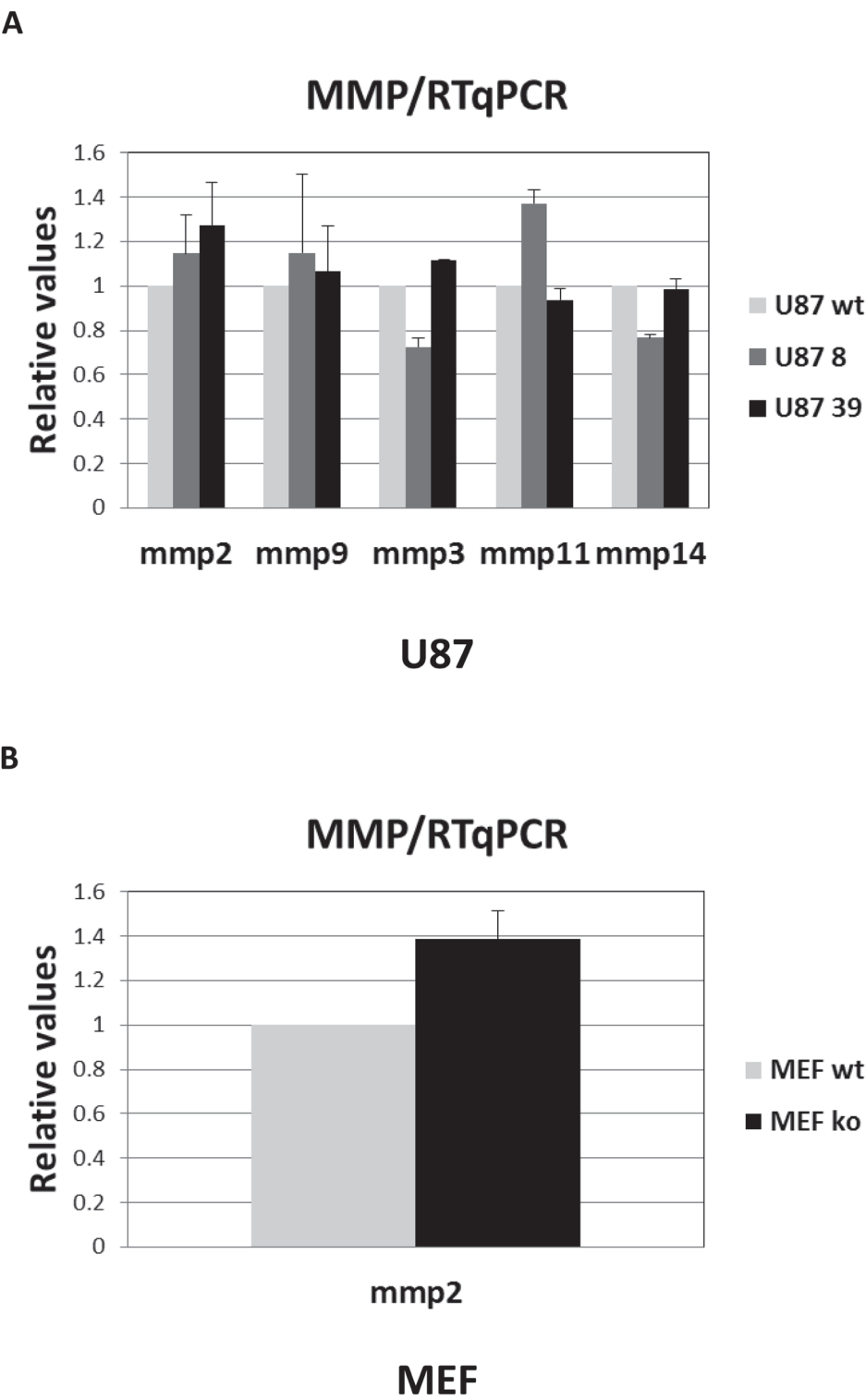


Figure 6: RT-qPCR analysis of MMP expression. A. Quantification of relative MMP expression in U87 wt vs *BSG*^{-/-} cells. B. Quantification of relative MMP expression in MEF wt vs *BSG*^{-/-} cells.

could not evaluate their expression in the extracellular medium, but only at intracellular level. Due to high variability in the expression level of MMPs in tumour cells, and to standardize the results, we choose to use RT-qPCR method rather than immunodetection to measure the expression of these other MMPs. In confirmation of the zymogel results, RT-qPCR analysis showed that MMP2 and MMP9 mRNA are barely detectable in wt LS174T cells (CT>35), while wt U87 cells expressed a

significant level of MMP2 and MMP9 mRNAs (Figure 6A). Concerning other MMPs, levels of MMP3, MMP11, MMP13 and MMP14 mRNA are too low in LS174T cells to be detected. In contrast, U87 cells expressed noticeable levels of MMP3, MMP11 and MMP14 as shown in Figure 6A. On the contrary to NHF, which express high level of all tested MMPs (data not shown), MEF do not express any of the tested MMPs, except MMP2 (Figure 6B). Nevertheless, the invalidation of the *BASIGIN* gene does

not significantly affect MMP2, MMP9, MMP3, MMP11 and MMP14 expression at intracellular level in any of the cells tested (Figure 6A and 6B). It is to be noted that the MMP1 collagenase expression present in NHF (data not shown) was not detectable in tumour cells.

DISCUSSION

In 1982, Biswas et al [17] showed for the first time that fibroblasts co-cultured with tumour cells, or treated by tumour cell conditioned media, produced an increased collagenase activity *via* a soluble factor present in tumour conditioned media. Then, they showed that this factor named tumour cell-derived collagenase stimulatory factor (TCSF) is present both as a soluble form and a tumour cell membrane component. Finally, in 1995 the same group identified TCSF as BASIGIN/CD147, renamed EMMPRIN for MMP inducer [31]. Since then, a large number of papers continue to be published establishing a correlation between BASIGIN and the tumorigenic capacities of cancer cells *via* MMPs induction.

BASIGIN and the control of bioenergetics

However, in the meantime Halestrap's group demonstrated that BASIGIN is a unique accessory protein allowing the expression of MCT1 and MCT4 at the plasma membrane [6]. Several publications, including ours, clearly demonstrated that BASIGIN is involved in cancer cells through the direct functionality of MCT1 and MCT4. By assuring lactic acid export, BASIGIN indirectly contributes to intracellular pH changes [32-34], the rate of glycolysis and tumour growth [14-16]. Therefore, the question was raised whether the pro-tumour action of BASIGIN is linked to its role in glucose metabolism and bioenergetics, MMPs induction, or both. Because of the strong structural and physiological interdependent relationship between BASIGIN and MCT1/MCT4 for plasma membrane expression, it was difficult to uncouple the function and the expression of these three proteins. So to answer this question, we performed an experiment in which we pharmacologically and genetically ablated MCT1 and MCT4 functions respectively, keeping high plasma membrane expression of BASIGIN [14]. This experiment revealed that tumorigenicity declined with the loss of MCT function, independently of BASIGIN level. We recently confirmed these findings by showing that the knockout of BASIGIN alone in LS174 and U87 tumour cell lines re-programmed tumour cell bioenergetics from glycolysis to oxidative phosphorylation [16].

BASIGIN/EMMPRIN and the control of Extracellular Matrix Metalloproteases

In parallel, as reported extensively in the literature, the role of BASIGIN in the process of cellular invasion and metastasis *via* the induction of metalloproteases has remained very active, and with few exceptions, ignores the metabolic component. However, these publications are mostly based on indirect proofs since the experiments were often made using either BASIGIN overexpression, siRNA invalidation or forced expression with recombinant soluble BASIGIN [1, 18, 23, 28]. In this manuscript, we addressed the question of the direct involvement of BASIGIN in MMPs induction by exploiting four *BSG* knockout (KO) cell lines derived from human and mouse. First, using three human cancer cell lines, LS174, A549, and U87 (wt *versus* *BSG*^{-/-}), we showed that *BSG* gene disruption did not significantly affect the MMPs production in extracellular media. The same results were obtained for intracellular expression of MMPs by using qPCR analysis. Then, we developed co-cultures experiments between fibroblasts and tumour cells, and measured MMPs production in the extracellular media. We showed that not only did BASIGIN disruption not affect MMPs production, but that co-cultures also did not significantly increase MMPs production in the media. This surprising finding indicates that, contrary to what has been previously reported, fibroblasts and tumour cells do not seem to cooperate to produce more MMPs in the extracellular medium. The use of mouse fibroblasts (MEF) lead to the same result, namely a lack of significant effects on MMPs production while comparing MEF-*bsg*^{-/-} vs MEF-wt, or in co-culture with tumour cells. How can we then explain these contradictory results? First of all as mentioned above, many previously published data are based on either forced expression (transfection or used of a purified recombinant soluble form of BASIGIN), or with incomplete invalidation (siRNA or antisense). Use of these methods showed therefore a correlation between BASIGIN and MMPs induction rather than a direct link. The results presented here stand out from previous reports because it is the first study conducted with *BASIGIN* KO where, in contrary to siRNA experiments, BASIGIN expression was totally abolished. In the same way, *BSG* KO performed in MEF by a neo box insertion and destruction of the *BASIGIN* gene function confirmed our results. Moreover, we pushed the study further by exploring the direct stimulation of fibroblasts with tumour cell-conditioned media as a more informative approach. Surprisingly, even under these conditions, where human fibroblasts acquired an activate profile quite similar to CAFs by presenting the α -SMA myofibroblastic marker, and activating TGF β pathway, we failed to demonstrate a link between BASIGIN expression and MMPs. Therefore, we must conclude from this work that the direct link between BASIGIN, tumorigenicity and MMPs expression

is not proven.

Thus if BASIGIN has a real effect on MMPs induction in cancer cells, this could be in an indirect way, and probably *via* its interaction with MCTs transporters. BASIGIN overexpression in tumour cells, that is usually associated with the overexpression of MCT1/MCT4, would be simply a reflection of a greater ability of the cells to export lactate and to grow in a hostile environment. The increased production of MMPs which accompany the tumour growth and the metastatic stage, would not be directly linked to the overexpression of BASIGIN, but more a consequence of the acquisition of aggressive capacities of the cells becoming best adapted to the acidic microenvironment by overexpressing MCTs.

Our findings complement and echo similar results obtained by Kolb's group [35] on mammary gland development. They showed that, contrary to what might be expected, BASIGIN has no direct effect on MMP gene expression during adult mammary gland development. Inactivation of *basigin* gene in mice [30] affected developmental processes that include defects in embryos implantation, arrested spermatogenesis, male and female infertility, abnormal behaviour deficits in vision and odour. However until now and despite many studies using BASIGIN-null mice, a direct role of MMPs and above all a direct link between basigin and MMP induction in these processes could not be clearly established. Furthermore, Philp's group demonstrated that vision deficits in *basigin*-null mice could be ascribed to the failure of MCTs in photoreceptor cells to properly locate in the plasma membrane [36]. Taken together these data reinforced our proposal that BASIGIN is involved in cancer progression through its MCTs chaperone function and not by MMP induction.

However, it remains to be explained by which mechanism soluble BASIGIN (from transfection or injection into the extra cellular media) manages to trigger MMPs production by treated cells, as it was reported in the literature. Many data from various studies indicate that the expression of MMPs is regulated at several levels, from gene transcription to activation, by multiple factors present in the extracellular matrix, such as TGF β , EGFR, TNF- α , prostaglandin E2 and many others bioactive molecules. Moreover, depending on the cells, hypoxia and hyperglycaemia have been reported to regulate MMPs expression. More interestingly, these data showed that cell-extracellular matrix and cell-cell interactions bring an important contribution to the regulation of MMPs production at each level of regulation (for review[37]). In conclusion, the profile of MMPs production is cell specific, and the regulation of MMPs expression is a very complex process in which BASIGIN could play a minor role as a cell recognition molecule in a cell dependant manner. Indeed, we could imagine that, in a context of over-expression, BASIGIN, which is a very reactive cell surface protein due to its highly glycosylated structure,

could have a cell recognition function, directly *via* cell-cell contact or indirectly *via* its soluble form and could activate MMPs production in a non-specific way *via* its contacts with the many extracellular matrix MMPs inducers.

At this stage we conclude that the induction of MMPs by BASIGIN as it has been initially hypothesized by Biswas [17, 31] is not proven and we propose to abandon the misleading acronym EMMPRIN.

EXPERIMENTAL PROCEDURES

Cell culture

Normal Human Fibroblasts (NHF), Mouse Embryonic Fibroblasts (MEF), colon adenocarcinoma (LS174T), glioblastoma (U87), lung carcinoma (A549) and breast carcinoma (MDA-MB231) human cell lines were grown in Dulbecco's modified eagle medium (DMEM, Gibco) supplemented with 10% FBS, penicillin (10U/mL) and streptomycin (10 μ g/ml) except for conditioned media preparations where cells were cultured in 2% FBS.

ZFN- mediated gene knockout of the BSG gene

LS174T, U87 and A549 BASIGIN expressing cell lines were transfected with the ZFN designed by Sigma-Aldrich (CKOZFN1227-1KT, CompoZr Custom ZFN) targeting exon 2 of *BSG* as previously described [16, 38]. Another LS174T *BSG* knockout cell line was generated with a ZFN targeting exon 7 of *BSG*, common to all spliced isoforms of the gene (Sigma-Aldrich, CSTZFN-1KT, CompoZr Custom ZFN). Transfected cells were detected with a CD147 (MAB972, R&D Systems) primary antibody and with a PE-conjugated anti-mouse IgG (115-115-164, Jackson ImmunoResearch) secondary antibody as described [14]. Several negative clones were detected by immunoblotting and only clones disrupted for the two alleles (DNA sequencing) were studied (cl18: *BSG*^{-/-} LS174T cells, cl8 and cl39: *BSG*^{-/-} U87 cells and cl153: *BSG*^{-/-} A549 cells).

Immunoblotting

Immuno-detection was performed as previously described [14]. Briefly, cells were lysed in 1.5X SDS buffer and protein concentration was determined by the BCA assay. Proteins (40 μ g) were separated on 8% SDS polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore). Blots were blocked in 5% milk in Tris-HCL/NaCl buffer and incubated with antibodies. Immunoreactive signals were revealed with horseradish peroxidase (HRP) antibodies (Promega)

using ECL system (Amersham Biosciences). The antibody against BASIGIN (MAB972) was purchased from R&D systems, antibodies against SMAD2 (3122), pSer465/467-SMAD2 (3108), were purchased from Cell Signaling technology, and alpha-smooth muscle actin (Ab569) from Abcam. α -tubulin (T4026, sigma) and Arrest Defective-1 protein (ARD1) (produced in our laboratory [39]) were used as loading controls.

Gelatin zymography (Zymogel) analysis

MMP activity was detected using zymogel technology [24]. Gelatin zymogels were performed as follows. After centrifugation, conditioned media samples from various cell lines were diluted (v/v) in loading buffer containing Tris-HCl pH 6.8, 2% SDS, 4% sucrose and bromophenol blue. Then, samples were separated by polyacrylamide gel electrophoresis (SDS free, 10% polyacrylamide gel containing 0.3% (w/v) gelatine). After electrophoresis, gels were first incubated in 2.5% triton for 90min, then in 50 mM Tris-HCl pH 7.5, 200mM NaCl, 2mM CaCl₂, and 1mM MgCl₂ buffer for 18h at 37°C. Gels were stained with Coomassie blue and washed in 10% acetic acid and 10% ethanol. MMP gelatinase activity was visualized by observing the light bands standing out against the dark background of the gel. The signal strength of these light bands was analyzed and quantified using the “gene tools” software from Syngene. Results were normalized to the amount of proteins.

Conditioned media (CM) preparation

Cancer cells were grown to confluence, washed twice with PBS and then incubated in 2% serum media at 37°C. After 48h, CM was collected, centrifuged at 5000g for 5min to remove cell debris and the supernatant stored at -80°C. For immunoblotting analysis, fibroblasts were stimulated with cancer cells CM for 48h.

Matrix remodelling assay

Matrix-remodelling assays were performed as previously described [40]. Fibroblasts were embedded (2.5×10^4) in 100 μ l of matrix gel [41]. After 1h at 37°C, matrices were overlaid with 100 μ l of CM from different conditions. The CM was changed every 2 days and at day 6 gel diameters were measured using image J. The contraction of the gel was calculated using the formula: $100 \times (\text{well diameter} - \text{gel diameter}) / \text{well diameter}$.

RT-qPCR analysis

Total RNA was extracted from various cell lines using the RNA extraction kit from Qiagen according to

the manufacturer's instructions. cDNA synthesis was performed by the Quantitect reverse transcription kit (Qiagen). The relative expression of various MMPs and BSG mRNA was determined by real-time quantitative PCR (qPCR) using TaqMan nucleotide primers probes (Life Technologies, references will be provided upon request) and qPCR master mix buffer (Eurogentec). Real-time qPCR experiments were performed on a “step one plus” system.

Statistical analysis

Data are expressed as mean \pm SEM. Each experiment was performed at least three times and the number of experiments is represented by n. statistical analysis was done with the unpaired student's test. Differences between groups were considered statistically significant when $p < 0.05$.

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CONFLICTS OF INTEREST

There is no conflicts of Interest.

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DISCUSSION AND PERSPECTIVES

Discussion and perspectives

Hypoxia and oncogenes induce metabolic reprogramming that has recently been recognized as an emerging “Hallmark” of cancer. The glycolytic switch in cancer cells not only provides cells with energy and biomolecules but also contributes to cell-cell communication. Recent evidence supports the notion of metabolic symbiosis within tumours, in which cancer and stromal cells use lactate as a metabolic fuel and signalling molecule, mimicking thus pre-existing and high-performance physiological mechanisms (Draoui and Feron, 2011). However, unlike the neurone-astrocyte shuttle or muscle fiber-red blood cells shuttle, the lactate circuit in tumours still remains poorly understood, mainly due to the complexity of the tumour microenvironment and interconnections between individual cellular subtypes. Thus, additional preclinical studies are needed to confirm and reinforce the available data. Moreover, although, issues concerning when and where lactate exchanges occur are unresolved, regulators implicated in its handling are being characterized. Therefore, we have shown through this work, that targeting MCT/BSG complexes offers a great potential for developing new anti-cancer therapies based on disruption of glycolysis and pH homeostasis.

A. The MCT4 pharmacological inhibitor: a dream come true

Transfer of MCT1 inhibitors developed by AstraZeneca from bench to clinical trials constitutes a first step in a long process validating the success of this strategy. However, as we have already shown in this work (see results A, publication 1) the MCT1 inhibitor alone is not efficient on most of cancer cells that also express the hypoxia-inducible MCT4. Consequently, combination of both MCT1 and MCT4 inhibitors is needed to achieve anti-tumour activity. Recently, using a molecular model of MCT4, Nancolas et al. (Nancolas et al., 2015) reported structural differences between this transporter and MCT1, and identified N147, R306 and S364 as key residues involved in MCT1 inhibitor (AR-C155858) binding and selectivity, which gives hope for development of selective small drugs inhibiting specifically MCT4. In the meantime, AstraZeneca succeeded in generating an MCT4 (AZ93) inhibitor. Our preliminary results show that this inhibitor is likely selective and highly efficient in blocking growth of a wide range of cancer cells in normoxia and hypoxia, but only

when the *MCT1* gene is disrupted or MCT1 is inhibited pharmacologically (Marchiq I et al, manuscript in preparation) (Fig. 19 and 20). However, it is worth noting that this effect is only cytostatic and not cytotoxic, confirming thus our previous findings on genetically disrupted cancer cells for MCT4, or both MCT1 and MCT4 (data not shown).

The availability of such specific inhibitors, which disrupt only the function and not the expression of MCTs, offers new perspectives in determining precisely the role of expression and function of MCT1 and MCT4 in cancer development. This includes also BSG, which has a metabolic function linked to the activity of MCTs. As we reported in publications 1 and 4, this metabolic function of BSG seems to predominate in tumour development, thus blocking both MCTs without affecting their expression and subsequently the expression of BSG, should be an efficient and more reliable way of confirming our results on the lack of induction of MMPs by BSG. However, it will be interesting to not only focus on the effect of these inhibitors on tumour cells, but also on different stromal cell types. Indeed, as I have mentioned in the introduction of this manuscript (see E1.3), lactate plays a key role in immunosuppression during tumourigenesis. Subsequently, it will be worth assessing the direct effect of MCTs inhibitors on T-lymphocytes and natural killer cell viability and activity, but also on macrophage polarization and plasticity by measuring the balance between M1-like (inflammatory and “tumour-suppressing” macrophages) and M2-like (alternative and “tumour promoting” macrophages) phenotypes under these conditions. Moreover, experiments seeking to understand the mechanisms and cell-signalling pathways behind the cytostatic effect of the inhibitors need to be developed to better understand the role of lactic acid export in cell physiology.

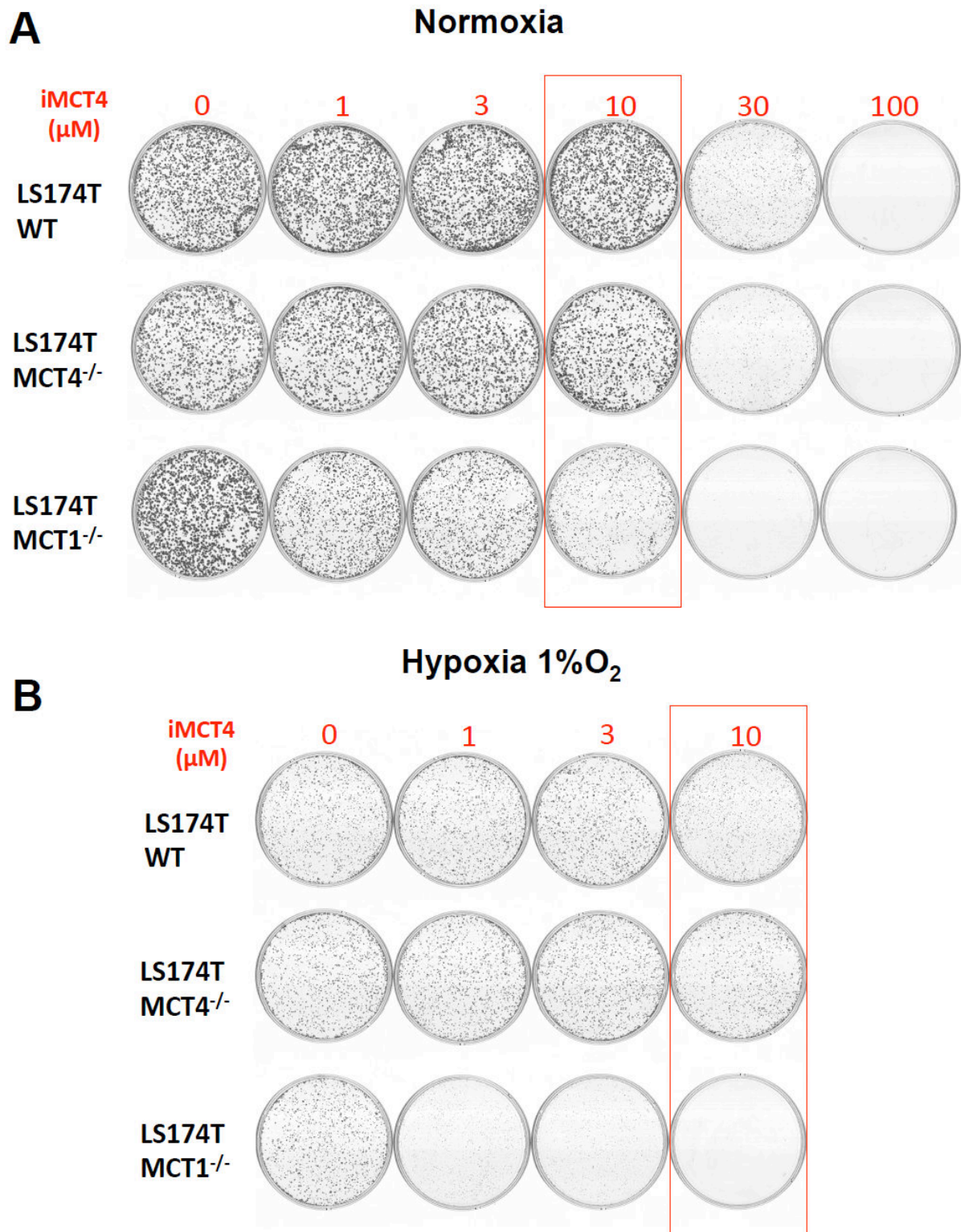


Figure 19. Specificity and efficacy of the pharmacological inhibitor of MCT4 (MCT4i) developed by AstraZeneca.

Clonal growth of WT, MCT4^{-/-} and MCT1^{-/-} LS174T cells treated with MCT4i (0, 3, 10, 30 and 100 $\mu\text{mol/L}$). Cells were plated in 60-mm dishes and incubated at 37°C, 5% CO₂. 24h after cell adherence, the medium was replaced with regular DMEM supplemented with MCT4i for 10d in **A**) normoxia or **B**) hypoxia. Dishes were stained with 5% Giemsa (Fluka) for 45min to visualize colonies. It is important to note that iMCT4 at 10 $\mu\text{mol/L}$ is non-toxic (no effect on LS174T MCT4^{-/-} cells) and highly efficient in blocking growth of LS174T MCT1^{-/-} cells in normoxia and hypoxia. The results are representative at least three different experiments.

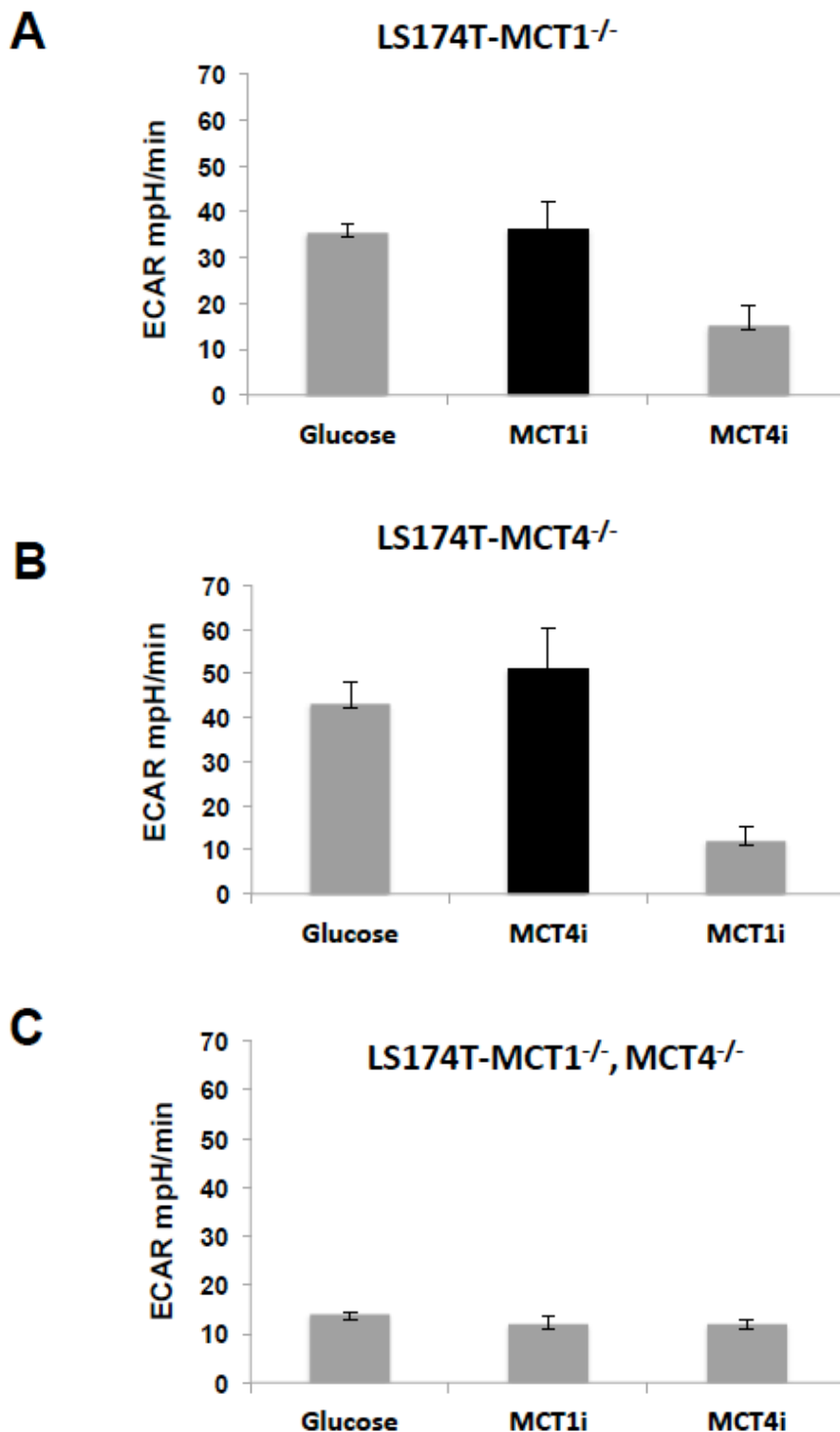


Figure 20. Functional determination of the specificity of MCT1 and MCT4 inhibitors.

Real-time analysis of the proton production rate (ECAR) with a Seahorse XF24 analyzer. The effect of addition of glucose (10 mmol/L), MCT1i (300 nmol/L) or MCT4i (10 μ mol/L) is shown; **A**) MCT1i has no effect on ECAR values of *MCT1^{-/-}* LS174T cells, while addition of MCT4i is highly affecting the ECAR. **B**) MCT4i is highly specific and has no impact on the ECAR of *MCT4^{-/-}* LS174T cells, which are highly sensitive to MCT1i. **C**) MCTs inhibitors are not affecting ECAR of MCT1/MCT4 disrupted LS174T cells.

B. The mystery of the residual expression of MCTs in *BSG*-null cells

During this project, we have also shown that genetic disruption of the *BSG* gene, in 5 different human cancer cell lines, resulted in substantial reduction of the expression and function of MCTs, up to 10-fold for MCT1 and 6-fold for MCT4 in the colon adenocarcinoma cell line LS174T. This result is, however, surprising as we were expecting total abolition of MCT function in the absence of the accessory protein BSG. We first hypothesized that other minor BSG spliced isoforms were present but not detectable with our antibodies. Indeed, Belton et al. have described the presence of potential BSG splicing isoforms, mainly BSG3 and BSG4, that are lacking the exon (exon 2) we targeted with the ZFN. To validate or refute this hypothesis, we designed additional experiments in LS174T cells in which we targeted the exon 7 that is common to all BSG isoforms. Our results showed that the new clones LS174T *BSG*^{-/-} clones displayed a similar MCT expression level, proliferation rate and metabolic phenotype than the former ones (Fig. 21).

We then investigated the role of the BSG paralogs, EMBIGIN (EMB) and NEUROPLASTINS (NP), in assisting MCTs at the plasma membrane, with low affinity compared to BSG. Thus we transfected rat BSG, EMB and NP into LS174T *BSG*^{-/-} cells and we assessed the activity of the MCTs by measuring their ability to take up ¹⁴C- lactic acid (Fig. 22). Our findings confirmed previous studies showing that EMB could play the role of an ancillary protein of MCT1 and MCT4 in the absence of BSG. However, although a recent study from Beesly group (Wilson et al., 2013) has reported that NP (55 and 65) were able to chaperone MCT2 at the neuronal cell surface, we did not observe any activity of MCT1/MCT4 after NP overexpression in LS174T *BSG*^{-/-} cells. However, these results do not solve the mystery around the residual expression of MCTs as our cells are obviously expressing NP, but are lacking endogenous EMB (Fig. 23).

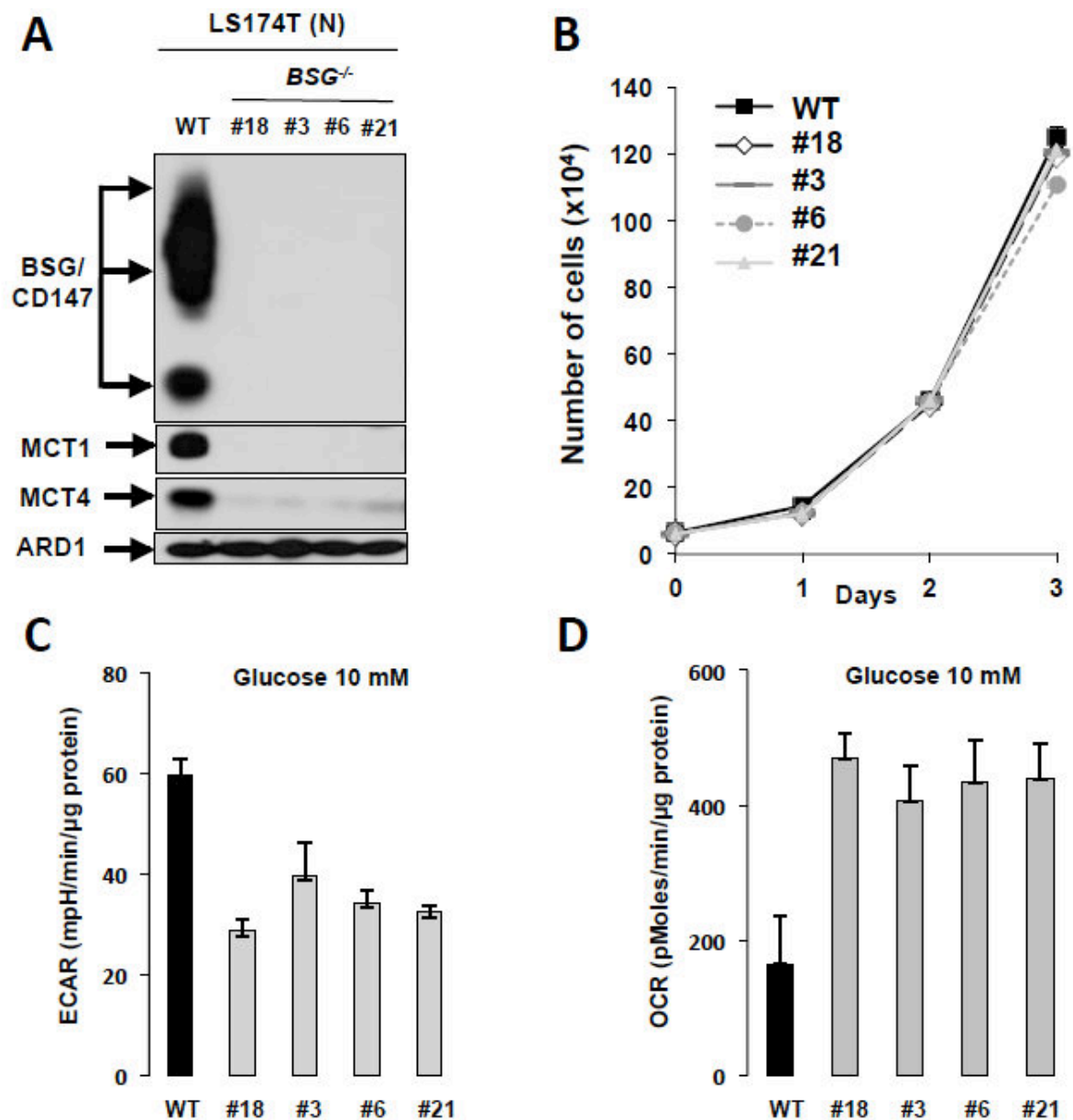


Figure 21. Disruption of *BSG* gene with ZFN targeting exon 7 encoding the transmembrane domain of all *BSG* splice variants has similar effects than using the ZFN targeting exon 2.

A) LS174T WT and knock out cells were maintained in normoxia for 48h and lysed. The cell expression of MCT1, MCT4, and *BSG* was analysed by immunoblotting. ARD1 was used as a loading control. #18 corresponds to *BSG*^{-/-} LS174T cells referred to in publication 2 and 4, while # 3, 6 and 21 are the clones obtained by the ZFN targeting exon 7 of *BSG* gene. **B)** Proliferation rate of LS174T WT and mutant cells incubated in normoxia for 72h. Real-time analysis of ECAR **C)** and OCR **D)** with a Seahorse XF24 analyzer. The effect of addition of glucose (10 mmol/L) to WT and *BSG*^{-/-} LS174T cells is shown.

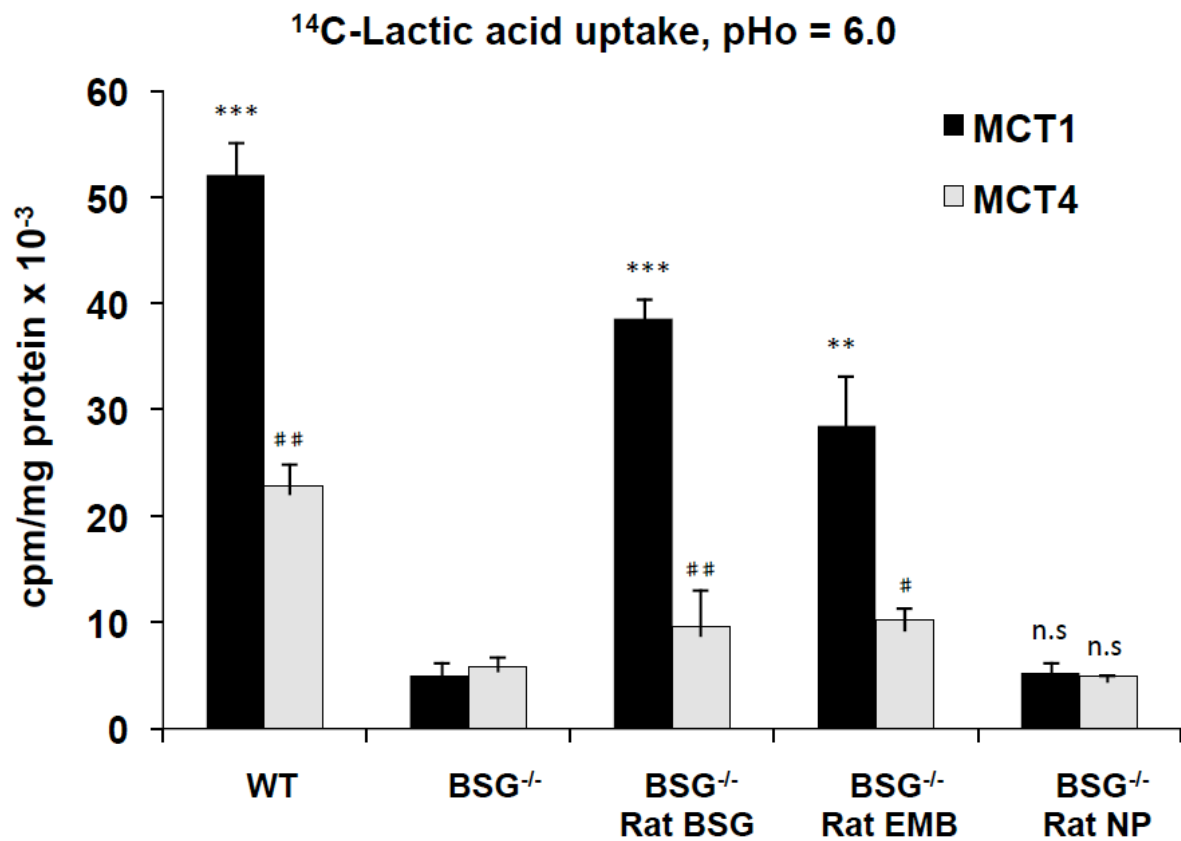


Figure 22. Effect of rat BSG, EMB and NP on MCT1 and MCT4 activity in LS174T cells lacking endogenous BSG.

[¹⁴C]-Lactic acid uptake in LS174T WT, BSG^{-/-} cells, and BSG^{-/-} cells transfected with rat Bsg, Emb or NP (NP 55). Discrimination of the total transport activity of MCT1 and MCT4 was possible by using the MCT1i (300 nmol/L); ** P < 0.001; *** P < 0.0001; n.s, not significant; and # P<0.05 ; ## P < 0.001 (comparison of the MCT1 and MCT4 activity, respectively, of LS174T WT and each mutant derived cell type vs. LS174T BSG^{-/-} cells).

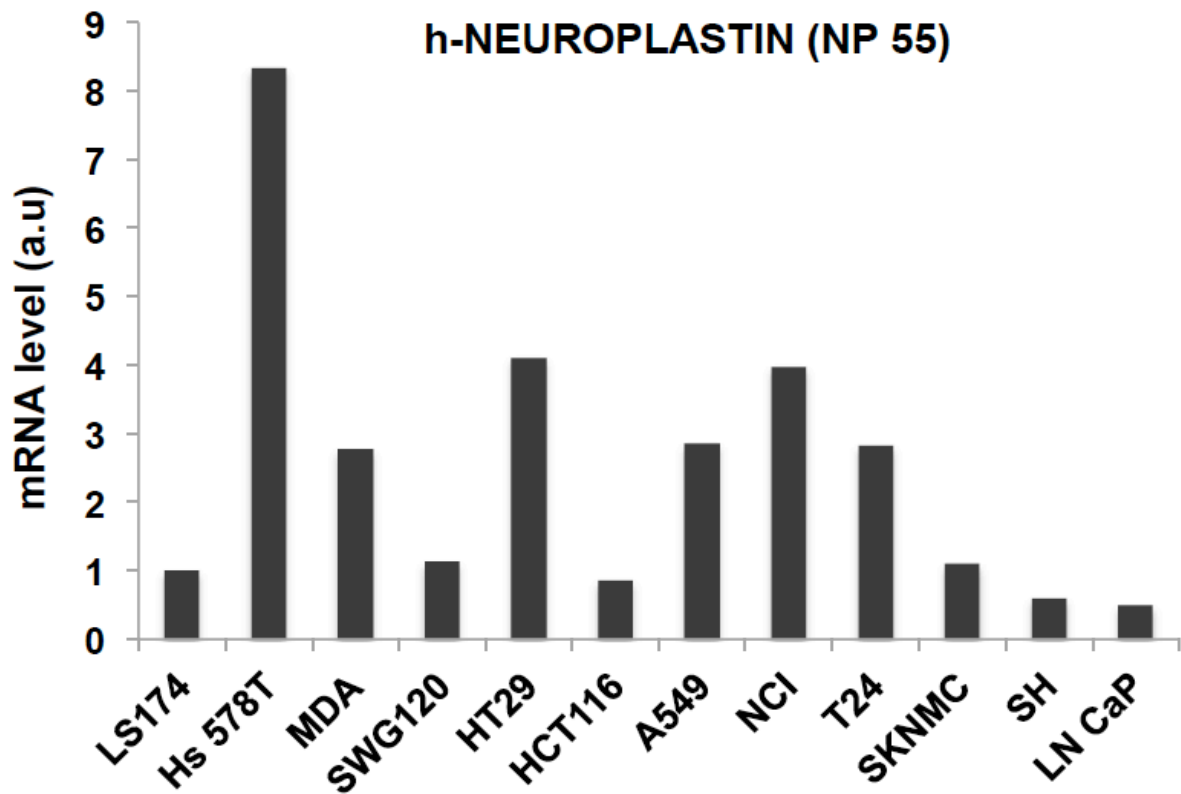
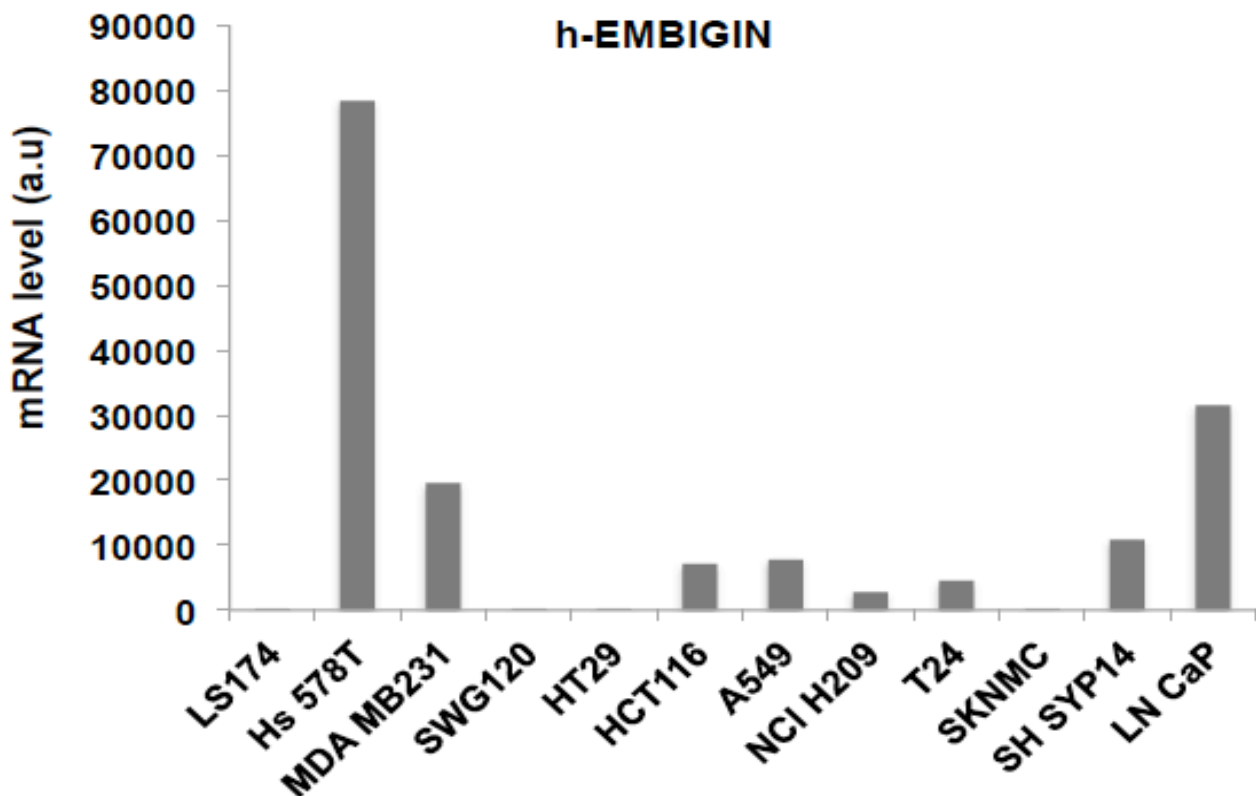
A**B**

Figure 23. Expression of endogenous NP and EMB in different human cell lines including LS174T cells

Expression of the mRNA level of NP-55 **A**) and EMB **B**) in LS174T cells and human cancer cell lines obtained from different tissues including colon, prostate, breast, lung and bladder.

Therefore, as I mentioned in the introduction of this thesis, the question regarding the presence of another unidentified component of the lactic acid transport complex remains unresolved. Nevertheless, a recent study has suggested a putative role of CD44 as an ancillary protein of MCTs (Slomiany et al., 2009). Further investigation is needed to determine the role of *CD44* knock out on MCT1, MCT4 and BSG expression and activity, and to also assess the expression of CD44 in *BSG*-null mice, specifically in tissues where MCT1 is still expressed.

Moreover, work from Jang et al. (Jang et al., 2008) has shown that LKB1 induces striking relocalization of Sln/dMCT1, the *Drosophila* homologue of mammalian MCT1, from the basolateral to the apical membrane, *via* an unidentified mechanism. Indeed, when inactive LKB1 (kinase-deficient) was expressed or LKB1 expression was reduced by siRNA, Sln/dMCT1 was mainly localized on the basolateral wing disc membrane. Therefore, it would be interesting to establish if LKB1 is involved in controlling MCT expression in our case, although the results obtained in NSCLC (publication 3) show that A549 cells, which are lacking LKB1 activity, are still expressing residual MCT1 and MCT4.

A recent study from Donaldson's group (Maldonado-Baez and Donaldson, 2013) has also reported an interesting mechanism of selective post-endocytic sorting of CD147, CD44 and CD98 that recycles these molecules directly to the plasma membrane after clathrin-independent endocytosis (CIE). In this study, the authors have shown that Hook1, microtubules, and Rab22a work in coordination to directly recycle CD147 and the two other proteins that express, at their cytoplasmic tail, two clusters of acidic amino acids, necessary for specifying the endosomal trafficking route. Such acidic clusters are also present at the C-terminus of MCT1 (amino sequences DDE and EEE), subsequently, it could be interesting to verify the implication of these residues but also Hook1 and Rab22a proteins in the residual expression of MCTs when BSG is lacking (Fig. 24).

Although all these suggestions are interesting, it remains the possibility that MCT1 and MCT4 are able to hijack some unknown chaperone or even that a small fraction of MCT1 and MCT4 adopts a right folding allowing them to traffic to the plasma membrane without chaperone assistance.

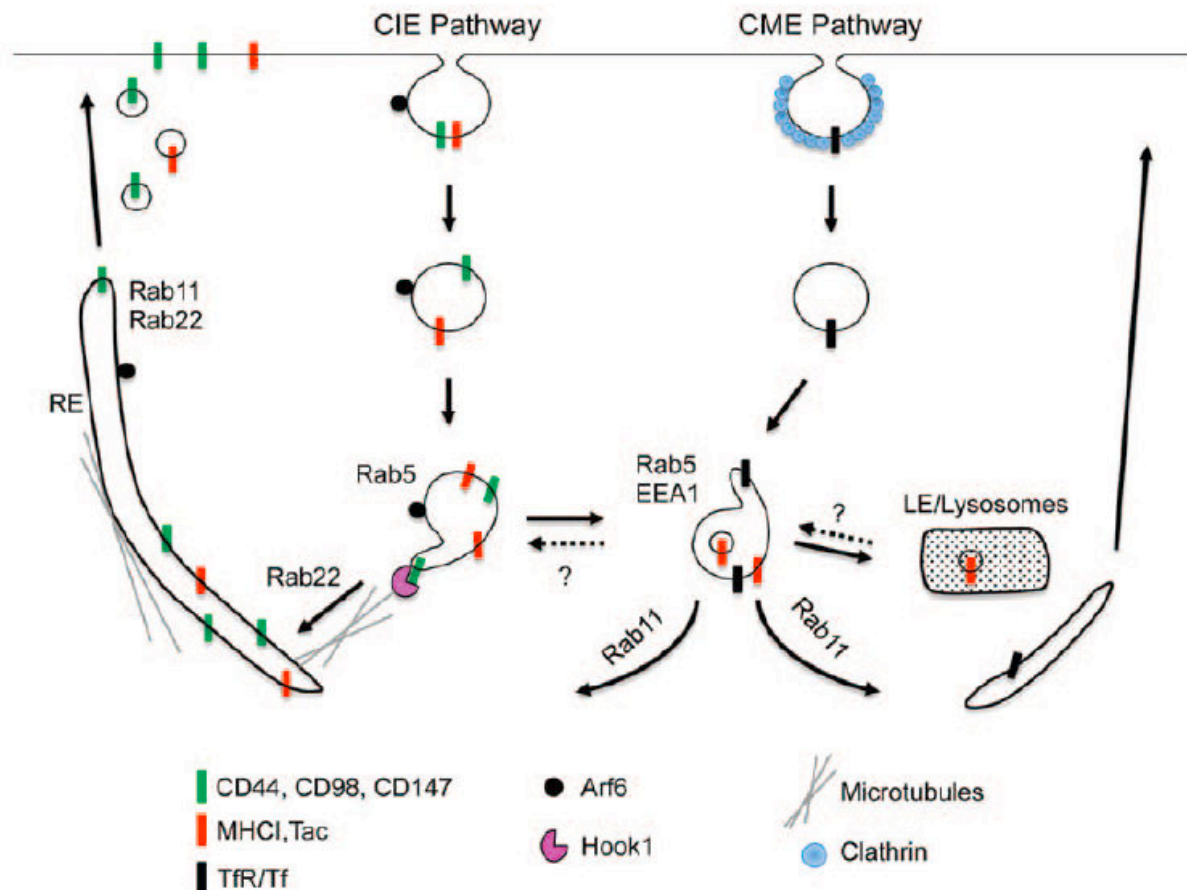


Figure 24. General model for the endosomal sorting of CIE cargo proteins

CIE cargo proteins are internalized by a pathway independent of clathrin, dynamin and associated with the small G protein Arf6. Prototypical CIE cargo proteins (red bars) enter the cell in Arf6-positive endocytic vesicles that either fuse with or mature into Rab, EEA 1- and transferrin-positive endosomes. The cargo proteins are then either targeted to late endosomes (LE) for degradation or recycled back to the plasma membrane in a Rab22/Rab11dependent manner. CIE cargo proteins harbouring cytoplasmic sorting motifs (green bars) avoid trafficking to EEA 1- and transferrin-(TfR/Tf; black bars) associated endosomes and traffic directly to the recycling endosomes (RE). Hook1 facilitates the directed recycling of CIE cargo proteins, such as CD98 and CD147, through its interaction with microtubules and their cytoplasmic sequences on sorting endosomes (adapted from (Maldonado-Baez and Donaldson, 2013)).

C. Pyruvate: the driver of the metabolic shift from glycolysis to OXPHOS?

Ultimately, the problem of targeting cellular metabolism is the interconnection between all the pathways and the ability of cancer cells to switch from one phenotype to another in order to produce energy and to sustain viability and slow growth, which raises the question of resistance to anti-cancer therapies and tumour recurrence. In the case of MCT/BSG inhibition, our studies show that blocking both MCT1 and MCT4 in human colon adenocarcinoma, glioblastomas and NSCLC cells causes a shift of their metabolism from glycolysis to OXPHOS, which sensitises them to biguanides, such as metformin and phenformin (Granja et al., 2015; Marchiq et al., 2015b). Similar observations were reported for Raji lymphoma and breast cancer cells (Doherty et al., 2014). However, the mechanism driving this switch still remains unclear. We hypothesised that accumulated pyruvate, after MCTs inhibition, increased, by mass action, the activity of PDH. To verify this hypothesis, it was necessary to determine whether when pyruvate accumulates in the cytoplasm more pyruvate is oxidized by PDH or not? To approach this question, we designed experiments without glutamine, as glutamine is another major substrate for respiration (publication 2). The results showed that the rate of respiration of the different LS174T cell lines increased in proportion to the decreased level of the MCT activity ($WT < MCT4^{-/-} < BSG^{-/-}$) and became maximal in the presence of MCT1i. We also found that the respiration fold-changes recorded for $MCT4^{-/-}$ and $BSG^{-/-}$ cells, paralleled the increase in intracellular pools of pyruvate, following the addition of MCT1i.

To further assess whether accumulated pyruvate is redirected to mitochondria, we collaborated with Martinou's group who has recently engineered a genetically encoded biosensor based on bioluminescence resonance energy transfer (BRET), which they called RESPYR (for REporter Sensitive to PYruvate). By monitoring the changes in BRET, it has been possible to monitor the activity of the mitochondrial pyruvate carrier (MPC) in real time with a high temporal resolution. Therefore, using LS174T WT and $MCT4^{-/-}$ cells, we have found that increasing the intracellular pool of pyruvate in LS174T $MCT4^{-/-}$ cells, and not WT cells, by pharmacological inhibition of MCT1, significantly increased MPC activity and cell respiration when cells were exposed to glucose, suggesting that accumulated pyruvate crosses the IMM and binds to MPC (Fig. 25).

However, further investigation using metabolomics with radio labelled glucose are required to determine if indeed accumulated pyruvate enters the mitochondria. Monitoring the PDH activity in our different WT and MCT/BSG disrupted cell lines, should also be done to validate our hypothesis. Finally, due to the emergent role of MPC in cancer, it would be interesting to assess the effect of blocking MPC, genetically by generating an MPC1 knock out or pharmacologically by using the specific inhibitor UK5099, on WT and MCT/BSG disrupted cells in the presence and absence of glutamine.

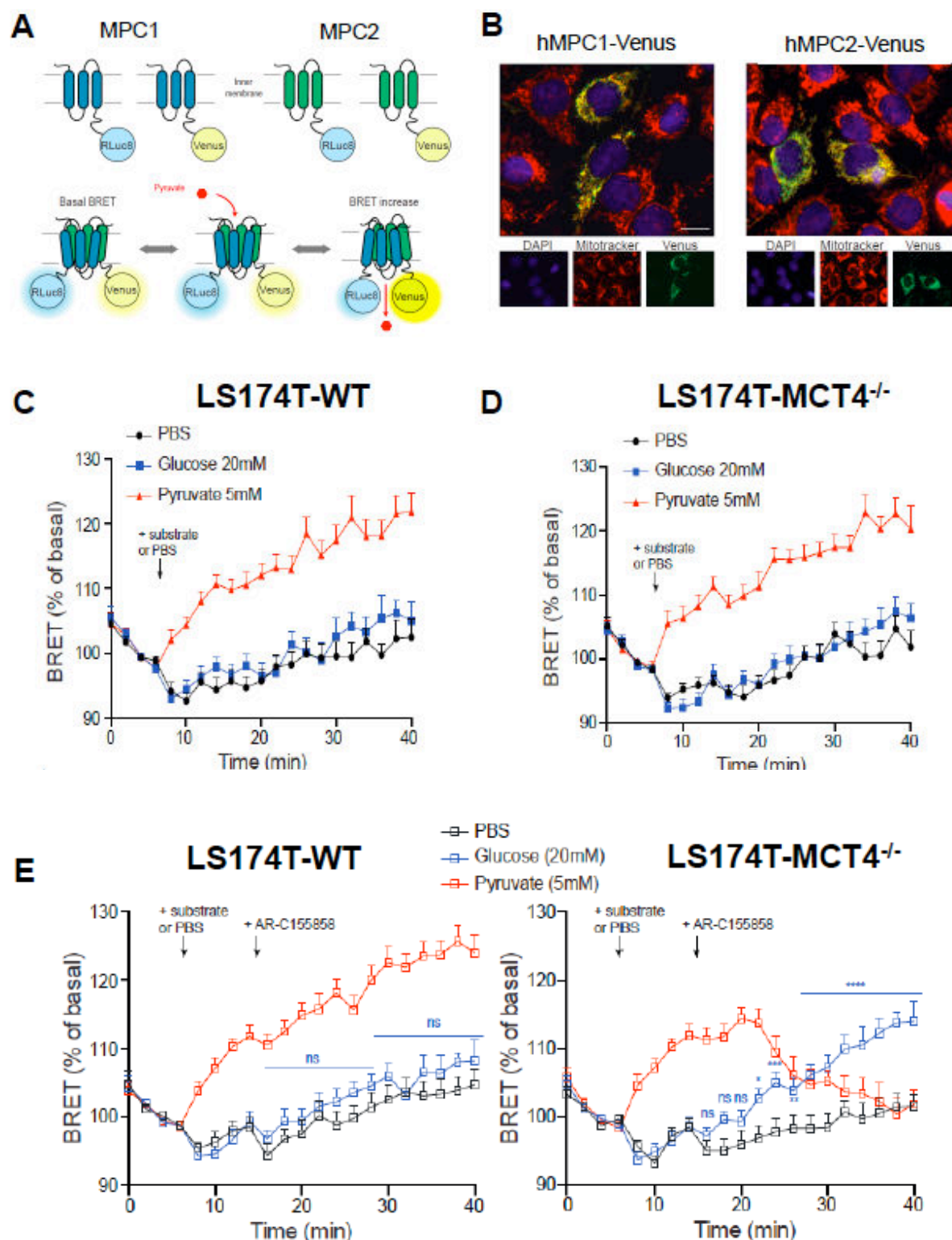


Figure 25. RESPYR, a BRET-based sensor to monitor MPC activity in WT and *MCT4*^{-/-}

A) Schematic representations showing MPC subunits tagged with either RLuc8 or Venus (top panel), and pyruvate-induced changes in MPC conformation leading to increase in BRET signal (bottom panel). **B)** Representative images showing the subcellular localization of hMPC1 and hMPC2 tagged with Venus. Two days after transfection with either MPC1-Venus or MPC2-Venus (green), HeLa cells were stained for mitochondria using Mitotracker (red) and for nuclei with Dapi (blue). Scale bar: 10 μ m. BRET kinetics in LS174T **C)** or LS174T-MCT4^{-/-} **D)** stably expressing RESPYR. Cells were stimulated with PBS (black), 5 mM pyruvate (red) or 20mM glucose (blue) at the time indicated by the arrows. **E)** BRET kinetics in LS174T cells (E, left panel) or LS174T-MCT4^{-/-} cells (E, right panel) stably expressing RESPYR. At the times indicated (arrows), cells were stimulated with PBS (black), 20 mM glucose (blue) or 5 mM pyruvate (red) prior to treatment with 1 μ M AR-C155858 (arrows) (adapted from (Compan et al., 2015)).

D. Benefits and risks of future anti-cancer therapies based on the blockade of lactic acid export

During this thesis, we have presented genetic and pharmacological proofs on the efficiency and promise of targeting MCT/BSG complexes in cancer. While the inhibition of MCT1 alone in malignancies that are highly expressing MCT1, such as lymphomas, was described to induce tumour cell arrest and an increased radiosensitivity (Bola et al., 2014; Doherty et al., 2014; Polanski et al., 2014) (Fig. 26A), we expect that MCT4 inhibitor alone will also show efficacy in treating cancers that are highly expressing MCT4, such as renal cell carcinomas. However, due to functional redundancy between MCT1 and MCT4, and according to our findings (Granja et al., 2015; Marchiq et al., 2015b) and to work recently published by Cleveland's group (Doherty et al., 2014), this mono-inhibition will not be sufficient to achieve a long term anti-tumour activity (Fig. 26B). Consequently, a concomitant inhibition of both MCTs will be required to completely inhibit glycolysis. If combining the two MCT inhibitors will be introduced for anti-cancer treatment, further preclinical investigations in mice assessing their toxicity in normal tissues will also be required. The wide spread expression of MCTs and BSG, in addition to the large spectrum of their functions (cellular metabolism, pH_i, proliferation, angiogenesis, immune response) imply necessarily the emergence of possible side effects, specifically in MCT/BSG highly expressing organs, such as heart, skeletal muscle, eyes, colon and genital organs (Fishbein et al., 2002; Nakai et al., 2006). AstraZeneca have already indicated that there may be possible undesirable side effects to some of these organs in the phase I clinical trial of AZD3965. Another major problem that will be facing such a strategy is mainly the resistance that will occur due to the metabolic plasticity of tumour cells. Indeed, we reported in this thesis that unexpectedly, tumour cells escaped the blockade of lactic acid export by shifting their metabolism from glycolysis towards OXPHOS, to maintain physiological ATP pools necessary for cell growth and survival (Fig. 26C). Concomitant inhibition of glycolysis (MCTs inhibition) and mitochondrial complex I (biguanides) induced then an "ATP crisis" or a "metabolic catastrophe" leading to rapid tumour cell death and tumour growth collapse (Fig. 26D). Similar synthetic lethality was described for prostate and colon cancer cells, in which combined treatment with 2-deoxyglucose and metformin or phenformin alone resulted in

extensive cell death (Ben Sahra et al., 2010a; Lea et al., 2011). However, such strategies now require further pharmacological evaluation in immune competent and genetically engineered mouse tumour models. Although blocking the last step of glycolysis, through the inhibition of MCTs, should be less toxic for normal tissues than 2-deoxyglucose, an acceptable therapeutic window of combined MCT1/MCT4 inhibitors needs to be determined. Then, a second acute treatment for 1- to 3-days with phenformin should be tested for toxicity and tumour eradication. Considering the potency and the selectivity of the two MCT1 and MCT4 inhibitors developed by AstraZeneca, and the large spectrum of growth arrest obtained in all of the human tumour cell lines analysed, we are very optimistic for future clinical development of these new drugs.

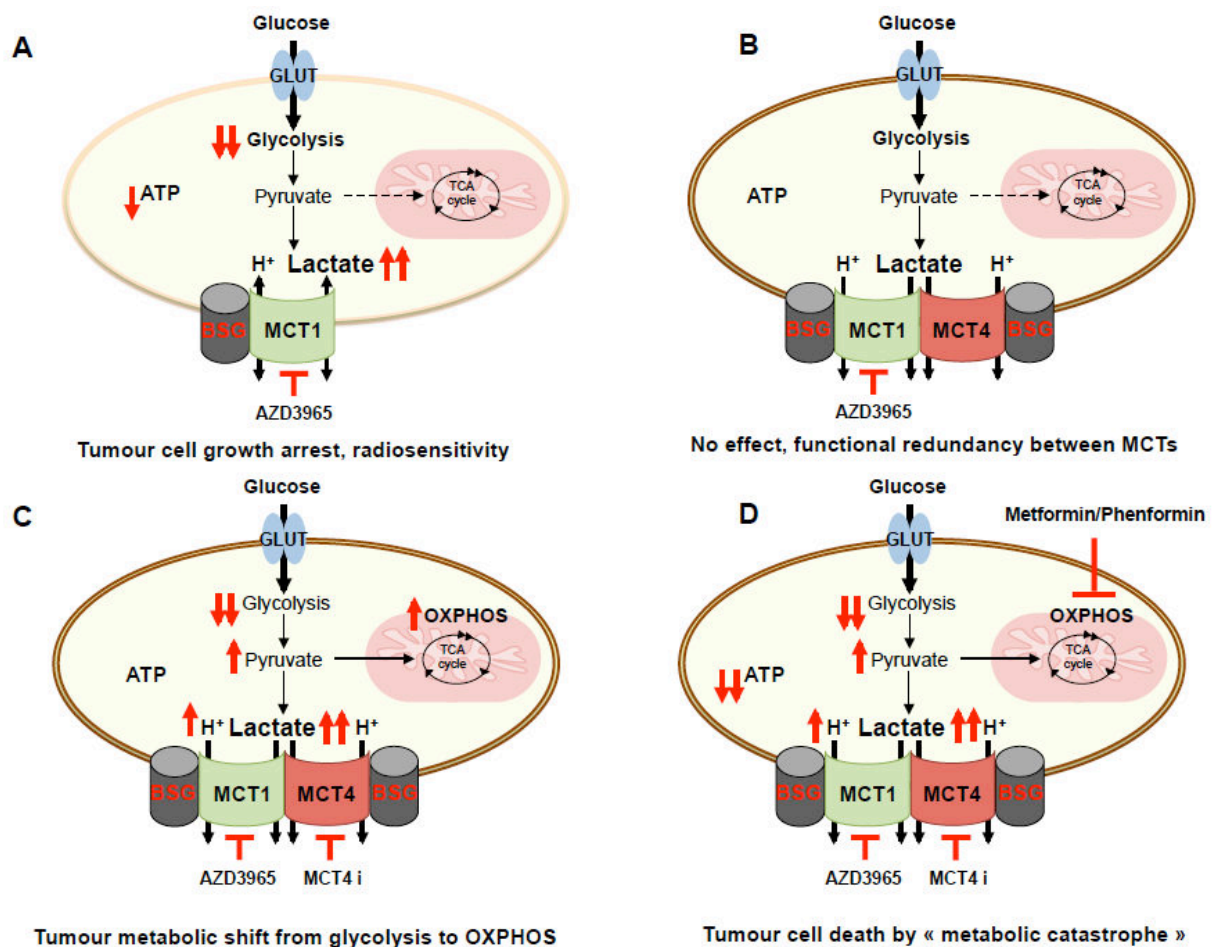


Figure 26. Efficiency of targeting lactate/H⁺ symporters for anticancer therap

A) Few oxidative cancer cells could use lactate to generate ATP, thus inhibition of monocarboxylate transporter 1 (MCT1) with AstraZeneca's specific inhibitor AZD3965 results in growth arrest. Other type of cancer cells, glycolytic and expressing only MCT1, will be also sensitive to MCT1 inhibitor showing growth reduction, cell death and radiosensitivity. **B)** Most of glycolytic cancer cells are expressing both MCT1 and MCT4. Due to functional redundancy between these two MCTs, AZD3965 will have no effect on hypoxic regions of the tumours. **C)** Combined inhibition of MCT1 and MCT4 results in decreased glycolytic rate and severe growth arrest. However increased intracellular lactic acid pool and subsequently increased intracellular pyruvate concentration, will fuel the tricarboxylic (TCA) cycle leading to metabolic shift from glycolysis towards OXPHOS. Therefore, tumour cells, although growing slowly will survive by keeping physiological ATP pool and escape lactate export blockade. **D)** Concomitant application of MCT inhibitors with metformin or phenformin, which inhibits OXPHOS, induces synthetic lethality resulting in "ATP crisis". Consequently, rapid tumour cell death occurs due to "metabolic catastrophe". Basigin (BSG); MCT4 inhibitor (MCT4 i).

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ANNEXES

Annexes

List of publications

- **Marchiq I**, Pouyssegur J. Hypoxia, cancer metabolism and the therapeutic benefit of targeting lactate/H⁺ symporters. *Journal of Molecular Medicine*. (invited review). 2015 June 24 (Epub ahead of print).
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Gene Disruption Using Zinc Finger Nuclease Technology

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Abstract

Zinc finger nucleases are reagents that induce DNA double-strand breaks at specific sites that can be repaired by nonhomologous end joining, inducing alterations in the genome. This strategy has enabled highly efficient gene disruption in numerous cell types and model organisms opening a door for new therapeutic applications. Here, we describe the disruption of CD147/basigin by this technique in a human cancer cell line.

Key words DNA double-strand breaks, Gene targeting, Knockout, Zinc finger nuclease

1 Introduction

Zinc finger nucleases (ZFNs) are a class of engineered DNA-binding proteins that have redefined genome engineering. The ZFN plasmids bind a specific DNA sequence, inducing a double-strand break (DSB). This break is repaired by the non-perfect endogenous repair mechanism called nonhomologous end joining (NHEJ). The DSB will be misrepaired by the addition and/or deletion of nucleotides. This disruption results in multiple mutations that could induce nonsense gene products in both alleles leading to a specific gene knockout. The ZFN structure consists of a DNA-binding domain—zinc finger protein (ZFP)—linked to a nuclease domain of the FOKI restriction enzyme [1].

The ZFP region contains a tandem array of Cys2–His2 fingers that each recognizes 3 bp of DNA. This domain can have from 3 to 6 fingers binding to a target of 9–18 bp, respectively. The catalytic domain composed by the enzyme FOKI requires dimerization to cut; thus, a pair of ZFNs is required. The dimerization restriction avoids cleavage at single binding sites [2]. Genomic modification with ZFN was first described more than 10 years ago, and a huge progress in this technique was observed in the last few years. This is now a well-established method in several organisms [3].

ZFN-targeted mutagenesis has been achieved in zebrafish [4], rat embryo [5], mouse [6], sea urchin [7], frog [8], *Drosophila* [9], plants [10], *C. elegans* [11] and human cells [12].

Applications of ZFNs represent a complementary strategy to siRNA enabling highly efficient gene disruption in human cells, opening a door for new therapeutic applications [1]. The aim of our study is to understand the tumorigenic influence of lactate transport by monocarboxylate transporters (MCT1 and MCT4) in human cells by inhibiting their chaperone CD147/basigin. Here, we demonstrate an efficient disruption of the gene CD147/basigin in the A549 human lung adenocarcinoma cell line. A similar procedure has already been described in our laboratory in the LS174T colon adenocarcinoma cell line [12]. In this chapter, we show a protocol used to knock-out CD147/basigin in A549 cell line.

2 Materials

2.1 ZFN

1. CompoZr Knockout ZFNs (Sigma-Aldrich): ZFN plasmids targeting basigin exon 2 encoding signal peptide of basigin splice variants 1 and 2 were designed and obtained from Sigma-Aldrich. Store at -80°C .
2. Each kit contains, besides the plasmid DNA for each ZFN, a pair of primers (forward and reverse) for screening of mutations in the DNA locus. Make aliquots of $10\text{ }\mu\text{M}$. Store at -20°C .

2.2 Plasmid Transformation and Isolation

1. Chemically competent *E. coli*.
2. LB medium: For 1 l of water dissolve 5 g yeast extract, 10 g tryptone and 10 g of NaCl. Adjust to pH 7.0. Autoclave and allow to cool to 55°C . Add the antibiotic kanamycin. Store at room temperature (RT). For LB plates, add to the LB medium 20 g agar before autoclaving. Fill 100 mm diameter plates, and allow to solidify.
3. Plasmid isolation/purification Maxi Kit (QIAGEN) [13].

2.3 Cell Lines

1. A549 human adenocarcinoma cell line.
2. Culture media: Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 % inactivated serum, penicillin ($10\text{ }\mu\text{g/ml}$), and streptomycin ($50\text{ }\mu\text{g/ml}$).

2.4 Reagents

1. PBS-10 %: For 1 l of water dissolve 80 g NaCl, 2 g KCl, 16.1 g K_2HPO_4 and KH_2PO_4 . Allow to dissolve, and adjust the pH to 7.4. Store at RT.
2. PBS-1 % serum and 1 mM EDTA: Dilute PBS-10 % with water to 1 %, add 1 % of serum (FCS) and 1 mM EDTA (*see Note 1*).
3. JetPRIME[®] DNA and siRNA Transfection Reagent (Polyplus-transfection SA): The kit contains a JetPRIME buffer to dilute DNA and the transfection reagent. Store at 4°C (*see Note 2*).

2.5 Antibodies

1. Mouse monoclonal EMMPRIN antibody (Santa Cruz Biotechnology): Store at 4 °C.
2. Mouse monoclonal antibody EMMPRIN/CD147 (R&D Systems): Store at -20 °C.
3. R-Phycoerythrin-conjugated AffiniPure Goat Anti-Mouse IgG (Jackson ImmunoResearch): Store at 4 °C in the dark.
4. Horseradish peroxidase anti-mouse antibody (Promega): Store at 4 °C.

2.6 Equipment

1. Centrifuge and microcentrifuge.

3 Methods

3.1 Plasmid Transformation and Isolation

This protocol should be carried out on ice unless otherwise specified.

1. Transform the ZFN plasmids into chemically competent *E. coli* cells.
2. Add 50 ng/μl of each plasmid into a vial of chemically competent *E. coli* cells separately.
3. Incubate on ice for 30 min.
4. Subject the cells to a thermal shock at 42 °C for 45 s, and put them on ice for 2 min.
5. Transfer into 900 μl of pre-warmed LB medium, and place to shake horizontally (200 rpm) for 1 h at 37 °C.
6. Plate 10–100 μl from each transformation on a plate with LB agar medium containing the antibiotic for selection (*see Note 3*), and incubate overnight at 37 °C (*see Note 4*).
7. Pick isolated colonies, and subculture them in a tube containing 5 ml of LB medium with the antibiotic of selection (*see Note 3*). Allow growing overnight (orbital incubator, 37 °C, 225 rpm).
8. Isolate the plasmid with the QIAGEN Plasmid isolation Maxi Kit (*see Note 5*).

3.2 Cell Line Transfection

All procedures should be carried out under sterile conditions in a laminar airflow chamber (*see Notes 6 and 7*).

1. Seed A549 cells with 60 % confluence (1×10^6 cells) in a 100 mm diameter dish.
2. After 24 h, transfect the cells using JetPRIME DNA transfection reagent.
3. Dilute 10 μg of DNA (5 μg of each ZFN plasmid) into 500 μl JetPRIME® buffer. Mix by vortexing (*see Note 8*).

4. Add 20 μ l of JetPRIME reagent vortex, and spin down briefly. Incubate the mixture for 10 min.
5. Add 500 μ l of the complex (drop by drop) into the cells, and mix gently.
6. Replace the transfection medium after 6 h by fresh medium.
7. Culture the cells for 1–2 weeks in a humidified atmosphere of 5 % CO₂ at 37 °C.

3.3 Fluorescence-Activated Cell Sorting

All procedures should be carried out under a laminar airflow chamber. During incubation times, place the cells and PBS–1 % serum on ice. Cell clumps should be avoided by resuspending the cells during long incubations (*see Note 9*).

1. Place up to 1×10^6 cells in a tube. Centrifuge for 5 min at $700 \times g$. Discharge supernatant (*see Note 10*).
2. Wash once with cold PBS–1 % serum. Centrifuge for 5 min at $700 \times g$. Discharge supernatant.
3. Per 1×10^6 cells add 100 μ l of CD147 antibody diluted 1/100 in PBS 1 \times 1 % serum. Incubate for 30 min on ice (*see Note 11*).
4. Wash once with PBS 1 \times serum 1 % by centrifugation at $700 \times g$ for 5 min. Aspirate supernatant.
5. Incubate the cells with the R-Phycoerythrin-conjugated AffiniPure Goat Anti-Mouse IgG (Jackson ImmunoResearch) diluted 1/1,000 in PBS–1 % serum. Incubate for 30 min on ice (*see Note 12*).
6. Wash once with PBS 1 \times FCS 1 % by centrifugation at $700 \times g$ for 5 min. Aspirate supernatant.
7. Add 500 μ l PBS 3 % serum 1 mM EDTA, and filter the samples in a fluorescence-activated cell sorting (FACS) tube.
8. Select and sort CD147-negative cells, and plate one cell per well in a 96-well plate with 100 μ l DMEM supplemented with 10 % inactivated serum and penicillin (10 μ g/ml). Allow the cells to grow in a humidified atmosphere of 5 % CO₂ at 37 °C (*see Notes 13 and 14*).
9. After growing in 96-well plates, each clone should be subcultured for the screening of CD147/basigin cells (*see Note 15*).

3.4 Screening of CD147/Basigin Cells

1. Freeze some cells of each clone in a vial with serum 1 % DMSO (*see Note 16*).
2. Subculture each clone in two different wells. Add 1 mM metformin to one well (for a preselection *see Note 17*), and let the other well growing to propagate the clone.
3. Extract proteins from the clones that die in the preselection with metformin.

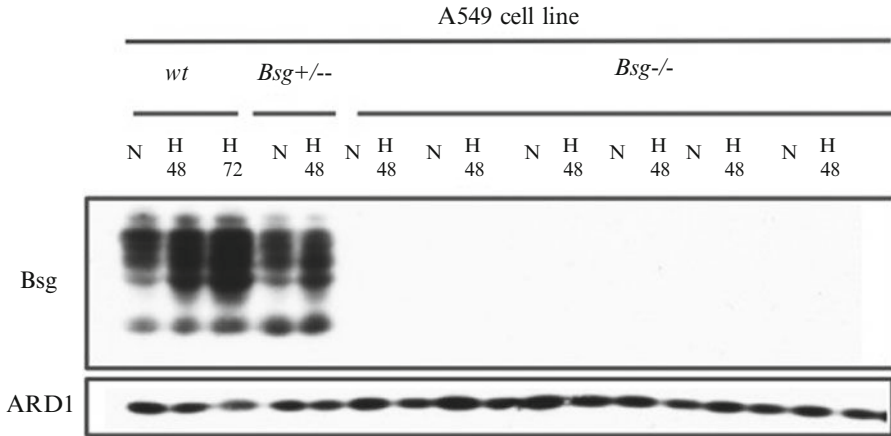


Fig. 1 Western blot analysis of Bsg in transfected cells. A549 wt cells (lanes 1–3) *Bsg^{+/-}* cells (lanes 4 and 5) and *Bsg^{-/-}* cells (lanes 6–17) exposed to normoxia (N) and hypoxia 1 % O₂ (H). ARD1 was used as a loading control

4. Perform a Western blot to analyze the expression of CD147/basigin in these clones as shown in Fig. 1 (*see Note 18*).
5. Select the clones that were completely negative by Western blot (*see Note 19*).
6. Extract genomic DNA from cells.
7. Confirm gene disruption by performing a PCR with the primers provided by ZFN kit (*see Note 20*). Send to sequence your PCR products (*see Note 21*).

4 Notes

1. Prepare this solution immediately before use. PBS should be cold and sterile.
2. The delivery of ZFN plasmids into the cells could be done with different transfection protocols. The choice of transfection technology can strongly influence transfection efficiency. From all the protocols we tested, we chose this transfection protocol because it is fast and easy to perform, causes minimal cytotoxicity for this cell line, and requires smaller amount of DNA to transfect.
3. The appropriate antibiotic is described in the datasheet of the zinc finger plasmids sent by the manufacturer. In this specific case, the antibiotic used for selection was kanamycin.
4. Plate two different volumes to ensure that at least one plate will have well-spaced colonies.

5. QIAGEN-tip 500 protocol was followed as described by the manufacturer. In the end, DNA was redissolved in approximately 100 μ l.
6. Aseptic techniques and the proper use of laboratory equipment are essential when working with cell cultures. Always use sterile equipment and reagents, and wash hands, reagent bottles, and work surfaces with surfanios or 70 % ethanol before starting the work.
7. Health and viability of the cell line can influence the efficacy of transfection. Check this before you start your experiment.
8. In order to control if the cells internalized the plasmids, you can also introduce during transfection a GFP plasmid (0.5 μ g). This will give you the information that the transfection went good and cells were properly transfected.
9. This approach cannot be used for all molecules. You need the cells viable so that the antibody should be able to recognize protein without internalization. In case this is not possible, transfection with GFP plasmid should be performed and the cells should be analyzed by FACS after 48 h of transfection. In this case, cells positive for GFP should be sorted.
10. Try to stain the maximum of cells possible to have the number of events necessary for FACS analysis (around 6×10^6 cells). Use separate tubes for staining of 1×10^6 each to make sure that all cells are uniformly stained.
11. Do not forget to have one tube as negative control for CD147/basigin expression. This tube should not be incubated with primary antibody. Incubation time can vary between 30 min and 1 h. Do not forget to resuspend the cells to avoid deposition that could lead to inefficient staining.
12. Protect all tubes from light so as not to degrade the fluorochrome.
13. There are some cell lines that are not able to grow as single cells. For that, 100 μ l of conditioned media should be added to the well. The growth factors available in the conditioned media can help cells to attach and to growth.
14. As single cells (clones) are plated in each well, cells will need approximately up 3–4 weeks to grow and generate a cell population. Thus, after 2 days of sorting the cells, make up the well volume to 200 μ l with fresh medium to ensure enough nutrients for cell growth.
15. Clones with different size and phenotypes are obtained. You should not forget that besides the selection of negative cells stained by the cell sorter, different cells are also originated as heterozygotic cells (with only one allele cut), homozygotic cells (with the two alleles cut), and cells that have not been

transfected. Each clone should be identified as desired (e.g., 1, 2, 3, ...). Select the maximum of clones, and subculture them. This step is critical. Manipulate carefully your cells to avoid contamination.

16. Freezing media depends on the cell line. It normally consists in growth medium (RPMI, DMEM, etc.) containing 5–10 % serum.
17. Metformin is a drug from biguanide class that impairs oxidative phosphorylation. We perform this treatment as a preselection for putative negative clones. The ones that have disruption of CD147/basigin and consequently a decrease in lactate export (inhibiting glycolysis) will die after blocking respiration.
18. Western blot can vary according to the lab. In our lab and for this study, we separated 40 µg of protein on 8 % SDS polyacrylamide gels and transfer onto polyvinylidene difluoride membranes (Millipore). Membranes were incubated with the monoclonal antibody of Bsg/CD147 (R&D Systems) diluted 1/1,000 and then with a horseradish peroxidase anti-mouse antibody (Promega) diluted 1/5,000. Bands were detected with ECL (Amersham Biosciences or Millipore).
19. Select only 5–8 clones to further characterize the decreasing amount of cells to manipulate.
20. PCR reaction was carried out in a total volume of 20 µl, consisting of 200 ng of gDNA, 0.5 µM of both sense and antisense primers (sent with the ZFN kit), 200 µM of dNTPs, 1.5 mM of MgSO₄, 1× Taq Buffer, and 10 U of KOD hot start DNA polymerase (all reagents from Novagene). The reaction consisted of an initial denaturation at 95 °C for 3 min, followed by 31 cycles with denaturation at 95 °C for 10 s, annealing at 68 °C for 30 s, and extension at 68 °C for 30 s, followed by a final extension during 3 min at 68 °C.
21. New primers targeting a smaller fragment in the targeted area should be designed and synthesized. These new nested PCR products should be sent for sequencing to confirm the disruption of your gene in your clones.

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