Universidade do Minho
Escola de Ciências da Saúde

Sara da Costa Granja

Role of Monocarboxylate transporter in lung development and lung cancer

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Role of Monocarboxylate transporter in lung development and lung cancer

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Trabalho efectuado sob a orientação da
Doutora Maria de Fátima Baltazar
Professora Auxiliar da Escola de Ciências da Saúde da Universidade do Minho, Braga, Portugal

E co-orientação do
Professor Doutor Jorge Correia Pinto
Professor Catedrático da Escola de Ciências da Saúde da Universidade do Minho, Braga, Portugal

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Declaração

Nome: Sara Costa Granja
Endereço eletrônico: saragranja@ecsaude.uminho.pt
Telefone: (+351) 253604837
Numero de Bilhete de Identidade: 12779490

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Orientação
Doutora Maria de Fátima Baltazar
Co-orientação
Professor Doutor Jorge Correia Pinto

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“Todas as riquezas do mundo não valem um bom amigo.”
Voltaire

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ABSTRACT/RESUMO

ABSTRACT

Some studies suggest that lung tumour cell ontogeny is determined by consequences of gene expression that recapitulate important events in embryonic lung development. Hypoxia plays a crucial role either in physiological or pathological conditions such in tumour growth, and in embryonic and fetal development, being one of the most important extracellular factors for lung morphogenesis. In order to adapt to environmental hypoxia, cells rely on anaerobic glycolysis, resulting in increased production of acids, mostly lactic acid. Some studies demonstrated that the control of intracellular pH (pHi) could be carried out by a family of transporters which are H^+-monocarboxylate co-transporters, known as monocarboxylate transporters (MCTs). These studies show evidence for the expression of MCTs during early development of human and mouse embryos and in embryo pre-implantation.

As in embryonic development, tumour cells take advantage of MCTs to maintain tumour intracellular pH, by promoting the efflux of accumulating acids. There are studies showing the upregulation of MCTs in several solid tumours such as colorectal carcinomas, cervix carcinoma and breast tumours. Regarding lung tumours the results published so far are controversial.

MCT family comprises 14 members however only MCT1-MCT4 are proton symporters that exhibit different affinities for lactate, leading to different levels of tissue expression. Ancillary protein, named CD147 is required to maintain the catalytic activity of and the translocation to the plasma membrane of MCT1 and MCT4.

Since tumourigenesis and embryogenesis exhibit some common features, such as low oxygen availability, this work aims to study and characterize the role of MCTs both in lung development and lung cancer and thus seek for the metabolic adaptations common to both embryo and tumour development.

In lung development, MCT1, MCT4 and CD147 expression was evaluated during human and rat fetal lung development and the effect of MCT inhibition was evaluated on the morphogenesis of rat fetal lung explants. Importantly, MCT1, MCT4 and CD147 were differently expressed during the different phases of lung development suggesting that these molecules are important for lung morphogenesis. Moreover we exposed rat fetal lung explants to the MCT inhibitor CHC and we observed an inhibitory effect on lung branching and viability, in a dose dependent way.

Moreover, in lung cancer, MCT1, MCT4 and CD147 were evaluated in a human lung cancer series and in order to understand the influence of MCTs inhibition in lung cancer we disrupted CD147 using the zinc finger technology. The effect the knockout (KO) of CD147 was evaluated using *in vitro* and *in vivo* models.

Regarding lung cancer, MCT1, MCT4 and CD147 were upregulated in tumour samples when compared with non-tumoural adjacent tissue. With the KO of CD147 we decreased the expression and activity of MCT1 and MCT4. Moreover, the clonal growth of KO cells is inhibited *in vitro* after oxidative phosphorylation (OXPHOS) inhibition with phenformin and we observed a more pronounced decrease in tumour growth *in vivo* of KO cells to the treatment with phenformin.

To conclude, this study suggests that MCTs are important molecules in the physiology of lung development and provide evidence supporting the exploitation of MCTs/CD147 as therapeutic targets. However, further studies, particularly using specific MCTs/CD147 blockers are urgently needed.

RESUMO

Alguns estudos sugerem que a ontogenia das células tumorais do pulmão é determinada por consequências na expressão genética que recapitulam eventos importantes do desenvolvimento embrionário do pulmão. A hipóxia tem um papel crucial tanto em condições fisiológicas como patológicas no crescimento tumoral e no desenvolvimento embrionário e fetal, sendo um dos factores extracelulares importante na morfogéneses do pulmão. Para se adaptarem ao ambiente de hipoxia, as células dependem da glicólise anaeróbica resultando num aumento da produção de ácidos; nomeadamente acido láctico. Alguns estudos demonstram que o controlo do pH intracelular (pHi) pode ser realizado por uma família de transportadores denominados Transportadores de Monocarboxilatos MCTs. Estes estudos mostram evidências para a expressão dos MCTs durante o desenvolvimento precoce do embrião humana e de ratinho e também na pré-implantação do embrião.

Tal como no desenvolvimento embrionário, as células tumorais utilizam os MCTs para manter o pHi tumoral, promovendo o efluxo de ácidos acumulados. Há estudos que demonstram o aumento de expressão dos MCTs em diversos tumores sólidos, tais como carcinomas colo-rectal, carcinoma do colo-uterino e tumores mamários. Quanto aos tumores de pulmão, os resultados obtidos até agora são controversos.

A família dos MCTs engloba 14 membros no entanto, apenas MCT1-MCT4 são transportadores de protões e apresentam afinidades diferentes para o lactato, levando a diferentes níveis de expressão tecidual. A proteína auxiliar, denominada CD147 é necessária para manter a atividade catalítica e a translocação para a membrana plasmática do MCT1 e do MCT4.

Uma vez que a tumorigênese e embriogénese apresentam algumas características comuns, como o baixo teor em oxigênio, este trabalho tem como objetivo estudar e caracterizar o papel dos MCTs, tanto no desenvolvimento pulmonar como em cancro do pulmão e, assim, identificar as adaptações metabólicas comuns a ambos os processos.

No desenvolvimento pulmonar, a expressão do MCT1, MCT4 e CD147 foi avaliada durante em pulmão fetal humano e de rato e o efeito da inibição dos MCTs foi avaliada na morfogénese pulmonar usando explantes pulmonares fetais de ratinho. MCT1, MCT4 e CD147 foram diferencialmente expressos durante as diferentes fases do

desenvolvimento pulmonar, sugerindo que estas moléculas são importantes para a morfogênese de pulmão. Além disso, após a exposição dos explantes pulmonares fetais de rato ao inibidor dos MCTs, o CHC, observamos um efeito inibitório na viabilidade e ramificação do pulmão, de um modo dependente da dose.

Além disso, no cancro do pulmão, o MCT1, o MCT4 e a CD147 foram avaliados numa série de cancro de pulmão humano e a fim de compreender o efeito da inibição dos MCTs, inibimos a CD147 utilizando a tecnologia de "zinc finger". O efeito do knock-out (KO) da CD147 foi avaliada em modelos *in vitro* e *in vivo*.

Neste modelo observamos a sobre-expressão do MCT1, do MCT4 e da CD147 nas células tumorais, quando comparado com o tecido adjacente não-tumoral. Com o KO da CD147 verificamos a diminuição da expressão e da atividade do MCT1 do MCT4. Estudos *in vitro* demonstraram que após a inibição da fosforilação oxidativa com a fenformina o crescimento clonal das células negativas para a CD147 foi inibido e para além disso observamos uma diminuição mais acentuada no crescimento tumoral destas celulas *in vivo* após tratamento com a mesma droga.

Em termo de conclusão, este estudo sugere que os MCTs são moléculas importantes na fisiologia do desenvolvimento pulmonar e fornece evidências que suportam a exploração dos MCTs/CD147 como alvos terapêuticos. No entanto, são urgentemente necessários mais estudos, principalmente utilizando inibidores específicos para MCTs/CD147

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ABBREVIATIONS

ABBREVIATION LIST

AE1: anion exchanger 1

AMPK: AMP-activated protein kinase

CA: carbonic anhydrase

CHC: α-cyano-4-hydroxycinnamate

DIDS: 4,4'-diisothiocyanostilbene-2,2'-disulphonate

ECM: extracellular matrix

ERK 1/2: extracellular-signal-related kinase 1/2

FdG-PET: 18F-fluorodeoxyglucose positron emission tomography

GLUT1: glucose transporter 1

HIF: hypoxia-inducible transcription factor

HK: hexokinase

LDH-A: lactate dehydrogenase A

MCT: monocarboxylate transporter

MMP: matrix metalloproteinases

mTORC1: mammalian target of rapamycin complex 1

NF-κB: nuclear factor-kappaB

NAD^+ Nicotinamide adenine dinucleotide

NHE1: Na+/H+ exchanger 1

NSAIDs: non-steroidal anti-inflammatory drugs

OXPHOS: oxidative phosphorylation

pCMBS: p-chloromercuribenzene sulphonate

PPP: pentose phosphate pathway

PDH: pruvate dehydrogenase

PDK1: pyruvate dehydrogenase kinase 1

PFK1: phosphofructokinase 1

PGC-1α: peroxisome proliferator-activated receptor gamma, co-activator 1 alpha

PI3K: phosphatidylinositol-3-OH kinase

PKC: protein kinase C

PKM pyruvate kinase isozymes M1/M2

pHe: extracellular pH

pHi: intracellular pH

PPARα: peroxisome proliferator-activated receptor alpha

REMP: retinal epithelial membrane protein

SCO2: synthesis of cytochrome c oxidase

siRNA: small-interfering RNA

TCA: tricarboxylic acid

TMD: transmembrane domain

TNF-α: tumour necrosis factor-alpha

VEGF: vascular endothelial growth factor

AIMS AND THESIS LAYOUT

AIMS AND THESIS LAYOUT

AIMS

It is suggested that gene expression in lung tumour cells recapitulate important events in embryonic lung development. Hypoxia is a common event in both conditions where cells rely on anaerobic metabolism for energy supply, leading to intracellular accumulation of acids. These acids have an important role in cell metabolism and their rapid transport across the plasma membrane is crucial for the maintenance of intracellular pH homeostasis. This transport is mediated by a family of transporters, designated by Monocarboxylate Transporters (MCTs), namely isoforms 1-4. Studies on MCTs contribution to lung development are still scarce and for the malignant behavior of a tumour cell is not really elucidated. Thus, more basic scientific efforts on this matter are needed, which will probably result in important findings, with clinical implications.

Therefore, the **general aim** of this thesis was to contribute to a better understand of the role of MCTs in both lung development and lung cancer.

THESIS LAYOUT

The present thesis is organized in 4 different chapters:

Chapter 1 contains a general introduction on the subject of the thesis. It is presented a briefly introduction on embryo and cancer cell metabolism with special emphasis on MCT family members.

Chapter 2 presents the study on MCTs in lung embryo. In this study we describe a complete characterization of the expression pattern of MCTs in human fetal lung development and we explore the effect of MCTs inhibition in rat fetal lung explant cultures.

Chapter 3 presents the study on MCTs in lung cancer. The current knowledge on MCT contribution to tumour microenvironment, lactate production and its contribution to the malignant phenotype and the potential of MCTs/CD147 as targets in cancer therapy

are also reviewed. This study reports the expression of MCTs in lung cancer samples, and their biological effect in lung tumourigenesis assessed by *in vitro* and *in vivo* experiments.

Chapter 4 is the last chapter where a general discussion of the thesis is presented as well as the final conclusions of the work.

Chapter 1: General Introduction

1.1 Cell metabolism

Metabolism impacts all cellular functions and plays a fundamental role in biology. Lately there has risen a great interest in cell metabolism and its involvement in several pathways. Metabolic requirements of each cell type are determined by their tissue function and microenvironment as nutrient availability and oxygen levels [1].

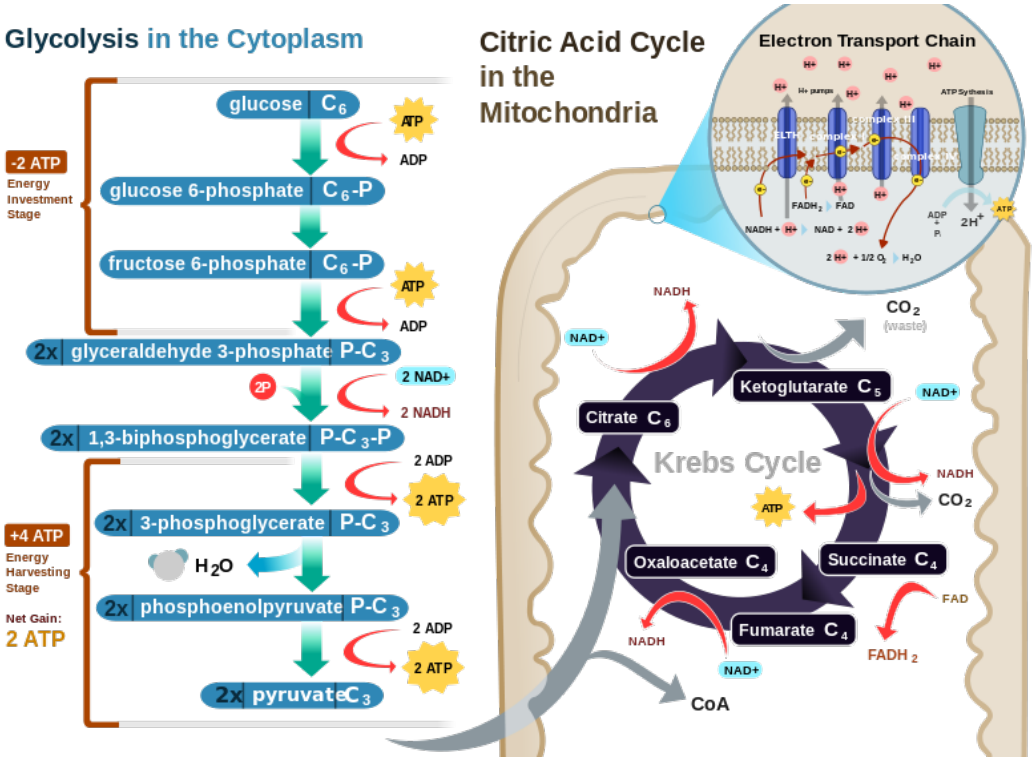


Figure 1: Cell respiration in eukaryotic cell in normal physiologic conditions [5]. Illustrative scheme of glycolysis, tricarboxylic acid (TCA) cycle, and the electron transport chain. Briefly glucose is converted into pyruvate, generating 2 molecules of ATP per glucose. Pyruvate is then oxidized into Acetyl-CoA, which enters the mitochondrial TCA cycle. The NADH generated is then fed into the oxidative phosphorylation pathway producing 36 molecules of ATP.

Metabolic pathways are well conserved among eukaryotes, where organisms use both glycolytic and mitochondrial metabolism depending on extracellular conditions, cues and cellular needs [2]. Glycolysis, occurs by the Embden-Meyerhof pathway and involves a series of cytoplasmatic enzymes that converts glucose into pyruvate [3]. Then, pyruvate enters the mitochondria to be oxidized by the Krebs cycle. These two processes in sequence generate the equivalent of 36 ATP units per glucose molecule (Figure 1) [4,5].

Such is the energetic fate of glucose in healthy tissue under normal physiological conditions. Under anaerobic conditions, where oxygen is limited, glycolysis may be the main energy source of an organism. Cells are able to switch from a state of low energy production that maintain basal functions to a state of activation of anabolic pathways to rapidly grow and proliferate [1]. More specifically, the differences in aerobic and anaerobic respiration depend on the different roles played by the NADH molecules produced. In both aerobic and anaerobic respiration, NADH must be restored to its oxidized state (NAD^+). Under aerobic conditions, meaning oxygen availability, NADH can be transported into the mitochondria where it can be immediately reoxidized to NAD, playing a role in the electron transport chain. However, under anaerobic conditions, NADH is reoxidized to NAD through anaerobic mechanisms, either by homolactic or alcoholic fermentation. Pyruvate, product of glycolysis, is converted into lactic acid by the enzyme lactate dehydrogenase (LDH). In this reaction, the hydrogen from NADH molecule is transferred to the pyruvate molecule, leading to production of lactate. From the lactate product, lactic acid can be formed. These anaerobic conditions lead to glycolytic products other than pyruvate. These different products are necessary for NADH reoxidization so that it allows the next round of glycolysis (Figure 2) [6].

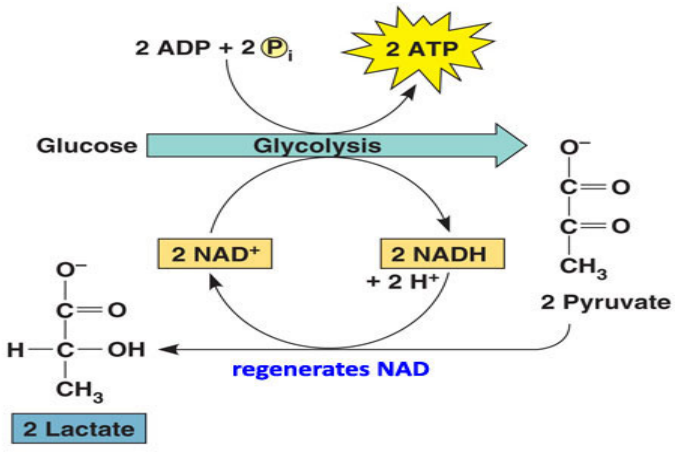


Figure 2: Anaerobic glycolysis. In the absence of oxygen, pyruvate is converted to lactate via lactate dehydrogenase A (LDH-A) [5]. This reaction allows the cell to regenerate NAD to continue glycolysis [5].

Several systems control and modulate metabolic flux in a cell such as hormones, small macromolecules and/or other extracellular factors that have an effect in different enzymes. AMP-activated protein kinase (AMPK) is a sensor that

coordinates diverse metabolic responses [7]. AMPK acts as a metabolic master switch regulating several intracellular systems including the cellular uptake of glucose, the β-oxidation of fatty acids and the biogenesis of glucose transporter 4 (GLUT4) and mitochondria [8]. The energy-sensing capability of AMPK can be attributed to its ability to detect and react to fluctuations in AMP: ATP ratio that takes place during rest and exercise [9]. Upon activation, AMPK increases cellular energy levels by inhibiting anabolic energy consuming pathways, such as fatty acid synthesis and protein synthesis and stimulating energy producing, catabolic pathways as fatty acid oxidation, glucose transport and others [4,10].

Low oxygen concentrations also control and induce a whole spectrum of cellular and systemic responses [11,12]. The oxygen level in the cells and organs is regulated by pathways that affect the expression and activity of numerous cellular proteins [13]. Sensing and responding alterations in oxygen tension are important variations in the physiology, and tissues have developed essential mechanisms of response to physiological oxygen reduction [14]. Hypoxia is commonly associated with pathologies such as tissue ischaemia and inflammation. However, hypoxic microenvironments also occur in both the developing embryo and the adult, and often create specific niches that regulate cellular differentiation [13,15]. Molecular mechanisms through which O_2 levels interfere with cell physiology have been elucidated by the characterization of hypoxia inducible factors (HIFs), which are dimeric transcription factors that regulate hypoxic responses in cells and tissues [11,16]. HIF-1 is considered a master switch that allows cells to respond to falling oxygen levels. The protein consists of two essential subunits, HIF-1α and HIF-1β, which heterodimerize [11], whereas HIF-1β is constitutively expressed, HIF-1α expression is induced in hypoxic cells with an exponential increase in expression as cells are exposed to O_2 concentrations of less than 6% [11]. The amino-terminal half of each subunit contains basic helix-loop-helix (bHLH) and PER-ARNT-SIM homology (PAS) domains [16]. The mechanism by which hypoxia increase HIF-1 activity was just discovered when evidence showed that HIF-1α ubiquitination required hydroxylation at two proline residues, which are Pro-402 and Pro-564 in human HIF-1α. Under normoxic conditions, hydroxylation of HIF-1 α is catalyzed by prolyl hydroxylase domain proteins (PHDs) using O_2 as substrate [17]. When O_2 is low, PHD activity decreases and hydroxylation of HIF-1α at the proline residues also decreases, resulting in protein

stabilization. HIF-1α then translocates into the nucleus, where it binds HIF-1β and recruits coactivator proteins to the HIF binding site within the hypoxia response element (HRE), activating the transcription of various target genes [18,19]. Many genes are transcriptionally activated by HIF-1 in response to hypoxia, such as glucose transporters, glycolytic enzymes, and vascular endothelial growth factor (VEGF) for angiogenesis [20].

Nutrient availability also control and influence cell metabolism. For cells inserted in an environment with glucose limitation, one known adaptation is to decrease glucose oxidation and utilize amino acids, fatty acids or lipids.

1.2 Embryogenesis vs tumorigenesis: cellular metabolism

1.2.1 Embryo metabolism

There still seems to be a controversy as to whether the developing embryo relies on glycolysis or respiration.

Embryonic and fetal development is strongly influenced by the levels of oxygen in the environment [21,22]. Studies related to placenta formation indicate that the human embryo and human placenta develop in a hypoxic environment during the first trimester. The placenta consists of a gestational sac, villi with few capillaries, a trophoblast layer and an exocoelomic cavity which does not contain oxygen transport system but the anti-oxidant molecules present protect the embryo from oxidative damage. Thus, these features are indirect evidence that the gestational sac limits the embryonic oxygen exposure [14,23]. Also, studies of metabolism in various stem cell populations have also highlighted a role for cell-specific metabolic pathways that are modulated during differentiation [24]. Some authors suggest that the initial low hypoxia levels normally present within the heart fields and neural tissue activate HIF1-inducible genes for normal development [14].

The fundamental effects of oxygen levels on embryo development, physiology, and disease pathophysiology are now being unveilled. It seems that the development of the embryo takes place in a physiologically low oxygen environment and energy demands rely on glycolysis [14,25]. However, studies indicate that metabolism is tightly controlled by glycolytic enzymes such as hexokinase (HK) and phosphofructokinase 1 (PFK1), thus glycolysis rates are initially low [26]. Thus, the

only energy and carbon sources are pyruvate analogs (Figure 3).

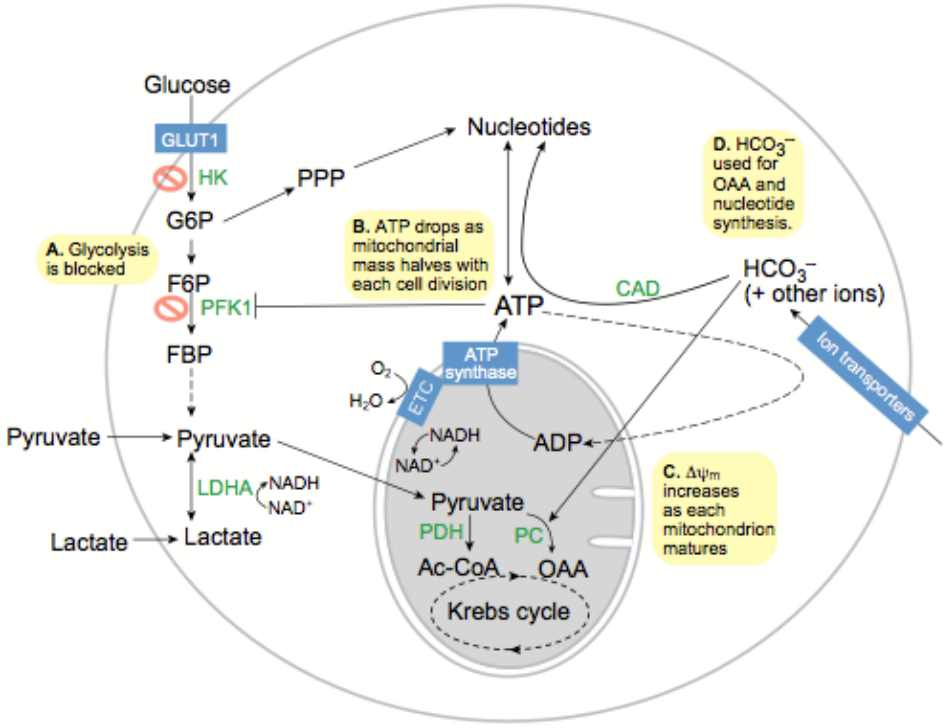


Figure 3: Metabolism in embryonic cells; Energy and carbon source are obtained by oxidative metabolism of pyruvate and lactate [24]. Ac- CoA, acetyl coenzyme A; HK, hexokinase; F6P, fructose-6-phosphate; FBP, fructose-1,6- bisphosphate; G6P, glucose-6-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDHA, lactate dehydrogenase A; OAA, oxaloacetate; OxPhos, oxidative phosphorylation; PDH, pyruvate dehydrogenase complex; PFK1, phosphofructokinase 1; TPI1, triosephosphate isomerase.

In fact, early embryo development is actually inhibited by high concentrations of glucose [24]. Thus, pre-implantation embryos *in vivo* develop in uterine fluid and derive their ATP predominantly from oxidative metabolism of pyruvate, lactate, and amino acids. The oxidation of pyruvate in mitochondria then fuels the Krebs cycle, which in turn generates carbon intermediates and fuels oxidative phosphorylation (OXPHOS). Therefore, pyruvate has the ability to react with hydrogen peroxide [27] and/or may serve as means of removing ammonia from the embryo, converting it into alanine [28]. The embryo only becomes more dependent on aerobic glycolysis after implantation, at the morula stage [25,29]. During this stage, there is an increase in growth and metabolic activity. Cells upregulate the expression of glucose transporters as GLUT1 and GLUT3 [30]. The glycolytic flux increase, leading to a higher synthesis of lactate [31]. The importance of glycolysis to blastocysts is evident *in vivo*, as mutations

in four glycolytic enzymes as glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase and lactate dehydrogenase A (LDH-A) result in early post-implantation lethality [24,32–34]. The mouse embryos at gestational day 6.5–9.5 that grow *in vitro* convert 90% of the catabolized glucose to lactate, even though the embryos are cultured in 20% oxygen, which should favour oxidative pathways [35]. For rat and mouse embryos, it appears that for the start of the organogenic period, including the period of neural tube closure, the embryo is largely dependent on glycolysis even when conditions favour more efficient pathways [36,37]. However, the availability of oxygen to the embryo *in vivo* is not really known [22].

Lactate formed by early human embryos derived from pyruvate [38] and its exchange with the extracellular space, seems to be important for the regulation of the intracellular pH (pHi) and / or to maintain the NAD^+/NADH ratio[39]. Cellular function depends on its energetic metabolism, and metabolism generates intracellular acid by the formation of H^+ ions. The ability of cells to control their ionic composition in response to external ionic stress is, therefore, likely to be an important determinant of the developmental progress, but there is limited knowledge on the ion transporters and channels at these early stages of development [29]. For example, H^+ ions can exert particularly powerful influences on cell function, and so understanding the nature and properties of the proton transport and buffering systems available to the conceptus is of particular significance [40,41]. The pHi may also play a role in developmental signalling. It has been described as an important trigger for the later developmental events of neural induction and posterior axial [42]. The importance of the pHi for successful early development was first demonstrated empirically in Bavister's studies on the pH dependence of monospermic fertilization in the hamster [43]. Cells regulate pHi through exchangers on the plasma membrane. These transports include the Na^+/H^+ exchanger, Na^+, HCO_3^-/Cl^- exchanger, however, the mechanism of pHi regulation during embryo development is not really elucidated [44]. Posterior studies demonstrated that control of pHi could be also carried out by a family of transporters designated by H^+-monocarboxylate co-transporters (MCTs).

1.2.2 Cancer metabolism

Targeting metabolic pathways in cancer has been an interesting area of research and has been the subject of study of several groups in the last decades.

Cancer cells have a higher demand for metabolic energy to grow and survive, thus studying cancer cell metabolism and the interplay between metabolic pathways contribute to a better knowledge in tumourigenesis [1].

Changes in nutrient metabolism are critical parts of tumour suppressor and oncogene networks to support cancer development [45]. Enhanced glucose uptake is observed in several tumours, being a significant fraction of glucose metabolized through glycolysis. Glucose-derived pyruvate can enter mitochondria to fuel the tricarboxylic acid (TCA) cycle, but in many cancer cells, a significant fraction of glucose ends up as lactate instead, a phenomenon known as aerobic glycolysis, since it occurs independently of the presence of oxygen.

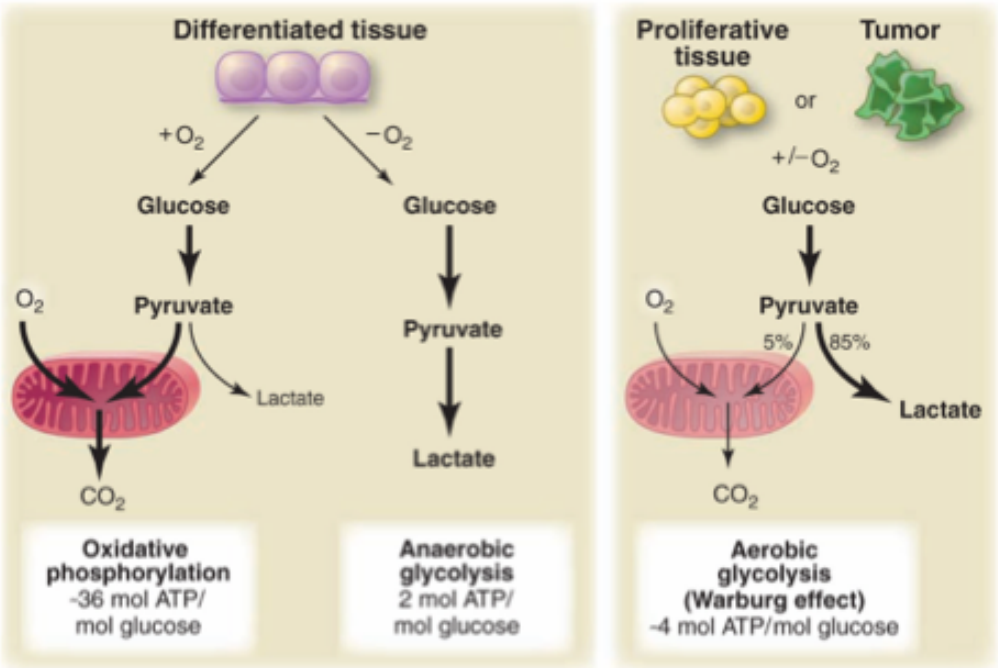


Figure 4: The Warburg effect or aerobic glycolysis [1]; Tumour/proliferative cells predominantly produce energy by a high rate of glycolysis followed by lactic acid fermentation in the cytosol, rather than by a comparatively low rate of glycolysis followed by oxidation of pyruvate in mitochondria as in most normal cells.

This increase in glucose uptake and conversion to lactate in the presence of oxygen is also known as "Warburg effect". Otto Warburg, a pioneer in the study of respiration, made a huge discovery in the 1920s. He found that, even in the presence of oxygen, cancer cells prefer to metabolize glucose by glycolysis a less efficient pathway for producing ATP when compared to oxidative phosphorylation (Figure. 4) [46]. The Warburg effect has since been demonstrated in different types of tumours and

the concomitant increase in glucose uptake has been exploited clinically for the detection of tumours by fluorodeoxyglucose positron emission tomography (FDG-PET) and also for an early indicator of drug efficacy (Figure 5) [47].

Although aerobic glycolysis has been introduced as a hallmark of cancer [48], its relationship with cancer progression can vary among tumours. The Warburg effect, meaning the switch of metabolism to glycolysis confers numerous advantages to cancer cells. For example, glycolysis alone produces ATP more rapidly than glycolysis coupled with OXPHOS and generates carbon intermediates used in biosynthetic pathways [49,50].

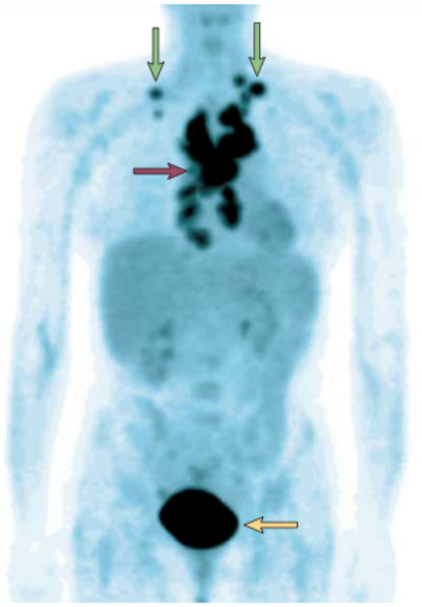


Figure 5: Positron-emission tomography (PET) imaging with 18 fluorodeoxyglucose (FDG) [52]. Analysis of a patient with lymphoma. The mediastinal nodes (purple arrow) and supraclavicular nodes (green arrows) show high uptake of FDG showing that this tumours have a high uptake of glycolysis. The bladder (yellow arrow) also has high activity, because of excretion of the radionuclide.

The upregulation of glycolysis triggers the accumulation of pyruvate and production of lactate [51], which results in an acidic microenvironment that also confers a selective advantage to cancer cells, being harmless for them but fatal to normal cells [52,53]. A low extracellular pH (pHe) together with hypoxia results in a escape to cell death through activation of intracellular pathways, for example the extracellular-signal-related kinase (ERK1/2) [54]. This pathway is involved in the activation of several cell phenotypes as proliferation, transformation, tumourigenesis, invasion, angiogenesis, differentiation and survival [55]. In fact, experiments demonstrate that the acidic pH upregulate the phosphorylation of ERK1/2 [56]. Thus, altogether, the activation of ERK1/2, acidification and hypoxia also lead to the increased release and

activation of acidic proteases such as cathepsin B [57], MMP2 and MMP9 [54,58] which are expressed in the extracellular matrix (ECM) of the tumour and promotes its degradation leading to invasion and metastasis [59]. The vascular endothelial growth factor (VEGF) is also upregulated inducing angiogenesis [60]. Moreover, pyruvate accumulation is redirected to lipids synthesis and OXPHOS decrease leads to citrate accumulation that will serve to promote fatty acid and cholesterol synthesis [50]. In some tumour cells, the utilization of the pentose phosphate pathway (PPP) is also reported to produce ribose and NADPH for *de novo* nucleic acid synthesis.

As the tumour starts to grow, the oxygen diffusion becomes limited, thus cells must sense and respond to these environmental changes [61]. HIF initiates a transcriptional program that provides multiple solutions to hypoxic stress. HIF induces the up-regulation of glucose transporters such as GLUT1 and GLUT3 during carcinogenesis of several tumours [62]. Also, it induces the expression of glycolytic enzymes as hexokinase II and LDH-A [63,64]. HIF also prevents the entry of pyruvate into the TCA cycle by inducing pyruvate dehydrogenase kinase 1 (PDK1) [65] driving tumour cell energy dependence mainly to glycolysis [53]. In addition, HIF induces angiogenesis by overexpression of angiogenenic factors such as VEGF [61,66]. The tumour recruits blood vessels creating a disorganized and often non-functional network that results in a microenvironment with fluctuating areas of oxygen and therefore with upregulation of glycolysis [61,67,68].

Besides HIF, there are other molecular mechanisms that are equally important and involved in metabolic changes inside a tumour. Several studies have demonstrated that the decrease of oxidative phosphorylation, the increase in glucose consumption and lactate production can also be driven by oncogene activation [61]. The major oncogene Ras, when mutated, can induce glycolysis, as well as the mammalian target of rapamycin complex I (mTORC1). Akt kinase activation by phosphatidylinositol-3-OH kinase (PI3K) results in increased glucose uptake, hexokinase targeting to mitochondria and glycolytic flux [69], while the transcription factor myc has been shown to increase glutaminolysis and also to promote the alternative splicing of the pyruvate kinase gene PKM, resulting in the overexpression of PKM2 embryonic isoform [70].

Similary to oncogenes, cell metabolism is also be influenced by tumour suppressor genes. Loss of the p53 protein prevents expression of the SCO2 gene (synthesis of cytochrome c oxidase) decreasing mitochondrial respiration [71]. Also

TIGAR (TP-53-induced glycolysis and apoptosis regulator) inhibits glycolysis by increasing the expression of fructose-2,6-bisphosphate and the availability of glucose-6-phosphate (G6P) to entry the pentose phosphate pathway (PPP) [72].

Initially thought as a metabolic waste, lactate the end product of glycolysis, can be a paracrine signalling molecule [61]. Acidosis often precedes angiogenesis and it has been shown that lactate can stimulate HIF expression independently of hypoxia [73]. Thus, instead of one event causing the Warburg effect, numerous factors play a role in determining the fate of glucose in cancer cells. HIF and other signalling pathways control glycolysis flux, which ends with the production of pyruvate. At the center of pyruvate metabolism lies the pyruvate dehydrogenase (PDH) complex, a multisubunit complex that catalyses the oxidative decarboxylation of pyruvate to Acetylcoenzyme A (ACoA) and conversion of NAD^+ to NADH in the mitochondria. This complex is regulated by PDKs and phosphatases that control phosphorylation of the E1a subunit of the PDH complex to control its activity. Besides hypoxia, these kinases are also controlled by nutrient levels [65]. Apart from phosphorylation, PDH complex activity is influenced by the mitochondrial NAD^+/NADH ratio and ACoA levels. The complex regulation of PDH activity illustrates how diverse metabolic inputs can affect pyruvate fate. Importantly, this relationship between pyruvate/lactate and NAD^+/NADH will only cooperate if the reaction is at equilibrium. The specific isoform of pyruvate kinase can also influence pyruvate to lactate conversion in cells [74]. The PKM2 isoform promotes anabolic metabolism with conversion of glucose into lactate, while high pyruvate kinase activity from the PKM1 isoform promotes efficient pyruvate oxidation in the TCA cycle [75].

Another enzyme playing a critical role in the fate of pyruvate is LDH that catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+. LDH-A is responsible for the rapid conversion of pyruvate into lactate with regeneration of NAD^+, supporting the high glycolytic rates of cancer cells, while the isoform B converts lactate into pyruvate to undergo the TCA cycle (Figure 6).

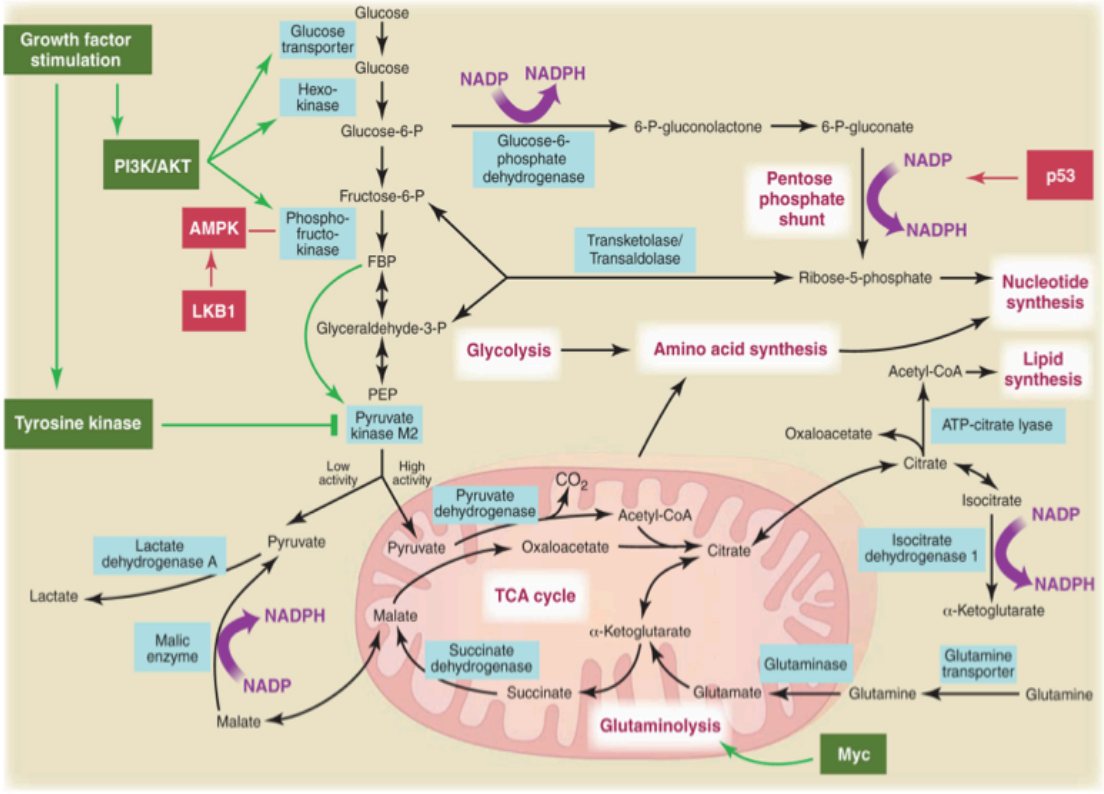


Figure 6: - Metabolic pathways active controlled by signaling pathways involving known oncogenes and tumor suppressor genes [1]. Tumor suppressors are shown in red, and oncogenes are in green. Key metabolic pathways are labeled in purple with white boxes, and the enzymes controlling critical steps in these pathways are shown in blue. Some of these enzymes are candidates as novel therapeutic targets in cancer. Malic enzyme refers to NADP+-specific malate dehydrogenase [systematic name (S)-malate:NADP+ oxidoreductase (oxaloacetate-decarboxylating)].

Upregulation of glycolysis requires additional adaptations to overcome the negative effects of extracellular acidosis through resistance to apoptosis or upregulation of membrane transporters to maintain normal intracellular pH (pHi) [76]. Thus, pHi is maintained by multiple families of H^+ transporters that cooperate to protect the cytosol from acidification, such as Na^+ /H^+ exchanger (NHE) [77] and its isoforms, the vacuolar-type H^+-ATPases (V-ATPase)[78], the monocarboxylate transporters (MCTs) [79] and HCO_3^- transporters and exchangers (NBCs and AEs) [80,81]. Several of these transporters have been shown to be upregulated in cancer. Moreover, in hypoxic cancer cells, HIF-1α upregulates pHi-regulating systems such as MCT4 [82] and the membrane-associated carbonic anhydrases (CA) [83] CAIX and CAXII, that catalyse the extracellular hydration of CO_2 into $HCO3^-$ and H^+. Then, $HCO3^-$ enters the cell through AE1 where, in combination with the accumulating H^+ derived from

the glycolytic metabolism, forms CO_2 and H_2O, that will exit the cell by diffusion, causing trapping of the H^+ in the extracellular milieu (Figure 7) [84].

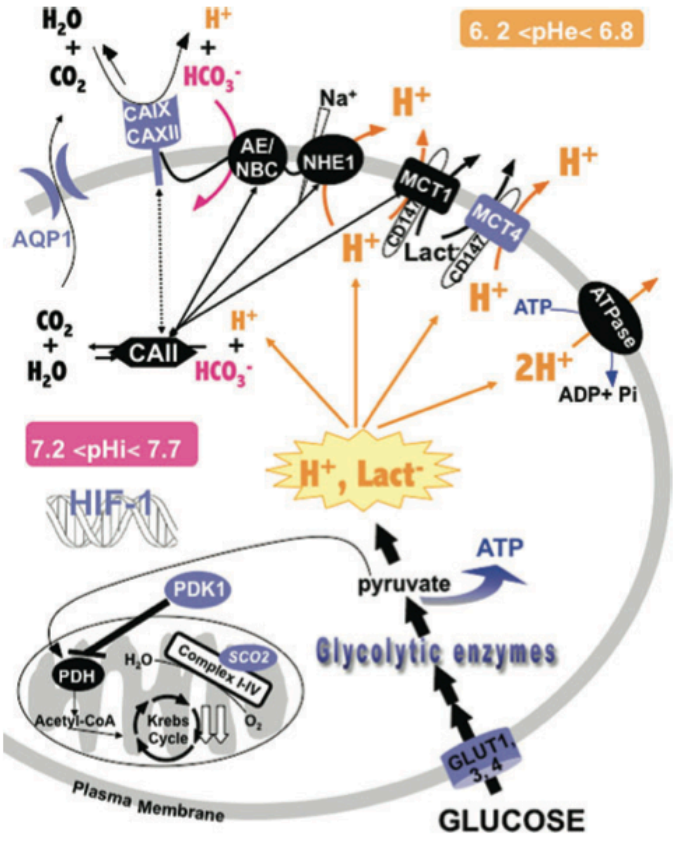


Figure 7: Uptake of glycolysis and pHi regulators. With high production of lactate and consequent acidification cells maintain a optimal intracellular pH expressing pH regulating systems as: Na^+/H^+ exchanger 1 (NHE1), Carbonic anhidrases (CA), Na^+- dependent HCO_3^- transporter (NBC) Monocarboxylate transporters (MCTs) bicarbonate transporter exchanger (AE) and the H^+ pump (V-ATPase) [76].

No single event can explain a metabolic phenotype such as the Warburg effect. It is mandatory a tight regulation and sensing mechanism that allow cells to switch their metabolic phenotype to meet their energy demands. In addition to glucose, also glutamine is an energetic fuel [85]. Furthermore, metabolites that participate in the glycerol-phosphate and malate-aspartate shuttle systems responsible for moving reducing equivalents across the mitochondrial membranes are also required for biosynthesis. Glycerol-3-phosphate is a side product of glycolysis and is involved in lipid synthesis, while aspartate is used to synthesize proteins and nucleotides. Most cells rely on lipids and some cancer cells have the ability to store triacylglycerides in lipid droplets that can be used as energy source [86].

In opposition to what Warburg and followers postulated, that enhanced glycolysis is due to mitochondrial dysfunction, the fact is that most cancer cells maintain functional mitochondria [70].

Mitochondria are essential for the production of cytoplasmic CoA, by citrate synthesis in TCA cycle, an important source of acetyl groups for protein acetylation and lipid synthesis [69,87]. They can also be required to restore the NAD^+ pool to support the high glycolytic fluxes [70]. Besides this fact, it is true and becoming more obvious that being a tumour a group of heterogeneous cells it is possible to observe a whole scale of bioenergetics types. On one hand, cells adopt the glycolytic phenotype during the highest proliferative phase and in response to hypoxic conditions and, on the other hand, where glucose is limited, cells use OHPHOS to derive energy from glutaminolysis. There is another hypothesis proposed by Sonveaux and his co-workers [88] for explaining the importance of these "different bioenergetic areas". They described the existence of a hypoxic tumour cell compartment that relies on a metabolic symbiosis in which glycolytic/hypoxic and oxidative/oxygenated tumour cells mutually regulate their access to energy metabolites. They describe that lactate is an important metabolic fuel for oxygenated tumour cells. Thus, even in presence of glucose, cells import large amounts of lactate that is used to fuel the TCA cycle for ATP production. This symbiosis involves lactate recycling: oxidative tumour cells prefer to use lactate in the presence of glucose as an oxidative fuel, allowing glucose to diffuse farther from blood vessels where it is taken up by glycolytic tumour cells, in turn producing lactate in large quantities [89]. However, this hypothesis is not yet completely proven and appears not to be observed in all tumours.

1.3 Monocarboxylate transporters

1.3.1 Characterization

Lactic acid transport across the plasma membrane is fundamental for metabolism and pH regulation of all cells, by removing the lactic acid produced by glycolysis and allowing uptake by those cells that utilize it as a respiratory fuel, as does the heart cells [90]. If lactic acid efflux does not occur, the increased intracellular concentration will lead to a decrease in pH inside the cell, leading to inhibition of glycolysis [91]. Although it is lactic acid that is both produced and utilized by

metabolism, the p*K* of lactic acid is 3.86, which ensures that it dissociates almost entirely to the lactate anion at physiological pH. This charged species cannot cross the plasma membrane readily by free diffusion, but requires a specific transport mechanism, provided by MCTs [79,90].

Monocarboxylate transporters or MCTs, constitute a family of proton-linked plasma membrane transporters that transport molecules (monocarboxylates), such as lactate, across biological membranes [92].

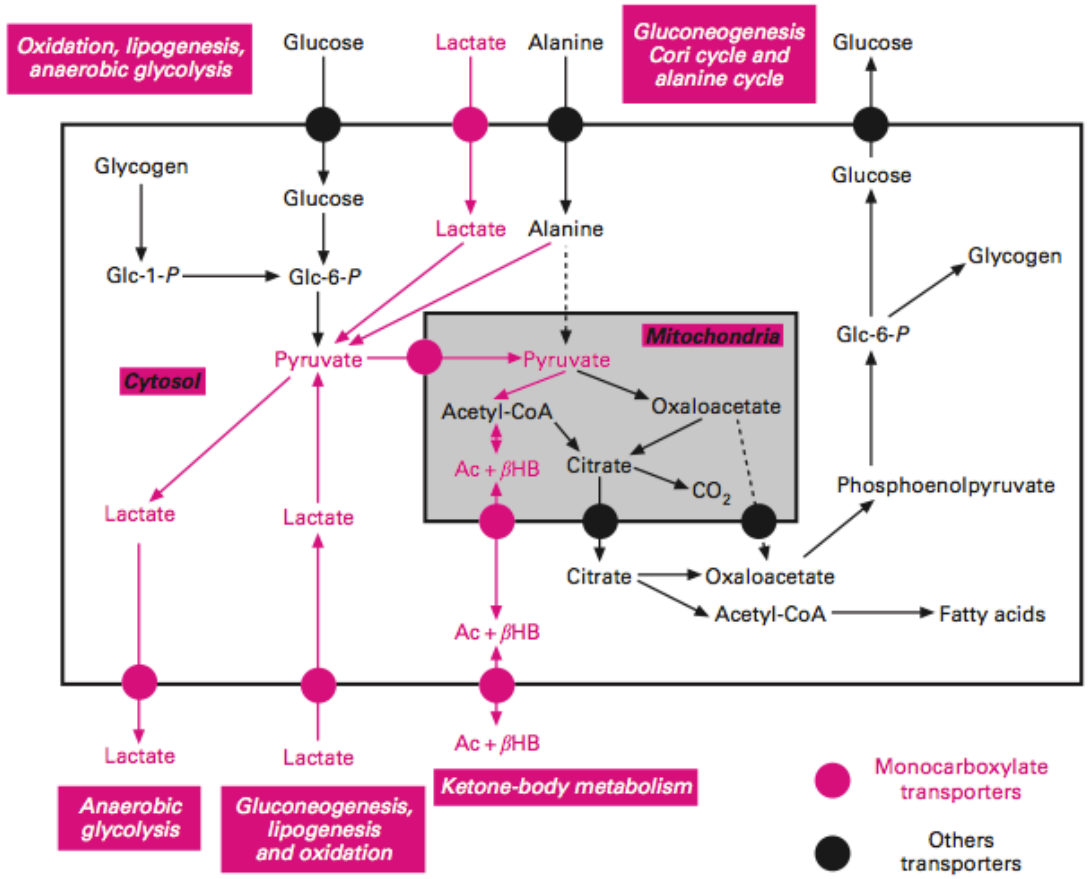


Figure 8: Pathways that involve the transport of monocarboxylates across the mitochondrial and plasma membranes [79]. Glc-1-P and Glc-6-P, glucose 1-phosphate and glucose 6-phosphate; Ac+bHB, acetoacetate plus b-hydroxybutyrate.

Although lactate is the monocarboxylate which transport through the plasma membrane takes place in highest quantities, MCTs are also essential for the transport of other metabolically important monocarboxylates such as pyruvate and ketone bodies. Being so, MCTs play a central role in mammalian metabolism and are critical for the metabolic communication between cells (Figure 8) [79].

The family of MCTs comprises 14 members already identified, each one with

properties connected with the metabolic requests of the tissues in which they are expressed. It is described that the topology of MCTs consists of 12 transmembrane α-helical (TM) domains with the N- and C-termini located within the cytoplasm and a large cytosolic loop between TM6 and 7, as illustrated in Figure 9 [79,93,94].

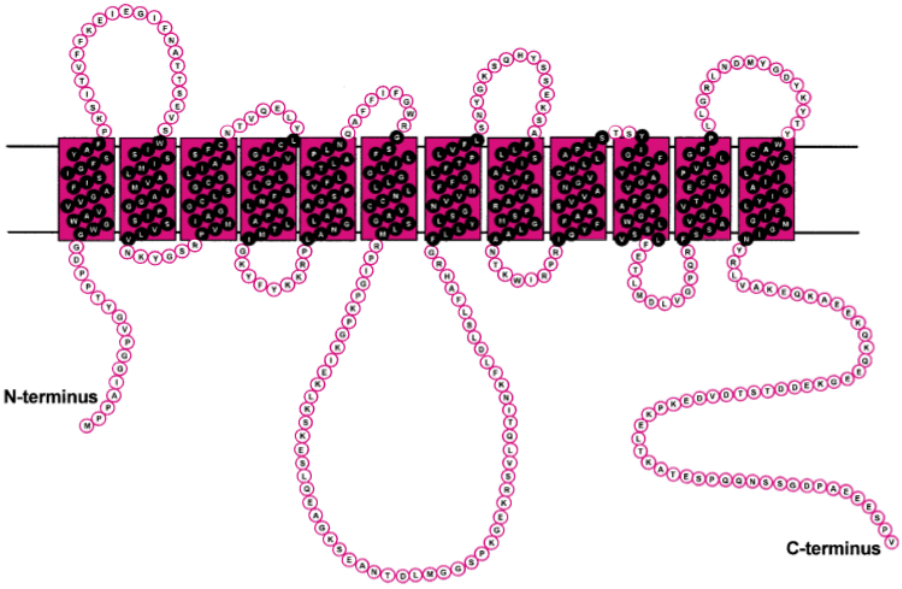


Figure 9: Membrane topology of the MCT family. The sequence shown is of MCT1[79].

Experimental evidence [95] indicates that none of the MCT family members is glycosylated. The first four isoforms are the best characterized. MCT1-4 catalyse the proton-coupled transport of monocarboxylates, playing an important role in cell metabolism. Other isoforms such as MCT6 were shown to transport synthetic drugs such as probenecid, bumetanide and the anti-diabetic drug Nateglinide [96], while MCT8 transports thyroid hormones [97,98]. MCT10, besides the transport of thyroid hormones [97,99], has been identified as a transporter of aromatic amino acids [100]. MCT9 was recently described to transport carnitine [101]. Recently MCT12 has been identified as a new creatinine tranporter [102]. However, the function of the other members is still unknown (Table 1) [103].

Since MCT1-4 isoforms are the ones which catalyse the proton coupled transport of monocarboxylates [100,104–107], they will be the ones to be boarded along this work.

The mechanism that predicts that transport of lactate into the cell has been already identified [108]. The transporter has a substrate-binding site open to the extracellular matrix, which firstly binds a proton, followed by the lactate anion [109].

The protein stucture change to a new conformation where both the proton and lactate (lactate first and then the proton) are exposed and released to the opposite surface of the membrane.

Table 1: Members of the monocarboxylate transporter (MCT) family [103] (adapted from Halestrap *et al* 2013)

Human gene name	Protein name	Predominant substrates	Transport type/ coupling ions	Tissue distribution and cellular/subcellular expression	Human gene locus	Sequence Accession ID	Splice variantes and their specific features
SLC16A1	MCT1	Lactate, pyruvate, ketone bodies	C/H+ or E/ monocarboxylate	Ubiquitous except b-cell of endocrine pancreas	1p12	NM_003051	Splice variants in non-coding region
SLC16A2	MCT8	T2, rT3, T3, T4	F	Most tissues including liver, heart, brain, thymus, intestine, ovary, prostate,pancreas, placenta, lung kidney, skeleta,l muscle	Xq13.2	NM_006517	
SLC16A3	MCT4	Lactate, ketone bodies	C/H+	Skeletal muscle, chondrocytes, leucocytes, testis, lung, ovary, placenta, heart	17q25.3	NM_004207	Splice variants in non-coding region
SLC16A4	MCT5		O	Brain, muscle, liver, kidney, lung, ovary, placenta, heart,	1p13.3	NM_004696	Multiple splice variants listed in ENSG00000168679
SLC16A5	MCT6	? Bumetanide probenecid nateglinide		Kidney, muscle, brain, heart, pancreas, prostate, lung, placenta	17q25.1	NM_004695	Several splice variants listed in ENSG00000170190
SLC16A6	MCT7		O	Brain, pancreas, muscle, prostate	17q24.2	NM_004694	
SLC16A7	MCT2	Pyruvate, lactate, ketone bodies	C/H+	High expression in testis, moderate to low in spleen, heart, kidney, pancreas, skeletal muscle, brain and leucocyte	12q13	NM_004731	Multiple splice variants listed in ENSG00000118596
SLC16A8	MCT3	lactate	C/H+ (pH dependent but cotransport not confirmed experimentally)	Retinal pigment epithelium, choroid plexus	22q112.3-q13.2	NM_013356	Several splice variants listed in ENSG00000100156
SLC16A9	MCT9	carnitine		Endometrium, testis, ovary, breast, brain, kidney, spleen adrenal, retina	10q21.1	NM_194298	Several splice variants listed in ENSG00000165449
SLC16A10	TAT1 MCT10	Aromatic amino acids, T3,T4	F	Kidney (basolateral), intestine, muscle, placenta, heart	6q21-q22	NM_018593	Several splice variants listed in ENSG00000112394
SLC16A11	MCT11		O	Skin, lung, ovary, breast, lung, pancreas, retinal pigment epithelium, choroid plexus	17p13.1	NM_153357	Two splice variants listed in ENSG00000174326
SLC16A12	MCT12		O	Kidney, retina, lung, testis	10q23.31	NM_213606	Several splice variants listed in ENSG00000152779
SLC16A13	MCT13		O	Breast, bone marrow stem cells	17p13.1	NM_201566	
SLC16A14	MCT14		O	Brain, heart, muscle, ovary, prostate, breast, lung, pancreas liver, spleen, thymus	2q36.3	NM_152257	Several splice variants listed in ENSG00000163053

C: Cotransport E: Exchanger F: Facilitated transporter O: Orphan transporter.

Moreover, Wilson and his co-workers described that the feature that is responsible for transport, is a lysine residue (Lys38) in TM1 that has only been

recently identified in the MCT1 from rat [110]. In this study the authors show that Lys38 is normally uncharged in the hydrophobic environment of the ion pore, however, can bind to an extracellular H^+ leading to the open conformation, and subsequently binds the lactate ion. Upon binding, both the H^+ and lactate ions are transferred to an aspartate–arginine ion pair at TM8 (Asp302–Arg306) [111]. A phenylalanine residue (Phe360) in TM10 that regulates the substrate selectivity of MCT1 was also identified [112]. However, in MCT3 and 4 instead of a Phe residue, a tyrosine residue is found in this position [95]. This switch can explain the differences in lactate affinities between the four isoforms, which correlates with their function when expressed in a specific cell types. [79,92,95,103]. Recently, a report showed that a arginine residue (Arg-278) in TMD8 is involved in substrate, L-lactate recognition by human MCT4 [113].

1.3.1.1 MCT1

MCT1 was the first MCT isoform identified after being cloned from Chinese hamster ovary cells and later functionally expressed in a breast tumour cell line. Similar transporters from human, rat and mouse have been cloned and sequenced. This isoform is encoded by the gene *slc16a1* and it has been mapped and located in the chromosomal band 1p13.2-p12 and it is found in the majority of tissues examined being the most well-studied and functionally characterized member of the MCT family. SLC16A1 comprises 5 coding exons with no evidence for splice variants within the coding region [79], however, 8 transcripts have been identified, 5 resulting in proteins of different sizes and 2 with no protein product (ENSG00000155380). The functional protein is composed by 500 amino acids and has a molecular weight of 53,944 Daltons (P53985). This is the isoform with the widest tissue distribution [92]. Different studies showed that MCT1 transport demonstrates Michaelis Menten kinetics with a broad specificity for short-chain monocarboxylates including those substituted on the 2 and 3 positions with with small groups such as hydroxyl, and carbonyl groups [104,114]. The transport of short-chain fatty acids, such as acetate, propionate, and butyrate, is also facilitated by MCT1 [115] however, these substrates can also enter cells by free diffusion in the undissociated form of the acid [93]. Natural substrates for MCT1 include L-lactate, pyruvate, b-hydroxybutyrate, and acetoacetate and K_m values for each one can vary (Table 2). To note that the transport of lactate is relatively stereoselective, with D-lactate being a poor substrate compared to L-lactate,

whereas such stereoselectivity is not demonstrated for 2-chloropropionate or b-hydroxybutyrate. Besides all these substrates, the main function of MCT1 is to transport lactate coupled with a proton across plasma membrane. This transport can be bidirectional depending on the pH gradient between the intracellular and extracellular space [39,116].

Table 2: K_m values for the various substract between the diferent MCTs isoforms. [103]

Substrate	MCT1 K_m	MCT2 K_m	MCT4 K_m	Plasma concentration
L-Lactate	3.5 (4.5)	0.74	28	1.5
D-Lactate	(27.5)	–	519	NA
Pyruvate	1.0 (0.7)	0.08	153	0.06
D-β-Hydroxybutyrate	(10.1)	1.2[a]	130	0.06
γ-Hydroxybutyrate	(7.7)	–	>500[b]	NA
Acetoacetate	(5.5)	0.8	216	0.02
α-Ketobutyrate	(0.2)	–	57	NA
α-Ketoisocaproate[c]	0.7	0.1	95	ND
α-Ketoisovalerate[c]	1.3	0.3	113	NA

NA – not assayed. ND – not detected.
[a] D, L Racemic mix used in these studies.
[b] Uptake too low to measure accurately.
[c] These substrates were transported slowly and acted better as inhibitors.

1.3.1.2 MCT2

The second isoform of MCT was sequenced and cloned [112] from Syrian hamster liver, a tissue where MCT1 is absent. Named MCT2, it shares 60% identity with MCT1 [79,117]. MCT2 is encoded by the gene *slc16a7* and it is located in the locus 12q14.1. It comprises 5 coding exons, and has 17 transcripts identified in a public database (ENSG00000118596) however only 8 result in protein. The functional protein is composed by 478 amino acids and has a molecular weight of 52,200 Daltons (O60669). There are studies showing alternative splicing in both human and rat [118]. This isoform has a higher affinity for monocarboxylates, increasing the affinity for the substrate relative to MCT1. K_m values for pyruvate and L-lactate were found to be about 0.1 and 0.74 mM, respectively, compared to values of about 1 and 3.5 mM for MCT1 (Table 2). Both Northern blot analysis and examination of the human expressed sequence tag (EST) database suggest that MCT2 has a lower expression in human tissues. This isoform is mainly present in cells where a rapid uptake of monocarboxylates is necessary, where these are in low concentrations and in cells which release of lactate is rare. Thus, it is expressed in proximal kidney tubules, neurons, sperm tails, cardiac myocytes and liver [39,112]. MCT2 has a special important function in the brain where its expression is largely confined to the postsynaptic

density of the neurons and may facilitate the uptake of lactate for oxidation as a respiratory fuel [119,120]

1.3.1.3 MCT3

MCT3 was first identified in the retinal epithelium cells of chicken [121]. The functional protein is composed by 504 amino acids and has a molecular weight of 52,319 Daltons (O95907). It is encoded by the gene *slc16a8* and it is located in the locus 22q13.1. It seems to be a very specialized MCT since its expression is restricted to the basal membrane of the retinal pigment epithelium (RPE) of the eye and on the Choroid Plexus epithelium [122,123]. It is thought to play an important role in facilitating the transport of glycolytically derived lactic acid out of the retina [93].

1.3.1.4 MCT4

Searching for novel members of MCT family in the EST database, Price and his co-workers identified MCT4 [116]. Initially called MCT3 based on its sequence homology with chick MCT3, it was renamed MCT4 after MCT3 had been identified. MCT4 is encoded by the gene *slc16a3* and it is situated in the locus 17q25.3. Also with 5 coding exons, MCT4 has already 20 transcripts identified (ENSG00000141526). The protein is constituted by 465 residues and a molecular weight of 49,469 Daltons (O15427). It is a transporter of low affinity adapted for the export of lactate of glycolytic cells like muscle cells, tumour cells and white cells [124]. It is expressed in chondrocytes, leucocytes, testicle, lung and in placenta where a quick transport of lactate is necessary from the fetus to the motherly circulation. MCT4 exhibits a lower affinity for most substrates and inhibitors than MCT1, with K_m and K_i values some 5–10-fold higher [104,105]. The high K_m for pyruvate may be especially significant as this avoids loss of pyruvate from the cell, which prevents removal of the reduced form of NADH produced in glycolysis [93].

1.3.4 MCTs: physiology and function

As already described above, the transport across the plasma membrane by MCTs is of extreme importance for metabolic function. For many years lactate was

considered nothing more than a waste product of glycolysis. The accumulation of lactate within cells and their microenvironment, such as in muscle fibbers during exercise, was thought to cause lactic acidosis and consequences to normal physiological function [95]. However, new discoveries during the last decades showed that lactate can be utilized as a metabolic substract for OXPHOS [89,125]. This important role of lactate brought a huge impact on studying the role of MCT1-4 in the metabolism of the muscle, brain, kidney, liver and retina. Thus, the uptake of lactate is of great importance in liver and kidney, where lactic acid is transported into the cell to support gluconeogenesis and lipogenesis, and in heart, skeletal muscle and brain, where lactate, together with ketone bodies, is used as a major respiratory fuel [79]. Both skeletal muscle and heart express MCT1 to meet this role, while in liver, kidney and brain both MCT isoforms 1 and 2 are expressed, with MCT2 with a higher affinity for lactate uptake [95]. Lactic acid formation and its subsequent distribution throughout the body is of major importance, being responsible for the coordination of intermediary metabolism in different tissues, and cells within those tissues. This role of lactate as oxidative and gluconeogenic substrate, as well as in cell signalling, is explained by the "cell-cell" and "intracellular lactate shuttle" concepts [89,95,125126]. This concept was introduced by Brooks in 1985 [127], and according to this hypothesis, lactate produced in glycolytic cells will be utilised continuously under fully aerobic conditions. This lactate shuttle is identified between glycolytic muscle fibbers and oxidative muscle fibbers [127,128], between glycolytic astrocytes and oxidative neurons [95,129] and between tissues of net release of lactate and gluconeogenic tissues. As mentioned before, the differential expression of MCT isoforms correlates with their roles within different cell types where they facilitate lactate transport in the direction that can be predicted by the metabolic profile of the cells. In the retina, there is a similar interplay between different cell types with the photoreceptor cells and other neurons oxidising lactate exported from glial cells (Müller cells). In addition, lactate must be transported across the retinal pigment epithelium (RPE) to avoid accumulation of lactate within the subretinal space that could lead to osmotic swelling and detachment of the retina from the RPE. MCT1 is located on the apical surface of the RPE and transports lactate and water from the sub-retinal space into the RPE from where MCT3, located on the basolateral surface transports the lactate and osmotically obliged water into the choroidal blood supply [95]. Two cell-cell lactate shuttles, in the brain and skeletal muscle, are described below with more detail (Figure 11).

- ✓ **Skeletal muscle lactate shuttle**

The best-established lactate shuttle is described in skeletal muscle. During intense exercise, fast-twitching glycolytic muscle fibbers (white muscle) become hypoxic and produce lactic acid, which is then released to the extracellular compartment. Then, this lactate can be taken up by slow-twitching muscle fibbers (red muscle) where it is utilized as a respiratory fuel, or shuttled into the blood stream for clearance in the kidney. Thus, for lactate transport, muscles express MCT1 and MCT4 with an isoform-specific pattern: tissues like heart and oxidative red muscle show higher expression of MCT1, for the uptake of lactic acid, whereas MCT4 expression is seen largely in glycolytic white muscles [103,128].

- ✓ **Brain lactate shuttle**

A fundamental principle in the physiology of brain energy metabolism is that neuronal activity is tightly coupled to blood flow and energy metabolism. Studies have shown that the brain tissue consumes lactate from blood [130]. A similar lactate shuttle is proposed between astrocytes and neurons in the brain [125]. MCT1 expression is found in astrocytes [131–133], as well as in brain endothelial cells [134,135] while MCT2 is exclusively found in neurons [131–133]. MCT4 presents a strong expression in the plasma membrane of astrocytes. Thus, a mechanism of cell-cell lactate shuttle between astrocytes and neurons is described, where astrocytes perform aerobic glycolysis, exporting a substantial amount of lactate through MCT1 and MCT4 into the extracellular space that is then taken up by the neighbouring neurons through the high affinity lactate transporter MCT2 expressed on the cell surface (Figure 10) [129,136]. Moreover, MCT activity is not limited to plasma membrane as neurons contain a mitochondrial lactate oxidation complex that has the potential to facilitate both intracellular and cell-cell lactate shuttles in brain [137].

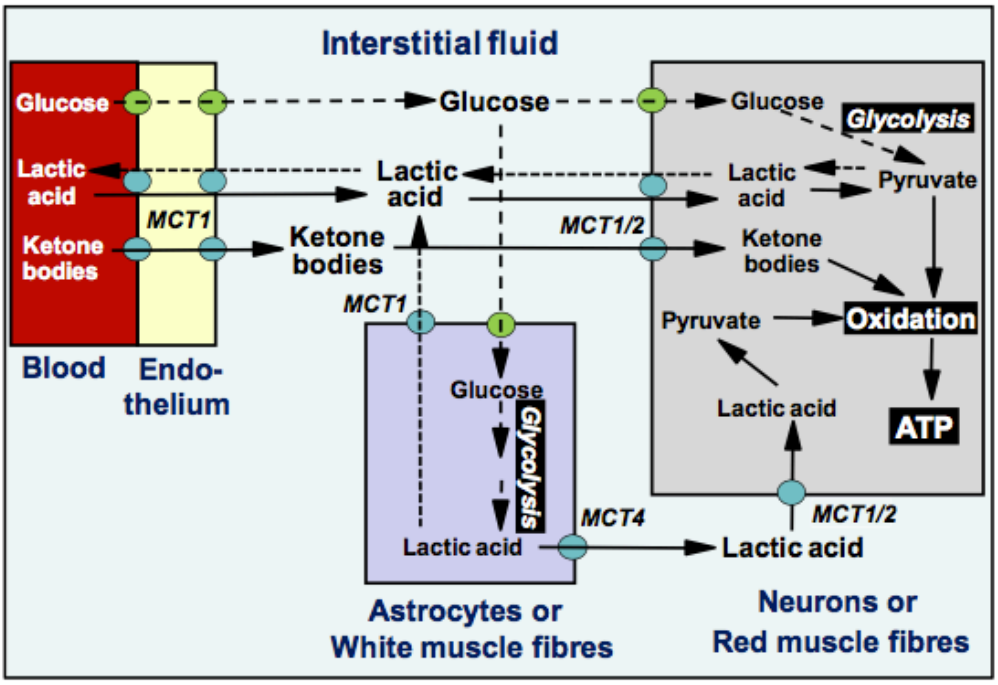


Figure 10: The role of different MCT isoforms in shuttling lactate between producing and utilizing cells in the muscle and brain [103].

1.3.4.1 MCTs as drug carriers

In addition to their roles in metabolism, MCTs may also act as drug transporters [138]. Few studies have been conducted assessing the contribution of MCT isoforms to overall drug disposition and the impact of MCT modulation on drug pharmacokinetics and disposition [138]. Thus, MCTs isoforms have been reported to mediate the transport of gamma-hydroxybutyrate (GHB) [139,140] a neuromodulator that binds to the GABA(B) receptor and is therapeutical use in in narcolepsy and alcohol withdrawal [141], however, abuse of GHB is widespread. Furthermore, MCTs are also involved in the transport of other drugs as salicylic acid [142], pravastatin [143] and atorvastatin [144], among others. Importantly, MCT1 was found to be the main determinant of 3-bromopyruvate (3-BrPA) uptake and sensitivity and also to be necessary for the uptake in cancer cells [145]. Further investigations are required to more clearly understand the role of MCTs in drug disposition for a wider range of drug substrates.

1.3.3 Regulation of MCTs

Importantly, MCT regulatory mechanisms vary among isoforms, which allow induction of expression of specific isoforms upon different stimuli, adapting cells to different energy demands. MCTs carry multiple splice variants and differ only at the 5′-UTR regions, suggesting that they have transcripts that can be subject to both transcriptional and post-transcriptional regulation [95]. This level of control may be necessary because MCT1 and MCT4 are widely expressed in many different cell types, thus requiring additional mechanisms to regulate their relative abundance in a cell-specific manner [146].

Expression studies showed that c-Myc amplification appeared to be coupled with the modulation of SLC16A1 expression [147,148] and the pro-inflammatory cytokines IFN-γ and TNF-α, have also been implicated in the transcriptional control of MCT1, by down-regulating *SLC16A1* transcription [149,150]. Another tumour suppressor gene that has been shown to regulate MCT expression is p53. Evidence shows that under hypoxic conditions, loss of p53 promotes MCT1 expression. p53 interacts directly with the MCT1 gene promoter and alters MCT1 mRNA stabilization [151]. The latter study also showed that in hypoxic conditions in p53 $^{-/-}$ tumor cells, NF-κB further supported expression of MCT1. The regulation of MCTs via NF-κB was also demonstrated by another group in human colonic Caco-2 cells. [152]

Hypoxia conditions, which are known to induce the glycolytic phenotype, are also associated with altered expression of MCTs. Several studies suggest that hypoxia regulates MCT1, MCT2 and MCT4 [153–155], however only Ullah et al demonstrated that the activity of the MCT4 promoter (but not that of MCT1 or MCT2) is stimulated by hypoxia and is mediated by HIF-1a binding to two hypoxia response elements (HRE) in the MCT4 promoter [82]. The overexpression in MCT4 expression in response to hypoxia is observed in several tissues and cell lines and is consistent with the role of MCT4 in mediating lactic acid production from glycolytic tissues [156].

During heavy exercise, the rapid increase in the energy demand of contracting skeletal muscles is associated with an increase in glycolysis and subsequent production and accumulation of lactate and protons [91]. Thus, evidence demonstrates that acute exercise alters human MCT expression [157–160], producing a greater increase in MCT1 than MCT4 content within the first 24h from the onset of exercise[128].

Recently, experiments showed that the overexpression of MCT1 and MCT4 mRNA after acute exercise may be mediated by AMPK activation [161].

Furthermore, carbonic anhydrase (CA) has been shown to facilitate lactate/proton exchange in rat skeletal muscle [162], and when MCT1 and CA are co-expressed in oocytes, they cooperate with binding of CA to MCT1, increasing the flux of protons via MCT1 [163,164], however, this effect is independent of the catalytic activity of CA. Similar results have recently been observed between MCT4 and CA [165]. In humans, inhibition of extracellular and intracellular CA activity has been demonstrated to be associated with a lower plasma lactate concentration during incremental exercise [166], which may due to lower activity of MCT. Thus, the activity of both MCT1 and MCT4 is enhanced by interaction with intracellular CAII, whilst that of MCT2 is stimulated by interaction with extracellular CAIV. MCTs can also interact functionally with sodium bicarbonate co-transporter (NBCs) in a cooperative mechanism to regulate pH, enhancing the activity of both MCT1 and NBC [167167].

MCTs 1–4 carry out their physiological function by mediating lactate transport, but they are just as capable of transporting other substrates, which can regulate their expression. Evidence has demonstrated substrate-induced upregulation of MCT expression and function via transcriptional mechanisms [168]. Moreover, exposure to butyrate resulted in a concentration- and time- dependent increase in MCT1 mRNA and protein expression, with a corresponding increase in butyrate transport [115]. Moreover, butyrate activates the monocarboxylate transporter MCT4 expression in breast cancer cells [169].

Furthermore, MCT1 transcripts can also be regulated by microRNAs, which target the mRNA at its 3'-UTR for translational repression or mRNA degradation [170]. This mechanism is particularly important in pancreatic β-cells, where MCT1 is not normally expressed. Studies show that promoter-activating mutations MCT1 expression in β-cells allow pyruvate uptake and pyruvate-stimulated insulin release, despite inducing hypoglycaemia [171]. Thus, MCT1 expression is silenced in pancreatic cells at least in part by miRNAs selectively present in this cell type.

Many membrane proteins are tightly associated with other glycosylated membrane proteins [172]. Kirk and his co-workers when trying to overexpress both MCT1 and MCT4 in a cell line, realized that the proteins accumulated in the perinuclear region, characteristic of the Golgi apparatus instead of being translocated to the plasma membrane [173]. However, when co-expressed with a cell surface

glycoprotein CD147/basigin, both MCT1 and MCT4 were correctly targeted to the plasma membrane, suggesting that MCTs rely on this chaperone for their correct location [173,174]. CD147/basigin, also known as EMMPRIN, is widely distributed and expressed in high levels in many tissues, mainly in metabolically active cells such as lymphoblasts and malignant tumour cells. Elevated CD147 stimulates matrix metalloproteinase (MMP)-production in stromal fibroblasts and endothelial cells, leading to extracellular matrix degradation, tumour growth promotion, and metastasis. CD147 also stimulates expression of VEGF [175].

Thus, acting as an essential chaperone, CD147 leads the correct activity and targeting of at least MCT1 and 4 to the plasma membrane [176]. MCT2 interacts preferentially with another ancillary protein called embigin (gp70), which is a CD147 homologue with an unreactive Ig-like V domain in place of the C2 domain [174]. Other basigin family members may bind different ligands through the extracellular Ig-like domains, which are less similar than the transmembrane and cytoplasmatic regions [172]. GP70, that shares the ability of CD147 to also interact with MCT1, is strongly and broadly expressed during the early stages of embryogenesis, but has a much more restricted distribution in adults [110,173]. (See a more complete review on the role of CD147 in lactate transporter in tumour cells in Chapter 3)

1.3.4 MCT Inhibition

There are numerous non-physiological competitive inhibitors described that are known to inhibit the function of MCTs [177]. Several studies have demonstrated the affinity and specificity of each inhibitor to each MCT isoform. These include aromatic compounds such as α-cyano-4-hydroxycinnamate (CHC) and phenylpyruvate (k_i value of 50- 500 μM), stilbene disulfonates such as 4,4'-di-isothiocyanostilbene-2,2'-disulfonate (DIDS) and 4,4'-dibenzamidostilbene-2,2'-disulfonate (DBDS). These inhibitors inhibit MCTs with higher affinity for MCT1 and 2, although they have the ability to also inhibit other transporters. CHC has been shown to be a potent inhibitor of the mitochondrial pyruvate transporter, whereas DIDS and DBDS inhibit the chloride/bicarbonate exchanger AE1 [110,178]. Bioflavonoids such as phloretin and quercetin and thiol reagents as p-chloromercuribenzenesulfonic acid (p-CMBS) are also non-specific MCT inhibitors [93,177]. MCT2 is not inhibited by p-CMBS because this compound inhibits MCTs by

targeting the chaperone CD147 and not GP70 [174]. For MCT4, a smaller inhibition by DIDS or CHC is observed due to the lower affinity for this drug when compared to MCT1 [93]. A new class of specific and high-affinity inhibitors of MCT1/2 have recently been developed by AstraZeneca. The authors found it to be active against MCT1 and MCT2 but not MCT4. AR-C155858 inhibitor seems to exert its function by binding MCT1 from the cytosolic side. The use of chimeric transporters combining different domains of MCT1 and MCT4 revealed that the binding site for the inhibitor is contained within the C-terminal of MCT1, and involves TM 7–10 [179].

1.3.5 Monocarboxylate transporters: embryo vs cancer

The importance of members of the SLC16 family, and especially the proton-linked monocarboxylate transporters MCT1-4, is clear in a wide range of physiological and pathological process and additional roles will probably be discovered.

Since the role of MCTs in embryo and cancer is the main subject of this thesis, a more detailed review of the state of the art is described in chapter 2 and 3, respectively.

1.3.6 Is inhibition of MCTs a promising strategy to improve the efficacy of cancer therapy?

It is described that the metabolic properties of cancer cells are different from those of normal cells. Metabolic reprogramming renders cancer cells dependent on specific metabolic enzymes or pathways that could be exploited for cancer therapy (Figure 12). However, the search for suitable targets may be complicated by the high plasticity of the metabolic network that can induce compensatory biosynthetic routes [70]. The development of treatments that target tumour metabolism is receiving renewed attention, with several potential drugs targeting metabolic pathways, already in clinical trials (Table 3, for review see [4]). Regarding MCTs, after the development of specific inhibitors for MCT1/2 by AstraZeneca, the compound AZD3965 is already being tested in a phase 1clinical trial in patients with advanced solid tumours (prostate and gastric) or lymphomas [180].

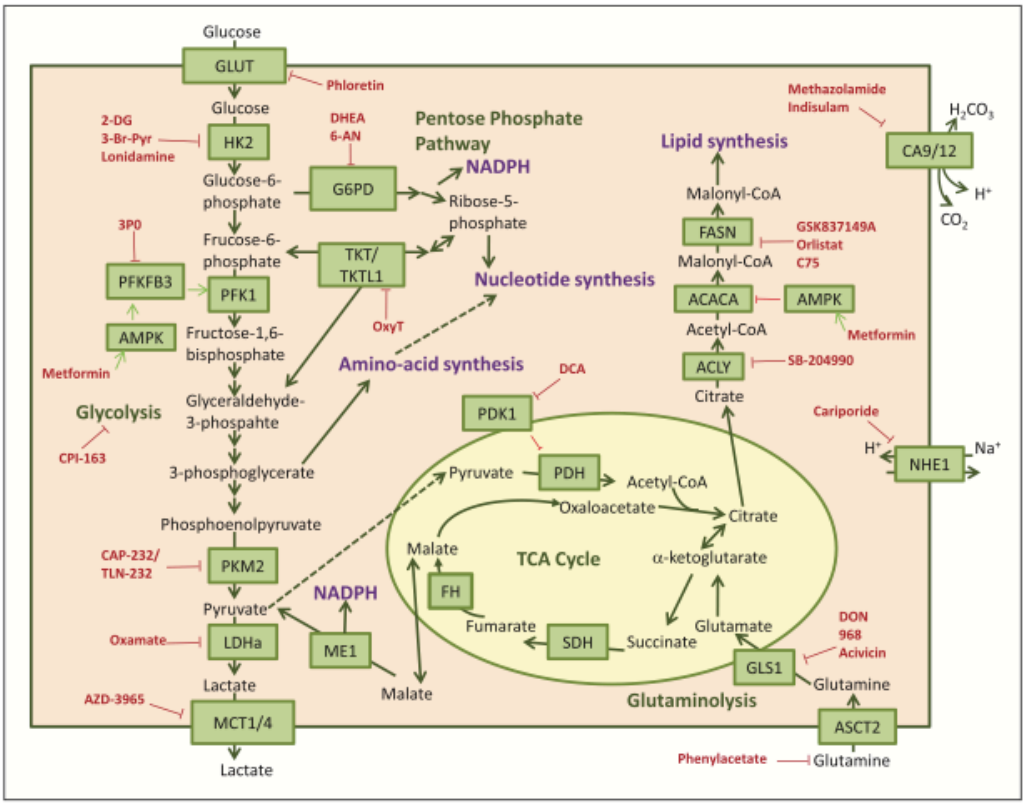


Figure 11: Actual therapeutic targets of tumour cell metabolism reported. Key enzymes are shown in green boxes and key biosynthetic endpoints listed in purple [1]. GLUT – Glucose transporter; HK2 – Hexokinase-2; PFK1 – Phosphofructokinase 1; PFKFB3 – 6-phosphofructo-2- kinase/fructose-2,6- biphosphatase 3; PK-M2 – pyruvate kinase-M2; LDHA – lactate dehydrogenase-A; MCT1/4 – Monocarboxylate transporters 1/4; AMPK – AMP activated protein kinase; G6PD – glucose-6-phosphate dehydrogenase;TKT – transketolase;TKTL1 – transketolase like -1; ASCT2 – solute carrier family 1, member 5 [SLC1A5]; GLS1 – Glutaminase 1; ME – malic enzyme; FH – fumarate hydratase; SDH – succinate dehydrogenase; PDH – pyruvate dehydrogenase; PDK1 – pyruvate dehydrogenase kinase 1; ACLY – ATP citrate lyase; ACACA – acetyl-CoA carboxylase alpha; FASN – fatty acid synthase; CA9/12 – carbonic anhydrase 9/12; NHE1- Na+-H+ exchanger [SLC9A1]

The inhibition of glycolysis seems to be tolerated remarkably well by normal tissues, but could have a disappointingly limited effect on tumourigenesis as monotherapy [4,181]. Cancer cells are characterized by their plasticity, being able to reprogramme their energetic metabolism, switching to other bioenergetic pathways to survive. Actually, there is already evidence showing that anti-glycolysis treatment could lead to increased glutaminolysis or oxidative phosphorylation to produce energy [182]. Thus, new strategies should be adopted and better and specific tumour cell therapies and treatment regimens need to be designed.

Table 3: Compounds targeting tumour metabolism currently in clinical studies. (adapted from Tennant *et a*l 2010[4])

Compound	Target	Effect	Stage as anti-tumour therapy	Tumour types targeted	Study number(s)
2-deoxyglucose	Hexokinase	Inhibits glycolysis	Phase I/II	Advanced solid tumours (e.g. lung, breast, prostate and gastric)	• NCT00633087 • NCT00096707 • NCT00247403
Lonidamine	Hexokinase	Inhibits glycolysis	Phase III	Benign prostatic hyperplasia	• NCT00435448 • NCT00237536
3-bromopyruvate	Hexokinase	Inhibits glycolysis	Preclinical	N/A	N/A
TLN-232	Pyruvate kinase	Inhibits glycolysis	Phase II	Metastatic melanoma and renal cell carcinoma	NCT00735332
Dichloroacetate	PDK1	Reactivates PDH	Phase I/II	Metastatic solid tumours, glioma and GBM	• NCT00540176 • NCT00566410 • NCT00703859
Phenylacetate	Glutamine	Reduces plasma glutamine levels	Phase II	Brain tumours (e.g. glioma, astrocytoma and medulloblastoma)	• NCT00003241 • NCT00006450 • NCT00001565
Acetazolamide, Indisulam and other sulfonamides	Carbonic anhydrases	pH regulation	Phase II	Solid tumours (e.g. pancreatic, lung, melanoma and metastatic breast)	• NCT00060567 • NCT00165594 • NCT00165880 • Others
Cariporide	NHE1	pH regulation	Preclinical	N/A	N/A
Metformin	AMPK and Complex I (mitochondrial)	Activates AMPK	Phase I/II	Solid tumours and lymphoma	• NCT00659568 • NCT00881725 • NCT00984490 • NCT00909506 • Others
PX-478	HIF1α	Inhibits HIF signalling	Phase I	Advanced solid tumours and lymphoma	NCT00522652
Acriflavine	HIF1α	Inhibits HIF signalling	Preclinical	N/A	N/A

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Chapter 2: Lung development

The results presented in this chapter are:

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Sara Granja, Filipa Morais-Santos, Manuel Viana-Ferreira, Rosete Nogueira, Cristina Nogueira-Silva, Jorge Correia-Pinto, Fátima Baltazar. *The monocarboxylate transporter inhibitor α-cyano-4-hydroxycinnamic acid disrupts rat lung branching*.

2.1 CHAPTER OVERVIEW

RATIONALE

The human embryo develops in a hypoxic environment. In this way, cells have to rely on the glycolytic pathway for energy supply, leading to an intracellular accumulation of monocarboxylates such as lactate and pyruvate [1]. These acids have an important role in cell metabolism and their rapid transport across the plasma membrane is crucial for the maintenance of intracellular pH homeostasis [2–4]. This transport is mediated by a family of transporters, designated by monocarboxylate transporters (MCTs), namely isoforms 1 and 4. MCT1/4 expression is regulated by the ancillary protein CD147 [5]. The general aim of this project was to characterize the expression pattern of MCT1/4, CD147 and the glucose transporter GLUT1 during human fetal lung development by immunohistochemistry and elucidate the role of MCTs in lung development by studying the effects of MCT inhibition in fetal lung viability and branching. Rat fetal lung explant cultures were exposed to different doses of CHC, an inhibitor of MCT activity. Our findings show all the biomarkers are differently expressed during fetal lung development and that CHC appears to have an inhibitory effect on lung branching and viability, in a dose dependent way. To conclude, it appears that MCTs play a role in embryo development, however to prove the dependence of MCT activity further studies will be needed.

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2.2 ROLE OF MONOCARBOXYLATE TRANSPORTERS IN LUNG DEVELOPMENT

2.2.1 INTRODUCTION

2.2.1.1Developmental biology

The process by which the embryo is formed and develops is known as embryogenesis. It is initiated by a process called fertilization that is the fusion of genetic material from the two gametes, the sperm and the egg. After that cleavage begins which consist on a series of rapid mitotic divisions wherein the zygote is divided into numerous smaller cells. These different cells derived from cleavage, up to the blastula stage, are called blastomeres. The blastula is usually a spherical layer of cells, the blastoderm, surrounding a fluid-filled or yolk-filled cavity, the blastocoel. The cells then migrate to the interior of the blastula, consequently forming three germ layers. During this process the embryo is called gastrula. The germ layers are referred to as the ectoderm, mesoderm and endoderm and give rise to all of an animal's tissues and organs through the process of organogenesis [1].

- ✓ **Lung Development**

The development of the lung appears on the day 14th of gestation [1]. The respiratory system arises from the ventral foregut endoderm. The lung buds consist of three cell layers: the inner epithelium (endodermal origin), which is surrounded by loosely packed mesenchyme and a thin outer mesothelial (squamous epithelial) layer. The bronchial tree develops by branching of the bronchial epithelium around the mesenchyme [2]. The mesenchymal cells of the lung are not as well characterized as the epithelial cells. The initial lung buds are composed of endodermal epithelium surrounded by mesenchyme. Relatively little is known about the development, maintenance, and lineage relationships of the mesenchymal cell types in the distal lung. It is controversial the number of different differentiated mesenchymal cell types in the adult lung, or the molecular markers which can be used to distinguish between them [3]. These include the endothelium of veins, arteries, capillaries, and lymphatic vessels as well as pericytes, fibroblasts, vascular and airway smooth muscle, myofibroblasts, lipocytes, and mesothelium [4].

The development of the lung can be divided into 5 stages: embryonic, pseudoglandular, canalicular, saccular and alveolar stage (Figure 1) [5].

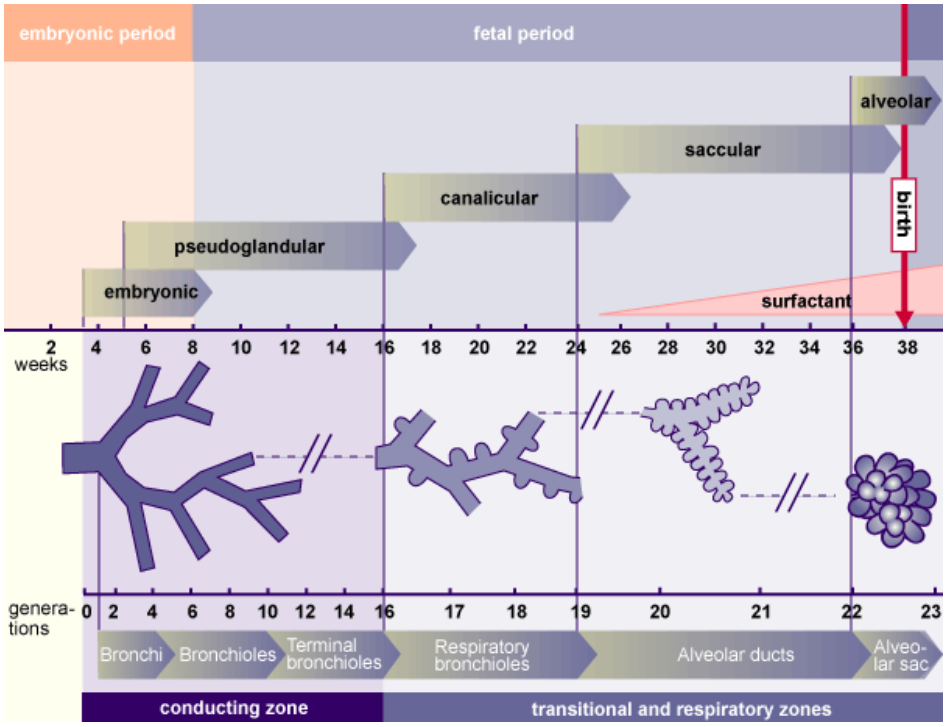


Figure 1: Overview of prenatal lung development: phases of the developing lung [5].

During embryonic development the epithelium of the lung bud undergoes branching morphogenesis given rise to the structure of the bronchioles and alveoli. Initial branches are mostly side-branches, which form by budding along the length of the tube [2]. The branching epithelium initially generates the future bronchioles (pseudoglandular stages of lung development). The distal epithelium continues to branch (canalicular stage), although budding morphology and frequency changes continuously occur giving rive to the future alveolar development, the terminal sacs. These sacs increase in number during saccular stage becoming during the alveolar phases mature alveoli and alveolar ducts. The alveolar epithelium consists of type 1 and type 2 alveolar epithelial cells. The type 2 cells contain abundant secretory vesicles called lamellar bodies that will produce the components for the surfactant media [6]. In contrast type 1 cells are flat or squamous and approximately 95% of the surface area of the lung even though they account only for a minor proportion of the total population [7]. Their high membrane to cytoplasm ratio, and the proximity alveolar capillary network, and expression of ion channels and pores support the notions that type 1 cells are the primary sites of gas exchange and that they also regulate fluid homoeostasis [8]. The lung vasculature forms, in part, by migration of blood vessels from the aortic arches and from the left atrium to the lung

(angiogenesis). Blood vessels also develop by vasculogenesis in the lung mesenchyme near developing epithelial buds; a rudimentary capillary network initially forms and expands, and later connects to the larger vessels to give rise to the lung vasculature.

✓ MCTs in embryo development

The ability to sense and respond to changes in oxygen concentration is a fundamental requirement for the survival of all organisms.

Localized hypoxia is a normal feature of embryo development [9], and this low fetal oxygen environment is one of the most important extracellular factors for lung morphogenesis [10,11], which is a complex process [12,13]. Given this idea that lung development occurs in a hypoxic environment, it is not surprising that HIFs would be important contributors to embryogenesis. Indeed, HIF mRNA and protein levels are quite high in the fetal lung [10,14], with the entire HIF system in place as early as 8 weeks of gestation in the human [10]. HIF- 1a protein expression is induced by hypoxia in cultured cells derived from all cell types found in the adult lung [15], whereas HIF-2a protein expression is restricted to the vascular endothelium and type II pneumocytes [16]. During fetal development, the lung of the normal embryo is hypoxic [11], and analysis of the spatial pattern of expression in human lungs during initial development of the pulmonary vasculature revealed high levels of HIF-1a protein localized predominantly in branching epithelium [17]. HIF-2a protein was also present in epithelium, as well as in mesenchymal structures, which give rise to the vascular endothelium [17]. Given the distinct expression pattern observed in the developing lung, HIF-1a and HIF-2a may serve different, and specific, functions in epithelial and vascular morphogenesis. In this context, HIF system plays a critical role in pulmonary development. While some studies suggest that low oxygen favours airway branching and bud formation [18,19] other suggest that the growth of buds occurs at higher oxygen consumption [20,21]. Thus, the role of hypoxia during lung branching is not really elucidated. On the other hand vascular branching, by contrast, is almost completely dependent on the low oxygen tension of the fetal lung and it is abolished experimentally when the levels increase [10,21,22]. These controversies in the fluctuant levels of oxygen during lung development it is also reflected on how do cell obtain

energy to grow and differentiate. Thus embryo cells may, at some stage, rely primarily on glycolytic metabolism for energy production [23,24], a necessary adaptation for the environmental hypoxia [25].

Several studies have been already demonstrated the expression of MCTs during pre-implantation development of the human and mouse. The authors [26,27] demonstrated that since the development of the zygote until the stage of 2 cells, there is an absolute requirement for pyruvate and that lactate could support the development of the embryo in stages of 4 and 8 cells. For that is required a system that is capable of transporting lactate, pyruvate and protons in order to counteract the decreasing of pH inside the cell. Thus, Hérubel et al [28] analyzed and compared the expression of MCT1, MCT2, MCT3, MCT4 and basigin during stages of the early embryonic development of the human and mouse. For both human and mouse the transcript of MCT1, MCT2 and basigin were detected during all stages studied. MCT3 was detected neither in human nor in mouse and MCT4 was detected during all stages but only for mouse. Also, Harding and co workers examined changes in the activity of the MCTs during early mouse embryo development and the relationship between MCTs and pHi [27]. They demonstrated the presence of mRNA for MCT1, MCT2 and MCT3 isoforms throughout pre-implantation of the mouse. Furthermore, they studied MCT activity by inhibition with pCMBS and they suggested that MCT1 is the predominant isoform on the regulation of the pHi during early development of the mouse**.** Thus, MCTs have a crucial role during the preimplatation of the embryo during early embryonic development of human and mouse [26–29]

Regarding organogenesis there are also some studies reporting the expression of MCTs. MCT-4 isoform and basigin are co-expressed in the early differentiating cardiomyocytes in the developing heart of chick embryos [24] and MCT1 and MCT2 are expressed in the forebrain of rat embryos between embryonic day 16 and postnatal day 14.

The role of MCTs in the lung development it is not elucidated. Troug and co-workers [12] evaluated the effect of hypoxia on lung development. They observed that chronic hypoxia at the end of gestation interrupted lung development and they detected upregulation of MCT4 in these tissues. However, there are no studies demonstrating the expression of MCTs during the process of embryogenesis and specifically lung morphogenesis. In this work, we evaluate for the first time the expression of MCT1/4, CD147, the glucose transporter 1 (GLUT1) and the biomarker

of hypoxia carbonic anhydrase 9 (CAIX) in a human fetal lung series and evaluate the effect of MCT inhibition on viability and branching morphogenesis of rat fetal lungs.

2.2.2 MATERIAL AND METHODS

2.2.2.1Human tissue specimens

All the cases were retrieved from the laboratory of pathology from CGC genetics in Porto. Fetal human tissues were selected from late spontaneous abortions between 12th and 37th weeks of gestational age. Placental abruption occurred in all cases, including one case of fetal focal defects of the distal extremities in a context of amniotic bands, and other case with clinical features suggestive of chromosomal anomalies confirmed by cytogenetic studies (trisomy 21), which had a cardiac ventricular septal defect. None of the other fetus had developmental abnormalities. All cases had permission for autopsy and were donated for pathologic/scientific research. All tissues were collected after informed consent in accordance with the Ethics Guidelines of CGC clinics.

2.2.2.2 Animal model

Animal experiments were performed according to the Portuguese law for animal welfare. Animals were housed in an accredited rat house and treated as specified in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Healths (National Institutes of Health Publication No.85-23, revised 1996). Sprague–Dawley female rats (Charles-River, Barcelona, Spain) were maintained in appropriate cages under controlled conditions, fed with commercial solid food. After mating, they were checked for intracoital plugging and all pregnant rats were sacrificed by decapitation. Fetal rat lung tissues were obtained from fetuses harvested by caesarean section at 15.5 (pseudoglandular phase), 17.5 (canalicular phase), 19.5 (saccular phase) and 21.5 (alveolar phase) day post-conception (dpc). Samples were fixed in formalin, embedded in paraffin and 4μm sections were used for immunohistochemistry (IHC).

2.2.2.3 Immunohistochemistry

IHC for MCT1 (1:200 dilution; AB3538P; Chemicon International) was performed according to the avidin-biotin-peroxidase principle (R.T.U. Vectastin Elite ABC kit; Vector Laboratories), as previously described by our group [30]. MCT4 (1:1000 dilution, H-90; sc-50329; Santa Cruz Biotechnology), GLUT1 (1:500 dilution; ab15309; Abcam) and CAIX (1:1000 dilution; ab15086; Abcam) IHC was

performed with the Ultravision Detection System Anti-polyvalent, HRP (Lab Vision Corporation), as previously described [30,31]. CD147 (1:300 dilution; sc-71038; Santa Cruz Biotechnology diluted) IHC was performed using the UltraVision ONE Detection System: HRP Polymer (Lab Vision Corporation). In brief, paraffin embedded sections were deparaffinized in xylene and hydrated in a graded series of ethanol solutions. For antigen retrieval, slides were incubated with 1mM EDTA buffered solution (pH 8.0) for 20 min in water bath at 98°C. Slides were incubated overnight at room temperature with the primary antibody CD147. Tissues were stained with 3,3'-diamino-benzidine (DAB + Substrate System, DakoCytomation) for 10 min and counterstained with haematoxylin. Colon carcinoma tissue was used as positive control for MCT1, MCT4, CD147 and GLUT1 and normal stomach tissue for CAIX. Stained slides were evaluated and then photographed under a bright field microscope.

2.2.2.4 Rat fetal lung explant cultures

Fetuses were removed by caesarean section at 13.5 dpc. Harvesting and dissection of the lungs was made in Dulbecco's Phosphate Buffered Saline (DPBS - Lonza) under a dissection microscope (Leica MZFLIII, Wetzlar, Germany). Lungs were transferred to Nucleopore membranes with 8 mm pore size (Whatman, Clifton, NJ, USA) and incubated in a 24-well culture plates from Costar (Corning, NY, USA). Membranes were pre-soaked in DMEM (Lonza) for 1 hour before the explants were placed on them. Floating cultures of the explants were incubated in 200 ul of 50% Dulbecco's modified eagle medium (DMEM), 50% nutrient mixture F-12 (Invitrogen, Carlsbad, CA, USA) supplemented with 100 mg/ ml streptomycin, 100 units/mL penicillin (Invitrogen), 0.25 mg/mL ascorbic acid (Sigma–Aldrich, St Louis, MO, USA) and 10% fetal calf serum (Invitrogen). After 1 hour incubation, 200 µl of medium with the inhibitor (CHC) at pH 7.4 was added. Fetal lung explants were incubated for 96 hours, at 37°C and 5% CO_2. The medium with inhibitor was replaced every 24 h for 4 days. Fetal lungs were photographed daily and D0 was considered the first day and D4 the last. At the end of the incubation time, explants were washed in PBS and stored at -80°C until use.

2.2.2.5 Morphometric analysis of lung explants

Branching morphogenesis was monitored daily by photographing the

explants. At day 0 (D0: 0 h) and 4 (D4: 96h) of culture, the total number of peripheral airway buds (branching), the epithelial perimeter and total explant area in all lung explants were measured using a specific software developed by the laboratory [32]. For all experimental conditions, results of branching and epithelial perimeter were expressed as D4/D0 ratio.

2.2.2.6 Western blot

Expression of MCT1 and MCT4 was also evaluated by Western blotting in rat embryo samples. Total protein was extracted from the lung explants at stages 16, 18, 20 and 22. After maceration of the lungs with lysis buffer, the homogenate was centrifuged at 13000 rpm for 15 min at 4°C. The supernatants were collected and protein concentrations were determined by the DC Protein Assay Kit (500-0113, BioRad). 40 µg of protein of each sample were separated on 10% (w/v) polyacrylamide gels and transferred onto nitrocellulose membranes (Amersham Biosciences). After that, membranes were blocked with 0.1% Tween, 1% BSA in TBS for 1h and then incubated overnight at 4°C with the primary polyclonal antibodies for MCT1 (1:200 dilution; AB3538P; Chemicon International) and MCT4 (1:1000 dilution, H-90; sc-50329; Santa Cruz Biotechnology). After washing with TBS-0.1%Tween, membranes were incubated with an anti-rabbit secondary antibody coupled to horseradish peroxidase (SantaCruz Biotechnology, 1/5000 in TBS-0.1% Tween, 1% BSA). At the end, bound antibodies were visualized by chemiluminescence (Supersignal West Femto kit; Pierce). For loading control, blots were probed with β-tubulin monoclonal antibody (1:200000 dilution, Abcam, UK).

2.2.3 RESULTS

2.2.3.1 Expression of MCTs along human lung embryo development

We evaluated the expression of MCT1, MCT4, CD147, CAIX and GLUT1 in in human fetal lung samples (Figure 2). Lung tissue samples were collected and divided into 4 groups, according to the lung development phases: pseudoglandular (11-17 weeks of gestation; n=7); canalicular (18-25 weeks; n= 8); saccular (24-36 weeks; n= 5) and alveolar (37-40 weeks; n=3). All proteins were expressed in the pseudoglandular phase of human lung gestation. MCT1 immunoreactivity was primarily detected in mesenchymal cells and in primitive epithelial alveolar cells. From 15 weeks of gestation, staining decreased in mesenchymal cells and started to appear also in the bronchial epithelium. In contrast, MCT4 and GLUT1 proteins were consistently early detected in bronchial and primitive alveolar epithelial cells and absent in mesenchymal cells. CD147 expression was only observed from week 16 in mesenchymal cells, primitive epithelial alveolar cells and on the bronchial epithelial cells, however staining was weak. At 17 weeks of gestation, CD147 was strongly detected in the basal region of the primitive epithelial alveolar cells and also on bronchial epithelium and mesenchymal cells. CAIX expression was restricted to the bronchial epithelial cells along this phase.

During the canalicular phase, MCT1 expression continues to decrease in mesenchymal cells and to become more intense in the apical region of bronchial epithelium cells and in the basal region of alveolar cells. MCT4 and GLUT1 have similar expression patterns, maintaining the expression in the bronchial epithelial cells from 18 to 25 weeks of gestation and becoming absent in alveolar cells from week 19. MCT4 was focally detected in the glands of the bronchial epithelium. CD147 and CAIX expression varies during this phase. First, CD147 is only detected in the epithelium of the principal bronchus while CAIX is absent. From week 22, CD147 is expressed in mesenchymal cells, and both CD147 and CAIX are present in epithelial alveolar and bronchial cells.

In saccular/alveolar phase, MCT1 expression decreased in the bronchial epithelium and is detected in pneumocytes type 2.

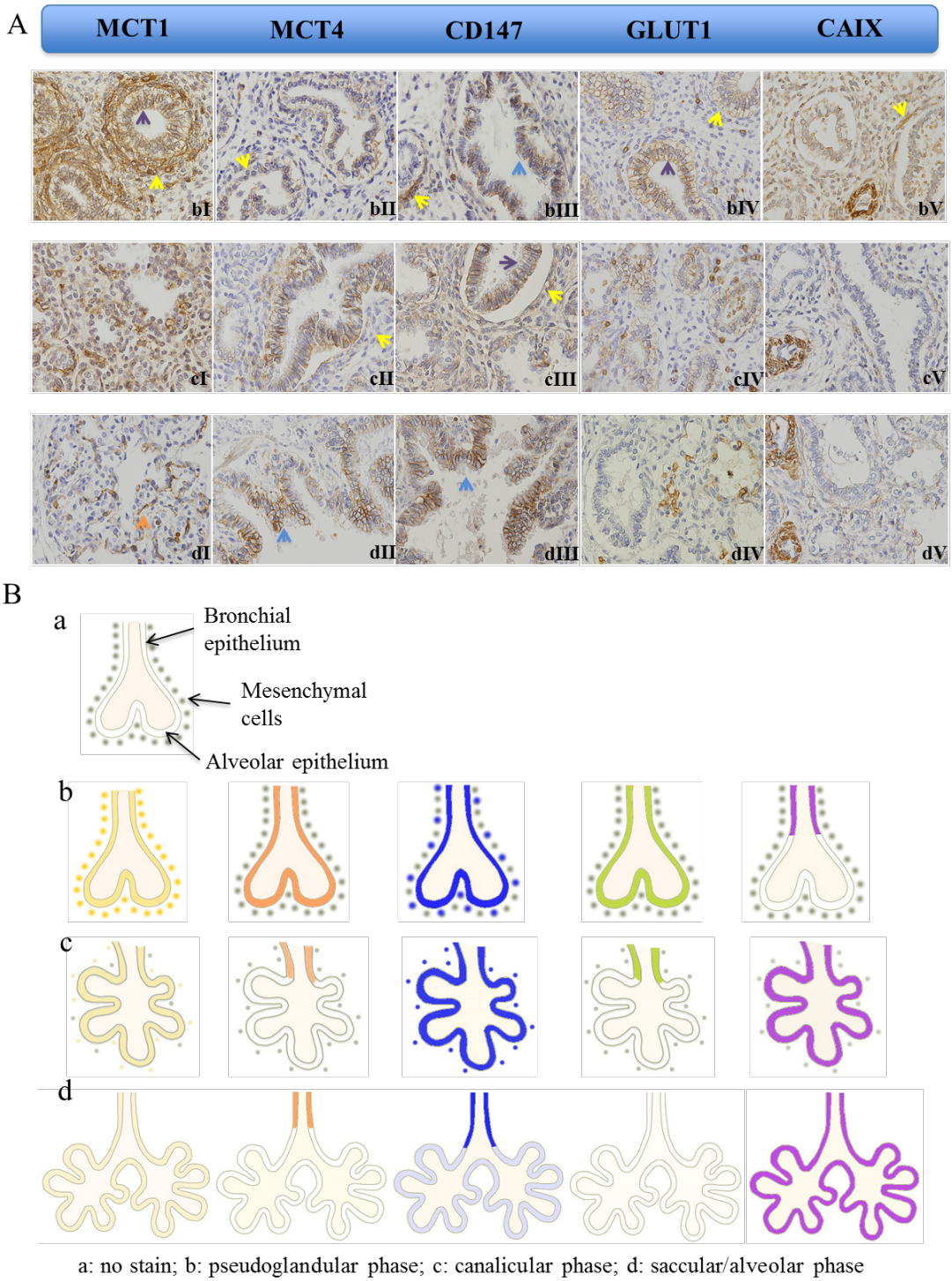


Figure 2: A - Immunohistochemical analysis of MCT1, MCT4, CD147, GLUT1 and CAIX during human fetal lung development. Immunoreaction was differently detected in mesenchymal cells (yellow arrow), primitive alveolar cells (purple arrow), bronchial epithelium (blue arrow) and alveolar type II cells (orange arrow). B- Illustrative scheme of the expression pattern of MCT1, MCT4, CD147, GLUT1 and CAIX during the lung embryo development phases.

MCT4 expression remained similar to canalicular phase while GLUT1 is absent. CD147 immunoreactivity is detected predominantly in the bronchi epithelium, being weak or even absent in the alveolar epithelium. CAIX expression is detected in both alveolar and bronchial epithelium.

CD147, CAIX and MCT1 were also detected in the mesothelium. CAIX is mainly expressed in vessels during all stages of gestation

2.2.3.2 Expression of MCTs in fetal rat lung

Firstly we confirmed the expression of MCTs in rat fetal lung tissues. Thus, we performed IHC on samples corresponding to pseudoglandular (15.5 dpc), canalicular (17,5 dpc), saccular (19.5 dpc) and alveolar phase (21.5 dpc). By analysis of Figure 3A, it is possible to see that both isoforms are expressed in fetal lung throughout all studied gestational ages. MCT1 and MCT4 were predominantly expressed in the plasma membrane of bronchiolar and alveolar epithelial cells from all studied gestational stages, while immunoreaction in mesenchymal cells was observed for MCT1 at 17.5 dpc and 19.5dpc and for MCT4 at 15.5, 17.5 and 19.5 dpc.

Western blot analysis for MCT1 and MCT4 revealed bands around 50 kDa and 43 KDa, respectively, consistent with the molecular weight described for these proteins (Figure 3B). Thus, we confirm that both MCT1 and MCT4 are expressed throughout all gestational stages in rat fetal lung.

2.2.3.3 Effect of MCT inhibition on rat lung morphogenesis

We aimed to understand the contribution of MCTs to lung morphogenesis. For that we cultured rat fetal lung explants and MCT activity was inhibited with CHC, a classical MCT inhibitor.

For this study, 30 lung explants were cultured with two doses of CHC (0.5 mM and 1mM). Representative pictures of fetal lung explants are illustrated in figure 4A. Comparing fetal lung explants treated with CHC at D4 with control (DMSO) it appears that CHC had an inhibitory effect in both doses studied (figure 4B).

Morphometric analyses showed that 1mM CHC had a significant inhibitory effect on branching morphogenesis ($p<0.05$). There was a decrease of the epithelial perimeter area and external perimeter of lung explants, in a dose dependent way, being significant for 1mM CHC ($p<0.05$).

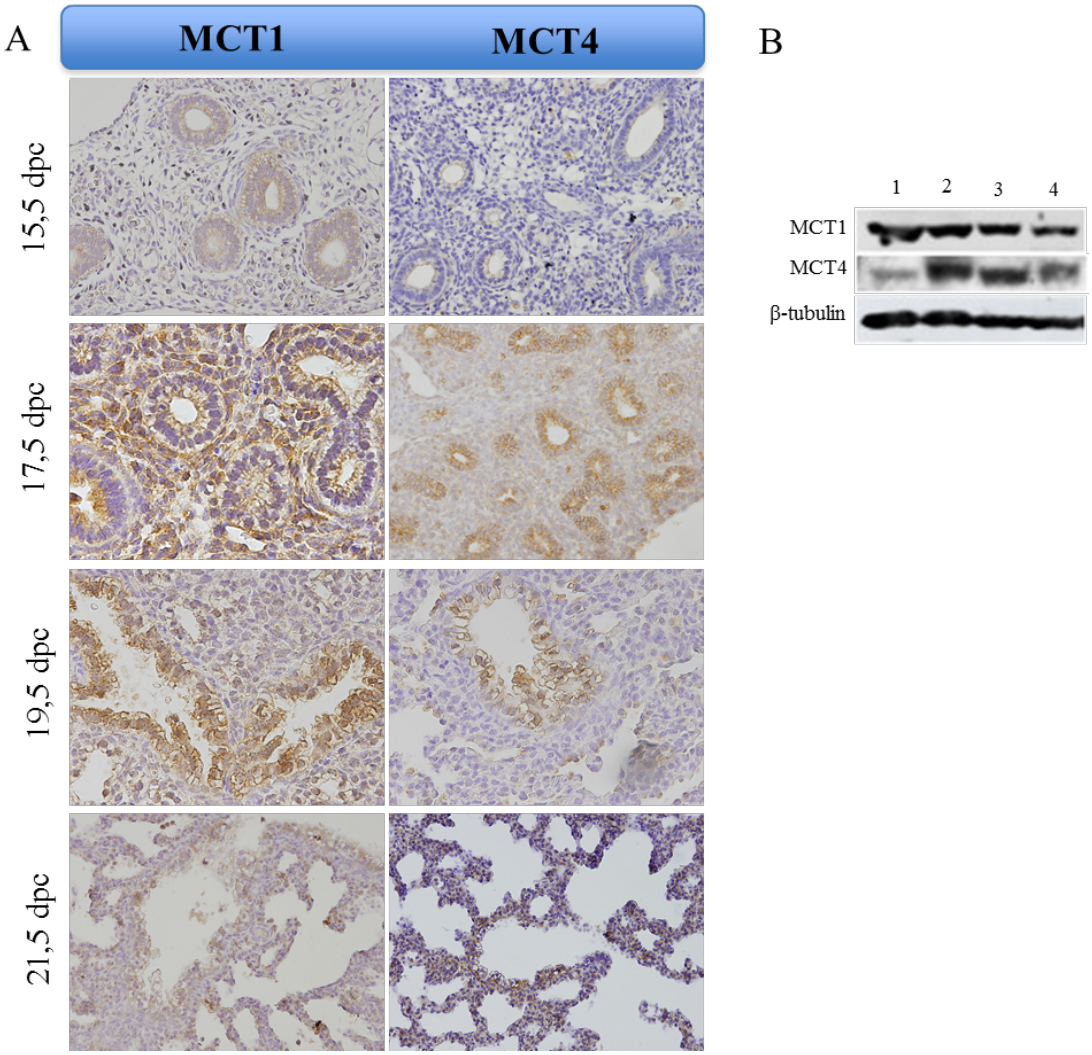


Figure 3: A – Expression pattern of MCT1 (left column) and MCT4 (right column) during rat fetal lung development. Both isoforms are expressed throughout all stages of lung development studied, from early 15.5 dpc until late 21.5 dpc. Original magnification ×200. B – Western blot analysis of both MCT1 and MCT4 during the lung development phases: (1) 15.5 dpc; (2) 17.5 dcp; (3) 19.5 dpc; (4) 21.5 dpc. β-tubulin was used as loading control.

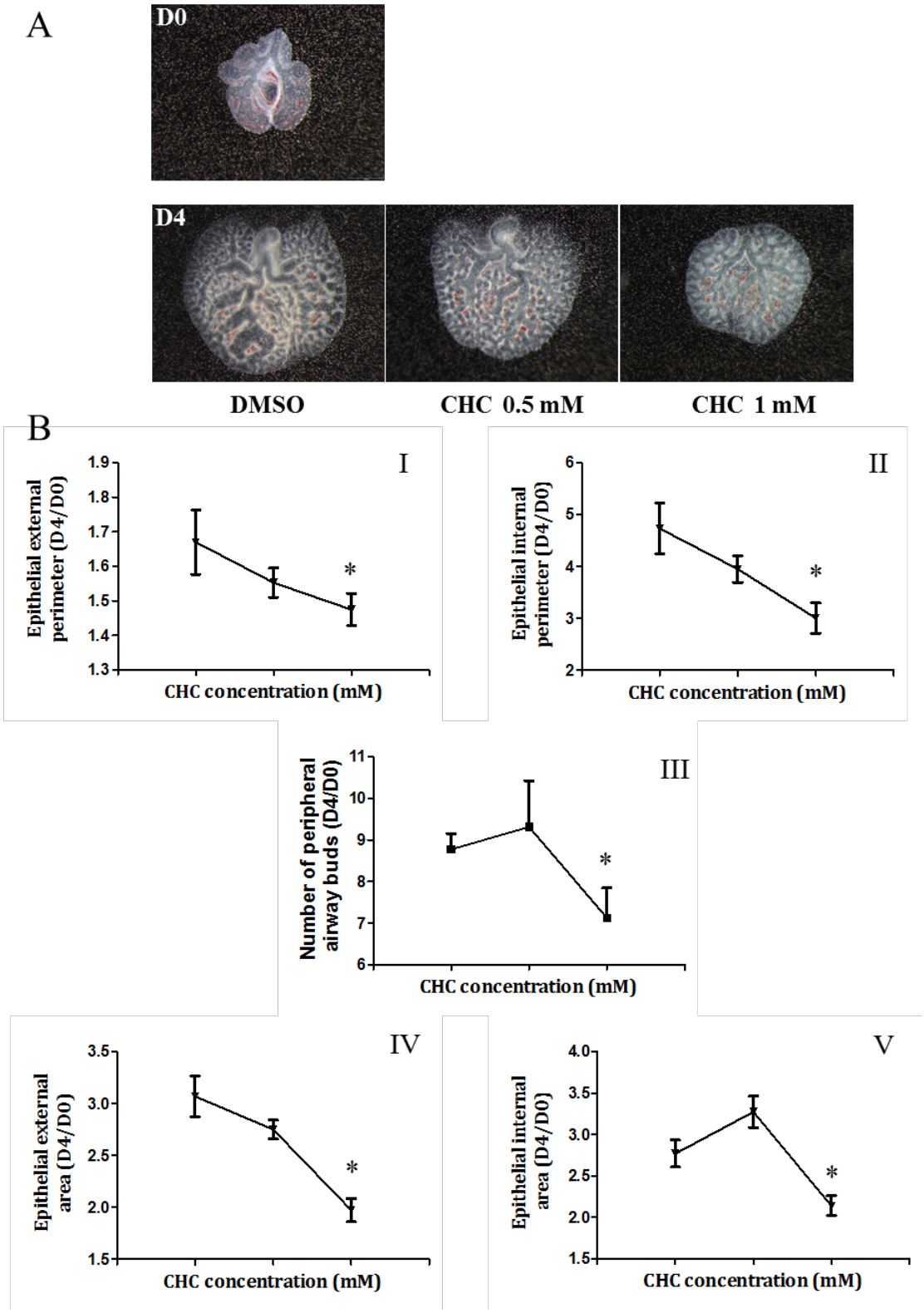


Figure 4: A - Branching morphogenesis in rat lung explants treated with CHC (0.5mM and 1mM). The first line represent lung explants at D0, the second line represent lung explants treated with different doses of CHC at D4. (n=12 per condition); B - Morphometric analysis; i/ii: epithelial external and internal perimeter; iii: total number of peripheral airway buds; iv/v epithelial area. Numeric results are expressed as D4/D0 ratio. $p<0.05$; * vs control (DMSO).

2.2.4 DISCUSSION

Hypoxia is a normal component of embryo development [33], and it seems to be one of the most important extracellular factors for lung morphogenesis [12,13] which is a complex process [13]. In most mammalian systems, the cellular responses to oxygen alterations are mediated through the hypoxia-inducible factor (HIF) family of transcriptional regulators [34,35]. HIF complex is a heterodimer composed of one of three α-subunits (Hif-1α, Hif-2α, or Hif-3α) and a β-subunit (ARNT; also known as aryl hydrocarbon receptor nuclear translocator). In fetal lung HIF mRNA and protein levels are quite high [17,34,36], being the Hif-1α protein localized predominantly in the branching epithelium [17]. These authors showed that induction of HIF-1 protein in fetal lung buds was sufficient to induce lung development even under non-hypoxic conditions, whereas its depletion, using antisense oligonucleotides, reduced lung branching morphogenesis and vascularization [37]. The enhancement of lung epithelial branching morphogenesis by low oxygen has also been reported by Gebb and Jones[18].

Hypoxic environment favors anaerobic glycolysis. This metabolic phenotype is characterized by the switch of cell metabolism from oxidative phosphorylation to glycolysis, being pyruvate metabolized to lactate [38] which is then exported by MCTs to avoid a decrease in pHi.

Although the contribution of MCTs to the glycolytic and acidic phenotype of tumours is well studied [39], their contribution to the embryo development is still not clear. Several studies have already suggested that embryos, as proliferating cells, express a metabolic phenotype similar to that of cancer cells [38]. We showed that lung cells develop in an intermittent hypoxia environment by the expression of the hypoxia marker CAIX. These results are in agreement with previous studies that report expression of HIF during lung development [17,34].

In order to adapt to intermittent hypoxia, cells need to create conditions to support embryogenesis, increasing the expression of genes related to the metabolic phenotype such as GLUT1 and MCT1/4 and CD147. It has also been described that HIF-1α up-regulates both GLUT1 and MCT4 [40]. Thus, with this study we show for the first time the expression pattern of MCT1/4 and their chaperone CD147 during human lung embryo development. We demonstrate that in normal fetal lung, MCT1/4 isoforms are expressed throughout the lung development phases suggesting that these molecules are important for lung morphogenesis. MCT1 was seen in the

pseudoglandular phase in all cellular types, becoming absent in mesenchymal cells along gestation, being detected only in alveolar epithelium cells in later gestation, while MCT4 was predominantly found to be expressed throughout the various phases in bronchial epithelium. These results suggest that MCT1 might have a role in alveolar epithelium and MCT4 is more important in bronchial epithelium. CD147 expression was similar to both MCT isoforms, however from week 18 to week 22 it is not detected in alveolar epithelium where MCT1 is present. This finding was unexpected since it has been described that MCT1/4 require this chaperone to be trafficked to the plasma membrane and perform their activity [41,42], which suggests the existence of another chaperone. We also analysed the expression of GLUT1, a glucose transporter widely distributed in fetal tissues that has been shown to be overexpress in tissues with high glycolytic rates, as well as CAIX. GLUT1 expression was similar to MCT4 through gestation but absent in the last phase (saccular/alveolar), while CAIX expression varies along gestation, starting to be expressed only in bronchial epithelium and being present in all epithelial cells in later phases. These results suggest that lungs develop in a hypoxic environment and that cells have the machinery to adapt to diferent levels of O_2. Our results show that the glycolytic metabolic phenotype is more pronounced in the early phases by the expression of glucose transporter (GLUT1) and lactate exporters (MCT1/4). This phenotype continues to be detected in the bronchial epithelium during the canalicular phase. However, in the late stage of gestation this phenotype is attenuated. Besides the expression of the hypoxia marker CAIX in most of the cells, expression of MCT4 is restricted to bronchial epithelium and GLUT1 is absent. At this level, lung tissue is close to complete maturation and the rates of proliferation decrease. The expression of CAIX at this time was not expected. Liao et al showed that CAIX was highly expressed during the second trimester of gestation but decreased after week 30 [43].

Several studies have already demonstrated the expression of MCTs during embryo development specifically during pre-implantation development of the human and mouse [27,28,44]. Some studies showed that since the development of the zygote until the stage of 2 cells, there is an absolute requirement for pyruvate [45] and lactate to support the development of the embryo in stages of 4 and 8 cells [26,27]. However, from stage 8 it has been observed that glucose consumption increases, being the main carbon and energy source to the embryo [46]. The importance of MCTs has been already described in other organs during rat embryo development [24,47,48]. Han et al [24] detected

MCT4 and the chaperone CD147 by immunohistochemistry during early cardiomyocyte differentiation and another study describes the immunoexpression of MCT1 and MCT2 in various cell populations of the rat forebrain, from the late embryonic period to the early post-natal period [47].

In order to understand the influence of MCT inhibition in lung morphogenesis, studies using fetal lung explant models cultured under CHC exposure were performed. First, in rat tissues we confirmed the expression of both MCT1 and MCT4 during rat lung development. We showed that MCT1 and MCT4 were expressed in primitive epithelial cells as early as the pseudoglandular stage and were also both detected in the bronchial and alveolar epithelium cells in the remaining phases. The immunohistochemical results were corroborated by Western blot. For in vitro studies, two different doses of CHC, MCT inhibitor, were added to lung explants and it was observed that lung branching was inhibited with increasing concentrations of CHC. Morphometric analysis was performed to determine the number of peripheral airway buds, epithelial perimeter and total area. We observed a significant effect for 1mM CHC in the number of peripheral airway buds. Although CHC is considered as a classical inhibitor of MCT activity, it can also inhibit the mitochondrial pyruvate transporter [49,50], This was described by Halestrap and co workers in 1974 [50], however in this study they use isolated mitochondria. On one hand, there are no studies reporting the entry of CHC into cells and, on the other hand, there is evidence showing that CHC acts only outside the cell [50–53]. Thus, we believe that effect of CHC in our model is mediated by inhibition of MCT activity at the plasma membrane. However, further experiments should be performed to clarify the true contribution of MCT inhibition in lung branching. Experiments with the specific inhibitor of MCT1/2 from AstraZeneca [54] or even RNA interference approaches could be used.

Although the role of MCTs in lung morphogenesis is still far from being well understood, with this study we showed that MCT inhibition disrupts lung branching, suggesting the dependence of these transporters for lung development. Embryonic developmental is strongly influenced by oxygen availability in the environment [23]. From our results, we hypothesize that lung embryonic cells are able to survive in hypoxia environments adopting a metabolic phenotype to supply energy and that MCTs/hypoxia may have different and specific functions in epithelial differentiation and vascular morphogenesis. Nevertheless, further studies are needed to better characterize this metabolic phenotype during lung differentiation.

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Chapter 3: Lung cancer

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Sara Granja, Elisabete Rios, Conceição Souto Moura, Fátima Carneiro, Fátima Baltazar. **Exploitating of Monocarboxylate transporters as therapeutic targets in lung cancer**. *39th meeting of ISOBM*, October 2011 Florence, Italy (published in Tumour biology 32 (suppl. 1): S97-8)

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Sara Granja, Ibtissam Marchiq, Renaud Le Floch, Fátima Baltazar and Jacques Pouysségur. **Targeting CD147/Basigin in A549 lung carcinoma sensitizes these cells to Metformin**. *Porto Cancer Meeting*, April 2012, Porto, Portugal

I Marchiq, S. Granja, R. Le Floch, D. Roux, J. Pouysségur **Genetic disruption of CD147/ Basigin, a subunit of lactate-H+ Symporters (MCTs), sensitizes glycolytic tumour cells to Phenformin**. *EMBO meeting 2012*, September 2012 Nice, France

Sara Granja, Ibtissam Marchiq, Renaud Le Floch, Fátima Baltazar and Jacques Pouysségur **Targeting CD147/Basigin sensitizes A549 lung carcinoma cells to Phenformin.** *Advances in Molecular Oncology: Translating Molecular Biology into Cancer Treatment*, February 2013, Sao Paulo, Brasil

Sara Granja, Ibtissam Marchiq, Renaud Le Floch, Fátima Baltazar and Jacques Pouysségur **Targeting CD147/Basigin sensitizes A549 lung carcinoma cells to Phenformin.** *TAT meeting 2013*, March 2013, Paris, France

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Sara Granja, Ibtissam Marchiq, Fátima Baltazar and Jacques Pouysségur. CD147/Basigin disruption using zinc finger nuclease technology. In Cancer Cell

Signalling" Second edition, Methods in Molecular Biology, in press

The results presented in this chapter will be submitted for publication in an international journal with peer review:

Sara Granja, Ibtissam Marchiq, Joffrey Pelletier, Fátima Baltazar and Jacques Pouysségur. *Targeting CD147/Basigin sensitizes A549 lung carcinoma cells to Phenformin.*, *in preparation*

3.1 CHAPTER OVERVIEW

RATIONALE

In contrast to normal differentiated cells, which rely primarily on mitochondrial oxidative phosphorylation to generate the energy needed for cellular processes, most cancer cells instead rely on aerobic glycolysis, a phenomenon termed "Warburg effect". This alteration allows tumour cells a rapid growth through higher rates of glucose consumption and lactic acid production leading to a cellular acidosis. To maintain high glycolytic rate and ATP production cells must efficiently export lactic acid [1,2]. This transport is mediated by a family of proton-coupled monocarboxylate transporters (MCT1/4) across the plasma membrane. MCT1/4 are distinguished by their kinetic properties and tissue distribution and they require association with an accessory protein, CD147/Basigin, for trafficking to the plasma membrane and activity [3].

Firstly, we analyzed the expression of these isoforms and also the chaperone CD147 and the glucose transporter (GLUT1) in 50 cases of lung cancer and we observed that all proteins were upregulated in tumour tissues when compared to non-tumoral adjacent tissue. Moreover, we propose a series of experiments aiming to understand the effect of MCT1/4 inhibition by disrupting CD147. Using the functional genomics approach 'Zinc-Finger Nuclease' (ZFN) we successfully isolated cells knocked out for CD147, decreasing the expression and activity of both MCT1 and MCT4. Furthermore, the disruption of CD147 sensitized tumour cells to the inhibition of OXPHOS by phenformin in vitro and in vivo.

Our results demonstrate that inhibiting MCTs via CD147 disruption sensitize A459 tumour cells to Phenformin-induced cell death. These findings support the exploitation of MCTs /Bsg as therapeutic targets in lung cancer, however further studies, particularly development of MCTs/Bsg blockers are urgently needed.

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3.2 THE ROLE OF MONOCARBOXYLATE TRANSPORTERS (MCTS) IN LUNG CANCER

3.2.1 Introduction

3.2.1.1 A brief introduction to cancer

Cancer is a leading cause of death worldwide [1]. Based on GLOBOCAN 2008, 12.7 million cancer cases and 7.6 million cancer deaths are estimated in 2008 (Figure 1) [1].

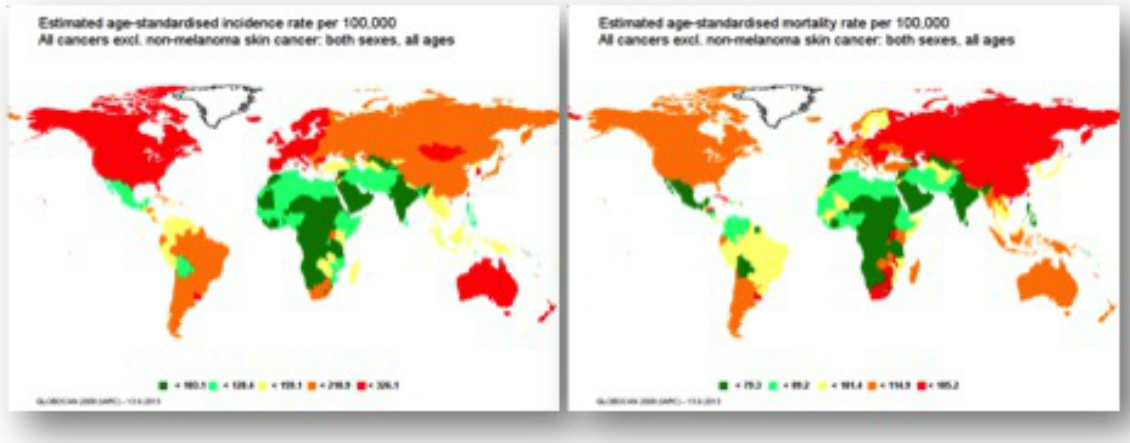


Figure 1: Age-standardised incidence (left panel) and mortality (right panel) of all cancers (per 100.000; all ages; excluding non-melanoma skin cancer), according to GLOBOCAN 2008 [1].

About 70% of all cancer deaths occurred in low- and middle-developed countries and death from cancer is predicted to continue to rise to over 13.1 million in 2030 worldwide. Lung (1.6 million, 12.7% of the total for men and women), female breast (1.4 million, 10.9% of the total for women), colorectal (1.2 million, 9.7% of the total for men and women) and stomach cancers (1 million, 7.8% of the total for men and women) where the most common types of cancer, with more than 40% of all cases diagnosed [1–3].

The principal feature of cancer is the rapid proliferation of abnormal cells that grow beyond their limits, and which can then invade adjacent parts of the body and spread to other organs. This process is referred as metastasis and this is the major cause of death from cancer[4]. Knowledge about the causes of cancer, and interventions to prevent and manage the disease is extensive. Strategies concerning cancer

prevention or early detection can reduce and/or control cancer progression. In fact, many cancers have a high chance of cure if detected early and treated adequately [5].

✓ Lung cancer: Epidemiology and Risk factors

Non-small cell lung cancer (NSCLC) is one of the most common cause of death from cancer worldwide, with 29% of all male cancer deaths and 26% of all female cancer deaths in 2009 (Figure 2) [3]. The overall 5-year survival rate of patients with metastatic disease is less than 15% [1,6]. This is due to the history of lung cancer, which has a rapid rate of growth when compared with other cancers[1].

Estimated New Cases*

Males				Females		
Prostate	241,740	29%		Breast	226,870	29%
Lung & bronchus	116,470	14%		Lung & bronchus	109,690	14%
Colon & rectum	73,420	9%		Colon & rectum	70,040	9%
Urinary bladder	55,600	7%		Uterine corpus	47,130	6%
Melanoma of the skin	44,250	5%		Thyroid	43,210	5%
Kidney & renal pelvis	40,250	5%		Melanoma of the skin	32,000	4%
Non-Hodgkin lymphoma	38,160	4%		Non-Hodgkin lymphoma	31,970	4%
Oral cavity & pharynx	28,540	3%		Kidney & renal pelvis	24,520	3%
Leukemia	26,830	3%		Ovary	22,280	3%
Pancreas	22,090	3%		Pancreas	21,830	3%
All Sites	**848,170**	**100%**		**All Sites**	**790,740**	**100%**

Estimated Deaths

Males				Females		
Lung & bronchus	87,750	29%		Lung & bronchus	72,590	26%
Prostate	28,170	9%		Breast	39,510	14%
Colon & rectum	26,470	9%		Colon & rectum	25,220	9%
Pancreas	18,850	6%		Pancreas	18,540	7%
Liver & intrahepatic bile duct	13,980	5%		Ovary	15,500	6%
Leukemia	13,500	4%		Leukemia	10,040	4%
Esophagus	12,040	4%		Non-Hodgkin lymphoma	8,620	3%
Urinary bladder	10,510	3%		Uterine Corpus	8,010	3%
Non-Hodgkin lymphoma	10,320	3%		Liver & intrahepatic bile duct	6,570	2%
Kidney & renal pelvis	8,650	3%		Brain & other nervous system	5,980	2%
All Sites	**301,820**	**100%**		**All Sites**	**275,370**	**100%**

Figure 2: Estimated new cases (upper painel) and estimated deaths (lower painel) of all types of cancers for men and women [3].

Since exposure to risk factors such as smoking, only results in disease after several years, lung cancer incidence and mortality for women and men tends to reflect prior and long-term exposure to risk factors. In developed countries, the incidence of

lung cancer in men has reached a plateau and is now decreasing, whereas the number of new cases in women continues to increase [1,3], while in less developed countries male lung cancer incidence is continuing to increase. Besides tobacco smoking, exposure to radon gas, asbestos and other chemicals, environmental pollution and employment-related risks in certain industrial settings, constitute also risk factors to develop lung cancer. However, tobacco smoking is the most important risk factor for cancer, causing 22% of global cancer deaths and 71% of global lung cancer deaths [7,8].

- ✓ **Lung cancer: Classification**

Based on the histologic features and response to conventional therapies, lung cancer has been divided into 2 major categories: non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC)[9]. NSCLC is further divided into three major types, squamous cell carcinoma (SCC), adenocarcinoma (AC) and large cell carcinomas. Large cell carcinomas represent poorly or undifferentiated forms and the criteria for its diagnosis vary widely, thus, for these reasons, we will focus on the squamous cell carcinoma and adenocarcinoma subtypes of NSCLC and combine large cell, poorly differentiated, undifferentiated and not otherwise specified NSCLC as "other types". [10]

- ✓ **Lung cancer: Treatment**

Lung cancer treatment requires a careful selection of one or more intervention, such as surgery, radiotherapy, and chemotherapy that can vary according to tumour stage [9]. The treatment options for most patients with NSCLC are not different between SCC or AC histologic subtypes. Only early-stage patients will benefit on having treatment based on surgery or radiotherapy/chemotherapy [2]. Advanced NSCLC may also have biological therapy such as erlotinib (Tarceva), gefitinib (Iressa) or crizotinib (Xalkori), however, this type of treatment requires certain parameters [11].

The goal is to cure the disease or considerably prolong life while improving the patient's quality of life. Genetic profiles or individual molecular characteristics of

lung cancer have been known for many years but have failed to make much impact on the clinical setting, until recently [12]. Important molecular drivers, that can characterize a subsets of lung cancers, mainly adenocarcinomas, have been identified in the last years [13]. Mutations in epidermal growth factor receptor (*EGFR*), *KRAS,* and anaplastic lymphoma kinase (*ALK*) are mutually exclusive in patients with NSCLC, and one of these mutations can predict the response to targeted therapy (18). Tyrosine-kinase inhibitors (TKIs), namely erlotinib and gefitinib, which act on EGFR have become important drugs in the treatment of advanced NSCLC and were approved in many countries for the treatment of locally advanced or metastatic NSCLC as a second- or third-line regimen [14]. Further investigation demonstrated that a better response to this therapy is observed in patients harbouring EGFR mutations, particularly exon 19 deletion, exon 21 L858R, and exon 18 G719X [7,15]. These mutations are commonly observed in female Asian descent patients, with adenocarcinomas and no prior history of smoking [7]. Other TKI used in NSCLC is crizotinib that acts on *ALK* rearrangements that are more common in nonsmokers or light smokers patients with adenocarcinoma [16]. Bevacizumab (Avastin), a monoclonal antibody which inhibits angiogenesis via acting on the vascular endothelial growth factor A (VEGF-A) was also approved by U.S. Food and Drug Administration (FDA) for use in first-line treatment for NSCLC combining with chemotherapy agents [17].

Moreover, inactivating point mutations, homozygous deletions or promoter hypermethylation at the tumour suppressors LKB1, BRG1, MYC, PTEN, P16, RB are now becoming part of the landscape of lung cancer classification [18] (figure 3).

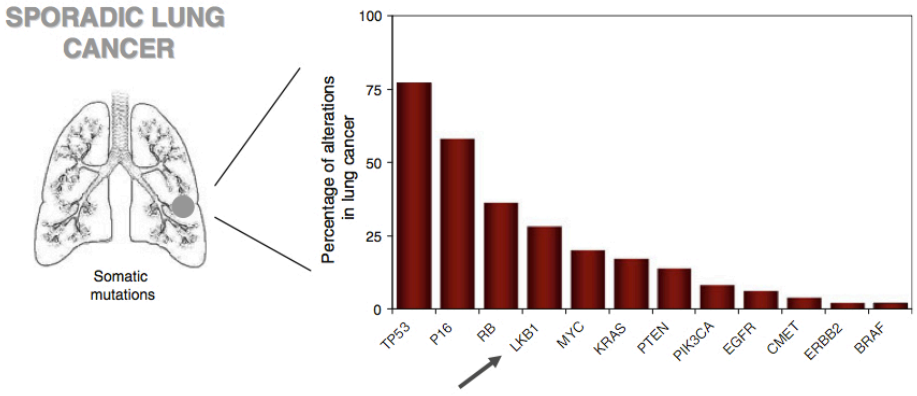


Figure 3: Frequency of mutations at the indicated genes in lung tumours (including all histopathologies) of sporadic origin [18].

The selection of individual patients for specific treatment strategies will then depend not only on clinical characteristics but also on tumour biology. A better understanding of the molecular mechanisms driving lung tumourigenesis is crucial and will provide enormous benefits in developing new pharmacological treatments.

3.2.1.2 MCTs in Cancer

Although lactate transport via MCTs is important in metabolism in a variety of biological systems under normal physiological conditions, MCT1 and MCT4 are commonly overexpressed by cancer cells to maintain intracellular lactate and pH homeostasis [19]. Taking in account the tumour microenvironmental scenario and molecular events that occur in carcinogenesis, it is possible to predict the importance of these lactate transporters in cancer. Tumours cells are characterized by an excessive anaerobic glycolysis that is a hallmark of cancer cells and leads to accumulating of glycolytic intermediates, including precursors of nucleic acids, lipids and proteins that contribute to rapid cell proliferation [20,21]. As already described in Chapter 1, the higher consumption of glucose and consequently a higher production of lactate is a constant feature in most cancers, therefore it is required an abundant expression of glucose transporters and glycolytic enzymes [22–24]. Also, an effective lactate extrusion mechanism to avoid lactic acidosis is necessary, which could act by a feedback mechanism to inhibit glycolysis [25]. In this context, many cancer cells overexpress MCTs (Table 1 [26]).

The first report on MCT expression in human tumour samples was described in 1998 by Ritzhaupt and his co-workers [27]. They observed that MCT1 expression decreased in the transition from normal colon to malign tissue. Later evidence showed a high expression of MCTs in colon carcinoma [28–30]. Pinheiro et al document MCT1 and MCT4 in the plasma membrane of colorectal cancer cells accompanied by a decrease in MCT2 at the plasma membrane [29]. This finding is in accordance with the dependence of glycolytic cancer cells in exporting lactate through MCT1 and MCT4, but not MCT2. Additionally, MCT1 and/or MCT4 expression was also detected in lung, breast, brain, prostate, head and neck, ovarian, cervical cancers and others [28,29,31–44]. Importantly, MCT expression was also correlated with several clinic-pathological data. Plasma membrane MCT1 expression in colon cancer was associated with vascular invasion [29].

Tumour site	MCT1 expression	MCT2 expression	MCT4 expression	CD147 expression*
Colon	↓ from normality to malignancy 27;38	Not detected in either normal or tumor tissues 38	Not detected in either normal or tumor tissues 38	(+) in tumor cells; no significant associations with MCTs 28
	(+) in tumor cells but (−) normal epithelium 30	(+) in tumor cells cytoplasm, but not in plasma membrane 30	Cytoplasm of cancer cells 30	
	↑ in tumor cells, compared to normal epithelium/ associated with vascular invasion 29	↑ in cytoplasm expression but ↓ in tumor cells plasma membrane compared to normal epithelium 29	↑ in tumor cells, compared to normal epithelium 29	
	(+) in tumor cells 28	(+) in tumor cells 28	(+) in tumor cells 28,43	
Central nervous system	Strongest in high grade glial neoplasms, compared to low grade glial neoplasms 39	↑ in glioblastoma, compared to normal tissue 40	(-) in glioblastoma 40	↑ glioblastomas, compared to nontumoral tissues 33
	(+) in glioblastoma and (−) in normal tissue 40		↑ diffuse astrocytomas and glioblastomas, compared to nontumoral tissues 33	
	↑ diffuse astrocytomas and glioblastomas, compared to nontumoral tissues 33			
	(+) in neuroblastoma/ associated with age >1 year at diagnosis, stage 4 disease, unfavorable Shimada histopathology, DNA diploid index, n-myc amplification and high-risk clinical group (COG criteria) 41			

Table 1: (cont)

Tumour site	MCT1 expression	MCT2 expression	MCT4 expression	CD147 expression*
Breast	↓ due to gene hypermethylation 42	(+) in tumor cells and normal epithelium cytoplasm, but not in plasma membrane 28	Tendency to be ↑ in tumor cells, compared to normal epithelium 45	(+) in tumor cells 28,45 and normal epithelium 45;
	↑ in tumor cells, compared to normal epithelium/ associated with basal-like subtype, high histological grade, estrogen and progesterone receptors, cytokeratins 5 and 14 and vimentin (alone or co- expressed with CD147) 45		↑ in tumor cells, compared to normal epithelium 28	Significantly associated with MCT1 39 and MCT4 28,45
			(+) in tumor stroma of type B mucinous carcinoma 37	
lung	Cytoplasmic accumulation in alveolar soft-part sarcoma 49	(+) in tumor cells but (−) normal epithelium 35	(+) in tumor cells but (−) normal epithelium 35	(+) in tumor cells; tendency to be associated with MCT1 and significantly associated with MCT4 28
	(+) in tumor cells but (−) normal epithelium 35	(+) in tumor cells and normal epithelium cytoplasm, but not in plasma membrane 28	↓ in tumor cells, compared to normal epithelium 28	
	(+) in tumor cells and normal epithelium 28		↑ tumor adenocarcinoma cells (AC) than in squamous cell carcinoma (SCC). ↑ in hypoxic areas in SCC and near vessels in AC 48	
Gynecologic tract	↑ from preinvasive to invasive cervical cancer/associated with metastases in AC (when co-expressed with CD147)44	No progressive change from preinvasive to invasive cervical cancer/↑ ASC 44	↑ from preinvasive to invasive cervical cancer/↑ AC 44	↑ from preinvasive to invasive cervical cancer; significant association with MCT1 and MCT4 but not MCT2 34

Table 1: (cont)

Tumour site	MCT1 expression	MCT2 expression	MCT4 expression	CD147 expression*
	(+) in ovarian tumor cells 44,48 but (−) in normal and benign epithelium / associated with low grade, high FIGO stage, residual tumor, lack of tumor relapse and presence of ascites 44	(+) in ovarian tumor cells cytoplasm, but not in plasma membrane 48	(+) in ovarian tumor cells 38,40 but (−) in normal and benign epithelium/ associated with high grade, high FIGO stage, residual tumor, tumor relapse and presence of ascites	(+) in ovarian tumor cells 44 but (−) in normal and benign epithelium
				Tendency to be associated with MCT1, significantly associated with MCT1 and MCT4 44
Prostate	(+) in tumor cells but (−) normal epithelium and PIN lesions/ associated with high pretreatment PSA, high Gleason score, high pathological grade and nodal involvement 46	↑ in tumor cells, compared to normal epithelium 31	(+) in tumor cells but (−) normal epithelium and PIN lesions/ associated with high pretreatment PSA, high Gleason score, high pathological grade and nodal involvement 46	(+) in tumor cells but (−) normal epithelium and PIN lesions; co-localization with MCT1 and MCT4 46
	↓ in tumor cells, compared to normal epithelium/ associated with high PSA, absence of perineural invasion and presence of biochemical recurrence 31		↑ in tumor cells, compared to normal epithelium/high PSA levels, advanced tumor stage, higher Gleason score, presence of perineural invasion, and presence of biochemical recurrence 31	(+) in tumor cells and normal epithelium; significantly associated with MCT1 and MCT4, but not MCT2 31
Stomach	(+) with no change along progression/ associated with advanced gastric cancer, Lauren's intestinal type, stage III+IVand lymph- node metastases when (co-expressed with CD147) 47		↓ from normal tissue, to primary tumor, to lymph-node metastases/ associated with early gastric cancer and Lauren's intestinal type 47	(+) with no change along progression; significantly associated with MCT1 and MCT4 47

In breast cancer MCT1, alone or in co-expression with CD147, was associated

with basal-like subtype (a more aggressive breast cancer group) and other poor prognostic variables, including high tumour grade [45]. Moreover, MCT1 and MCT4 were significantly associated with high FIGO stage, presence of residual tumour, lack MCT4 expressions were associated with high PSA levels, high Gleason score, high pathological stage, and nodal involvement [31,46]. In contrast to what was found in the previous types of tumours, neither MCT1 nor MCT4 were found to be up-regulated in gastric adenocarcinomas [47]. Regarding lung cancer the literature is controversial. Koukourakis and co-workers firstly observed overexpression of MCT1, 2 and 4 in cancer cells [35]. No expression of MCTs in normal lung was found, while MCT1, MCT2 and MCT4 were expressed in cancer cells. This study also analyzed the metabolic cooperation between lung cancer cells and tumour-associated stroma, however, tumour-associated stroma expressed MCTs weakly. In opposition, a recent study by our group showed that normal lung presents a high frequency of MCT expression and, in fact, MCT4 is less expressed in tumour samples than in normal epithelium [28]. However, as this last study was performed in a small number of cases, further work is needed to confirm these results [28]. Another study showed the spatial distribution of MCT4 in 90 NSCLC cases. They observed that MCT4 expression was relatively high in the first cell layers around the vessels in adenocarcinomas whereas its upregulation was stronger in hypoxic areas in squamous cell carcinomas [48]. MCT1, in association with its chaperone CD147, was also described in the cytoplasm of alveolar soft part sarcomas [49].

- ✓ **Targeting MCTs in cancer**

Considering the crucial role of MCTs in cancer, these lactate transporters are appealing targets for cancer therapy. Sonveaux and co-workers [50] described the cell-cell lactate shuttle in cancer, where MCT1 has a crucial role by importing lactate into oxidative cells. Thus, targeting MCT1 will have important implications in cancer homeostasis (Figure 4). Also, inhibition of MCTs will have a direct effect in lactate transport, interfering with pHi homeostasis and also with acidic microenvironment.

In fact, there are already *in vitro* studies showing inhibition of MCTs in several human cancer cell lines. Thus, MCT1 inhibition has been shown to decrease intracellular pH [41,50], cell proliferation and migration [33] cell invasion [33,36], leading to cell death [33,40,41,50,51] and inducing cancer cell radiosensitivity [51].

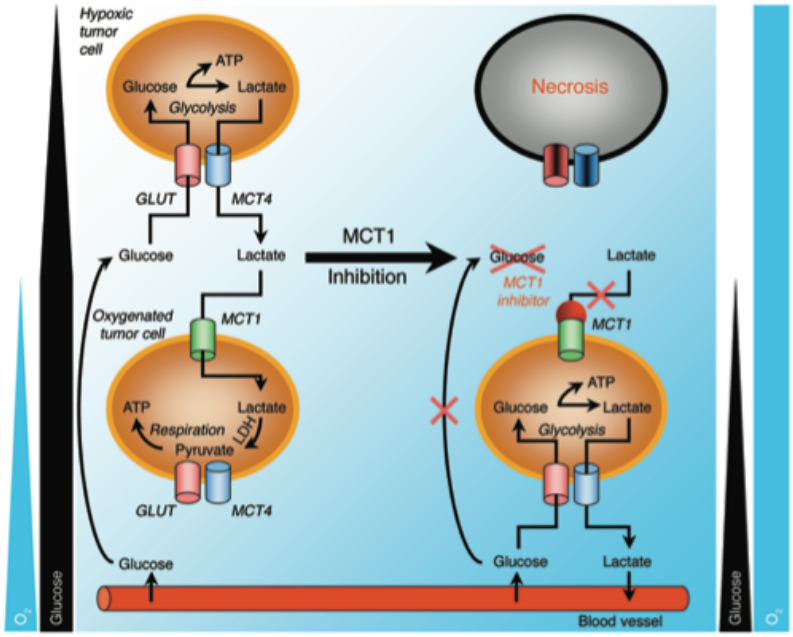


Figure 4: Model for therapeutic targeting of lactate-based metabolic symbiosis in tumors [46]. Hypoxic tumor cells depend on glucose and glycolysis to produce energy. Lactate, the final product of glycolysis, diffuses toward blood vessels and oxygenated tumour cells import lactate by MCT1 that is located at the cell plasma membrane and oxidize it to produce energy. In the respiration process, lactate is a substrate preferred to glucose. As a consequence, glucose freely diffuses through the oxygenated tumor cell sheath to fuel glycolysis of distant, hypoxic tumor cells. This metabolic symbiosis can be disrupted by MCT1 inhibition. Upon MCT1 inhibition, oxidative tumor cells switch from lactate oxidation to glycolysis, thereby preventing adequate glucose delivery to glycolytic cells, which die from glucose starvation. This glycolytic switch is associated with a decrease in oxygen consumption of surviving tumor cells, which is responsible for increased tumor pO_2. GLUT, glucose transporter.

Downregulation of MCT4 results in decreased cancer cell migration [52], by mechanisms that also involve its interaction with with β1-integrin in breast cell line [52]. Promising results using *in vivo* models have already been reported. Administration of CHC, MCT1 inhibitor (Chapter 1), retards tumour growth [33,50], renders tumour cells sensitive to radiation [50], induces tumour necrosis and decreases tumour invasion [53]. Moreover, targeting both MCT1 and MCT4 by themselves or through the chaperone CD147, also reduced tumour growth and glycolytic flux *in vivo* [54]. In what concerns lung cancer cells, Izumi and co-workers showed that inhibition of MCT1 or MCT4 inhibited cancer cell invasion but did not influence cell migration [55].

✓ MCT regulation by chaperones in cancer

As mentioned in Chapter 1, accessory proteins, such as CD147, regulate the functional expression of MCTs. CD147 also known as extracellular matrix metalloproteinase inducer (EMMPRIN), HAb18G/CD147 or basigin (BSG) belongs

to the immunoglobulin (Ig) superfamily, is a heavily glycosylated type I transmembrane glycoprotein [56,57] and is widely distributed and expressed in high levels in many tissues, mainly in metabolically active cells such as lymphoblasts and malignant tumour cells [56,58]. CD147 is also involved in reproduction and development. Its best characterized function is stimulation of matrix metalloproteinase (MMP)-production [57] in stromal fibroblasts and endothelial cells, leading to extracellular matrix degradation and metastasis [57,59]. CD147 has also an impact on tumour growth, mediates chemoresistance [60–63] and stimulates VEGF expression, inducing angiogenesis [57,64]. The crystal structure of CD147 indicates that it is composed by an extracellular domain, a transmembrane domain (TMD) and a cytoplasmic domain [57]. The extracellular portion of basigin comprises an N-terminal IgC2 domain and a membrane-proximal IgI domain [65]. The IgC2 domain is required for counter-receptor activity, which is involved in MMP induction and oligomerization [66,67] while, the IgI domain is required for association with caveolin-1, that regulates cell surface clustering [68].

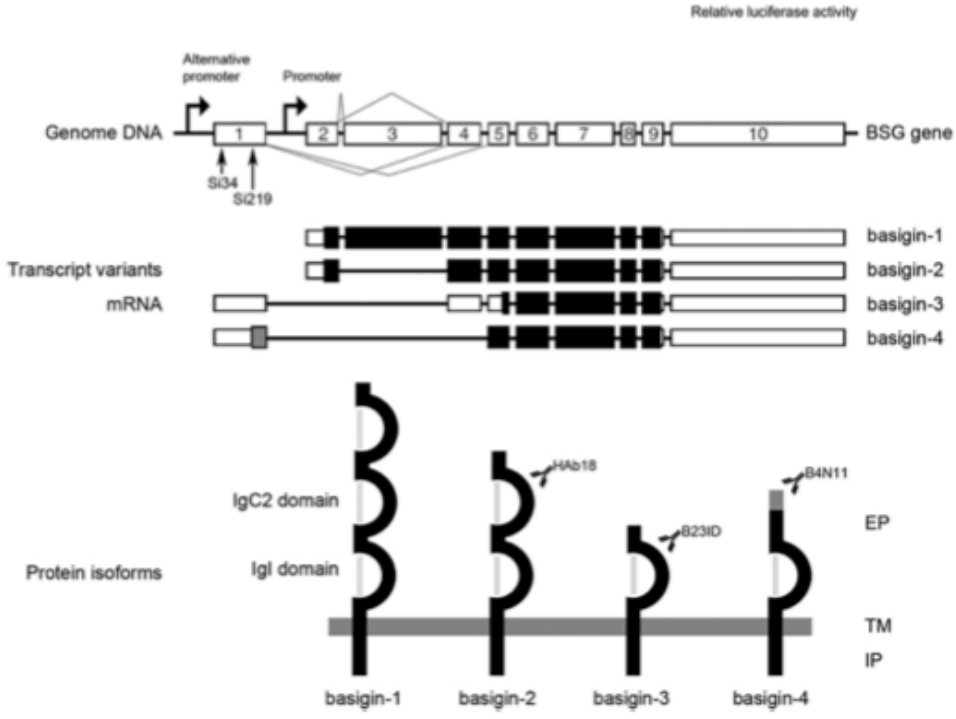


Figure 5: Structures of the basigin gene and protein isoforms. The basigin gene expresses 4 splice variants and contains an alternative promoter. Black, coding sequence; white, noncoding sequence. The double lines represent disulfide bonds. Si34 and Si219 represent the RNA interference target sites. HAb18, B23ID, and B4N11 indicate the binding sites of the antibodies [62].

Currently, four basigin alternative splicing transcript variants are described, encoding four different protein isoforms (basigin 1, 2, 3 and 4) (Figure 5).

The basigin-1 transcript (NM_001728) encodes the retina-specific isoform that is distinguished by an additional Ig-like domain (three Ig-like domains in total) in the extracellular portion. The basigin-2 transcript (NM_198589) encodes the well-known basigin/ CD147/EMMPRIN [69].

Basigin-3 (NM_198590) and basigin-4 (NM_198591), the isoforms less common, were first identified in human endometrial stromal cells and cervical carcinoma cell lines [70]. Basigin-3 is a short isoform comprising only one Ig-like domain (Ig-I domain) and interacts with the internalized basigin receptor-ligand complex. This isoform exerted an inhibitory function in proliferation and invasion of hepatocellular carcinoma cells, probably via hetero-oligomerization with basigin-2 [71]. Basigin-4 is overexpressed in cervical cancer and promotes proliferation of cervical cancer cells. Evidence shows that basigin-2 is highly expressed in normal heart, kidney, skeletal muscle and testis. Basigin-3 is highly expressed in the bone marrow, fetal liver, lung, testis and thymus, and the tissue expression profile of basigin-4 is similar to that of basigin-3 [66].

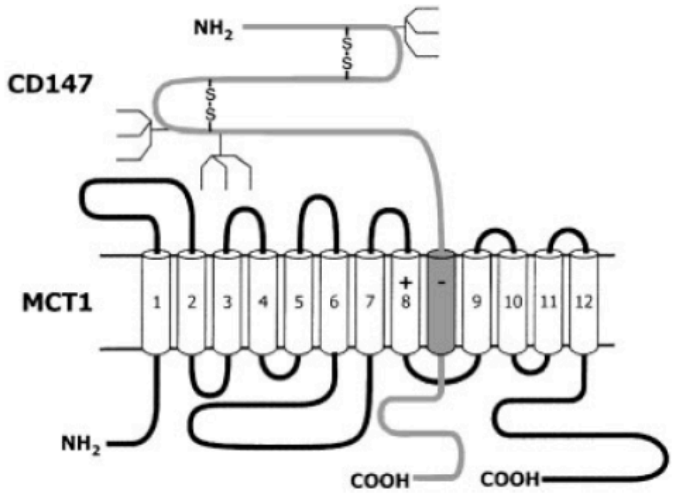


Figure 6: Monocarboxylate transporter 1 (MCT1) and CD147 predicted interaction. This interaction is proposed to involve an arginine residue within the transmembrane segment 8 of MCT1 and a glutamic acid residue in the transmembrane segment of CD147 [68].

The TMD of CD147 contains a glutamic acid residue, which is involved in the association of the protein to the plasma membrane (Figure 5)[72]. This residue forms a charged-pair with a positive residue in a TMD of MCTs [73]. Evidence implicates Arg306 in TMD8 (numbering based on rat MCT1) as a potential candidate for such a charged-pair. Acting as an essential chaperone, CD147 leads to the correct activity and targeting of MCTs to the plasma membrane [74].

CD147 is a multifunctional protein and its elevated expression is reported in various cancers such as in lung, breast, brain, prostate, ovarian and others [31,75–87]. It has been shown that higher expression of basigin was associated with poor prognosis of cancer patients and could serve as an independent predictor of poor survival in cancer [82,85].

Le Flock and co-workers demonstrated that the major pro-tumoural action of CD147/Basigin is mediated by its cardinal function of chaperoning MCTs, a central action in energy metabolism particularly in highly glycolytic tissues [54]. In fact, there are several studies showing the regulation of MCTs by CD147. Several results show that CD147 and MCTs are co-expressed in human cancer tissues [28,31,33,34,45,47,86]. Moreover, results published by our group showed that the prognostic value of CD147 appears to be associated with its co-expression with MCT1 in breast cancer [47]. Therefore, targeting CD147 to inhibit MCTs appears to be a rational approach. In this context, CD147 silencing has been reported to inhibit MCT1/MCT4 function, decreasing lactate export [88] and consequently decreasing pHi [54,89,90] and reducing the malignant potential of cancer cells *in vivo* [54,90].

An enhancement of glycolysis, a cellular pathway that produces both energy and precursors of macromolecules, is a classic metabolic hallmark of cancer, making this pathway an attractive target for therapy [21]. With the established roles of MCTs in lactate extrusion and pH homeostasis, it is perhaps not surprising that blocking MCT1 and MCT4 in cancer cells impacts tumour growth. However, tumour cells have the ability to adapt to alterations and switch to a different metabolic program. It is therefore important to understand cell metabolism mechanisms, since a single molecule may not be an important piece of the puzzle. Therefore, efforts to explore new strategies to target these metabolic pathways are crucial to develop effective anticancer therapies, namely the inhibition of more than one bioenergetic pathway.

This Chapter describes the experiments performed to clarify the importance of MCT1 and MCT4 in lung cancer. First we analyzed the expression of these isoforms and also the chaperone CD147 and the glucose transporter (GLUT1) in 50 cases of lung cancer. We observed that all proteins were upregulated in tumour tissues when compared to non-tumoural adjacent tissue. Afterwards, we evaluated the effect of MCTs inhibition through targeting CD147 on cancer cell viability. Preliminary results in the laboratory using RNA interference approach showed that knockdown of CD147 or MCT4 in the presence of iMCT1/2 inhibitor sensitized cells to the ablation of oxidative phosphorylation (OXPHOS) with olygomicin. However, these effects were reversed in hypoxia with the upregulation of the remaining protein of MCT4 and CD147. Thus, we decided to adopt an approach to knockout (KO) CD147 gene. Using the zinc finger technology we knocked-out CD147 in an adenocarcinoma lung tumour cell line. We observed that the disruption of CD147 decreased the expression and activity of both MCT1 and MCT4 and this disruption sensitized tumour cells to the inhibition of OXPHOS by phenformin *in vitro* and *in vivo*.

3.2.2 Material and Methods

3.2.2.1 Tissue samples

Representative formalin-fixed paraffin-embedded samples from primary lung tumour tissues were retrieved from pathology archives of the Department of Pathology of Hospital S. João, Porto, Portugal. The tumours were classified according to the WHO criteria [9]. This cohort includes 50 cases of non-small cell lung cancer (NSCLC), being 8 squamous cell carcinomas (SCC), 29 adenocarcinomas (AC) and 13 from other types. Non-tumoural adjacent tissue from each case was also selected.

3.2.2.2 Cell Line

In the present study we used the lung adenocarcinoma cell line A549 obtained from ATCC (American Type Culture Collection), which was maintained in Dulbecco's modified eagle medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS - Gibco), penicillin (10 units/mL) and streptomycin (10 μg/mL), in a humidified atmosphere of 5% CO_2 at 37 °C.

3.2.2.3 Immunohistochemistry (IHC)

Sections of 3-μm were used for Immunohistochemical analysis. IHC for MCT1 and CD147 was performed according to the avidin-biotin-peroxidase principle (R.T.U. Vectastin Elite ABC kit; Vector Laboratories), as previously described by our group [33]. MCT4, GLUT1 and CAIX IHC was performed with the Ultravision Detection System Anti-polyvalent, HRP (Lab Vision Corporation), as previously described [32,33]. 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 (MCT1, MCT4, GLUT1 and CAIX) for 20 min in water bath at 98°C. After endogenous peroxidase inactivation, incubation with the primary antibody was performed overnight for MCT1 and CD147 and 2 h for MCT4, GLUT1 and CAIX at room temperature. Tissues were stained with 3,3'-diamino-benzidine (DAB+ Substrate System, DakoCytomation) and counterstained with haematoxylin. Colon carcinoma tissue was used as positive control for MCT1, MCT4, CD147 and GLUT1 and normal stomach tissue for CAIX. 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 25% positive cells; 2, 26% 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 [29].

3.2.2.4 Generation of bsg-null cells using Zinc Finger Nuclease

Zinc-finger nucleases (ZFNs) are a class of engineered DNA-binding proteins that have redefined genome engineering. The ZFNs plasmids bind a specific DNA sequence inducing a double strand break (DSB). This break is repaired by the non-perfect endogenous repair mechanism called non-homologous end joining (NHEJ). The DBS will be misrepaired by the addition and/or deletion of nucleotides. This disruption results in a knockout, in a non-sense gene product and the loss of gene function [91].

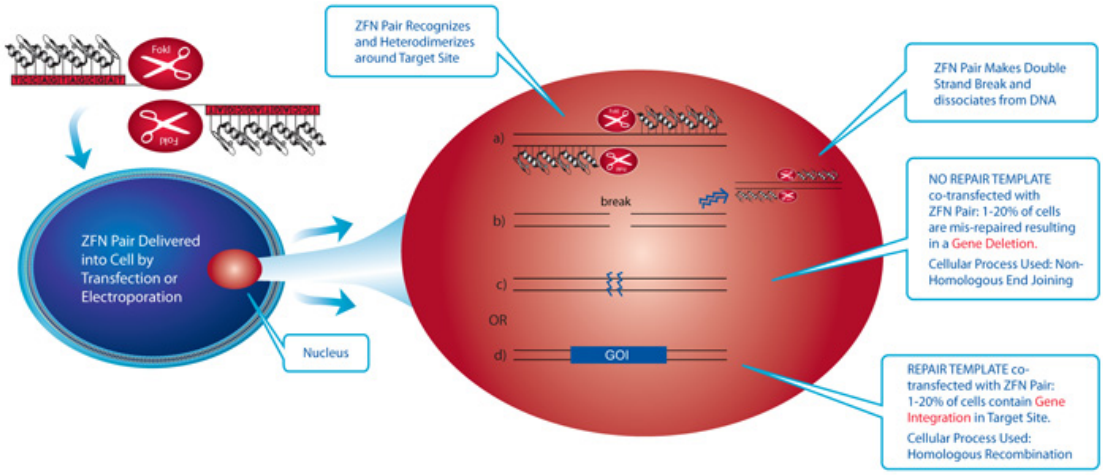


Figure 7: ZFN-mediated genome editing takes place in the nucleus when a ZFN pair targeting the user's gene of interest is delivered into a parental cell line, either by transfection, electroporation or viral delivery [86].

The ZFN structure consists of a DNA binding domain – zinc finger protein (ZFP) - linked to a nuclease domain of the FOK1 restriction enzyme [92]. The ZFP region contains a tandem array of Cys_2-His_2 fingers that each recognizes 3bp of DNA.

This domain can have from 3 to 6 fingers binding to a target of 9bp to 18bp respectively. The catalytic domain composed by the enzyme FOKI requires dimerization to cut thus, a pair of ZFNs is required. The dimerization avoids cleavage at single binding sites (Figure 7) [93].

To generate null cells, first two plasmids targeting basigin exon 2 encoding signal peptide of basigin splice variants 1 and 2 were designed and obtained from Sigma-Aldrich. A549 cells ($1x10^6$ cells/100-mm dishes) were transfected using JetPRIME® DNA & siRNA Transfection Reagent (Polyplus-transfection SA), according to the manufacturer's instructions. Briefly, 10 µg of DNA (5µg of each ZFN plasmid) were diluted in 500µl of jetPRIME® buffer and mixed. 20 µl of the jetPRIME transfection reagent were added to the first. After vortexing and a spin down, the mixture incubated for 10 min was added drop-by-drop to the cells and mixed gently. After 6 hours of transfection, the media was replaced by fresh media. One week after, transfected cells negative for bsg were selected and sorted by Fluorescence-activated cell sorting (FACS). First, cells were stained for bsg and passed through the FACS. Individual clones were plated in 96 well plates and allowed to grow until confluence. Single colonies were trypsinised and expanded in duplicate wells, one of which was frozen and the other was allowed to grow to propagate the clone. A pharmacologic pre-selection was performed by adding 1 mM of the drug metformin to each clone. Metformin is a drug from the biguanide class that impairs OXPHOS. Thus, the putative negative clones, the ones that have disruption of CD147/basigin and consequently decreasing of lactate export (glycolysis inhibited), will die after blocking respiration. For these clones, sensitive to metformin, protein was extracted with Laemmli buffer 1,5x (Bio-Rad Laboratories, CA) and negative expression for basigin was confirmed by Western blot.

3.2.2.6 Sequencing of the ZFN Cutting Site

Genomic DNA from A549 wt and bsg -/- cell was extracted and amplified by PCR with primers that surround the ZFN cutting site. The primers are: (sense) 5'-TTGGAGGTTGTAGGACCGGCGA-3', (anti-sense) 5'-CAAGCTCCATGCTGGCGC-3'. 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 anti-sense primers (sent with the ZFN kit), 200 µM of dNTPs, 1,5 mM of $MgSO_4$, 1 × Taq Buffer and 10

U of KOD hot start DNA polymerase (all the 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 3 min at 68 °C.

3.2.2.7 [14C]-Lactate Uptake

The [^{14}C]-lactate uptake was measured to determine lactate transport, according to the previously published [54]. Briefly, cells were seeded in 35-mm dishes and equilibrated for 5 min in 20 mM buffered glucose-free and $HCO3^-$-free saline solution containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, adjusted to pHe 6.0 in the presence or absence of iMCT1/2 (300 nM). The solution was then replaced by the same solution containing 0.2 μCi /mL of [^{14}C]-L-lactate (100 μM; Amersham Biosciences). Uptake was stopped by washing four times with ice-cold PBS. Cells were lysed with 250 μL of 0.1 M NaOH. The radioactivity was determined by mixing 750 μL of scintillation liquid with 200 μL of cell lysate and counted with a liquid scintillation counter (Wallac). The protein concentration was determined to normalize radioactivity to the protein content of the cells in each dish. [^{14}C]-L-lactate uptake was expressed as counts per million per milligram of protein.

3.2.2.8 Determination of Intracellular Lactate Concentration.

Cells ($3x10^5$) were seeded in 12-well plates and allowed to adere overnight. After incubation either in normoxia or hypoxia of 1% O_2 for 24h, cells were washed twice and maintained in DMEM without glucose, pyruvate and serum for 2 hours with either DMSO or MCT1/2 inhibitor (300nM). After that, regular DMEM 5% serum with either DMSO or MCT1/2i cells were collected at the indicated times (0h; 1h; 3h; 6h). For estimation of intracellular lactate, cells were washed once with cold PBS1x and cold water and lyzed with 200 μl cold water, while for extracellular lactate, the supernatant of each condition was collected at the same time points. Lactate concentration was determined using the Lactate colorimetric assay kit (Biovision Incorporated).

3.2.2.9 Western blot

Cells were lyzed in Laemmli 1,5x buffer and incubated for 15min at 95°.

Protein concentrations were determined using the BCA Assay. 40 μg of protein of each sample were separated on 8% SDS polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore). After that, membranes were blocked with 5% milk in TN buffer (50mM Tris-HCl pH7.4, 150mM NaCl) for 1h and then incubated overnight at 4°C with the primary antibodies for Bsg/CD147 (1/500; R&D Systems; MAB972), HIF-1α,ref MCT4 (1:1000; H-90; sc-50329; Santa Cruz Biotechnology) and for MCT1 (rabbit polyclonal antibodies against the C-terminal last 15 residues prepared by the laboratory). ARD1 was used as loading control. After washing with TN buffer with 1% Triton-X100 and incubated with secondary antibody coupled to horseradish peroxidase, bands were detected with the ECL system (Amersham Biosciences).

3.2.2.10 Clonogenicity assay

A549 wt and bsg -/- cells ($2x10^3$) were seeded in 60-mm dishes and incubated for 24h to attached. Medium was replaced by regular DMEM 10% serum with and without iMCT1 (300 nM) or Phenformin (50uM) or both for 10 days in normoxia and 15 days in hypoxic (1% O2) conditions. Cells were stained with Giemsa (Fluka).

3.2.2.11 Measurement of Glycolysis and Oxygen Consumption

Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured with a Seahorse XF analyser (Seahorse Bioscience). $8x10^4$ cells were seeded in seahorse plates and allowed to grow for 24h. Plates were incubated for 45 min in a non-CO_2 incubator at 37°C with seahorse medium without glucose and pyruvate before measurements. Then glucose (10 mM), MCT1i (AZ) (1uM, phenformin 50uM and olygomycin were injected and three measurements after each addition were undertaken determining oxygen and proton concentration in the medium. In the end, all media were removed and protein concentration was determined to normalize OCR and ECAR values.

3.2.2.12 Tumour Xenografts

Immunodeficient female nude mice with approximately 22 g body weight

were housed in groups of 5 under specific pathogen-free, controlled ambient conditions. Animal studies were conducted according to Centre National de la Recherche Scientifique institutional guidelines. A549 wt and bsg-/- cells ($2x10^6$/mouse) suspended in 500 μL of serum-free DMEM supplemented with insulin-transferrin-selenium (Gibco) were sub-cutaneously injected into the backs of the mice. Water containing 6% sucrose with and without 200mg/Kg phenformin was added to the drinking water, and the daily intake fluids were monitored. Fresh phenformin was administered every two days. Tumours were measured every 2–3 days using a calliper and 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 cm^3.

3.2.2.13 Statistics

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

3.2.3 Results

3.2.3.1 MCTs expression in lung tumours

In the present study we aimed to evaluate the effect of MCTs/CD147 inhibition in lung tumour cell lines. First of all, we went to analyse in a series of human lung cancer the expression of these biomarkers. We studied 50 cases of non-small cell lung cancer (NSCLC). The series comprehend squamous cell carcinoma tumours (n=8), adenocarcinomas (n=20) and others types (12). All the cases have non-tumoural adjacent tissue available that was also studied. We observed that MCT1, MCT4 and CD147 were significantly overexpressed in tumour samples when compared to non-tumoural adjacent tissue (Figura 8).

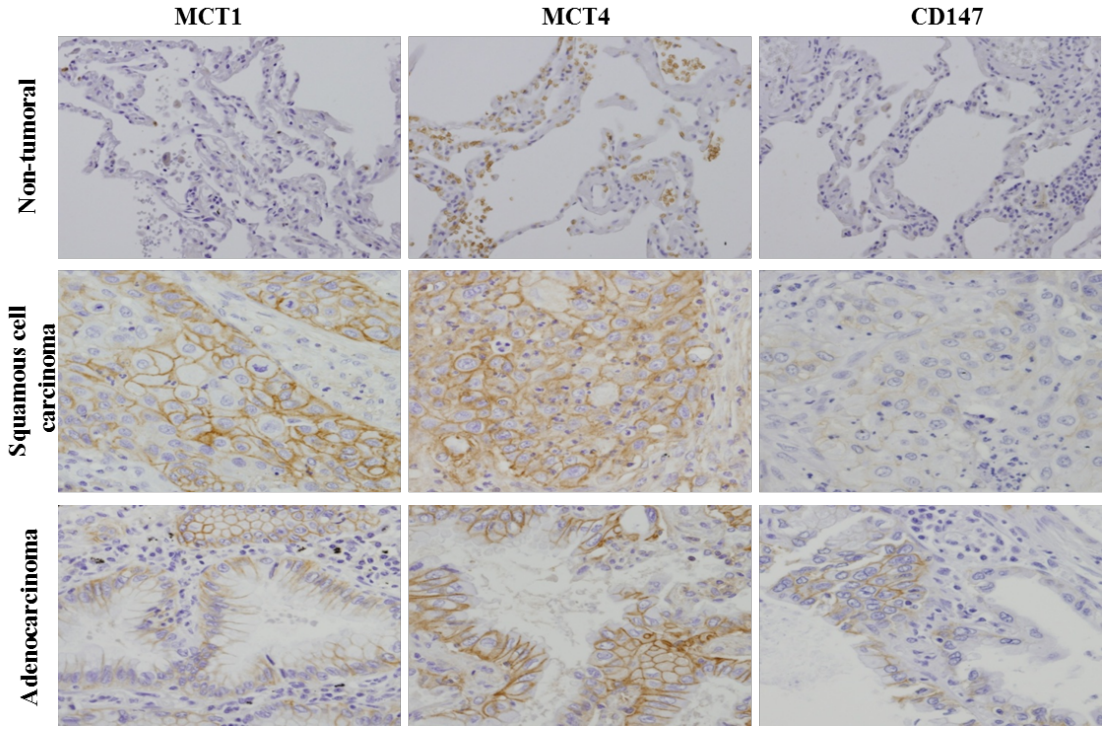


Figure 8: Immunohistochemical expression of monocarboxylate transporters, MCT1 and MCT4, and their chaperone protein CD147 in lung cancer samples. All markers were upregulated at the plasma membrane of tumour cells. Pictures were obtained using the microscope Olympus BX61, at 400× magnification

According to the WHO, the histological types more common are the squamous cell carcinomas and adenocarcinomas. Protein levels were distinct between these two hystological types. MCT1 and MCT4 were expressed in all squamous cell

carcinoma type cases, being CD147 expressed in 60% of the cases. Regarding adenocarcinoma tumours we observed that MCT1 was expressed in 30%, MCT4 in 20% and CD147 in 40% (Table 2). 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 of MCTs inhibition by disrupting their chaperone CD147 in a lung cancer cell line.

Table 2: Monocarboxylate transportes (MCT1 and MCT4) and CD147 expressions in non-tumoural and lung cancer tissues

Isoform	n	Plasma membrane expression		
		Positive	%	p*
MCT1				
Nontumoral	50	0		
SCC	8	8	100	
AD	29	9	31	<.001
Other types	13	1	7,7	
MCT4				
Nontumoral	50	0		
SCC	8	8	100	
AD	29	15	51,7	<.001
Other types	13	2	15,4	
CD147				
Nontumoral	50	0		
SCC	8	5	62,5	
AD	29	16	55,2	<.001
Other types	13	2	15,4	

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

3.2.3.2 Expression of MCT1, MCT4, and CD147 in A549 adenocarcinoma cell line

A549 cells expressed MCT1, MCT4 and CD147, however, with distinct levels, as detected by Western blot. Under normoxia conditions, cells express lower levels of MCT4 and CD147. Under hypoxia conditions (1% O_2), cells overexpress MCT4 and consequently the chaperone CD147 (Figure 9A). The expression of all markers was mainly present in the plasma membrane (Figure 9B).

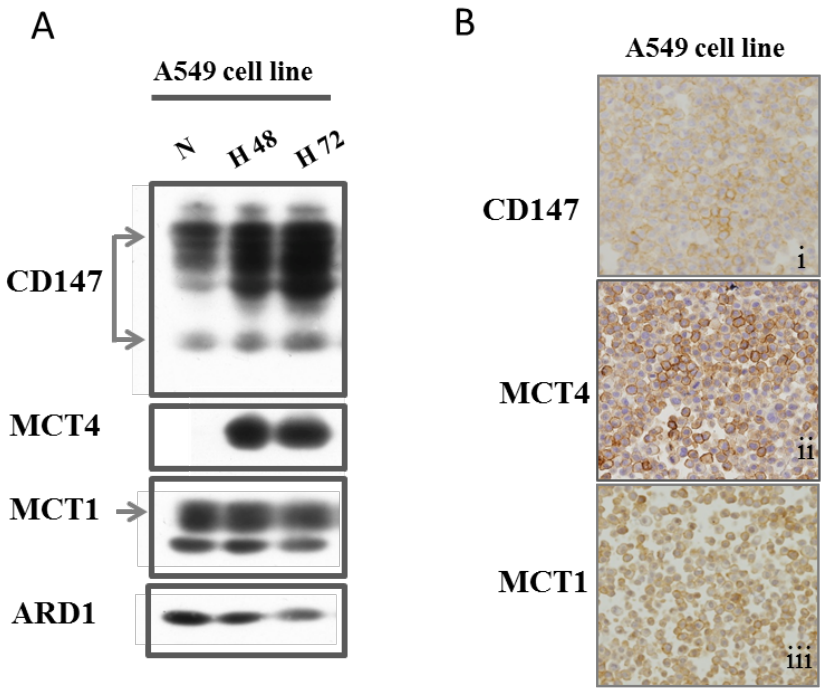


Figure 9: Monocarboxylate transporters (MCT1 and MCT4) and CD147 expressions in A549 cell line A: Western blot analysis of MCT1, MCT4 and CD147 in A549 cell line exposed to normoxia (N) and hypoxia 1% O_2 (H) for 48 and 72 hours. ARD1 was used as a loading control. B: Immunocytochemistry analysis of MCT1, MCT4, and CD147 expressions in A549 wt cells (400× magnification). All the markers were mainly present at the plasma membrane.

3.2.3.3 Generation of CD147 $^{-/-}$ Cell Lines using ZFNs

To assess the role of MCTs we decided to disrupt CD147. Thus CD147 was knocked out in the A549 lung adenocarcinoma cell line using CompoZr^{TM} ZFNs custom-designed to introduce double-strand breaks at a genomically unique target site in exon 2 encoding signal peptide of CD147 splice variant 2. Clones isolated were screened by Western blot for the absence of CD147 expression. From around 60 clones screened, in approximately 10 CD147 protein was not detectable and some appeared to show reduced CD147 protein expression, possibly due to the cut of a single allele (Figure 10A lines 4 and 5)). Two candidates knocked-out (Clone 153 and 113) (Figure 10A) were identified and selected for further characterization. The cut site was amplified by PCR and sequences and mutational status was confirmed by sequencing. First, we analysed the expression of MCT1 and MCT4 isoforms in normoxia and hypoxia 1% O_2 conditions. By immunoblotting, we observed that MCT1 protein was not detectable in CD147$^{-/-}$ cells compared to wild type, while

MCT4 expression was reduced. We also observed that, besides in a lesser extent, the remaining MCT4 in CD147 $^{-/-}$ cells is induced in hypoxia. By qPCR, we demonstrated low transcript expression of CD147 but no evidence for changes in expression of MCT1. MCT4 expression appeared to increase 2-fold in CD147$^{-/-}$ cells (Figure 10B). There were no changes in cell morphology and proliferation between parental and CD147$^{-/-}$ cells (Figure 11A and 12).

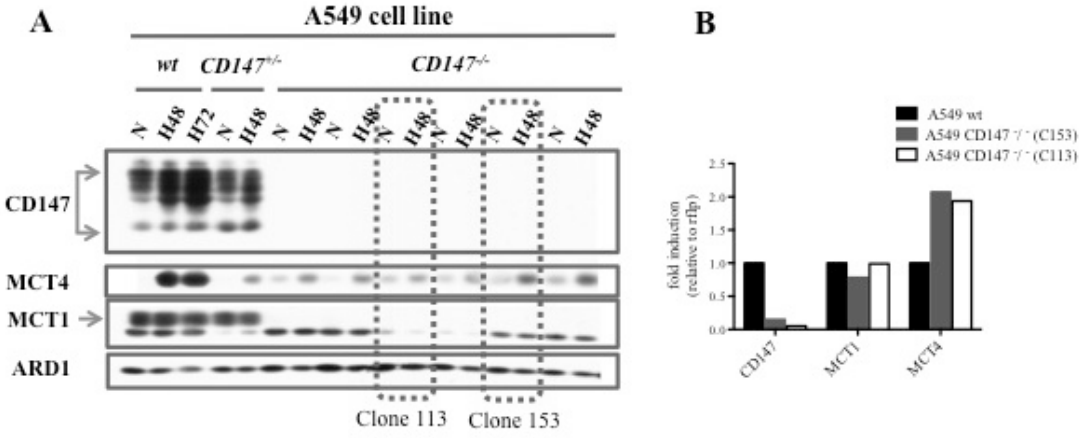


Figure 10: : Monocarboxylate transporters (MCT1 and MCT4) and CD147 expressions in A549 wt and CD147$^{-/-}$ cells. A: Western blot analysis of MCT1, MCT4 and CD147 in A549 cell line wt and CD147$^{-/-}$ exposed to normoxia (N) and hypoxia 1% O_2 (H) for 48 hours. ARD1 used as a loading control. B: mRNA levels of MCT1, MCT4 and CD147 in wt and CD147$^{-/-}$ cells (Clone 153 and 113) in hypoxia 1% O_2. The KO of CD147 did not influence with the transcritption of MCT1 and MCT4.

3.2.3.4 CD147 disruption inhibits MCTs activity

We then went to measure the activity of lactate transport in both conditions. The uptake of ^{14}C-lactic acid was measured with and without the presence of the MCT1/2 inhibitor AR-C155858 (Figure 11B). We demonstrated that the ability of MCT1 to transport lactate is completely suppressed in CD147 KO cells while for MCT4 decreased 58% when compared to wt cells. The transport activity was also evaluated by analysing the intracellular lactate accumulation (Figure 11C). Both cells lines were cultured with 25mM of glucose and in the presence or absence of the MCT1/2 inhibitor and intracellular lactate was measured for 1h, 3h and 6h. Results for CD147 cells without treatment showed a higher intracellular lactate accumulation and upon MCT1 inhibitor exposure this significantly increased when compared with wt cells. These results indicate that CD147 disruption, in addition to decrease in MCT expression, more importantly disturbs their activity.

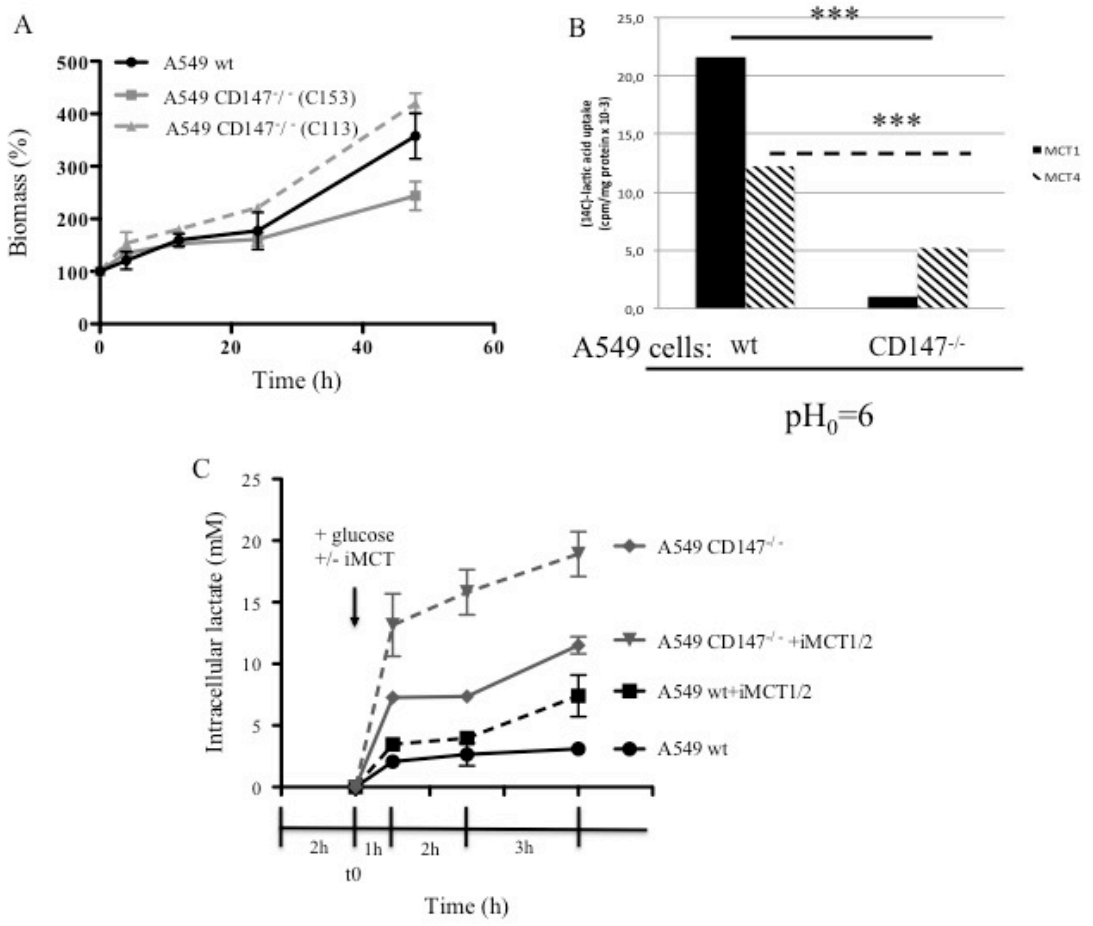


Figure 11: Effect of CD147 disruption on total cell biomass, MCTs activity and cellular metabolism. A: Total biomass of A549 wt and CD147$^{-/-}$ (Clone 153 and 113) cells was evaluated over time by the sulphorhodamine B assay. B: [14C]-Lactic acid uptake of A549 wt and CD147$^{-/-}$ cells in the absence or presence of iMCT1/2 (300nM) in hypoxia 1% O_2 . C: Time-course of intracellular lactate concentration in response to glucose (25 mM) addition in the presence of DMSO or iMCT1/2 (300nM). (*** p <0.0001)

3.2.3.5 MCTs inhibition sensitize cells to phenformin in hypoxia

To understand the energy metabolism of tumour cells and the interplay between the two main bioenergetic pathways, glycolysis and OXPHOS were inhibited with the inhibition of MCTs and phenformin, respectively. There is evidence that phenformin and metformin have anticancer effects. Regarding proliferation, there were no differences between A549 wt and CD147 $^{-/-}$ cells and colony morphology was also similar (Figure 12). In the same way, the growth and survival were not affected by the MCT1/2 inhibitor AR-C155858 in normoxia and hypoxia 1% for both cell populations. However, when in normoxia conditions, OXPHOS was inhibited with both phenformin and metformin wt cells growth was suspended and for CD147 $^{-/-}$ cells a significant loss of clonogenicity was observed, with no further change in

colony size (Figure 12). In hypoxia, where MCT4 is upregulated, only wt cells grew and survived (Figure 12). The combination with MCT1/2 inhibitor did not give any additional result. To further confirm these results we used the mitochondrial ATP synthase inhibitor olygomicin (data not shown). To note that wt cells were more sensitive to the phenformin effect. To support these effects on the bioenergetic pathways, we also evaluated aerobic glycolysis and OXPHOS using a Seahorse XF24 Extracellular Flux Analyser (Figure 13A and B).

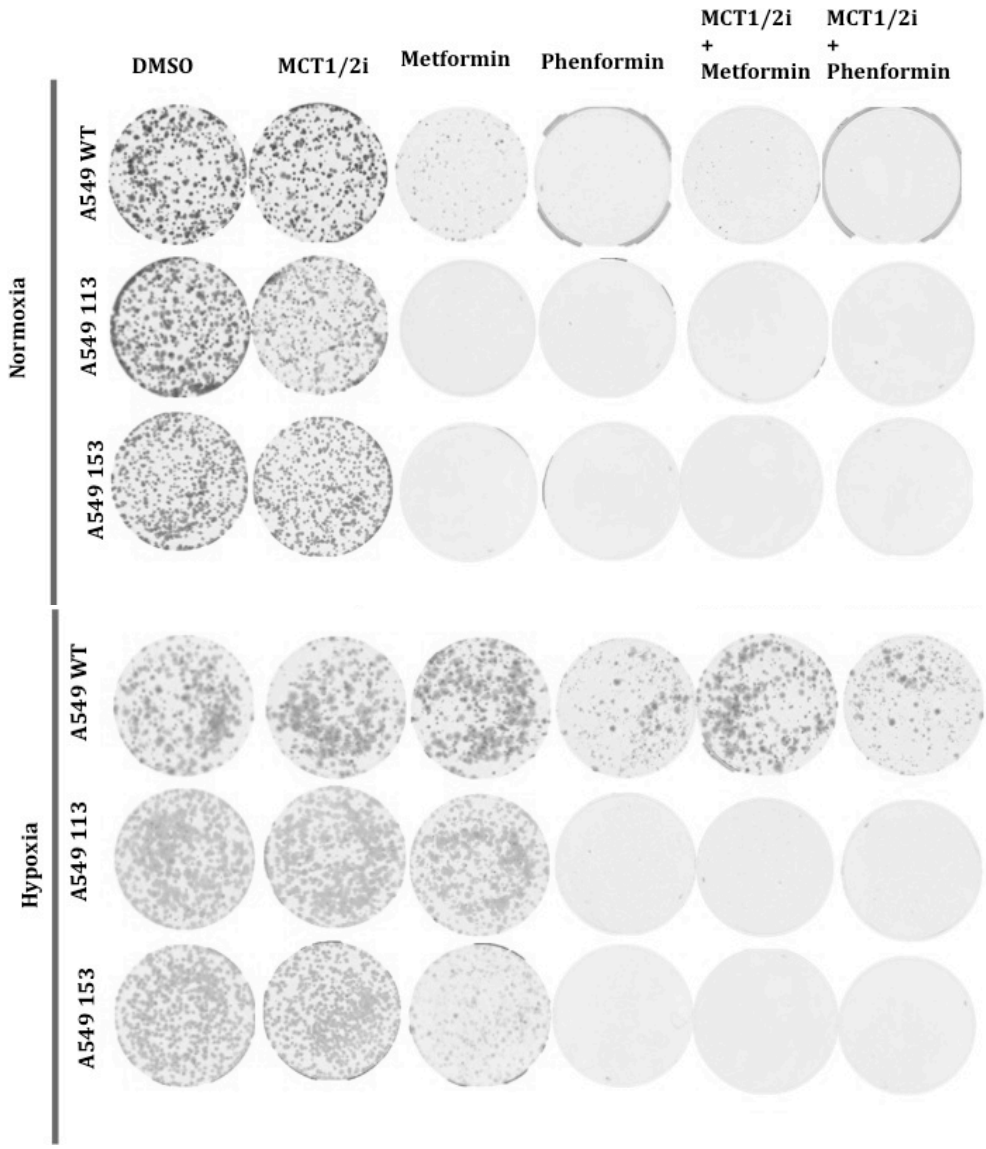


Figure 12: Clonal growth of A549 wt and Bsg-/- cells in the presence or absence of MCT1/2i (300nM), Metformin (1mM), Phenformin (50uM) or in combination in normoxia (N) for 10 days (upper panel) and in hypoxia 1% O2 for 15 days (lower panel).

A549 wt and CD147$^{-/-}$ cells were assayed in unbuffered seahorse media and extracellular acidification rates (ECAR) and oxygen consumption rates (OCR) were

measured simultaneously. When 10 mM glucose was added to the medium CD147$^{-/-}$ cells showed a small but statistically significant reduction in ECAR when compared to wt cells while for OCR no significantly alterations were observed. Both wt and CD147$^{-/-}$ cells significantly decreased ECAR after MCT1/2 inhibitor AR-C155858 injection. Finally, when we inhibited mitochondrial respiration with phenformin, and while OCR is reduced to low levels, wt cells almost maximize their ability to perform glycolysis but CD147$^{-/-}$ cells are not able to respond in the same levels as wt. These results suggest that A549 cells are sensitive to OXPHOS inhibition, and with the lack of MCTs, cells are not able to entirely switch to glycolysis.

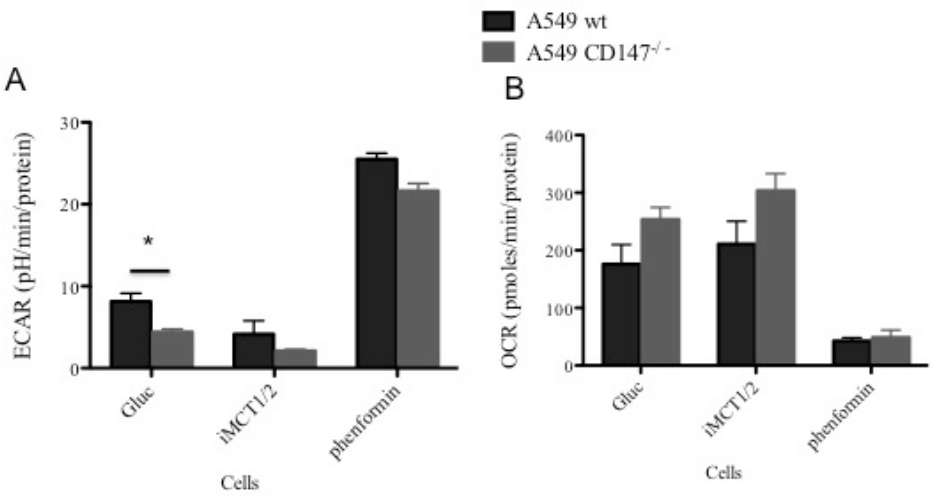


Figure 13: Real time analysis of bioenergetic pathways in A549 wt and CD147 $^{-/-}$ cells. A: Extracellular acidification rate (ECAR) and B: oxygen consumption rate (OCR) measurement after injection of glucose (10 mM), MCT1i (1 μM) and phenformin (50 μM).

3.2.3.6 A549 cells are more sensitive to phenformin

Analyzing the clonogenicity results we detected that A549 wt cells were more sensitive to phenformin than to metformin in hypoxia. Measurement of OCR by Seahorse XF analyser indicated a 30% decrease of cellular respiration rate in cells

exposed to phenformin when compared to metformin, suggesting that A549 cells, are more sensible to phenformin (Figure 14A and B). Recently, Shackelford and co-workers [94] showed that LKB1 mutated cells were more sensitive to phenformin than metformin and that this effect was explained by the fact that with lacking phosphorylation of AMPK these cells accumulate defective mitochondria. Thus, we went to analyse the expression of AMPK. We observed that the A549 cells express

phospho-AMPK and the downstream target phospho-Acetyl-CoA Carboxylase (pACC) (Figure 14C).

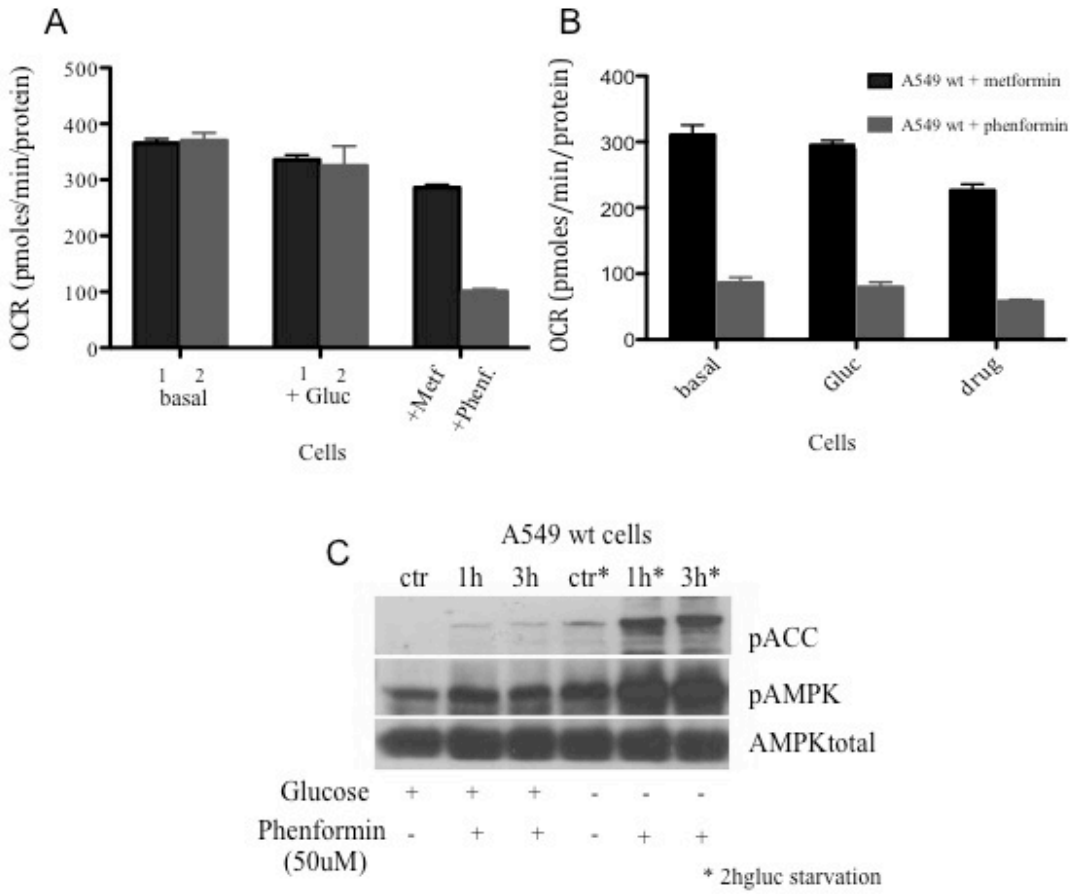


Figure 14: A549 cells are more sensitive to phenformin. A and B: Oxygen consumption rate measurement in A549 wt cells basal levels and after injection of glucose (10mM) and metformin (1mM) black column or phenformin (50 uM) grey column. A: cells without pre-treatment, B: cells were exposed to metformin or phenformin for 2h before measurement. C; Western blot for pACC, pAMPK and AMPK total in A549 cells. Cells were treated with phenformin for 1h and 3h with/without glucose. * Cells were maintained in glucose starvation for 2 hours extra before phenformin exposure.

3.2.3.6 Combination of Glycolysis and OXPHOS inhibition decreases tumour growth *in vivo*

To assess the effect of CD147 disruption *in vivo,* we compared the tumourigenecity of A549 wt and CD147$^{-/-}$ cells. Nude mice were s.c. injected with both cell populations. Surprisingly, CD147$^{-/-}$ cells generated bigger tumours than wt cells (Figure 13-black lines). However, phenformin greatly reduced tumour burdens in both cells with a major impact in CD147$^{-/-}$ cells (Figure 13A and B). These results reinforce the fact that a combined therapy, between glycolysis and OXPHOS, should be used as a strategy to treat lung cancer.

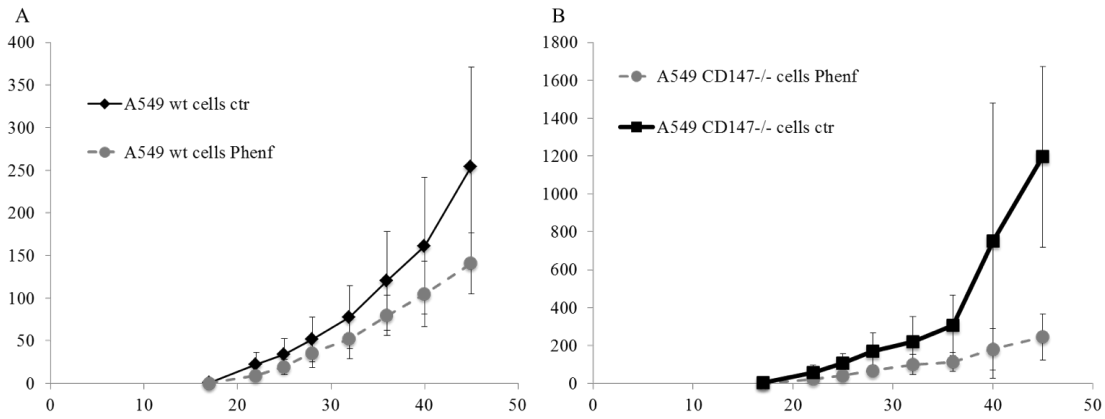


Figure 15: Xenograft tumor growth of A549 wt (A) and CD147$^{-/-}$ cells (B) s.c. injected into the back of athymic nude mice. 6% sucrose with and without phenformin (200 mg/kg) was added to the drinking water for all the mice. Ten mice were studied per condition.

3.2.4 DISCUSSION

Upregulation of glycolysis and adaptation to acidosis are key events in the transition from *in situ* to invasive cancer [4] and MCTs may play an important role through their involvement in exporting lactate [95]. MCTs have been described to be upregulated in several cancers [26] however, there are still some controversies namely with regard to lung cancer [28,35,48]. In the present study, we showed that MCTs are upregulated in tumour tissues when compared to non-tumoural adjacent tissue. Moreover, we detected MCT1 and MCT4 in the plasma membrane of all histological types studied. These results were in accordance with the ones of Koukourakis and co-workers [35] however, in their work they did not describe the cellular localization of the MCT staining. Interestingly, we found that MCT1 was overexpressed in squamous cell carcinoma subtype and MCT4 in the adenocarcinoma subtype, suggesting a different expression pattern between the histological types. However, due to the low number of cases this study should be replicated with a bigger number of cases to possible and better correlations.

To understand the biological role of MCTs, we inhibited their activity. MCT1/2 AR-C155858 compound did not affect cell growth in either normoxia and hypoxia conditions. In normoxia, A549 cell lines mainly rely on oxidative phosphorylation than on glycolysis [96], thus inhibiting MCT1, even with low expression of MCT4 did not affect cell clonogenicity. In hypoxia, were OXPHOS is compromised, cells are also able to survive due to the HIF1α-induced MCT4. Although regulation of MCT expression is still far from being well understood, one of the best characterized MCT regulation mechanisms is through the co-expression with CD147. As there is no pharmacological inhibitor available for MCT4 and as previous results published [54] showed that CD147 major tumoural action is mediated by its action in energy metabolism controlling lactate transport, we decided to disrupt this chaperone to interfere with both lactate symporters. We observed that the KO of CD147 results in a decrease in MCT1 and MCT4 expressions. Regarding mRNA levels, we observed that these remained unchanged for MCT1 but increased 2-fold for MCT4 in the absence of CD147. The disruption of CD147 with the ZFN approach did not interfere with the transcription of MCTs. Regarding the MCT4 increase, the data

could suggest that in the absence of CD147, MCTs are synthesized but not trafficked out of the ER.

Besides expression, we also observed that, under hypoxic conditions lactate efflux is compromised and a higher intracellular accumulation is observed in KO cells when compared with wt cells. These results confirmed the effect of CD147 silencing on the lactate transporters MCT1 and MCT4, as demonstrated by other groups in other cell lines [54]. To note is the fact that, despite the KO of CD147 we still have a functional activity of MCT4 in these cells. These results revived again the idea that MCTs may have an additional chaperone to be trafficked to and active at the plasma membrane. Some studies suggest that CD44 can also play a role as a chaperone for MCTs. The widely distributed transmembrane glycoprotein CD44 and its main ligand, hyaluronan, were implicated in the regulation of lactate efflux and membrane localization of MCTs, in human breast and prostate carcinoma [28,46,88,97]. In fact, CD44 co-immunoprecipitates with MCT1, MCT4 and CD147 and co-localizes with these proteins at the plasma membrane. Perturbation of endogenous hyaluronan, using hyaluronan oligosaccharides, induced intracellular accumulation of CD44, MCT1 and MCT4. These observations suggest that constitutive interactions between hyaluronan, CD44 and CD147 can regulate localization and function of MCT1 and MCT4 [97]. In fact, a previous report from our group describe an association of MCT expression with CD44 in a small series of lung tumours [28]. Moreover, the other basigin isoforms can also function as chaperones for MCTs, since ZFN targeted only isoform 2. Thus, further studies are needed to determine the mechanism by which MCTs could be trafficked and perform their activity in the absence of CD147.

Several reports show that tumour cell lines with downregulation of CD147, via RNA interference, exhibit a decrease in cancer cell properties such as cell proliferation [62,98], invasiveness [79,80,98,99], anchorage-independent growth [79], and drug resistance [60,63,79] *in vitro* and a decrease in tumour growth *in vivo* [79,99] when compared to those expressing high levels of cell-surface CD147. Our results are not in accordance with some of the evidence as for example we did not observe a decrease in cell proliferation and cell viability on CD147 KO cells.

Targeting a metabolic pathway can be tricky due to the high plasticity of the metabolic network of a cancer cell. Thus, we decided to test the effect on tumourigenicity inhibiting both glycolysis, by disrupting CD147, and OXPHOS.

The anti-diabetes biguanide compounds metformin and phenformin have been shown to inhibit complex I of the mitochondria [100,101], resulting in increases in intracellular AMP and ADP that bind to the gamma regulatory subunit of AMPK and trigger LKB1-dependent phosphorylation of AMPK [102]. Consistent with activation of a low energy check-point, metformin treatment has been found to reduce tumour growth [103] and epidemiological studies revealed that diabetic patients taking metformin show a statistically significant reduced tumor incidence [104]. Thus, this evidence point at both metformin and phenformin as promising anti-cancer agents. We evaluated the effect of both compounds on clonal growth and we observed that A549 cells are sensitive to them in normoxia. As shown by Wu and co-workers, A549 cell line rely mainly on OXPHOS than on glycolysis [96]. This evidence along with the results obtained from the seahorse analysis explained this sensitivity. In hypoxia, with the upregulation of MCT4, clonal growth remains unchanged for metformin and slightly decreased for phenformin. On the other hand, KO cells did not grow. The difference in response to both phenformin and metformin can be explained by the fact that phenformin is a 50-fold more potent inhibitor of mitochondrial complex I than metformin [100] and that uptake of metformin, but not phenformin, into tissue appears to require the expression of the Organic Cation Transporter 1 (OCT1), which is highly expressed in hepatocytes but not elsewhere reported [105]. Moreover, Shackelford and co-workers showed that inactivation of the suppressor gene LKB1 dictated therapeutic response of NSCLC to phenformin [106]. In this context, they showed that cells with mutated LKB1 (as A549 cell line) were more sensitive to phenformin and that this effect was dependent on AMPK activation. They hypothesized that without phosphorylation of AMPK trough LKB1, these cells lack the mitophagy process induced by the protein ULK1, a downstream target of AMPK. With this, cells accumulate defective mitochondria and therefore are unable to neutralize ROS, effectively leading to apoptosis. Taking this concept into account we went to analyse the expression of AMPK in A549 cell line. In contrast to other reports we detected expression of phospho-AMPK and the downstream target phospho-ACC under metabolic stress as glucose starvation or OXPHOS inhibition with phenformin. It is known that AMPK can also be activated by calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2) [107], however, further studies are needed. These results

have also led us to check the LKB1 *status* in our cell lines. Thus, studies on sequencing are underway to determine if LKB1 is mutated in these cells.

In vitro studies of cells cultured in monolayer give information about cell-autonomous metabolism however, they do not reflect the real physiological microenvironments in tumours. *In vitro* studies surprisingly showed that CD147 disruption did not reduce tumour growth as shown by other groups [99,108]. In fact, tumour growth increased approximately 4-fold when compared to wt cells. In the first place, these results could reflect a clonal growth effect of the clone used for this experiment. However, *in vitro* studies showed that these KO cells have the same proliferation rates as wt cells. Importantly and besides these results, we observed that phenformin drastically inhibited tumour growth, having a stronger impact in $CD147^{-/-}$ cells when compared to wt cells. Further experiments *in vivo* will be undertaken comparing wt cells and two $CD147^{-/-}$ clones to elucidate the real contribution of CD147 KO to tumourigenesis.

The results obtained by MCT/CD147 inhibition are promising however, we are far from assume MCTs as effective targets for cancer therapy. Thus, more efforts are 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/CD147 as targets in lung cancer therapy.

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Chapter 4: General Discussion

4.1 General discussion

In the light of the major findings achieved throughout this thesis, in the present section a general discussion will be given on how these findings can contribute to the link between basic research and the clinical scenery.

4.1.2 Overview on the contribution to the state of the art

4.1.2.1 Lung development

Embryonic development is characterized by the spatial and temporal differentiation of stem cells, leading to the generation of tissue diversity. This process of differentiation is dictated by microenvironmental mediators such as morphogen gradients and oxygen availability [1–3]. Hypoxic environment favours anaerobic glycolysis [4–6] and this metabolic phenotype is characterized by the metabolization of pyruvate into lactate [7] which is then exported by MCTs to the extracellular milieu to avoid a decrease in the intracellular pH (pHi) [8]. Although the contribution of MCTs to the glycolytic and acidic phenotype of tumours is well studied [8,9], their contribution to the embryo development is still not clear. In the Chapter 2, we provided evidence that could give some contribution for the understanding of the metabolic phenotype that lung developing cells harbour. Several studies have already suggested that embryos, as proliferating cells, express a metabolic phenotype similar to that of cancer cells [7]. Firstly, by the expression of the hypoxia marker CAIX, we propose that lung cells develop in an environment with low O_2 levels. Moreover, we showed that both MCT1/4 isoforms, CD147 and glucose transporters are expressed throughout the lung development phases, suggesting that these molecules are important for lung morphogenesis. Importantly, we described the expression pattern of these molecules in the different cells subtypes of the developing embryo. Stimulation of glycolytic metabolism in cells leads to lactate production. However, lactate is more than merely a by-product of glycolysis as it can be used as a metabolic fuel by oxidative cancer cells [10–12]. The branching morphogenesis of the lung growth process occurs through fundamental cross-talk interactions between epithelial and mesenchymal tissues,

involving a multitude of effectors [13,14]. Can we suggest a lactate shuttle between epithelial and mesenchymal cells as observed in other systems [10,15–17]? In the early phases of lung development epithelial cells express mainly GLUT1 and MCT4 and mesenchymal cells express MCT1. This led us to hypothesize that epithelial cells are glycolytic and produce lactate that could be utilized by mesenchymal cells. Along development, mesenchymal cells differentiate and the expression of MCTs is restricted to the alveolar and bronchial epithelia (Figure 1). However, studies are needed to support this theory. More sensitive techniques can be employed, such as single cell qPCR on laser-dissected cells in order to quantify the expression of each target in each cell population.

Pulmonary development is a complex process and two mechanisms, growth and maturation, are crucial for the developing lung [18–20]. Cell growth occurs through a cross-talk between epithelial and mesenchymal tissues, airway and vasculature, involving several effectors including growth factors, hormones, extracellular matrix interactions, and mechanical stimuli [13,21]. During the last decades, the regulating mechanisms of lung growth have been unravelled [18,22].

In Chapter 2 we also showed the inhibition of MCTs in lung morphogenesis, using rat fetal lung explant models cultured under CHC exposure. We observed that lung branching was inhibited with increasing concentrations of CHC, suggesting that MCTs are required for lung morphogenesis. The main limitation to this work is the fact that the most widely used MCTs inhibitors may also have other targets in the cell. For example, CHC, the inhibitor used in this work, can also inhibit the mitochondrial pyruvate transporter [23,24]. This was described by Halestrap and co-workers in 1974 [24], however in this study they used isolated mitochondria. On one hand, there are no studies reporting the entry of CHC into cells and, on the other hand, there is evidence showing that CHC acts only outside the cell [24–27]. Thus, we believe that the effect of CHC in our model is mediated by inhibition of MCT activity at the plasma membrane. However, further experiments should be performed to clarify the true contribution of MCT inhibition in lung branching. Experiments with the specific inhibitor of MCT1/2 from AstraZeneca [28] or even RNA interference approaches could be used. Other fact that should be taken into account was the hiperoxic conditions where explant cultures were performed. In fact, beyond the controversy existent in the literature about the levels of oxygen during gestation, the truth is that several evidence point to the existence of a hypoxic environment. However, due to the impossibility to

have adequate equipment to cultivate explants with low levels of oxygen, we performed the cultures under normoxic conditions (21% O_2), which may not mimic the real *in vivo* environment.

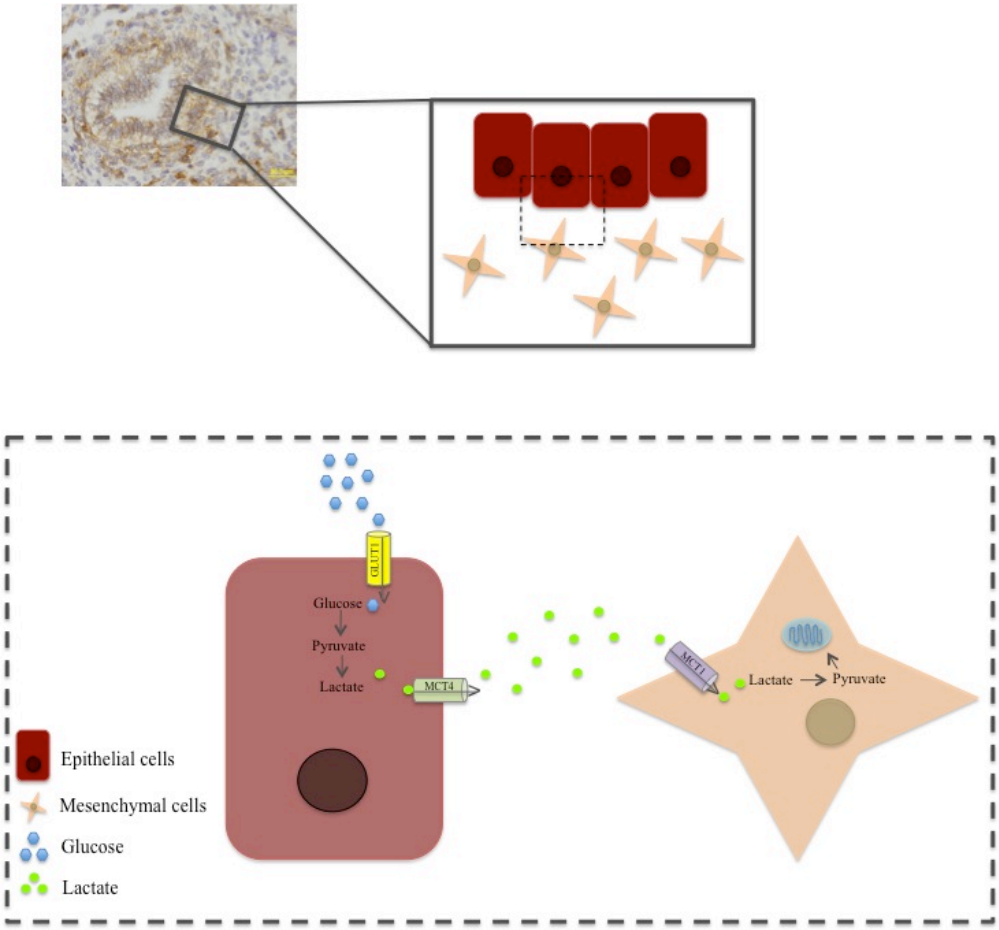


Figure 1: Schematic model for the lactate shuttle between epithelial cells and mesenchymal cells hypothesized in this work.

Although the role of MCTs in lung morphogenesis is still far from being well understood, with this study we showed that MCT inhibition disrupts lung branching, suggesting the dependence of these transporters for lung development. Embryonic developmental is strongly influenced by oxygen availability in the environment [29]. From our results, we hypothesize that lung embryonic cells are able to survive in hypoxia environments adopting a metabolic phenotype to supply energy and that MCTs/hypoxia may have different and specific functions in epithelial differentiation and vascular morphogenesis. Nevertheless, further studies are needed to better characterize this metabolic phenotype during lung differentiation.

The knowledge of all effectors and regulators of lung development is of great

importance [30]. The better we know and understand the normal physiology of the developing lung, better we will perceive lung development defects and also adult lung diseases [31].

4.1.2.2 Lung cancer

Upregulation of glycolysis and adaptation to acidosis are key events of invasion and metastization cancer [32–34] and MCTs play an important role through their involvement in exporting lactate [27,35–37]. MCTs have been described to be upregulated in several cancers [8], however, there are still some controversies namely with regard to lung cancer [38–40]. In the present study we showed that MCTs are upregulated in lung tumour tissues when compared to non-tumoural adjacent tissue. Moreover, we detected MCT1, MCT4 and CD147 at the plasma membrane of all histological types. One limitation of this work was the low number of lung cancer samples obtained from the hospital files to perform this retrospective study. In order to substantiate these results, a higher number of cases should be collect with the respective clinic-pathological data for further correlations.

To understand the biological role of MCTs we inhibited their activity, disrupting the chaperone CD147 to interfere with both lactate symporters, MCT1 and MCT4.

For the accomplishment of this work we used a class of engineered DNA-binding proteins named zinc finger nucleases that create double strand breaks in a specific gene [42]. With this technique we permanently knocked-out CD147 in the lung adenocarcinoma cell line A549. The KO of a gene is used to understand the role of a specific gene [43]. With this work, we tried to understand the role of CD147 on the inhibition of lactate transport. However, our results were not in accordance with several reports that study the effect CD147 downregulation, via RNA interference (iRNA). Inhibition of CD147 by iRNA leads to a decrease in cancer cell proliferation [44,45], invasiveness [45–48], anchorage-independent growth [46] *in vitro* and a decrease in tumour growth [46,47] *in vivo* when compared to cells expressing high levels of cell-surface CD147. Our results are not in complete agreement with the latter results, as for example we did not observe a decrease in cell proliferation and cell viability with CD147 KO cells *in vitro.* Moreover, we observed an increase in tumour growth with $CD147^{-/-}$ cells. So far, we do not have any justification for this fact. A characteristic of

this technique is that only the cells that are able to grow without CD147 will form clones and be selected for further characterization. Moreover, during clonal growth, cells may have time to adopt an advantageous phenotype to overcome the absence of this molecule and thus maintain the same proliferation rates as wt cells. Thus, with this approach we were able to decrease MCTs expression and activity, but this was not enough to kill cancer cells, probably due to the high plasticity of metabolic network inherent to these cells. Thus, a more drastic treatment should be adopted and tested. We proved that inhibiting two important bioenergetic pathways, glycolysis and mitochondrial respiration, decreases cell growth *in vivo* and *in vitro*.

Therefore, the work presented in Chapter 3 provides new data supporting in the exploitation of MCTs/CD147 as targets in lung cancer therapy. A better understanding of the molecular mechanisms driving lung tumourigenesis is crucial and will certainly contribute to the developing of new pharmacologic treatments. However, further studies involving the development of specific MCTs/Bsg blockers in particular, are urgently needed.

4.1.2.3 MCTs as promising therapeutic targets

With the revival of Warburg effect hypothesis, several reports have been published in the past years [49–52]. Many intervenients have been associated with cellular metabolic phenotype and MCT expression has been described in several solid tumours [8]. However, functional studies characterizing the overall role of MCTs as therapeutic agents are still very scarce. This fact may be due to the limitation on MCT inhibitors. The classical drugs used to inhibit MCT activity are not specific. Some studies have been published showing the effect of MCT inhibition by CHC [36,53,54] or DIDS [55,56], among others [56–58] however, these drugs also inhibit other targets in the cell [59,60]. In a search for immunosuppressive drugs, MCT1 was identified as a promisor target [61] and thus, specific inhibitors were designed by AstraZeneca [62,63]. Different compounds were developed, some inhibiting MCT1 [64] and others both MCT1 and MCT2 [28]. Moreover, direct strategies are the use of siRNAs, which have already shown promising results in other models however, the use of this approach in cancer therapy needs to be studied cautiously.

4.1.2.4 Evidence for MCT regulation

In this thesis, MCT expression was characterized of in a small series of human lung tumours, providing evidence for MCT upregulation in these cancer cells. These and similar results observed in other tumour types [36,53,65–67] lead us to suggest that these transporters are promising therapeutic targets for cancer therapy, however, further studies are needed. Importantly, although CD147 is described to be associated with both MCT1 and MCT4 the fact is that, besides the KO of CD147 we still have a functional activity of MCT4 in these cells. These results revived the idea that MCTs may have other chaperones to be trafficked and active ate the plasma membrane. Some studies suggest that CD44 can also play a role as a chaperone for MCTs. The widely distributed transmembrane glycoprotein CD44 and its main ligand, hyaluronan, were implicated in the regulation of lactate efflux and membrane localization of MCTs, in human breast and prostate carcinoma [38,68–70]. In fact, CD44 co-immunoprecipitates with MCT1, MCT4 and CD147 and co-localizes with these proteins in the plasma membrane. These observations suggest that constitutive interactions between hyaluronan, CD44 and CD147 regulate MCT localization and function of MCT1 and MCT4 [69]. In fact, a previously report from our group showed an association of MCTs expression with CD44 in a small series of lung tumour [38]. Further, in the tumour screening, this absence of CD147 was not overcome by CD44. Also, other basigin isoforms can play a role as chaperones for MCTs. The ZFN plasmids only target exon 2 that is common to both isoform 1 and 2 but not to 3 and 4. Thus, further studies are needed to determine the mechanism by which MCTs are trafficked and perform their activity in the absence of CD147. These findings suggest that a not yet identified chaperone may be involved in MCT trafficking to the plasma membrane.

4.1.3 How does a tumor compare to an embryo?

Several groups of genes that drive embryogenesis normally go silent during the rest of life, but these genetic programs can be turn on in many tumour cells [71]. Moreover, some reports document that a molecular signature of lung cancer recapitulates lung development [72,73]. Indeed, they hypothesized that a subset of a genes are associated with the different histologic types of lung cancer. Lung

adenocarcinoma tumours showed a similar signature of the earliest embryonic development stages, while squamous cell carcinoma type recapitulates later development events [72–74]. Exploring the results obtained in this thesis and with the background that hypoxia is a shared event in both embryogenesis and tumourigenesis process, we suggest that MCTs have a crucial role in both processes. However, our results have limitations and a clear relationship between lung development and lung cancer cannot yet be determined.

The idea of shared molecular mechanisms between tumours and embryos is not new. Thus, with a better understanding of the physiologic mechanisms governing lung development, comes the possibility to identify genes/pathways involved in tumourigenesis, opening new opportunities for treatment.

4.2 Conclusion and future perspectives

In general, the results here presented suggest an important role of MCTs in the physiology of the developing lung and in the phenotype of lung cancer. Besides the achievements of this work, many other doors are now open that should be explored.

Regarding lung development, a more specific inhibition should be use in rat explant cultures to determine the true role of MCTs in lung branching. Moreover, to understand the window of action of MCTs in lung morphogenesis, MCTs inhibitors should be tested at different time points to assess time-specific effects.

In lung cancer, characterization of MCTs expression in more comprehensive tumour series would be of great value, as additional promising results are anticipated. Further, correlations with the clinic-pathological data are anticipated, since MCT1 and/or MCT4 have been already reported to be associated with poor prognostic variables, invasiveness, aggressiveness and other tumour features in other tumour types [36,65,66,75,76].

Since with the KO of CD147 we were not able to completely inhibit both MCT1 and MCT4, and with the lack of a pharmacologic inhibitor specific for MCT4, a similar approach using zinc finger nuclease could be done. Moreover, these studies could be complemented in a different lung cell line with a more glycolytic phenotype. MCTs are pointed as important molecular players in cancer and promising therapeutic

targets however, it should be always kept in mind that the main goal to inhibit MCTs is the future clinical application, so MCT inhibitors should be subjected to clinical studies, including the study of their cytotoxic effects. As possible alternative chaperones are suggested to be involved in MCT trafficking to the plasma membrane, additional studies assessing protein interactions with MCTs will provide new knowledge on MCT regulation. For that, databases of protein interactions would be an important tool to explore for the first hints.

In conclusion, the results herein presented provide evidence for the involvement of MCTs in lung development and encourage the exploitation of MCTs, as potential targets for cancer therapy.

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Chapter 5: Appendix

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CD147/Basigin disruption using Zinc Finger Nuclease technology

Sara Granja, Ibtissam Marchiq, Fatima Baltazar and Jacques Pouysségur

Summary

Zinc Finger Nucleases (ZFNs) are reagents that induce DNA double-strand breaks (DSDs) at specific sites that can be repaired by non-homologous end joining (NHEJ), 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 ZFNs plasmids bind a specific DNA sequence, inducing a double strand break (DSB). This break is repaired by the non-perfect endogenous repair mechanism called non-homologous end joining (NHEJ). The DBS will be misrepaired by the addition and/or deletion of nucleotides. This disruption results, in multiple mutations that could

induce non-sense gene products in both alleles leading to a specific gene knockout. The ZFNs structure consists of a DNA binding domain – Zinc Finger Protein (ZFP) - linked to a nuclease domain of the FOK1 restriction enzyme[1].

The ZFP region contains a tandem array of Cys2-His2 fingers that each recognizes 3bp of DNA. This domain can have from 3 to 6 fingers binding to a target of 9bp to 18bp respectively. The catalytic domain composed by the enzyme FOK1 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 then 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 and 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 been already described in our laboratory in the LS174T colon adenocarcinoma cell line[12]. In this section we show a protocol used to knockout

CD147/Basigin in A549 cell line.

2. Materials

2.1 ZFN components

1. CompoZr™ Knockout Zinc Finger Nucleases (CKOZFN1227-1KT): 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 uM. Store at -20°C.

2.2 Plasmid transformation and isolation components

1. Chemically Competent *E. coli*
2. LB medium: Prepare according to the manufacturer's
3. Kanamycin
4. QUIAGEN Plasmid isolation/purification Maxi Kit

2.2 Cell line

1. A549 human adenocarcinoma cell line.
2. Culture media: DMEM supplemented with 10% inactivated FCS, penicillin (10 μg/mL) and streptomycin (50 μg/mL).

2.3 Reagents

1. PBS-1% serum (see note 1)
2. JetPRIME® DNA & siRNA Transfection Reagent from

Polyplus-transfection SA. Store at 4°C (See note 2)

2.4 Antibodies

1. Mouse monoclonal EMMPRIN antibody (Santa Cruz Biotechnology - sc-21746). Store at 4°C.
2. Mouse monoclonal antibody EMMPRIN/CD147 (R&D Systems; MAB972). Store at -20°C.
3. R-Phycoerythrin-conjugated AffiniPure Goat Anti- Mouse IgG (Jackson ImmunoResearch; no. 115–115-164) Store at 4°C in the dark.
4. Horseradish peroxidase anti-mouse antibody (Promega). Store at 4°C.

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3. Methods

3.1 Plasmid transformation and isolation

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

1. Transform the ZFNs plasmids into a 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 seconds and place them on ice for 2 min.
5. Transfer into 900 µl of pre-warmed LB medium and placed to

shake horizontally (200 rpm) for 1h at 37 °C.

6. Plated 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 antibiotic of selection (see note 3). Allow growing overnight (orbital incubator, 37 °C, 225 rpm).
8. Isolate the plasmid with the QUIAGEN Plasmid isolation Maxi Kit. (See note 5)

4.2 Cell line transfection

All procedures should be carried out under laminar air-flow chamber. (see note 6 and 7)

1. Seed A549 cells with 60% confluence (1x10^6 cells) in a 100 mm diameter dish.
2. After 24h transfect the cells using JetPRIME DNA transfection reagent.
3. Dilute 10 µg of DNA (5µg of each ZFN plasmid) into 500 µl jetPRIME® buffer. Mix by vortexing. (see note 8)
4. Add 20 µl of jetPRIME reagent vortex and spin down briefly. Incubate the complex for 10 min.
5. Add 500 µl of the complex (drop by drop) into the cells and mix gently.
6. Replace the transfection medium after 6 hours by fresh medium.

7. Culture the cells for one to two weeks in a humidified atmosphere of 5% CO_2 at 37 °C.

5.3 Fluorescence-activated cell sorting (FACS)

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 avoid by resuspending the cells during long incubations. (See note 9)

1. Place up to 1 X 10^6 cells in a tube. Centrifuge for 5 min at 1200 rpm. Discharge supernatant (see note 10)
2. Wash once with cold PBS 1% serum. Centrifuge for 5 min at 1200 rpm. Discharge supernatant.
3. Per 1 x 10^6 cells add 100 µl of CD147 antibody (sc-21746) diluted 1/100 in PBS1x 1% FCS. Incubate for 30 min on ice. (See note 11)
4. Wash once with PBS1x FBS 1% by centrifugation at 1200rpm for 5 min. Aspirate supernatant.
5. Incubate the cells with the R-Phycoerythrin-conjugated AffiniPure Goat Anti- Mouse IgG (Jackson ImmunoResearch; no. 115–115-164) diluted 1/1000 in PBS 1% serum. Incubate for 30 min on ice. (See Note 12)
6. Wash once with PBS1x FCS 1% by centrifugation at 1200 rpm for 5 min. Aspirate supernatant.

7. Add 500 µl PBS 3% serum 1mM EDTA and filter the samples in a FACS tube.
8. Select and sort CD147 negative cells and plate one cell per well in a 96 well plate with 100 µl DMEM supplemented with 10% inactivated FCS and penicillin (10 µg/mL). Allow the cells to grow in a humidified atmosphere of 5% CO_2 at 37 °C. (see note 13 and 14)
9. After growing in the 96 well plate, each clone should be subculture for the screening of CD147/Basigin cells. (See note 15)

4.3 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 diferent wells. Add 1mM metformin to on well (for a pre-selection see note 17) and let the other well growing to propagate the clone.
3. Extract proteins from the clones that die in the pre-selection with metformin.
4. Perform a Western blot to analyse the expression of CD147/Basigin in these clones. (See note 18)
5. Select the clones that were completely negative by western blot. (See note 19)
6. Extract genomic DNA from your cells.
7. Confirm the gene disruption performing a PCR with the

primers provided by ZFNs kit. (See note 20).

8. Send to sequence your PCR products (see note 21).

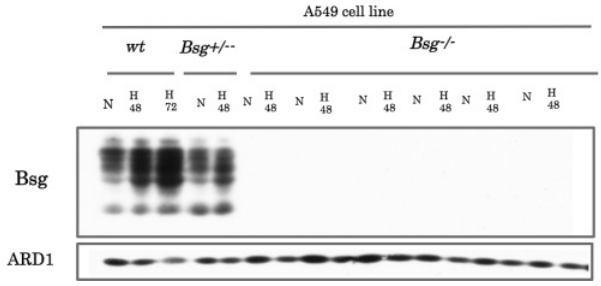


Figure 1: Western blot analysis of Bsg in transfected cells. A549 wt cells (lane 1,2 and 3) Bsg+/- cells (lane 4 and 5) and Bsg-/- cells (lane 6-17) exposed to normoxia (N) and hypoxia 1% O2 (H) . ARD1 used as a loading control.

4. Note

1. Prepare this solution immediately before use. The PBS should be cold and sterile.
2. The delivery of ZFNs plasmids into the cells could be done with diferent 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, cause minimal cytotoxicity for this cell line and required smaller amount of DNA to transfect.
3. The appropriate antibiotic is described in the datasheet of the zinc finger plasmids send by the manufacture. In this specific case the antibiotic of selection used was Kanamycin.
4. Plate two different volumes to ensure that at least one plate will

have well spaced colonies.

5. QIAGEN-tip 500 protocol from the kit was followed as described by the manufacturer. In the end, DNA was redissolved in approximately 100 µl
6. Aseptic technique 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 the 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 µg). This will give you the information that the transfection went good and cells were properly transfected.
9. This approach can not be used for all molecules. We need the cells viable so the antibody should be able to recognize our protein without internalization. In case this is not possible transfection with GFP plasmid should be performed and the cells should be analysed by FACS after 48h 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 6x10^6 cells). Use separate tubes for staining of 1 X 10^6 each to make sure that all cells are uniformly stained.

11. Do not to 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 to 1h. Do not forget to ressuspend the cells to avoid deposition of cells that could lead to an inefficient staining.
12. Protect all the tubes from light to not degrade the fluorochrome.
13. There are some cell lines that are not able to growth as single cells. For that, 100 µl of conditioned media could be added to the well. The growth factors available in the conditioned media can help cells to attach and growth
14. As single cells (clones) are plated in each well, cells will need approximately up 3 to 4 weeks to growth and generate a cell population. Thus, after 2 days of sorting the cells make up the well volume to 200 ml 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 be transfected. Each clone should be identified as desired (for example 1,2,3...). Select the maximum of clones and subculture them. This step is critic. Manipulate carefully your cells to avoid contamination.
16. Freezing media depends on the cell line. Normally consists in

Growth medium (RPMI, DMEM, etc.) containing 5-10% FBS

17. Metformin is a drug from biguanid class that impaired oxidative phosphorylation. We perform this treatment as a pre-selection 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. The Western blot can vary according to the lab. In our lab and for this study we separate 40 μg of protein on a 8% SDS polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were incubated with the monoclonal antibody of Bsg/CD147 (R&D Systems; MAB972) diluted 1/1000 and after with a horseradish peroxidase anti-mouse antibody (Promega) diluted 1/5000. Bands were detected with ECL (Amersham Biosciences or Millipore).
19. Select only 5 to 8 clones to further characterize decreasing the 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 anti-sense primers (sent with the ZFN kit), 200 μM of dNTPs, 1,5 mM of $MgSO_4$, 1 × Taq Buffer and 10 U of KOD hot start DNA polymerase (all the 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 3 min at 68 °C.

21. New primers targeting a smaller fragment in the targeted area should be designed and synthetized. These new nested PCR products should be sent for sequencing to confirm the disruption of your gene in your clones.

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