Universidade do Minho
Escola de Ciências da Saúde

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Master Thesis
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This work was performed under supervision of
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and
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ABSTRACT

During the hyperplasic growth of tumors, there is an impairment in both nutrient and oxygen supply to the neoplastic cells located far away from blood vessels, which would influence tumor progression. Thus, tumors have acquired the ability to assemble their own vasculature, mainly through the pre-existing vessels – tumor angiogenesis. However, tumor blood vessels exhibit structural and functional abnormalities, leading to the development of hypoxic regions, which are responsible for the metabolic reprogramming towards glycolysis, regardless of oxygen availability – "Warburg effect". The end-product of the pathway, lactic acid, is readily released to the tumor milieu through MCTs, contributing to malignant progression. Thus, the aims of the current work are to investigate 1) the role of MCTs on endothelial cell response to hypoxia and 2) the role of MCTs on the angiogenic stimulation by tumor cells.

Hence, our experiments demonstrated that MCT1 and MCT4 isoforms, their molecular chaperones, CD147 and CD44, as well as other key metabolic markers are expressed in human brain endothelial cells, mainly under hypoxia, contributing to the increased glycolytic phenotype. Further inhibition of MCT activity, using CHC, as well as MCT downregulation impaired endothelial cell viability and the development of capillary-like structures, which seems to be independent on lactate transport activity, under hypoxic environments. Upon endothelial cell growth in glioma cells' conditioned media (CM), metabolic adaptations in HBMEC cells were observed, which may contribute to the maintenance of endothelial cell survival, in spite of a decrease in endothelial cell proliferation and, consequently in the development of capillary-like structures that were observed *in vitro*. *In vivo* experiments showed a similar phenotypic alteration in chick chorioallantoic membrane vascularization, after exposure to MCT4- and MCT1/4-silenced glioma cells' CM from both normoxia and hypoxia, relatively to scramble groups.

Thus, besides its role in tumor cells, our data point out the importance of MCT1 and, to a lower extent MCT4, on the maintenance of endothelial cell function, under normoxia. Under hypoxia, the absence of these both isoforms seems to be counterbalanced, which may be due to the overexpression of other transporters at the plasma membrane of endothelial cells. In addition, MCTs seem to be players in tumor microenvironment, acting as essential mediators in tumor-endothelial cell interplay.

RESUMO

Durante o crescimento hiperplásico dos tumores, há um défice no transporte de nutrientes e de oxigénio para as células distantes dos vasos sanguíneos, comprometendo o desenvolvimento do tumor. Assim, as células tumorais desenvolveram a capacidade de construir a sua própria rede vascular, recorrendo à do tecido - angiogénese tumoral. Todavia, a vasculatura tumoral possui anomalias que promovem o desenvolvimento de regiões de hipóxia. Consequentemente, as células neoplásicas são capazes de reprogramar o seu metabolismo, favorecendo a glicólise, independentemente da disponibilidade de oxigénio – "efeito de Warburg". O ácido lático resultante é libertado para o microambiente tumoral, via MCTs, promovendo a progressão maligna. Assim, pretende-se avaliar 1) o papel dos MCTs na resposta das células endoteliais à hipóxia, bem como 2) o seu papel na estimulação da angiogénese.

Foi demonstrado que MCT1 e MCT4, as suas proteínas auxiliares, bem como marcadores importantes na via glicolítica são expressos em células endoteliais cerebrais, nomeadamente em hipóxia, contribuindo para o aumento do fenótipo glicolítico. A inibição da atividade dos MCTs, bem como a inibição da sua expressão, diminuiu a viabilidade celular e o desenvolvimento de estruturas do tipo capilar que, sob hipóxia, parece ser independente do transporte de lactato. O crescimento de células endoteliais em meio condicionado proveniente de células tumorais, cuja expressão dos MCTs foi inibida, induziu adaptações metabólicas em células endoteliais, contribuindo para a manutenção da viabilidade celular, apesar da diminuição da proliferação celular e do número de estruturas do tipo capilar desenvolvidas. Estudos *in vivo* demonstraram alterações fenotípicas na vascularização na membrana corioalantóide do embrião de galinha após a adição de meios condicionados, produzidos em normoxia e hipóxia após silenciamento individual do MCT4 ou em combinação com o MCT1.

Em suma, além do seu papel em células tumorais, os nossos resultados sugerem a importância do MCT1, e em menor extensão do MCT4, na manutenção da função endotelial, em normoxia. Em hipóxia, a inibição do MCT1 e do MCT4 parece ser compensada, pela sobre expressão de outros transportadores na membrana plasmática de células endoteliais, sob condições de hipóxia. Além disso, os MCTs parecem também desempenhar um papel importante no microambiente tumoral, atuando como proteínas essenciais nas interações tumor-endotélio.

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5-FU: 5-Fluorouracil

AE1: Anion Exchanger 1

Agn: Angiopoietin

AMP: Adenosine-monophosphate

AMPK: AMP-activated Protein Kinase

ATP: Adenosine-triphosphate

BBB: Blood Brain Barrier

BrdU: 5-bromodeoxyuridine

CAM: Chorioallantoic Membrane

cAMP: cyclic Adenosine-monophosphate

CAIX: Carbonic Anhydrase IX

CHC: α-hydroxy-4-cianocinnamate

CM: Conditioned Medium

CSCs: Cancer-stem Cells

DBDS: 4,4'-benzamidostilbene-2,2'-dissulfonate

DIDS: 4,4'-di-isothiocyanostilbene-2,2'-dissulfonate

ECs: Endothelial Cells

ECM: Extracellular Matrix

FGF: Fibroblast Growth Factor

GBM: Glioblastoma Multiform

GLUT: Glucose Transporter

HBMEC: Human Brain Microvascular Endothelial Cells

HKII: Hexokinase II

HIF: Hypoxia-inducible Factor

HRE: Hypoxia-responsive Elements

IL-8: Interleukin-8

LDH: Lactate Dehydrogenase

MCTs: Proton-linked Monocarboxylate Transporters

MMPs: Matrix Metalloproteinase

NADH: Nicotinamide Adenine Dinucleotide

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NFAT: Nuclear Factor in activated T-cells

NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells

NHE: Na^+/H^+ Exchanger

NRP: Neuropilin

OXPHOS: Oxidative Phosphorylation

***p*CMBS:** *para*-chloromercuribenzenesulfonic acid

PDGF-B: Platelet-derived Growth Factor B

PDH: Pyruvate Dehydrogenase

PDK: Pyruvate Dehydrogenase Kinase

PHD2: Prolyl Hydroxylase Domain Protein 2

PlGF: Placental Growth Factor

RPE: Retinal Pigment Epithelium

RTKs: Protein-tyrosine Kinase Receptors

SHH: Sonic Hedgehog

SMCs: Smooth Muscle Cells

SMCTs: Sodium-linked Monocarboxylate Transporters

TGF-β: Transforming Growth Factor β

VEGF: Vascular Endothelial Growth Factor

VEGFR: Vascular Endothelial Growth Factor Receptor

VHL: Von Hippel-Lindau

FIGURES AND TABLES

1 Introduction

1.1 Angiogenesis

1.1.1 Development of the vascular network: an overview

According to their size, humans and other vertebrate organisms require a specialized circulatory system [1], which comprises heart, major vessels (arteries and veins), as well as a subset of thin vessels known as capillaries [2]. This vascular network mainly functions to deliver an adequate nutrient and oxygen flow to whole body (arteries) and also to withdraw all the waste products from cellular metabolism (veins) [1-3]. Furthermore, it allows immune cells to travel through the body to carry out their function of protection [4].

In developing mammalian embryos, the heart and blood vessels are the first organs to be assembled and the recently formed vasculature is continuously remodeled in order to achieve a functional system [5].

During embryogenesis, there are two different ways of blood vessel formation: vasculogenesis and angiogenesis [1, 3, 5]. Vasculogenesis is thought to take place only throughout early stages of embryo development, where new blood vessels are assembled *de novo*. In opposition, angiogenesis promotes blood vessel assembling from the pre-existing vasculature. It occurs during wound healing and repair processes in adulthood [5, 6]. The formation of primitive vasculature during embryogenesis have been well described by Ema and Rossant, using mouse models [7]. Indeed, they showed key evidence for a common progenitor for both vascular and hematopoietic cell lineages, demonstrating that both cell subsets lack vascular endothelial growth factor receptor 2 (VEGFR-2 or *Flk-1*) in mutants. Accordingly, Choi and colleagues have previously described that *Flk-1*-positive cells, from differentiating embryonic stem cells, were able to differentiate into both endothelial and hematopoietic colonies [8]. Nevertheless, not all endothelial cells are derived from those progenitor cells, as have been demonstrated using different animal models [7, 9].

As embryo develops, the hemangioblasts in the posterior primitive streak can differentiate into both hematopoietic cells and angioblasts (Figure 1a), according to the signaling

pathway involved [7, 10]. These precursors then migrate to the embryo and yolk sac, where early blood vessels develop by aggregation of angioblasts into a primitive network of simple endothelial tubes (Figure 1b) [5, 11]. In the yolk sac, the angioblasts aggregate into a vascular structure known as blood islands, including endothelial cells in the outer side, whereas the inner side is composed of hematopoietic cells. Next, blood islands fuse themselves into a primary capillary plexus (Figure 1c). Contrarily, in the embryo, angioblasts aggregate into the dorsal aorta or cardinal vein, in a process mediated by vascular endothelial growth factor-A (VEGF-A), Sonic Hedgehog (SHH) and Notch signaling (Figure 1c). The final event of vasculogenesis is known as arterial-venous specification, which is reported to be dependent on the Eph-Ephrin system. EphrinB2 is expressed in both arterial endothelial cells (ECs) and smooth muscle cells (SMCs), while its receptor, EphB4, is only expressed in veins [11].

Altogether, both intra- and extra-embryonic plexuses assemble a *de novo* vascular network [5, 11, 12]. Further remodeling of the primary vascular network, through recruitment of mural cells or pericytes, gives rise to a stabilized and mature vascular system. This process, known as arteriogenesis, is mediated by several molecules, including platelet-derived growth factor B (PDGF-B), angiopoietins (Agn) and transforming growth factor β (TGF-β) (Figure 1d) [13].

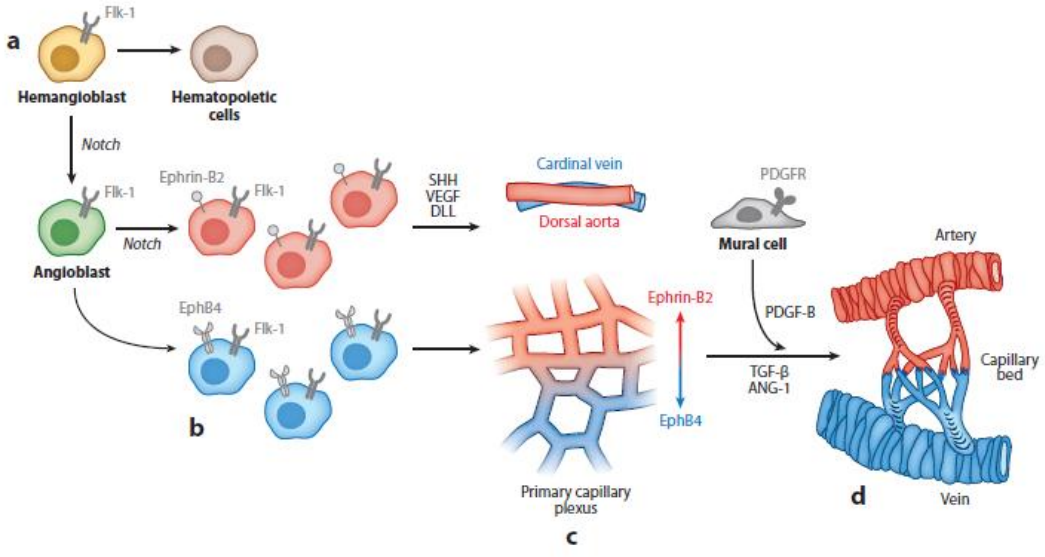


Figure 1 | Development of embryonic vascular network in mammals. (a) In the developing embryo, hemangioblasts become restricted to hematopoietic or angiogenic fate. **(b)** The activation of Notch signaling leads to the aggregation of angioblasts either in the intra- or extra-embryonic tissues. **(c)** In the extra-embryonic side, the angioblast aggregation assemble a primary capillary plexus whereas, in the embryo itself, the angioblasts fuse into dorsal aorta or cardinal vein in process mediated by VEGF-A, SHH and Notch signaling. **(d)** Several markers are involved in the maturation of the established nascent vessels by recruitment of mural cells or pericytes. *From [5].*

After the vasculogenic process, new blood vessels are formed from the preexisting ones, in a process known as sprouting angiogenesis (Figure 2A), which includes localized extracellular matrix (ECM) degradation and subsequent proliferation, migration and tissue infiltration by endothelial cells. At the end, new capillary tubes are assembled and a new ECM is reconstituted [12,14, 15].

Under normal physiological conditions, there is a balance between angiogenic activators and inhibitors. While these pro-angiogenic factors induce angiogenesis, the anti-angiogenic ones are released to counterbalance angiogenesis induction promoting its downregulation [16]. The most well studied angiogenic regulator of vascular development is the VEGF/VEGFR system [17]. However, there are other molecules, like angiopoietin/Tie system [18], Eph/Ephrin system [19] as well as acidic and basic fibroblast growth factors (FGF) [20], playing a central role as angiogenic activators. In opposition, the most well described anti-angiogenic regulators are angiostatin [21], endostatin [22] and thrombospondin [23].

In healthy organisms, the greater part of ECs are quiescent or in a non-dividing phase, collectively assembling a monolayer of interconnected phalanx cells covered by mural cells, SMCs or pericytes, which together constitute the basement membrane. The establishment of this tightly organized cell layer inhibits endothelial cell proliferation and promotes endothelial cell survival through the release of VEGF and Ang-1 [24]. In response to an angiogenic stimulus, the quiescent vessel becomes enlarged due to mural cells detachment from the vessel wall and their subsequent release from the basement membrane in a matrix metalloproteinase (MMPs)-mediated process [24]. As a result, the endothelial cell layer becomes more permeable, enabling plasma protein extravasion that, in turn, is responsible for the assembling of a transient ECM [24]. Then, some signaling molecules induce endothelial cell adherence to the ECM, allowing its migration [24].

Importantly, to avoid the endothelial cell movement *en masse* onto the angiogenic signal, a tip-cell is selected to guarantee the new vessel branching. Behind the tip-cell, other endothelial cells, the stalk cells, proliferate and elongate, consequently establishing the vessel lumen. Stalk cells, through the release of Agn-1, PDGF-B and TGF-β, recruit mural cells, giving rise to functional and mature vessels. Finally, a new basement membrane is synthesized and the endothelial junctions are re-established to ensure optimal flow distribution [24].

In healthy tissues, besides sprouting angiogenesis, there is another mechanism of angiogenic events known as intussusceptive angiogenesis (Figure 2B) [24, 25]. The intussusceptive angiogenesis was firstly described in postnatal remodeling of capillaries in the lung [26]. Here, endothelial cells from opposite walls make a kissing-like contact, creating a transluminal bridge which, in turn, is further remodeled, giving rise to two daughter vessels from the progenitor one (Figure 2B) [27, 28]. The intussusception process is faster than sprouting-like angiogenesis because it does not require endothelial cell proliferation. Instead, endothelial cells increase their volume becoming thinner [27].

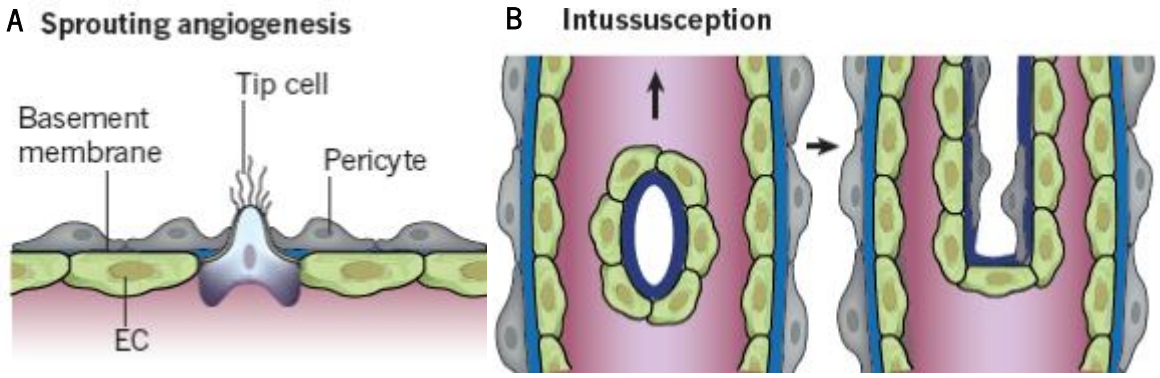


Figure 2 | Mechanisms of angiogenesis in health tissues. During sprouting angiogenesis **(A)**, the daughter vessels are formed through endothelial cell proliferation in two opposite progenitor vessels while, in the intussusceptive-like angiogenesis **(B)** a transvascular structure is assembled into the progenitor vessel after the kissing-like contact between endothelial cells in opposite walls. *From [4].*

1.1.2 Angiogenesis and disease

Besides developmental angiogenesis, some angiogenic events may occur at specific stages of adulthood, as wound healing and repair processes, pregnancy, as well as skeletal growth, which are strikingly regulated [5]. Nevertheless, the balance between pro- and anti-angiogenic factors may be, somehow, disrupted, giving rise to pathological conditions, the "angiogenic switch" [12, 29, 30].

In general, those pathologies arise from excessive angiogenesis, inadequate vessel growth or abnormal vessel regression, as well as abnormal vessel remodeling. For instance, heart and brain ischemia, neurodegeneration, hypertension and respiratory distress are a result

of insufficient angiogenesis rate. In opposition, there are other diseases like psoriasis, arthritis and blindness, which are caused by the upregulation of the angiogenic process. However, the best example of excessive angiogenesis is still cancer [5, 12].

1.1.3 Angiogenesis in cancer

The first observation of angiogenic events in tumors was made about 100 years ago [31]. Afterwards, in 1971, Judah Folkman and collaborators suggested that tumor growth and metastasis are angiogenesis-dependent processes, and that angiogenesis blockade could be a great strategy to prevent tumor progression [32]. Few years later, Gullino *et. al.* described that pre-cancerous lesions acquire angiogenic capacity during their evolution to malignant phenotype [33].

During the early stages of tumorigenesis (Figure 3a), there are several genetic and epigenetic modifications which, in turn, lead to activation of oncogenes or inhibition of tumor suppressors. Subsequently, the balance between cell proliferation and apoptosis rates is disrupted and tumors enter in a stage of early hyperplastic growth (Figure 3b) [34].

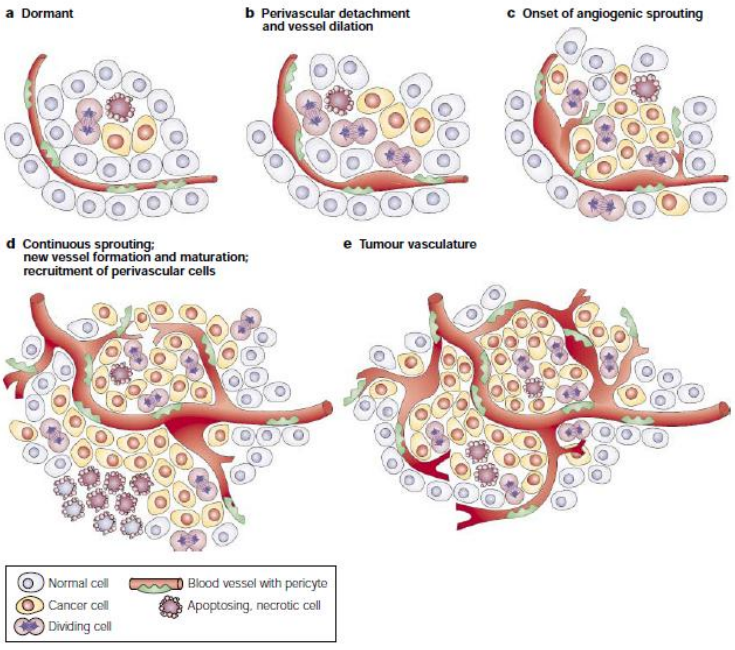


Figure 3| The onset of tumor angiogenesis. (a) Initially, most tumors develop in avascular sites. **(b)** The disruption between cell proliferation and apoptosis rates triggers early hyperplasic growth of the tumor. **(c)** When tumor cells, located far away from vessels, become deprived of nutrients and oxygen, the onset of tumor angiogenesis occurs **(d)** Tumor vasculature is further remodeled by recruitment of mural cells. **(e)** Tumor vasculature becomes mature and functional. *From [29]*

When the tumor reaches around 1 – 2 mm of diameter, there is an impairment in both nutrient and oxygen supply to the neoplastic cells, located far away from blood vessels, which will influence tumor progression (Figure 3c) [5, 34]. Nevertheless, through evolution, tumor cells acquired the ability to escape to growth inhibition, developing their own vasculature, commonly from the preexisting blood vessels – tumor angiogenesis (Figure 3c-e) [34]. Besides its role of supporting tumor growth, tumor angiogenesis also provides a key mechanism through which cancer cells disseminate via blood stream to distant organs to develop metastases [34].

In opposition to normal tissues, where the mechanisms of angiogenesis are limited to vasculogenesis, sprouting angiogenesis and intussusception, tumors exhibit three more modes of angiogenesis including vessel co-option, vascular mimicry and transdifferentiation [25]. Vessel co-option mainly occurs in well-vascularized tissues like brain and lung [35, 36], where tumor cells recruit the existing blood vessels to sustain their growth [27]. The first experiments describing the occurrence of vessel co-option in tumors were made upon the implantation of C6 glioma cells in the rat brain [37]. Few weeks after implantation, tumors became well-vascularized with blood vessels, exhibiting similar features to functional vessels of normal brain. However, these blood vessels start to retract themselves along time, without any angiogenic response as a compensatory mechanism. Consequently, in the center of the tumor, extensive areas of cell death were found due to the lack of functional vessels around glioma cells. Contrarily, in the tumor periphery, a burst of angiogenesis was further detected [37]. Indeed, these findings indicate that some tumors are able to incorporate the existing host blood vessels to support their growth, at least during early stages of tumor development [37, 38].

Later, the concept of vasculogenic mimicry was described in melanomas. Aggressive melanoma cells assembled vascular channels *de novo,* in an angiogenesis-independent mechanism, i.e., without the interference of endothelial cells [39]. Furthermore, tumor blood vessels lined by tumor-derived cells were discovered in mice transplanted with human glioma tumors - a process also known as vasculogenic mimicry. Altogether, these observations demonstrate that, due to their plasticity, tumor cells on the vicinity of the nascent blood vessel are recruited for the angiogenic process, acquiring some endothelial cells' features [25].

The discovery of cancer-stem cells (CSCs) in solid tumors, mainly in glioblastoma, has contributed to the better understanding of the biology of cancer. Recently, it was shown that glioma-stem cells and endothelial progenitors share some characteristics and, therefore, CSCs

have the ability to differentiate into functional endothelial cells, giving rise to tumor endothelium – cancer-stem cell transdifferentiation [40-42].

Independently of the mechanism by which tumor vasculature is built up, tumor blood vessels are normally irregular, dilated and tortuous. Additionally, the standard organization into well-defined venules, arterioles and capillaries is not found in tumor-associated vessels. Also, they are sometimes hemorrhagic, partially due to the VEGF overexpression, and leaky, because mural cells become more loosely associated or less abundant [43]. Furthermore, the blood flow in tumors is irregular, moving slower comparatively with normal tissues which, in turn, lead to dysfunctional capillaries [29].

Despite their structural and functional abnormalities, tumor endothelial cells are believed to be genetically normal [25]. However, recently, cytogenetic aberrations such as aneuploidy and the existence of multiple chromosomes were found in uncultured tumor endothelial cells. The aneuploid phenotype was exacerbated in culture, indicating that the cytogenetic abnormalities might be acquired while in the tumor microenvironment [44].

Taking together, these abnormalities in tumor endothelium are likely to occur as a result from an imbalanced expression and function of pro-angiogenic factors [29].

1.1.4 Regulatory mechanisms of angiogenesis in cancer: molecular insights

As already mentioned, in health adult organisms, blood vessel growth is a well-regulated process, in which endothelial cells remain quiescent. Thus, some physiological factors, such as hypoxia, as well as numerous endogenous molecules, mainly VEGF, are likely to perform an active role on angiogenesis induction [3, 17, 24].

1.1.4.1 Hypoxia

Hypoxia – a decrease in the normal oxygen levels within a tissue – is a hallmark of several vascular diseases, including cancer. There are essentially two types of hypoxia - acute and chronic hypoxia. Acute hypoxia occurs when abnormal blood vessels are shut down, leading to a reversal blood flow, while chronic hypoxia appears as a result from uncontrolled cell proliferation, overpassing the oxygen diffusion limit [45].

Hypoxic regions are frequently found within tumors, namely because of the abnormal features of tumor blood vessels. These hypoxic environments confer to cancer cells the ability to survive and proliferate in hostile conditions, contributing to the malignant phenotype and tumor aggressiveness [45]. Indeed, hypoxia has emerged as a primary regulator of angiogenic switch [46], facilitating tumor progression and metastasis [47].

The cellular response to hypoxia is mediated by the hypoxia-inducible factor (HIF) family [48, 49]. So far, three different transcription factors belonging to this family (HIF-1, HIF-2 and HIF-3) were identified [50]. HIF-1 is ubiquitous and is considered the major factor regulating responses to hypoxia. It is a heterodimeric protein composed of two subunits, an oxygen-inducible subunit, HIF-1α, and a constitutively expressed one, HIF-1β (Figure 4) [45, 48, 49].

In the presence of oxygen, the activity of the prolyl hydroxylase domain protein 2 (PHD2) promotes hydroxylation in two HIF-1α proline residues which, in turn, facilitates its binding to a tumor suppressor protein, Von Hippel-Lindau (VHL). This interaction results in polyubiquitination of HIF-1α, targeting it for proteossomal degradation (Figure 4) [51]. In opposition, in the absence of oxygen, HIF-1α is stabilized and, consequently, dimerized with HIF-1β. Then, the recently formed complex is translocated from the cytoplasm to the nucleus, where it binds to the hypoxia-responsive elements (HRE) in the promoter region of several target genes (Figure 4) [48, 49].

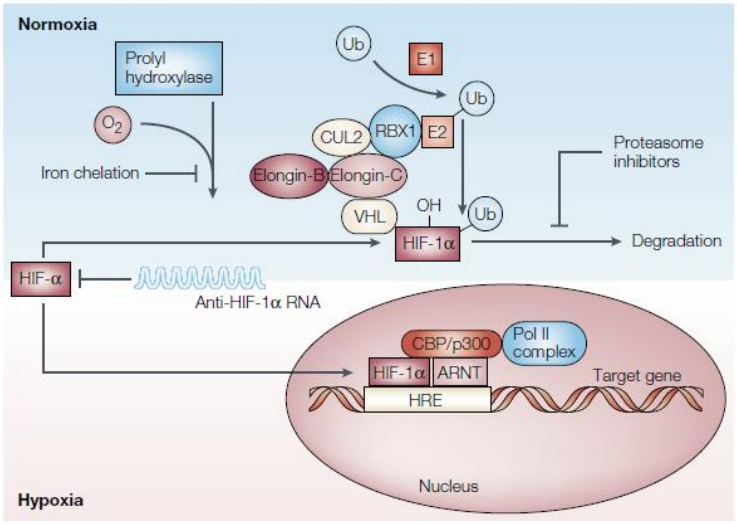


Figure 4|HIF-1 signaling pathway. Abbreviations: Ub: Ubiquitin; E1: Ubiquitin-activating enzyme; E2: Ubiquitin-conjugating enzyme; RBX1: RING-box protein 1; CUL2: Cullin-2; VHL: Von Hippel-Lindau; CBP/p300: CREB-binding protein/p300 co-activator family; ARNT: Aryl hydrocarbon receptor nuclear translocator protein *From [45].*

In addition, two more proteins have been described to mediate the cellular response to hypoxia, HIF-2α and HIF-3α. Similarly with HIF-1α, HIF-2α also interacts with HRE in the nucleus, enhancing gene transcription [52]. However, it has not a ubiquitous distribution, being expressed mainly in lung, carotid body and endothelium [52, 53]. There are few studies concerning HIF-3α, but it has been reported as an inhibitor of HIF-1α activity [52].

HIFs are essential molecules, performing a role in several aspects of cancer biology, including angiogenesis [54]. In fact, the angiogenic process is regulated by alterations in oxygen levels, and endothelial cells (ECs) have oxygen sensors, as well as HIF-related proteins, which, collectively, allow them to detect differences in oxygen levels [55].

HIF-1α directly activates the transcription of the vascular endothelial growth factor (VEGF) gene and the one of its receptor, vascular endothelial growth factor receptor 1 (VEGFR-1) [52], stimulating endothelial cell proliferation, migration and vascular sprouting [24, 55]. Indeed, HIF-1α and VEGF overexpression was correlated with more aggressive lesions in both breast cancer and gliomas [56, 57]. Additionally, HIF-1α knockdown in endothelial cells disrupted the autocrine loop required for the hypoxic induction of both VEGF receptors (VEGFR1 and VEGFR2) through VEGF signaling. Further impairments in endothelial cell proliferation and tube formation were observed *in vitro.* Similarly, a decrease in tumor angiogenesis and a consequent reduction of tumor growth were also observed *in vivo* [58].

Although further studies concerning HIF-2α function should be performed, it has been associated with the maintenance of vascular integrity [24, 54, 55].

1.1.4.2 VEGF family

It is widely accepted that VEGF signaling pathway is the major cascade regulating angiogenic events. The VEGF family consists of only few homologous members, including VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and also PlGF [59, 60]. VEGF-A is the main component of this family, regulating physiological angiogenesis during embryogenesis and at specific stages in adulthood. Furthermore, VEGF-A has also been implicated in pathological angiogenesis, being associated, for instance, with tumor growth [61].

Besides its role in angiogenesis induction, VEGF-A has other activities including vascular endothelial cell mitogenesis, endothelial cell survival and induction of hematopoiesis [62].

Additionally, Dvorak and colleagues described VEGF-A as a tumor-secreted molecule that increases tumors' vascular permeability [63].

The human *VEGFA* gene is located on chromosome 6 and is organized into eight exons, separated by seven introns [61, 64, 65]. Since VEGF-A pre-mRNA undergoes alternative splicing, two different families of proteins are produced, one pro-angiogenic and another anti-angiogenic [66]. Concerning the pro-angiogenic family, the alternative splicing of *VEGFA* gene generates seven different variants ($VEGF_{121a}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{165a}$, $VEGF_{183}$, $VEGF_{189a}$, $VEGF_{206}$), whereas only three anti-angiogenic variants ($VEGF_{165b}$, $VEGF_{121b}$ and $VEGF_{189b}$) were identified, so far. Those spliced variants are termed according with their aminoacid content after signal sequence cleavage [61, 64, 66].

Importantly, VEGF is a homodimeric glycosylated protein containing heparin-binding sites. The potential binding between each VEGF-spliced variant and heparin derivatives determines its diffusion degree [54, 61, 62], as well as contributes for the mitogenic activity of VEGF [67]. In fact, the larger VEGF variants, $VEGF_{206}$ and $VEGF_{189}$, are highly basic, exhibiting high affinity for heparin and, hence, are tightly bound to cell surface and extracellular matrix (ECM) [54, 61]. By contrast, the intermediate variants ($VEGF_{165}$, $VEGF_{148}$ and $VEGF_{145}$) are moderately diffusible, because of their weak affinity for heparin/heparin sulphate. Indeed, although these variants are secreted, a significant part of them remained attached to the ECM [61, 64]. Additionally, the shorter VEGF-spliced variant, $VEGF_{121}$, has acidic properties and is freely diffusible, due to the lack of heparin binding sites [61]. Importantly, the VEGF-A-spliced variants are differentially expressed in tissues. $VEGF_{165a}$ is expressed in several normal and transformed cells. Furthermore, both $VEGF_{121}$ and $VEGF_{189}$ variants are found in the majority of cells and tissues expressing the *VEGF* gene. In opposition, the largest variant ($VEGF_{206}$) in only expressed in human fetal liver [64]. Among all the pro-angiogenic subtypes, $VEGF_{165a}$ has been reported as the most important spliced variant of the *VEGF* gene [68]. Indeed, some authors described that the homolog protein in mice ($VEGF_{164}$), *per se,* was enough to trigger the angiogenic process, as transgenic $VEGF_{164}$ mice in a VEGF-A null genetic background were essentially healthy [69].

All the VEGF-A variants bind to specific protein-tyrosine kinase receptors (RTKs), the vascular endothelial growth factor receptor 1 (VEGFR1) and vascular endothelial growth factor receptor 2 (VEGFR2) (Figure 5) [17, 61]. Firstly, these RTKs were identified in vascular endothelial cells [17, 61]. However, their expression was also reported on bone marrow-derived

cells [61], macrophages [64, 70], some malignant cells [70] and vascular SMCs [71]. Structurally, both VEGF receptors contain seven immunoglobulin-like extracellular domains, a single transmembrane domain and a consensus tyrosine kinase sequence, which is interrupted by a kinase insert domain of 70-100 aminoacids [72, 73]. Additionally, VEGF-A binds to the specific non-enzymatic co-receptors neuropilin-1 (NRP1) and -2 (NRP2) [17, 61], which are believed to enhance VEGF-A signaling through VEGFR2, ultimately increasing angiogenesis [17].

Although VEGFR1 had been firstly identified, its exact function still remains unknown [61]. VEGFR1 has higher affinity for VEGF-A than VEGFR2. However, this interaction ligand-receptor triggers a weak tyrosine kinase phosphorylation [17]. Actually, VEGFR1-mediated signaling cascade does not induce significant mitogenic effects in endothelial cells. Nevertheless, during early embryonic development, VEGFR1 seems to sequestrate VEGF-A, preventing its binding to VEGFR-2 and consequently inhibiting further angiogenic events [61]. Contrarily to VEGFR1, VEGF-A binding to VEGFR2 induces a powerful tyrosine kinase activity, stimulating endothelial cell migration, proliferation, survival and enhancing vascular permeability [17, 61]

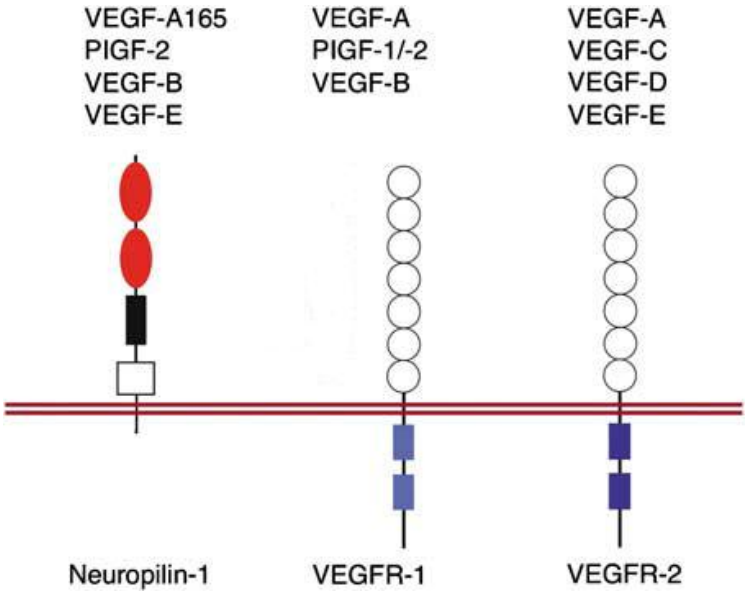


Figure 5 | Interactions between VEGF family ligands and their receptors involved in the angiogenic process. Abbreviations: VEGF: vascular endothelial growth factor; PlGF: placental growth factor; VEGFR: vascular endothelial growth factor receptor. *Adapted from [74].*

VEGF-A, mainly $VEGF_{165}$, is believed to be the major inducer of pathological angiogenesis [65]. It is overexpressed in the majority of solid tumors, including lung, thyroid, breast,

gastrointestinal tract, kidney, bladder, ovary and uterine cervix carcinomas, angiosarcomas and intracranial tumors like glioblastoma multiform (GBM) [64, 65]. Furthermore, increased expression of VEGF-A was also reported in some hematological malignancies and multiple myeloma [65]. Indeed, an association between increased VEGF-A mRNA levels and malignant progression has been identified [64].

Tumor cells exhibit higher mRNA levels of VEGF-A than tumor-associated endothelial cells, where its expression is negative. In opposition, both VEGFR1 and VEGFR2 are upregulated in tumor-endothelial cells [75]. These observations suggest that VEGF-A act as a paracrine molecule [64]. Interestingly, angiosarcoma cells co-expressed both VEGF-A and VEGFR1, indicating that an autocrine VEGF-A loop may also occur in some tumor types. However, angiosarcomas cannot be considered such a good model because these tumor cells are endothelial-derived ones [64]. Importantly, higher levels of VEGF-A have been correlated with decreased relapse-free survival rates of breast cancer patients [76]. Also, in gastric carcinomas, VEGF-A upregulation was associated with vessel involvement, lymph node metastasis and liver metastasis which, collectively, contribute for poor clinical outcomes [77].

Recently, the anti-angiogenic VEGF-A variants have gained focus in cancer. These molecules are highly expressed in normal tissues, but they are reported to be downregulated in certain tumor types including renal-cell, prostate and colorectal carcinomas, as well as in malignant melanoma [66]. In fact, overexpression of $VEGF_{165b}$ in those tumor specimens efficiently impaired tumor growth in xenografted mouse models [66].

1.1.5 Anti-angiogenic Therapy

Judah Folkman firstly identified the involvement of angiogenesis in tumor development and metastasis through VEGF-A. Hence, blocking such powerful pro-angiogenic factor might be an excellent strategy for cancer therapy [78].

Numerous preclinical studies concerning angiogenesis inhibition have been made using VEGF-A blockers. These strategies efficiently decreased tumor progression in a range of cancer models [79]. In patients with metastatic colorectal cancer, the combined administration of a monoclonal antibody against VEGF-A (bevacizumab) and 5-fluorouracil (5-FU) demonstrated

benefits for the patients [79]. Consequently, in 2004, bevacizumab was approved by Food and Drug Administration (FDA) for the treatment of metastatic colorectal, metastatic non-squamous non-small lung carcinoma, metastatic breast cancer, recurrent GBM and metastatic renal cell carcinoma [24].

Additionally, multi-targeted RTKs inhibitors (e.g. sorafenib, sunitinib and pazopanib), which prevent several signaling pathways such as the one induced by VEGF-A, were further approved for the treatment of different tumor types. Sorafenib is indicated for the treatment of metastatic renal cell carcinoma and hepatocellular carcinoma whereas, both sunitinib and pazopanib, are currently used only in the treatment of metastatic renal cell carcinoma [24, 79]. Recently, sorafenib has also been proposed for the treatment of advanced pancreatic neuroendrocrine tumors [24].

The blockade of VEGF-A signaling has been demonstrated as effective in a range of tumors. However, some patients with metastatic disease have acquired resistance to VEGF-A signaling inhibitors [24, 79]. Such evidence raises the question whether cancer cells become more malignant upon treatment with these drugs. In fact, the inhibition of vascular supply might increase tumor hypoxia, increasing local invasion and distant metastasis. Furthermore, the impairment of tumor vasculature results in decreased effectiveness of conventional radio- and chemotherapy [24, 80].

In spite of all the advances targeting tumor vasculature, anti-angiogenic therapy must be improved, optimizing the dosage, as well as the treatment periods of VEGF-A blockers. In addition, the combination of these VEGF-A blockers with other pharmacological agents targeting, for instance, the escape pathways (e.g. PlGF) might be further implemented [24].

1.2 Monocarboxylate Transporters

1.2.1 Monocarboxylate Transporters family

Monocarboxylates such as pyruvate, ketonic bodies (acetoacetate and β-hydroxybutyrate) and lactate have been described as key players in cellular metabolism (Figure 6). Some mammalian tissues, such as white muscle fibers, erythrocytes and the majority of neoplastic cells, rely on glycolysis for energy production, synthesizing lactate as end product. Thus, in order to maintain high glycolytic rates, lactate efflux becomes essential. Contrarily, other tissues like brain, heart and red skeletal muscle rapidly metabolize lactate, which constitutes the major fuel for these organs. Hence, large amounts of lactate must be canalized into these cells [81-83].

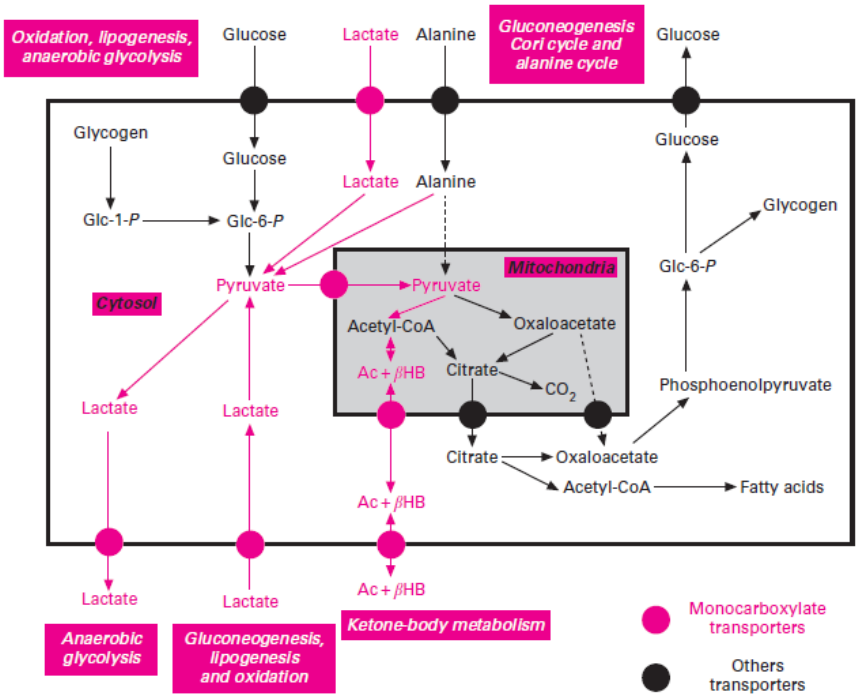


Figure 6 | The role of monocarboxylate transporters in cellular homeostasis. Abbreviations: Glc-1-*P* – Glucose-1-phosphate; Glc-6-*P* – Glucose-6-phosphate; Ac+βHB – acetate + β-hydroxybutyrate. *From [81]*

Due to their negative charge, monocarboxylates, including lactate, cannot freely diffuse through plasma membrane in large amounts, requiring a specialized transport system [81, 82, 84, 85]. Accordingly, cells express membrane transporters belonging to both proton-linked monocarboxylate transporters (MCTs) (Figure 6) and sodium-linked monocarboxylate transporters

(SMCTs) families, which carry those simple organic carboxylates coupled with protons (H^+) or sodium ion (Na^+), in an equimolar manner through a symport mechanism, respectively [81, 86].

Regarding the MCT family, it comprises 14 members of proteins encoded by the *SLC16* gene family, which is highly conserved between different species like rodents (e.g. rat and mouse) and chicken [87, 88]. So far, solely seven members of MCT-family have been functionally characterized and, from these seven only four (MCT1-MCT4) have been shown to perform the proton-coupled monocarboxylate transport [86, 87, 89]. The major differences between each of these MCT isoforms are their affinities for substrates and inhibitors, the regulation of their expression and tissue distribution, as well as their cellular localization [90].

Poole and colleagues, using hydropathy analysis, first described the amino acid sequence of rat MCT1, demonstrating a structure of twelve transmembrane (TM) helices, with both the amino and carboxyl termini located in the cytoplasm [91]. Further studies were performed, corroborating the previous results and, additionally, identifying a large hydrophilic loop between the 6 and 7 TM domains, extended from the plasma membrane into the cytoplasm [91]. Furthermore, it must be highlighted that these TM regions are highly conserved between MCT isoforms, with the greatest sequence variations located in the C-terminus and in the loop [86, 89]. Such variation is thought to be related with either the substrate affinity or the regulation of transport activity [86]

1.2.1.1 MCT1

Although MCT1 expression has been described in almost all tissues in all species studied, its major expression has been found in the heart and muscle [82, 84, 87]. Additionally, it is the only MCT isoform expressed in erythrocytes [87]. The main function of this transporter is to perform both the L-lactate entry into and efflux out of cells, depending on the intracellular and extracellular substrate concentration, as well as the pH gradient across plasma membrane [89, 90]. However, some studies in mouse tumor cell lines and *Xenopus* oocytes demonstrated that MCT1 isoform exhibits high affinity for a wide range of short-chain monocarboxylates (lower K_m value) [92, 93].

Besides its plasma membrane expression, MCT1 has also been reported in mitochondria of the heart, skeletal muscle and brain, enabling these tissues to oxidize lactate to pyruvate

though mitochondrial lactate dehydrogenase (LDH) activity [90, 94]. However, such hypothesis is still not well accepted by some researchers because oxidation of lactate within the mitochondria is both energetically unfavorable and incompatible with the nicotinamide adenine dinucleotide (NADH) redox compartmentalization in cells [94].

1.2.1.2 MCT2

Comparatively with MCT1 isoform, MCT2 exhibits higher affinity for pyruvate and lactate [89, 90]. This isoform was firstly isolated and functionally characterized from hamster liver [95]. Subsequently, a homolog protein was identified in humans [96]. However, there are several differences in expression patterns of this specific isoform among species [89, 90]. MCT2 expression in human tissues is relatively low compared with other isoforms and its expression is confined to those tissues with high lactate uptake rates (e.g. postsynaptic membrane of neurons) or gluconeogenic tissues (e.g. liver and kidney).

1.2.1.3 MCT3

Concerning all MCT isoforms, MCT3 is thought to have the most restricted distribution. Its expression has been reported in specific tissues like retinal pigment epithelium (RPE) and choroid plexus epithelia, facilitating the lactate efflux of the retina [86, 89, 90]. However, some evidence for MCT3 expression in other tissues, such as vascular smooth muscle cell lines, human aorta [97] and human kidney [98], have arisen, indicating a more widely expressed MCT3 mRNA.

1.2.1.4 MCT4

MCT4 isoform exhibits several similarities with MCT1, but the differences in both tissue distribution and substrate affinities must be highlighted [86]. MCT4 is widely expressed in glycolytic tissues, including skeletal muscle, astrocytes, white blood cells and also in some mammalian cell lines [90, 99]. Thus, MCT4 plays an important role in lactate efflux, which is clearly corroborated by the high K_m value for monocarboxylates, such as pyruvate and lactate. The low affinity, namely for pyruvate, ensures that there is no pyruvate loss from the cell, which is essential for the maintenance of the NADH pool, allowing the maintenance of the glycolytic pathway [83, 84].

1.2.2 Monocarboxylate transporter regulation

Despite the lack of studies concerning MCT regulation, some groups have recently been examining the regulation mechanisms of these transporters at several levels, including both transcriptional and post-transcriptional regulation, as well as regulation of transport activity.

Although putative N-glycosylation sites have been described for some members of the MCT family [85], sequence analysis demonstrate that MCTs are unlikely to be glycosylated [91, 100]. Additionally, membrane expression of MCTs become essential to perform their transport activity; thus, an ancillary protein, belonging to immunoglobulin (IgG) superfamily, is required for the correct translocation of MCTs to the plasma membrane [84, 85, 89, 90]. These molecular chaperones consist of a single transmembrane domain containing a conserved glutamate residue, a short intracellular C-terminus, and a large glycosylated extracellular domain with the immunoglobulin domains attached [101]. The correct association between MCTs and its chaperone supports MCT folding and stability in cell membranes, besides assisting MCT activity [102].

In a first study, using erythrocytes, interactions between MCT1 and an integral plasma membrane glycoprotein, gp-70 or embigin, were reported [103]. Secondly, an embigin-related protein, CD147 or basigin, was shown to coprecipitate with both MCT1 and MCT4 isoforms, emerging its potential role on MCT modulation [81, 85]. Afterwards, Kirk and collaborators, using both MCT1- and MCT4-transfected cells, demonstrated cytoplasm compartmentalization, which was clearly reverted upon CD147 overexpression, trafficking MCTs towards the plasma membrane [102]. In addition to MCT1 and MCT4, also MCT3 expression in plasma membrane is dependent on its association with the mature and glycosylated form of CD147 [104].

When available, CD147 is the favorite chaperone binding MCT1 [105] but, if CD147 is absent, an interplay between embigin and MCT1 may occur, at least in rat erythrocytes [103]. Contrarily, MCT2 expression in plasma membrane colocalize with embigin in mammalian cells, suggesting that embigin is involved in MCT2 regulation rather than CD147 [106]. Thus, it is currently accepted that both MCT1 and MCT4 isoforms have CD147/basigin as ancillary protein, while gp-70/embigin perform the molecular chaperone activity for MCT2 isoform [81].

Recently, another molecular chaperone, CD44, has been implicated in MCT regulation [107]. Indeed, CD44 was associated with both MCT1 and MCT4 at the plasma membrane in human cancer specimens. Accordingly, CD44 and MCT1 were co-expressed in lung carcinoma [108], whereas in prostate cancer, CD44 expression was associated with both MCT1 and MCT4 [109]. Considering recent findings, there is a reciprocal regulation between MCTs and their chaperones recognizing MCTs as indirect players in tumor growth and angiogenesis, tumor cell migration and invasion, as well as mediators in chemotherapy resistance [88].

Additionally, in recent years, some groups have reported MCT regulation through gene expression. The analysis of *slc16a1* (MCT1) promoter showed the absence of the classical TATA-box region. However, it contains several binding sites for a variety of transcription factors, including those related with the regulatory effects of butyrate [110]. The transcriptional *slc16a1* regulation has been well described in skeletal muscle. In this model, MCT1 appears to be upregulated after chronic stimulation or exercise in rats and humans, which seems to be associated with increased levels of calcium and adenosine-monophosphate (AMP), leading to the activation of calcineurin and AMP-activated protein kinase (AMPK), respectively. Calcineurin is believed to activate the transcription factor nuclear factor in activated T-cells (NFAT), which in turn binds to enriched-NFAT binding sites located in the promoter region of *slc16a1* gene, increasing its expression. In addition to calcineurin, the activation of AMPK stimulated the activity of *slc16a1* promoter, in both L6 myoblasts and hepatoma cells (HepG2). However, MCT4 expression upon AMPK activation is still controversial. In both L6 myoblasts and HepG2 cells, the promoter activity of *slc16a3* (MCT4) was decreased by AMPK [90] while, in skeletal muscle MCT4 expression was revealed as increased upon AMPK stimulation [111].

Beyond the above-mentioned regulatory mechanisms, hypoxia, through hypoxia-inducible factor 1-α (HIF-1α) remains the major regulatory factor concerning MCT4 expression [112], which is consistent with the role of this MCT isoform in lactate efflux. Thomas C. and colleagues demonstrated upregulation of both mRNA and protein levels of MCT4 upon hypoxic stimulation in several cell types, and such effect might be mimicked using cobalt chloride ($CoCl_2$) implying transcriptional regulation via HIF-1α. By contrast with MCT4, in this study, neither MCT1 nor MCT2 expression were found to be regulated by hypoxia [113], which might be associated with the absence of hypoxia response elements (HRE) in their 5' untranslated regions (UTR) [87]. The promoter activities of *slc16a1* (MCT1), *slc16a7* (MCT2) and *slc16a3* (MCT4) were measured

using luciferase promoter constructs, indicating that hypoxia stimulates *slc16a3* promoter activity, but no effect was observed in both *slc16a1* and *slc16a7* promoter activity [113]. Despite this evidence, MCT1 regulation by hypoxia still remains unclear. Recently, both MCT1 and MCT4 were found to be upregulated after hypoxia induction in human adipocytes [114].

In addition to transcriptional regulation, concomitant measurements of mRNA and protein levels have suggested that MCTs might be regulated in a post-transcriptional manner. For instance, post-transcriptional MCT1 regulation might involve regulation of its translation. The 3'UTR region of MCT1, where post-translational modifications may occur, is very long and it is thought to be involved in the control of MCT1 expression [90]. Wilson and Halestrap demonstrated a post-transcriptional upregulation of MCT1 protein expression throughout cell cycle. According to these authors, the MCT1 protein levels increased in both post-mitotic and G1 phases of the cell cycle, where mRNA levels remained unchanged [90]. These results might be explained because of the existence of a putative cytosolic polyadenylation element in the 3'UTR of MCT1 which is responsible for the regulation of polyadenylation process in the post-mitotic phase [90, 115].

Finally, another regulatory mechanism must be considered, upon the discovery of a C-terminus truncated form of MCT4, which was the major form in a variety of tissues according to Western Blot analysis. Immunohistochemistry procedures revealed the existence of two cellular compartments from distinct cell types, lymphocyte cytoplasm and the capsule of muscle spindle, containing the truncated form, but not the full-length one. This evidence might indicate the existence of a specific function for this shorter MCT4 isoform, which is still unknown [116].

1.2.3 MCT inhibitors

Due to their association with specific glycosylated molecular chaperones, MCTs are correctly targeted to the plasma membrane, in order to perform their transport activity. Thus, they are attractive targets for systemic therapy [117]. Indeed, several chemical compounds have been developed as non-physiological competitive MCT inhibitors [85, 89]. The best well-studied classes of compounds are the cinnamic acid derivates (e.g. α-hydroxy-4-cianocinnamate (CHC)), the stilbene dissulfonates (e.g. 4,4'-di-isothiocyanostilbene-2,2'-dissulfonate (DIDS) and 4,4'-

benzamidostilbene-2,2'-dissolfunate (DBDS)) and thiol reagents (e.g. *para*-chloromercuribenzenesulfonic acid (*p*CMBS)) (Figure 7) [82].

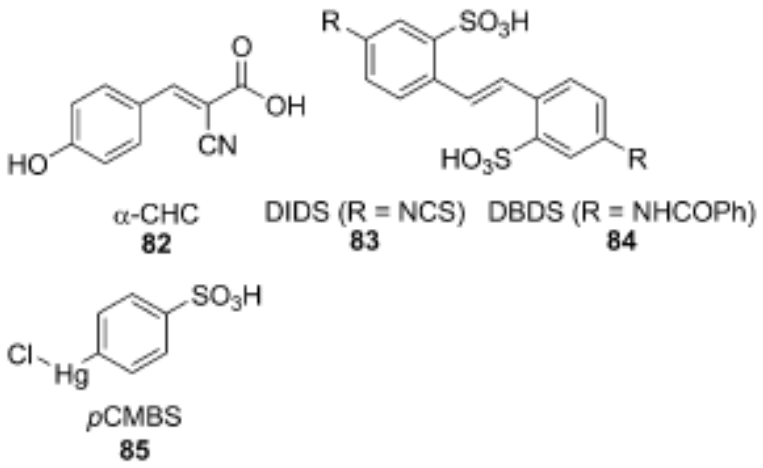


Figure 7 | Classical MCT inhibitors. Abbreviations: α-CHC – α-hydroxy-4-cyanocinnamate; DIDS - 4,4'-di-isothiocyanostilbene-2,2'-dissulfonate; DBDS - 4,4'-benzamidostilbene-2,2'-dissulfonate; *p*CMBS - *para*-chloromercuribenzenesulfonic acid. *Adapted from [118].*

CHC was firstly described as an inhibitor of the mitochondrial pyruvate transport [119] and also as an inhibitor of lactate transport in Ehrlich ascite tumor cells (K_i=0.5mM) [120]. Taking into account its potential inhibition of lactate transport, CHC was further evaluated for MCT1 inhibition [118]. Actually, CHC inhibits MCT1 isoform (K_i=166μM), in spite of lacking MCT1 specificity, as revealed by K_i=24μM and $K_{0.5}$≅1mM for MCT2 and MCT4, respectively [121].

In mouse models, CHC exhibited antitumor activity, *per se* or in combination with radiotherapy, without relevant side effects [122]. Furthermore, CHC induced a decrease in intracellular pH of human melanoma cells, leading to impaired cancer cell survival [123]. Treatment of glioma cells with CHC disturbed their glycolytic metabolism, re-sensitizing tumor cells to radiotherapy [124].

Stilbene dissulfonate compounds (e.g. DIDS and DBDS) are competitive inhibitors for many anion channels, including the chloride/bicarbonate exchanger AE1 and MCTs (Ki=22.4μM) [118]. Once again, MCT2 was reported to be more sensitive than MCT1 for both DIDS and DBDS [89]. DIDS may be considered an irreversible inhibitor upon long-lasting incubation periods, probably due to its reactive isothiocyanate groups which covalently bind to the transporters [125].

Also, noncompetitive inhibitors, as the organomercury-related compounds were examined as potential inhibitors for MCT activity. Indeed, *p*-CMBS inhibited MCT1 activity with a K_i value around 112μM [126]. More recently, Wilson *et al.* revealed that basigin was the real target for *p*-CMBS, through its thiol groups on cysteine residues, thus blocking the anion transport [106]. Furthermore, this organomercurial derivative did not demonstrate specificity for MCT1, also inhibiting MCT4 isoform ($K_{0.5}$=21μM) [121].

Some natural compounds, e.g. quercetin and phloretin, have also been reported as potential inhibitors of MCT activity. Actually, in quercetin-treated glioma cells (50μM), there was an increase in intracellular lactate accumulation, which was corroborated by a decrease in intracellular pH of glioma cells [127]. Also, phloretin decreased MCT activity in a non-specific manner (MCT1: K_i=5μM; MCT2: K_i=14μM; MCT4: $K_{0.5}$=41μM) [121].

Recently, a new class of MCT inhibitors has been developed by AstraZeneca. These compounds were firstly synthesized for immunosuppression, thanks to the relevance of MCT1 for T-lymphocytes [128]. These inhibitors demonstrated high affinity for MCT1 with K_i values in the nanomolar (nM) range [128, 129]. Further structure optimizations were performed, in order to overcome their solubility problems, giving rise to a new compound, AR-C155885, which efficiently binds to MCT1 in human erythrocytes [130].

1.2.4 MCTs in cancer

According to the World Health Organization (WHO), cancer has been the main cause of death worldwide, accounting with around 7.6 million deaths in 2008. Furthermore, this number is estimated to increase to over 13.1 million in 2030 [131].

Firstly, six hallmarks of cancer had been proposed as biological abilities acquired during the multi-step tumorigenic process, contributing for malignant progression, such as self-sufficiency in growth signals, insensitivity to growth suppressor signals, resistance to programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Figure 8), which are consequence of genomic instability underlying tumors [132]. However, more recently, the concept of tumor microenvironment has gained focus. In fact, tumors are not only insular masses of proliferating cancer cells [133]. They

are made up from several cell types including fibroblasts, endothelial and immune cells, which collectively give rise to tumor-associated stroma and perform an active role in tumorigenesis [133, 134]. Taking together such evidence, novel hallmarks were recently introduced, namely reprogramming of energy metabolism and evading immune destruction, which are believed to promote the development and progression of human cancers (Figure 8) [133].

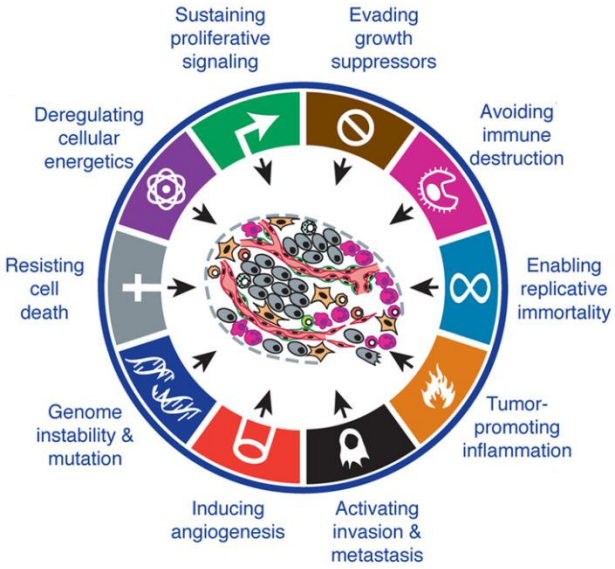


Figure 8 | The hallmarks of cancer. Here are depicted a range of functional abilities that cancers acquired during the multistep-tumorigenic process. *From [133]*

In normal tissues, cells are exposed to different oxygen levels, depending on their distance to the blood vessels, constraining cells to anaerobic glycolysis – the Pasteur effect [135, 136] . However, when the oxygen levels are re-established, non-malignant cells are able to convert glucose into pyruvate, via glycolysis in the cytoplasm, and then canalize it to the mitochondria for further oxidation into carbon dioxide (CO_2) and water through oxidative phosphorylation (OXPHOS) [133]. In contrast, tumors present an alternative metabolism to accomplish their high energetic demands and fulfill the biosynthetic requirements of proliferating cells [117, 137]. During the development of malignant lesions, which mostly occurs in avascular regions, tumor cells are both glucose- and oxygen-dependent. Hence, these substrates must diffuse from the closest blood vessel through the carcinoma basement membrane, as well as across the layers of tumor cells (Figure 9) [138].

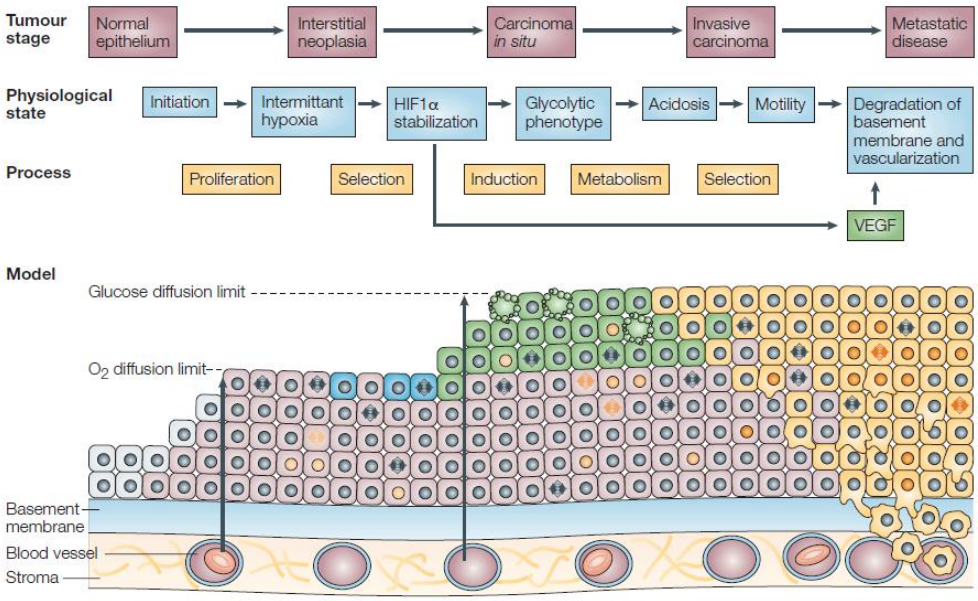


Figure 9 | Schematic representation of cell-microenvironment interactions occurring during tumorigenesis. Briefly, normal epithelial cells are represented in grey, hyperproliferative cells are pink, hypoxic cells are blue, glycolytic cells are green and motile/metastatic cells are depicted in yellow. Orange color in cell nuclei represents the appearance of mutations (light orange for one mutation and dark orange for more mutations). Apoptotic cells on the diagram exhibit irregular plasma membranes i.e. blebbing membranes. Abbreviations: HIF-1α, hypoxia-inducible factor 1α; VEGF, vascular endothelial growth factor. *From [138]*

As a consequence of high proliferation rates, solid tumors frequently overpass the oxygen-diffusion limit giving rise to hypoxic regions, where cells fail to receive enough oxygen amounts to perform an adequate oxidative metabolism, as well as the oxygen-dependent non-metabolic redox reactions [117, 138, 139]. Such abnormal characteristic of tumor cell metabolism is maintained, even when oxygen levels are restored, as discovered by Otto Warburg and his collaborators in 1920 [140-142]. Indeed, even in the presence of sufficient oxygen amounts, neoplastic cells rely on glycolysis – aerobic glycolysis or the Warburg effect. Consequently, cancer cells effectively convert around 85% of glucose into lactate, instead of driving the glycolysis-derived pyruvate into mitochondria through OXPHOS (Figure 10) [137]. The upregulation of glycolysis may be considered an adaptive consequence of microenvironmental tumor hypoxia [138]. The cellular response to hypoxia is mediated by the activation/stabilization of HIF-1α, which is closely related with tumor progression [143, 144]. In addition to the *VEGFA* gene, some glycolytic enzymes, as well as other essential players in glycolytic metabolism, are also upregulated in a HIF-1α-dependent mechanism [145].

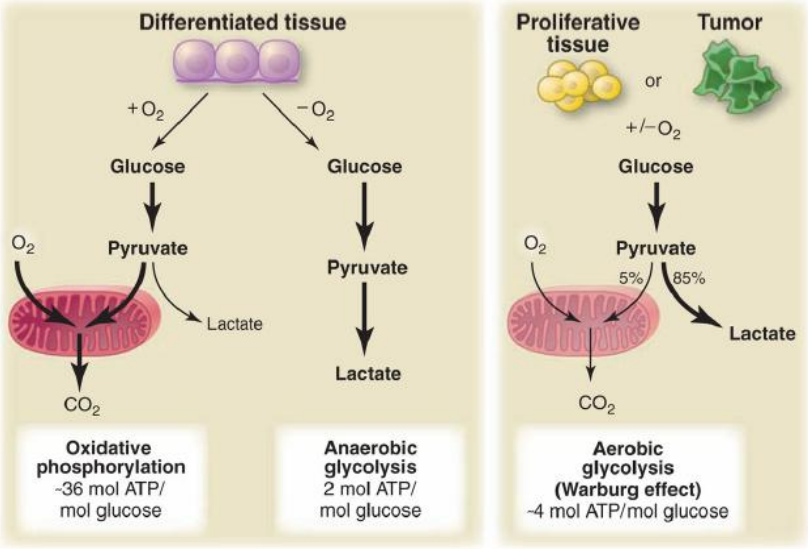


Figure10 | Differences between oxidative phosphorylation, anaerobic glycolysis and aerobic glycolysis (Warburg effect). *From [138]*

For instance, pyruvate dehydrogenase kinase 1 (PDK1) is upregulated by HIF-1α [146]. As a result, pyruvate dehydrogenase (PDH) activity will be inhibited, preventing the entry of pyruvate into the tricarboxylic acid (TCA) cycle and, consequently, oxidative phosphorylation [147]. Thus, hypoxic cancer cells exhibit an increased glycolytic metabolism to ensure sufficient ATP production. Taking into account such evidence, glucose transporters should also be upregulated under hypoxia. In fact, Ebert and colleagues revealed an increased expression of both glucose transporter 1 (GLUT-1) and 3 (GLUT-3) under hypoxia, in a HIF-1α-dependent manner [148, 149]. In addition, some downstream glycolytic enzymes (e.g. hexokinase II (HKII)), as well as lactate dehydrogenase 5 (LDH-5) were also reported to be upregulated by HIF-1α [150, 151]. In spite of its role on the maintenance of glycolytic metabolism, HIF-1α is also involved in the acidic-resistant phenotype by upregulating several pH regulators, such as carbonic anhydrase IV (CAIX) and monocarboxylate transporters (MCTs), mainly MCT4, as previously mentioned [112, 152, 153].

Although an inefficient pathway in terms of ATP synthesis [133], the increased glycolytic phenotype gives several advantages to cancer cells. It generates glycolytic intermediates that may be diverted to biosynthetic pathways [137]. Furthermore, increased glycolysis leads to acidification of the tumor milieu [138]. Indeed, a range of studies in both human and animal tumors demonstrated that the extracellular pH of these tumors are acidic and could reach pH values around 6 [138]. In opposition to normal cells, cancer cells exhibit a range of mutations,

including those in *p53* gene, which promote tumor cell adaptation to acidosis. Additionally, acidosis *per se* is likely to be mutagenic, leading to impairments in DNA repair, inhibition of GAP-junction conductance and spontaneous transformation of normal diploid fibroblasts [138, 154]. Some studies have suggested that the acidic phenotype contributes for tumor cell invasion *in vitro* [155] and tumor metastasis *in vivo* [156], accelerating malignant progression and promoting therapy resistance [138].

Despite their contribution to pH regulation, MCTs are not the only pH regulatory systems. Thus, other pH regulating systems, such as Na^+/H^+ exchanger (NHE), CAIX and anion exchanger 1 (AE1), exist on the cell plasma membrane [88, 157]. However, the role of MCTs on cellular homeostasis is unquestionable. They prevent intracellular lactate accumulation, which would lead to cell damage and, consequently, apoptosis through cellular acidosis [158]. Additionally, lactate extrusion through MCTs contributes to the maintenance of glycolytic flow, allowing the hyper-glycolytic phenotype [88].

The importance of lactate as tumor acidifier, mutagenesis inducer, as well as its role in both radio- and chemoresistance induction, due to its antioxidant activity [138, 159], are currently well accepted. However, this metabolite exhibits other properties which individually or collectively are essential in the development of malignant lesions [88]. For instance, the tumor milieu is highly lactate-enriched due to both tumor cell and T-lymphocyte metabolism. Importantly, this microenvironmental acidification appears to be detrimental for T-lymphocyte activity, both decreasing its immune response against neoplastic cells and favoring cancer cell escape from immune system [160, 161]. Also, both pyruvate and lactate have been reported as regulators of HIF-1α gene expression in a hypoxia-independent mechanism [162], indicating the intrinsic role of lactate on the induction of the hyper-glycolytic metabolism [88]. Additionally, lactate was also implicated in cellular motility [163], upregulation of VEGF [164-166], and both hyaluronan and its receptor, CD44, which perform an active role on tumor cell invasion and metastasis [167, 168]. Taking together, these findings suggest that lactate contributes for the malignancy of cancer, and its accumulation within tumors has been correlated with poor prognosis [157]. Indeed, studies concerning lactate accumulation in human tumors revealed that cervical tumors, head and neck cancers and rectal adenocarcinomas showing metastatic potential, exhibited increased lactate levels comparatively with the non-metastatic counterparts [169-171]. Additionally, increased lactate levels were further associated with poor prognosis,

poor disease-free survival and poor overall disease survival in human cervical carcinomas [172], head and neck cancer [173], high-grade gliomas [174] and non-small lung cancer [175].

Besides being an end-product in a range of metabolic pathways (e.g. glycolysis and glutaminolysis [176]), lactate may also be used in the mitochondria, as reported in both skeletal [177] and cardiac [178, 179] muscle as well as in the brain [180, 181]. Additionally, cell-cell lactate shuttles were also recently suggested in tumors. Accordingly, a metabolic symbiosis is established between hypoxic and oxidative tumor cells. In fact, Sonveaux and collaborators showed that human cervical cancer cells cultured under hypoxia effectively convert glucose into lactate, extruding it through MCT4, whereas oxidative tumor cells take up lactate, via MCT1, utilizing it for OXPHOS [122].

Taking into account their role on lactate transport, MCT involvement in tumor progression has also been hypothesized. Hence, it is not surprising that MCT expression appears to be increased in human tumors.

In colon tumors, MCT expression still remains controversial. First, a decrease in MCT1 expression was reported in the colonic transition from normal epithelium to malignant lesions. However, no differences in both MCT2 and MCT4 expression were found [182, 183]. Afterwards, high MCT expression was found in colon adenocarcinomas [108], as well as in the cytoplasm of tumor cells comparatively with normal colonic samples [184] [185]. Importantly, both MCT1 and MCT4 were reported as highly expressed on the plasma membrane, contrarily to MCT2 [185]. Additionally, the correlation between MCT expression and the clinico-pathological data revealed MCT1 expression in the plasma membrane, which was strongly associated with vascular invasion [185].

Regarding brain tumors, several studies point to a strong MCT1 expression in ependyomas, hemangioblastomas and high grade gliomas including glioblastoma multiform (GBM) [186], while its expression is clearly lower or even absent in low-grade gliomas [187]. Recently, an overexpression in the plasma membrane of MCT1 and MCT4, as well as its molecular chaperone, CD147, was reported in GBM comparatively with diffuse astrocytomas and non-neoplastic tissue [188].

Differential expression patterns have also been demonstrated in breast cancer. Actually, the hypermethylation of the promoter region of *slc16a1* (MCT1) gene was suggested to induce a

decrease in both mRNA and protein levels in breast cancer samples, compared to normal epithelium [189]. However, Pinheiro *et al.* reported a significant increase in MCT1 expression in both cytoplasm and plasma membrane of breast carcinomas in comparison with normal epithelium. Other MCT isoforms were also assessed, but only MCT4 was found to be upregulated in the cytoplasmic compartment of breast cancer cells, with no significant differences in plasma membrane expression [108, 190]. Furthermore, both MCT1 and CD147 were correlated with poor prognosis parameters, such as basal-like subtype and high grade tumors [190].

Despite few studies addressing MCT expression in lung cancer, some evidence point to MCT upregulation in lung cancers whereas, at least MCT1 expression was negative in normal lung [191]. In contrast, a recent study revealed high MCT expression in normal lung, while MCT4 was clearly downregulated in cancer cells [108]. In addition, both MCT1 and its chaperone were also described in the cytoplasm of sarcoma cells belonging to the alveolar soft region [192].

MCT expression has also been characterized in both cervical and ovarian cancers, which collectively are known as gynecologic tract tumors. Concerning cervical carcinomas, both MCT1 and MCT4 expression appear to be upregulated in the plasma membrane in either invasive squamous lesions or adenocarcinomas comparatively with pre-invasive lesions and normal glandular epithelium, respectively. MCT2 analysis was quite inconclusive, but it was frequently expressed in squamous cell carcinomas, while MCT4 expression was increased in adenocarcinomas. It is important to highlight that HPV-positive pre-invasive cases exhibited either high expression of both MCT1 and MCT4 or more MCT1 in plasma membrane than HPV-negative pre-invasive lesions [193]. Furthermore, the same group revealed that CD147 was frequently expressed in MCT1 and MCT4 positive cases and that MCT1/CD147 coexpression was correlated with lymph node metastasis in adenocarcinomas [194]. In ovarian cancer, MCT1 and MCT4, as well as its chaperone, appeared to be negative in normal ovarian tissues and benign ovarian tumors. On the other hand, upregulation of these molecular markers was described in both primary and metastatic ovarian neoplasms [195]. Also, Pinheiro *et al.* demonstrated upregulation of MCTs in ovarian carcinoma, being MCT2 the most expressed isoform (around 95%) [108].

In prostate cancer, around 90% of the cases were positive for both MCT1 and MCT4, whereas no MCT expression was detected in normal prostate tissues, prostate intraepithelial neoplasms (PIN) and non-tumor regions adjacent to primary tumor prostate tissues. Also, MCT

expression in prostate cancer cells was associated with high pathological stage and nodal involvement [109]. Contrarily, a subsequent study described MCT1 and CD147 expression in normal prostate tissue, while MCT1 appear to be downregulated in tumor specimens. The other MCT isoforms, MCT2 and MCT4, appear to be highly expressed in the cytoplasm of prostate tumor cells than in their normal counterparts. In addition, both MCT1 and MCT4 were associated with poor prognosis variables [196]. Also, overexpression of MCT2 was found in PIN lesions, suggesting a role on prostate cancer initiation. Furthermore, in addition to other markers (e.g. alpha-methylacyl-CoA racemase (AMACR)), MCT2 has a role as a putative biomarker for prostate cancer diagnosis [197].

MCT expression has also been studied in gastric carcinomas. This type of tumors showed distinct MCT expression patterns comparatively with other tumor types. Indeed, MCT4 was more frequently encountered in normal gastric mucosa than in gastric carcinomas, and even less frequently expressed in lymph-node metastasis, suggesting a loss in expression along tumor progression. However, the MCT1/CD147 co-expression was associated with poor prognosis, since it was associated with advanced gastric carcinomas and lymph node metastasis [198].

Concerning MCTs' role on the maintenance of the hyper-glycolytic and acid-resistance phenotype, as an adaptive consequence to tumor hypoxia, it seems to be clear that MCTs are attractive targets for cancer therapy. Actually, *in vitro* studies performing inhibition of MCT1 activity reported a drop in intracellular pH [122], inducing cell death [122, 186] and improving tumor cell radiosensitivity [124]. In addition, MCT4 downregulation decreased cancer cell migration, through impairment on the interactions between MCT4 and β_1-integrin [199]. By contrast, Izumi *et al.* suggested that downregulation of both MCT1 and MCT4 decreased tumor cell invasion with no effect on cancer cell motility [200].

Furthermore, CHC administration *in vivo* induced tumor retardation, enhanced tumor cell radiosensitivity [122], increased tumor necrosis and reduced tumor invasion [201]. Other experiments, specifically inhibiting both MCT1 and MCT4 or their molecular chaperone, CD147, revealed a decrease in the glycolytic flux and tumor growth, corroborating the previous results with CHC [202].

Importantly, taking into account its effective binding to MCT1 in lymphocytes, the new class of drugs developed by AstraZeneca is thought to be suitable for cancer treatment. Thus, a

new drug was developed, AZD3965, which is currently in Phase I clinical trials for advanced solid tumors, such as prostate cancer and non-Hodgkin lymphoma expressing MCT1 [117, 118].

1.2.5 MCTs in endothelial cells

In healthy tissues, endothelial cells rarely migrate or proliferate. However, under stressful environments (e.g. hypoxia), ECs have the ability to rapidly proliferate, migrate and assemble new blood vessels [55]. Concerning those features, dividing endothelial cells resemble tumor cells by easily sustaining their growth in hypoxic environments [55]. Interestingly, also like tumor cells, despite having direct contact with oxygen, dividing endothelial cells rely on aerobic glycolysis, mostly converting glucose into lactate [55, 203]. Thus, it is likely that MCTs play a key role in endothelial cell metabolism, as suggested for tumor cells.

Despite the lack of studies addressing MCTs in endothelial cells, there are some reporting the existence of these transporters in the endothelium of blood brain barrier (BBB) and more recently in the corneal endothelium.

A full array of studies, such as stereospecific and saturable transport of both lactate and pyruvate through the brain endothelium, suggest the presence of MCTs at the BBB [204, 205]. Interestingly, other simple organic compounds (e.g. acetate and butyrate) were proved to be transported across the BBB and the role of MCTs in this transport was hypothesized [204]. MCT1 was clearly identified on the luminal membrane of brain endothelial cells, being around 25 times higher in the neonatal brain than in the adult stage [206]. Further studies in cultured MCT1-depleted brain endothelial cells corroborated the previous *in vivo* evidence indicating that MCT1 mediates the transport of exogenous monocarboxylates into the brain [207]. Altogether, these data suggest that MCT1 might be responsible for the high rates of lactate and ketone bodies transport across the brain endothelium, namely under starvation periods [81].

Recently, intracellular pH was reported as participating in the control of MCT1 function in cerebrovascular rat endothelial cells. Not surprisingly, a drop in intracellular pH strongly inhibited lactate transport into the brain endothelial cells, while as intracellular pH increased, lactate transport was significantly enhanced [208]. Furthermore, the regulation of MCT1 function by cyclic adenosine monophosphate (cAMP) was also recently described in a rat brain endothelial

cell line (RBE4). Indeed, it was demonstrated that cAMP induced both MCT1 dephosphorylation and internalization from the plasma membrane into early endossomes [209]. Contrarily to MCT1, MCT2 expression in brain endothelial cells is still unclear. Its expression had been detected in one [210] but not in all studies [211].

The accumulation of lactate and fluid in the corneal stroma, which collectively may be harmful for the cornea homeostasis, has been reported along years [212, 213]. Indeed, the corneal endothelium plays an important role facilitating lactate exchanges between the corneal stroma and the anterior chamber, through MCTs [214]. However, the specific localization and identity of MCTs in corneal endothelial cells require further exploitation. In a rat model, both MCT1 and MCT2 are present in both basolateral and apical membranes, whereas MCT4 was not detected [215]. In opposition, bovine corneal endothelium also exhibited MCT2 in both membranes, while both MCT1 and MCT4 were only found in the basolateral and apical membranes, respectively [216]. In spite of all these efforts, there are still some controversies regarding MCT expression in animal models. Importantly, studies addressing MCTs in human endothelial cells, namely, tumor-associated endothelial cells are still required.

1.2.6 Tumor microenvironment: endothelial and tumor cell crosstalk

Over the last years, the biology of tumors has not been clearly understood. Hence, studies concerning both the tumor-proliferating cells as well as the surrounding tumor microenvironment became essential and, therefore, tumors started to be recognized as complex organs [133]. The tumor microenvironment is a complex system encompassing several cell types such as endothelial cells and their precursors, pericytes, SMCs, fibroblasts and a range of immune cells (e.g. macrophages), which collectively perform an active role in carcinogenesis [217].

Tumor microenvironment is highly acidic, mainly due to tumor cells-derived lactate [157], whose pro-angiogenic effects have been described: it induces VEGF secretion by macrophages, endothelial cell migration, vascular morphogenesis and the recruitment of circulating vascular progenitor cells [218]. However, it is still poorly understood whether lactate triggers the same effects in tumor-associated endothelial cells.

So far, the most exhaustive study concerning the role of lactate on the induction of angiogenesis in cancer is attributed to Végran and colleagues. Indeed, they described that MCT1 mediates lactate uptake in endothelial cells. Inside the cell, lactate stimulates NF-κβ/IL-8 signaling pathway, promoting endothelial cell migration and tube formation. They also documented, in mouse xenograft models of human colorectal and breast carcinomas, that lactate release from tumor cells through MCT4 was enough to induce IL-8-dependent angiogenesis and tumor growth [219].

Lactate has also been implicated in tumor aggressiveness, partially because of its role in HIF-1α activation through PHD inhibition in a hypoxia-independent manner [162]. Accordingly, it was hypothesized whether lactate directly activates HIF-1α in endothelial cells, consequently stimulating the angiogenic process. In fact, blocking the entry of lactate into endothelial cells inhibited HIF-1-dependent angiogenesis. Furthermore, it was reported that endothelial-MCT1 inhibition prevents angiogenesis, through HIF-1 inactivation in both *in vitro* and *in vivo* models [220].

In spite of the advances concerning tumor metabolism and angiogenesis, the role of MCTs in tumor-endothelial cell interplay, mainly under a hypoxic environment, is still an unanswered question, which should be further explored.

1.3 Rationale and Aims

Besides their function in tumor metabolism, MCTs seem also to be important in endothelial cell metabolism. Despite all the efforts in addressing the role of MCTs in angiogenesis, some questions, like how hypoxic environments may influence the angiogenic process in cancer, are still unanswered. In addition to a 2D culture system, which has some limitations, it becomes important to develop systems, which will fulfill the fundamental microenvironmental cues, essential for tumor initiation, progression, invasion and metastasis [221].

Thus, we intended to investigate in more detail the tumor-endothelial cell interactions occurring in tumors. We selected a glioma cell line (U251) as tumor model, since previous results from our group demonstrated MCT expression in brain tumor-associated vessels (Baltazar, F., unpublished data). Hence, the role of MCTs in the maintenance of brain endothelial cell (HBMEC) function was assessed, under normoxia and hypoxia. The influence of hypoxia and MCT downregulation on the angiogenic capacity of glioma cells was also evaluated. Ultimately, we intended to study the influence of a 3D *in vitro* culture system on the maintenance of tumor and endothelial cells' function.

The specific aims of this master thesis were:

- **Characterization of the metabolic behavior of brain endothelial cells, under normoxia and hypoxia**

    - Characterization of MCT expression, their molecular chaperones (CD147 and CD44), as well as other key glycolytic markers under normoxia and hypoxia;
    - Evaluation of endothelial cell metabolism (glucose consumption and lactate production), up to 24 hours, under normoxia and hypoxia.

⦿ **Assessment of the role of MCTs in key endothelial cell features, under normoxia and hypoxia**

- *In vitro* evaluation of the role of MCTs on endothelial cell survival, metabolism, proliferation, death, cell cycle, and tube formation, after inhibition of both MCT activity and expression, under normoxia and hypoxia

⦿ **Evaluation of the influence of hypoxia and MCT downregulation on the angiogenic capacity of human glioma cells**

- Assessment of the effect of glioma cells' conditioned media, upon tumor cell growth, under both normoxia and hypoxia, in combination with MCT downregulation, in a range of endothelial cell features including cell survival, metabolism, proliferation, cell cycle and tube formation;
- Evaluation of the effect of glioma cells' conditioned media in blood vessel assembling *in vivo*;
- Characterization of the expression of total VEGFA in glioma cells in the above-mentioned conditions.

⦿ **Assessment of the influence of an *in vitro* three dimensional (3D) culture system on cell function**

- Assessment of cell viability and metabolism in both glioma cells (U251) and human brain endothelial cells (HBMEC), upon seeding into hierarchical starch-based fibrous scaffold;
- Comparison between 2D and 3D culture systems, in terms of cell viability.

2 Materials and Methods

2.1 Cell Culture

Human Brain Microvascular Endothelial Cells (HBMECs), kindly provided by Professor Kwang Sik Kim (John Hopkins School of Medicine, Baltimore, USA), were maintained in Roswell Park Memorial Institute 1640 medium (RPMI, GIBCO, Invitrogen), supplemented with 10% (v/v) fetal bovine serum (FBS, GIBCO, Invitrogen) and 1% (v/v) penicillin/streptomycin (10μg/mL) (GIBCO, Invitrogen) at 37 °C, 5% CO_2. Glioblastoma cell line (U251) was purchased from American Type Cell Culture (ATCC) and grown in Dulbecco's Modified Eagle´s medium (DMEM, GIBCO, Invitrogen), supplemented with 10% (v/v) FBS (GIBCO, Invitrogen) and 1% (v/v) penicillin/streptomycin (10μg/mL) (GIBCO, Invitrogen) at 37 °C, 5% CO_2.

2.2 Hypoxia

In some experiments, endothelial and glioblastoma cells were grown under hypoxic conditions. For that, cells were placed into hypoxic chambers, where atmospheric air was replaced by N_2 (10L/min) and CO_2 (5cc/min), for 10 minutes and, then, incubated at 37 °C for 24 hours. Control cells were cultured under normoxic conditions (37 °C, 5% CO_2), during the same period.

2.3 Reagents

Alpha-cyano-4-hydroxycinnamate (CHC, Sigma-Aldrich) was dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich) to a 2.9M stock solution. Further concentrations (0.25 – 2M) were obtained through dilution in 100% DMSO. The working concentrations (2.5 – 20mM), used in HBMEC treatment, were obtained upon dilution in RPMI 1640 medium.

2.4 Small interfering RNA (siRNA) transfection

Downregulation of MCT1 and MCT4 was performed in both endothelial and glioblastoma cells, using siRNA (Invitrogen). A control group was also performed, using scrambled siRNA (Invitrogen). For that, different master mixes were prepared in Opti-MEM (GIBCO, Invitrogen) medium, including specific siRNA for each MCT isoform and lipofectamine RNAiMAX reagent (Invitrogen), according to manufacturers' instructions. Fifteen minutes after, the previous mixtures were distributed in different wells (6-well plate) and cells were seeded in antibiotic-free medium (HBMEC: 2×10^6 cells/well; U251: 1×10^6 cells/well). Twenty four hours after transfection, the medium was replaced by fresh complete medium.

2.5 Conditioned media

U251 cells were transfected and seeded into a 6-well plate, as described previously. Three days after plating, when MCT silencing was observed, complete DMEM medium was replaced by serum-free medium and cells were incubated either in normoxic or hypoxic environments, for an additional period of 48 hours. After, the conditioned media (CM) was harvested, centrifuged (900 rpm, 5 minutes), and, then, transferred to HBMEC in 2:3 proportions, in which two parts correspond to CM and the another to fresh medium.

2.6 Protein Extraction

HBMEC were grown, under both normoxia and hypoxia, until confluence was reached. Then, cells were harvested and homogenized with lysis buffer (1% Triton-X100; 1% NP-40; 0.1mM EDTA; 50mM Tris-HCl, pH=7.5; 150mM NaCl and protease inhibitors), for 15 minutes. After that, homogenized samples were centrifuged (13000 rpm, 15 minutes, 4 °C) and stored at -80 °C. All samples were quantified using the Bradford method.

2.7 Western blotting

Twenty micrograms of protein were loaded into SDS-PAGE gels, and, then, transferred to a nitrocellulose membrane (Amersham Biosciences), using a wet system (Bio-Rad). Subsequently, membranes were incubated overnight, at 4 °C, with specific primary antibodies: mouse anti-MCT1 (1:500, Santa Cruz Biotechnology), rabbit anti-MCT4 (1:2000, Santa Cruz Biotecnology), mouse anti-CD147 (1:200, Santa Cruz Biotecnology), mouse anti-CD44 (1:1000, Serotec) rabbit anti-GLUT1 (1:800, Abcam), rabbit anti-CAIX (1:1000, Abcam), mouse anti-PDK (1:2000, Abcam), rabbit anti-LDHV (1:2000, Abcam), mouse anti-PDH (1:300, Abcam), mouse anti-HXII (1:4000, Abcam), mouse anti-HIF-1α (1:1000, BD Biosciences), rabbit anti-HIF-2α (1:500, Abcam) and goat anti-β-actin (1:300, Santa Cruz Biotecnology). Afterwards, membranes were incubated with the adequate secondary antibodies: mouse anti-rabbit (1:5000, Santa Cruz Biotechnology), rabbit anti-mouse (1:5000, Santa Cruz Biotechnology) and donkey anti-goat (1:5000, Santa Cruz Biotechnology). Chemiluminescence was used as detection method using the Supersignal West Femto reagent (Thermo Scientific) and image capture was performed by X-ray Amersham Hyperfilm ECL exposure (GE Healthcare). Finally, band quantification was performed using ImageJ software (National Institutes of Health).

2.8 Cytoblock preparation

Endothelial cells were allowed to grow until around 70% of confluence was reached. Then, cells were grown either under normoxia or hypoxia, for 24 hours. After that, cells were harvested, centrifuged at 1300 rpm for 5 minutes, and fixed with 3.7% of paraformaldehyde (PFA) for 48 hours. Subsequently, cells were re-centrifuged (1300 rpm, 5 minutes), processed, and embedded into paraffin blocks.

2.9 Immunocytochemistry

Briefly, deparaffinized and rehydrated sections were exposed to an adequate heat-induced antigen retrieval treatment (Table 1), washed in phosphate buffered saline (PBS (1X)) solution, and, then, incubated with 3% hydrogen peroxide (H_2O_2) in methanol, to inactivate

endogenous peroxidase activity. Next, non-specific binding sites were blocked, using an appropriate blocking solution (Labvision, Thermo Scientific or Vector kit, Vector Laboratories), and, then, cytoblocks were incubated with specific primary antibodies (Table 1). Depending on the primary antibody, two different detection systems, Ultravision Detection System Anti-polyvalent HRP (Labvision Corporation, Thermo Scientific) or R.T.U. VECTASTAIN Elite ABC kit (Vector Laboratories), were used. Incubation conditions for each antibody are specified in Table 1. Subsequently, after a washing period, sections were incubated with the secondary biotinylated antibody (Labvision, Thermo Scientific or Vector kit, Vector Laboratories) and, then, with Steptavidin/Avidin peroxidase solution (Labvision, Thermo Scientific or Vector kit, Vector Laboratories). Immunocytochemical reactions were developed with 3, 3'-diamino-benzidine (DAB + Substrate system, Labvision, Thermo Scientific). Finally, cytoblocks were counterstained with hematoxylin/eosin (HE) and permanently mounted with Entellan®. Adequate positive controls were included in each reaction to verify the efficiency of immunoreaction (Table 1).

Table 1 | Details of the immunocytochemical reactions.

Protein	Positive Control	Antigen Retrieval	Detection System	Primary Antibody (company; dilution; incubation conditions)
MCT1	Colon Carcinoma	EDTA (1mM, pH=8) 20 min.; 98°C	Vector Laboratories	Santa Cruz Biotecnology; 1:500; Overnight, RT
MCT4	Colon Carcinoma	Citrate Buffer (10mM, pH=6) 20 min.; 98°C	LabVision Corporation	Santa Cruz Biotechnology; 1:200; 2 hours, RT
CD147	Colon Carcinoma	EDTA (1mM, pH=8) 20 min.; 98°C	Vector Laboratories	Santa Cruz Biotechnology; 1:400; Overnight; RT
CD44	Colon Carcinoma	Citrate Buffer (10mM, pH=6) 20 min.; 98°C	LabVision Corporation	Serotec; 1:1000; 2 hours, RT
GLUT-1	Head and Neck Carcinoma	Citrate Buffer (10mM, pH=6) 20 min.; 98°C	LabVision Corporation	Abcam; 1:500; 2 hours, RT
CAIX	Stomach	Citrate Buffer (10mM, pH=6) 20 min.; 98°C	LabVision Corporation	Abcam; 1:2000; 2 hours, RT

LDH-V	Colon	EDTA (1mM, pH=8) 20 min.; 98°C	LabVision Corporation	Abcam; 1:1000; 2 hours, RT
HXII	Colon Carcinoma	EDTA (1mM, pH=8) 20 min.; 98°C	LabVision Corporation	Abcam; 1:750; 2 hours, RT
PDH	Stomach	EDTA (1mM, pH=8) 20 min.; 98°C	Vector Laboratories	Abcam; 1:300; Overnight, RT
PDK	Stomach	EDTA (1mM, pH=8) 20 min.; 98°C	Vector Laboratories	Abcam; 1:500; Overnight, RT
HIF-1α	Glioblastoma	EDTA (1mM, pH=8) 20 min.; 98°C	Vector Laboratories	BD Biosciences; 1:100; Overnight; RT

RT – Room Temperature

2.10 Cell Viability

Cell viability was assessed using the *in vitro* toxicology sulforhodamine B (SRB)-based assay. Non-silenced or silenced HBMEC were plated in 96-well plate, at a cellular density of 5×10^3 cells/well, and allowed to adhere for 24 hours. After that, cells were incubated under normoxia or hypoxia, as described previously. Subsequently, non-silenced HBMEC were treated with different concentrations of CHC (2.5 – 20mM) or incubated with glioma cells' CM, for 24 hours. Furthermore, silenced HBMEC cells were incubated in serum-free medium, for an additional period of 24 hours. Then, cells were washed with PBS (1X) and, then, incubated with 10% of trichloroacetic acid (TCA) (Sigma-Aldrich), for 1 hour at 4 °C. Wells were rinsed, five times, with tap water, and allowed to dry overnight. On the next day, cells were incubated with sulforhodamine B (SRB) (Sigma-Aldrich) for 30 minutes, at room temperature, and, then, wells were rinsed, four times, with acetic acid (1%). When wells were completely dried, cells were incubated with 10mM of Tris-base (Sigma-Aldrich) for 5 minutes, at room temperature, under shacking. The absorbance was measured, at 570nm, with background absorbance of 655nm, using the Infinite M200 NanoQuant apparatus (Tecan). The IC_{50} values for both CHC-treated cells and respective controls were estimated either under normoxia or hypoxia, using a sigmoidal dose-curve response (GraphPad Prism 5).

2.11 Cell metabolism assay (glucose consumption and lactate production)

Metabolic activity of HBMEC cells was evaluated through quantification of extracellular glucose and lactate, using different colorimetric assays. Endothelial cells (2.5×10^4 cells/well) were seeded into 48-well plates and incubated in complete RPMI 1640 medium, for 24 hours. Then, cells were incubated under normoxia or hypoxia, as already mentioned. Upon the incubation period, non-silenced HBMEC were treated with different concentrations of CHC (estimated IC_{50} value or half-IC_{50} value) or incubated with CM, for further 24 hours. Additionally, silenced HBMEC cells were grown in serum-free medium, during 24 hours. Aliquots of supernatants (100μL) were harvested for glucose (Roche) and lactate (Spinreact SA) quantification, at 490nm (Infinite M200 NanoQuant, Tecan), using well-established protocols. All results were normalized for total cell biomass, using the SRB assay described above.

2.12 Cell proliferation assay

Cell proliferation measurements were assessed using the colorimetric cell proliferation ELISA, 5-bromodeoxyuridine (BrdU) kit (Roche). Both silenced and non-silenced HBMEC (5×10^3 cells/well) were plated into 96-well plates, incubated in complete RPMI 1640 medium, and allowed to adhere overnight. Then, cells were cultured under normoxia or hypoxia, for 24 hours. After that, non-silenced HBMEC were treated with working concentrations of CHC, referred on the previous section, or incubated with CM from glioblastoma cells, for 24 hours. Furthermore, silenced HBMEC cells were incubated with serum-free medium, also for 24 hours. Additionally, 20μM of BrdU was added to the medium and incubated at 37 °C, overnight. Then, cells were fixed for 30 minutes, followed by incubation with a specific antibody (anti-BrdU, 1:100), for 90 minutes. Cells were then washed five times with PBS (1X), and incubated with a colorimetric substrate, for further 5 minutes. The reaction was stopped using 25mM of sulfuric acid (H_2SO_4, Sigma-Aldrich). BrdU incorporation by proliferating cells was measured at 450nm, with 655nm as reference, using Infinite M200 NanoQuant apparatus (Tecan).

2.13 Cell death assay

Apoptotic and necrotic cell populations were estimated using Annexin V-FLOUS Apoptosis kit (Roche), according to the manufacturer's instructions. For that, both non-silenced and silenced HBMEC were seeded into 6-well plates (2×10^5 cell/well), and allowed to adhere overnight. Then, cells were incubated under normoxia and hypoxia, for further 24 hours. Afterwards, non-silenced HBMEC were treated with working CHC concentrations, during 24 hours. Additionally, silenced HBMEC cells were allowed to grow for additional 24 hours, in serum-free RPMI medium. Upon 24 hours of treatment, cells were harvested, and Annexin V/Propidium Iodide (PI) staining was performed according to manufacturer's instructions. The percentage of cell death was assessed by flow cytometry (LSRII model, BD Bioscience), reading a total of 20000 events, and the results analyzed using FlowJo software (Tree Star).

2.14 Cell Cycle analysis

Both non-silenced and silenced HBMEC were plated into 6-well plates (2×10^5 cell/well) and allowed to grow overnight. Then, cells were incubated under normoxia and hypoxia, for 24 hours. After that, all cells were starved for 6 hours. Six hours later, non-silenced HBMEC were incubated for additional 24 hours with working CHC concentrations, described previously, or with glioma cells' CM, whereas silenced HBMEC were allowed to grow in serum-free RPMI medium, for the same period. Then, cells were collected, fixed in ethanol (70%) for 30 minutes, under shacking, and, finally, PI (Sigma-Aldrich) staining was performed according to manufacturer's instructions. The relative amount of cells at each stage of the cell cycle was evaluated using flow cytometry (LSRII model, BD Bioscience), reading a total of 15000 events, and results analyzed with FlowJo software (Tree Star).

2.15 Tube formation assay

Plates (48-well) were coated with 150 μL of growth factor reduced (GFR) Matrigel® (BD Bioscience). Coated plates were then incubated at 37 °C, for 30 minutes, to induce Matrigel polymerization. Both non-silenced and silenced HBMEC cells were incubated, under normoxia

and hypoxia, for 24 hours. To evaluate the effect of both CHC and MCT downregulation, as well as the effect of glioma cells' CM on capillary-like structures assembling, HBMEC were incubated with working media, under normoxia and hypoxia, for further 24 hours. During this period, images were captured at 0, 8 and 24 hours, using an inverted phase-contrast microscope (Olympus IX61), and subsequent qualitative analysis of capillary-like structures assembling was performed.

2.16 Chick chorioallantoic membrane (CAM) assay

Fertilized eggs (*Gallus gallus*) were incubated for 4 days at 37 °C. At embryonic day (ED) 4, a small hole was opened in the eggs, and the viability of the embryo was checked. Viable eggs were then incubated for additional 4 days. At ED 8, a silicon ring was implanted and 40 μL of glioma cells' CM were placed inside ring. At ED 9 CAM was harvested, and the effects of CM in CAM vascularization were assessed through image acquisition, using a dissecting microscope (Olympus SZX16). It is important to highlight that *in vivo* images were captured at the time of the injection, as well as at 24 hours, after the injection of glioma cells' CM. To evaluate the effects of scramble groups, a CTR group was included, where only the silicon ring was placed without any injection.

2.17 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from glioblastoma cells (U251), upon MCT downregulation, under normoxia and hypoxia, using Trizol (Invitrogen) method, according to manufacturer's instructions. Isolated RNA was then quantified using Nanodrop ND-1000 apparatus (Thermo Scientific). Nanodrop, was also used to estimate the purity of the isolated RNA by optical density (O.D.) 260/280 and O.D. 260/230 ratios. For a pure RNA solution, the ratio O.D. 260/280 should be around 2. By contrast, if this ratio remains below than 1.8, there is contamination with proteins or solvents. Furthermore, the ratio O.D. 260/230 is also important as it allows the evaluation of potential contaminations with polysaccharides. After confirming the quality of the RNAs, U251 RNA was converted into cDNA (200ng/μL), using a specific cDNA synthesis kit (Fermentas, Thermo Scientific). This newly-synthesized cDNA was amplified by Polymerase Chain Reaction

(PCR) (My Cycler Thermal Cycler, BioRad). RT-PCR was performed using the following primer sequences: VEGFex3_F: ATCTTCAAGCCATCCTGTGTGC, VEGFex8_R: GCTCACCGCCTCGGCTTGT, β-actin_F: GGACTTCGAGCAAGAGATGG and β-actin_R: AGCACTGTGTTGGCGTACAG. Annealing temperatures of the above-mentioned primers were: VEGFex3_F/VEGF8ex_R - 61.2 °C and β-actin_F/ β-actin_R: 57°C. Then, RT-PCR products were separated in 2% agarose gel, and bands were further visualized in an UV chamber with GreenSafe Premium loading dye (Nzytech).

2.18 Three dimensional (3D) *in vitro* culture of both glioma and brain endothelial cells, using hierarchical starch-based fibrous scaffolds

We are beginning to implement a 3D co-culture system in our research model, which replicates the complexity and hierarchical organization of ECM in tumors, using hierarchical starch-based fibrous scaffolds. For each cell line, 5×10^5 cells/scaffold were individually seeded into hierarchical starch-based fibrous scaffolds and allowed to adhere for 6 hours. After this period, U251 and HBMEC cells were incubated in DMEM or RPMI 1640 medium (1mL), respectively, up to seven days. In parallel, U251 (8×10^3 cells/well) and HBMEC (1×10^4 cells/well) were cultured in monolayer into 96-well plates, up to 3 days. After 1, 3 and 7 days, cell-containing scaffolds, as well as one cell-free scaffold, were transferred to another plate, for evaluation of cell viability, using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega). The absorbance was measured at 490nm, using the Infinite M200 NanoQuant apparatus (Tecan). Five samples *per* scaffold, in a total of 3 scaffolds *per* time point, were analyzed. Metabolic assays were only performed in the 3D culture model, where supernatant aliquots (100μL) were harvested at 24 and 72 hours, for glucose and lactate quantification, as described previously.

2.19 Statistical analysis

All experiments were analyzed using student's t-test (GraphPad Prism 5). Results are expressed as mean ± SEM of at least two independent assays. Differences were considered statistically significant when $p \leq 0.05$.

3 Results

3.1Characterization of the metabolic behavior of brain endothelial cells, under normoxia and hypoxia

3.1.1 Characterization of MCT expression and their molecular chaperones

To evaluate the influence of hypoxia on the expression and cellular localization of MCT1 and MCT4 isoforms, as well as their molecular chaperones, CD147 and CD44, both WB analysis and immunocytochemistry were performed, respectively.

Although both HIF-1α and HIF-2α were already expressed under normoxic conditions, WB analysis showed, as expected, an increase in the expression of HIF-related proteins, under hypoxia in HBMEC cells (Figure 11).

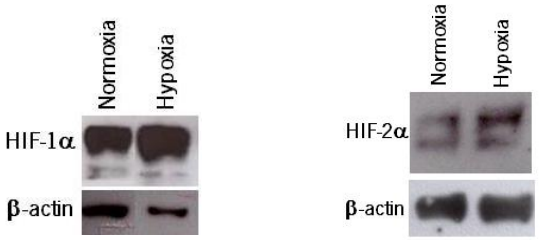


Figure11 | HIF-1α and HIF-2α expression in human brain endothelial cells, under normoxia and hypoxia, using Western blotting. Both HIF-related proteins are expressed under normoxic conditions in HBMEC cells. As expected, their expression is increased under hypoxia.

The expression of both MCT isoforms, as well as their chaperones, appeared to be slightly increased, under hypoxia, in HBMEC cells (Figure 12A and 12B). Furthermore, in brain endothelial cells, hypoxia induced an increase in plasma membrane expression of MCT1, CD147 and CD44 (Figure 12C). Interestingly, hypoxia had different effects on MCT4 expression. Under hypoxia, HBMEC cells seem to express MCT4, mainly in cytoplasm, although some plasma membrane expression was observed (Figure 12C).

3.1.2 Characterization of the expression of key glycolytic proteins

In addition to MCTs and their molecular chaperones, the effects of hypoxia on the expression and cellular localization of several other key metabolic markers were also assessed.

Similarly to MCTs and their accessory proteins, CD147 and CD44, also GLUT-1 appeared to be slightly upregulated, under hypoxia (Figure 13A and 13B), while other key glycolytic markers, including HKII, LDH-V, PDH, PDK and CAIX exhibited similar expression levels under normoxia and hypoxia (Figure 13A and 13B). Indeed, immunocytochemical analysis showed only a slight increase in plasma membrane expression of GLUT-1 and CAIX, under hypoxia (Figure 13C). Furthermore, HKII cytoplasm expression was found to be weak under both normoxia and hypoxia (Figure 13C), whereas LDH-V exhibited high expression levels in cytoplasm, under both conditions (Figure 13C). In addition, no alterations were found concerning PDH and PDK cytoplasm expression between normoxia and hypoxia (Figure 13C).

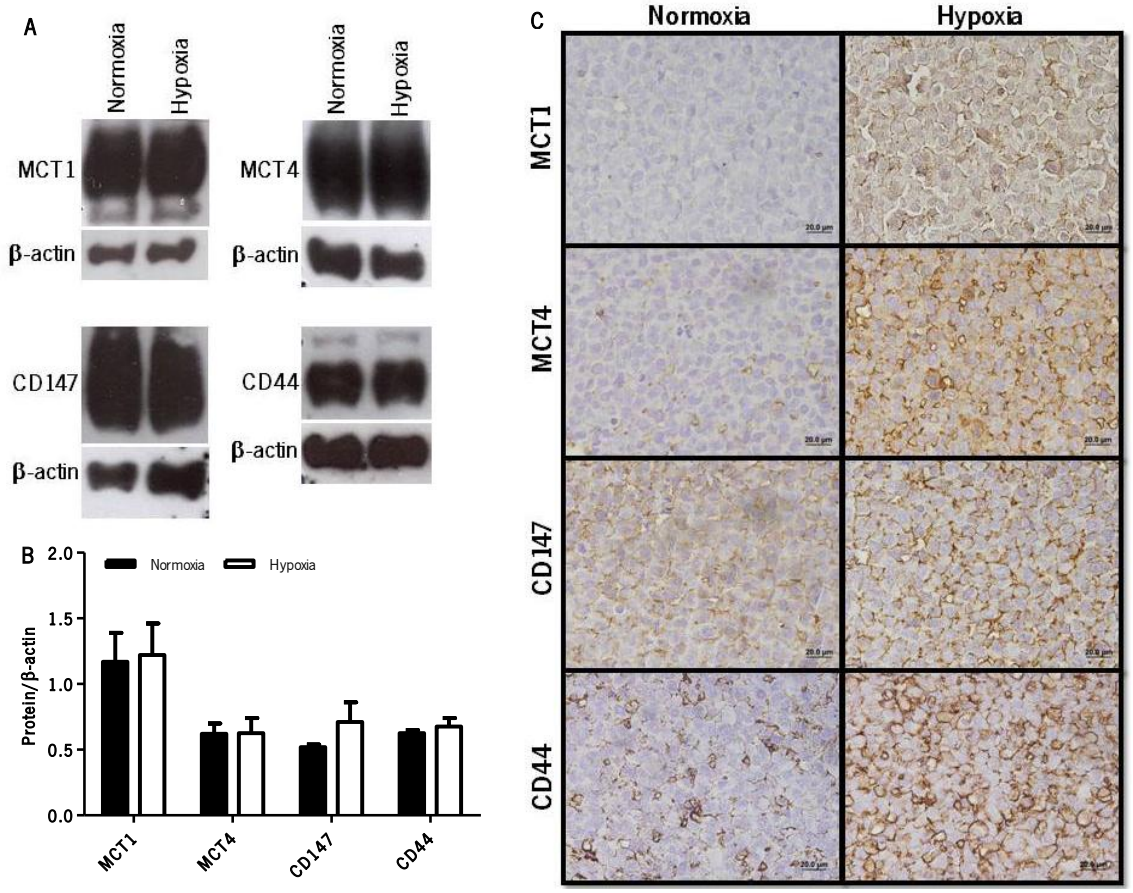


Figure 12 | Monocarboxylate transporters (MCT1 and MCT4) and their molecular chaperones (CD147 and CD44), under normoxia and hypoxia, in human brain endothelial cells. Western Blot analysis **(A)** and respective quantification **(B)** indicate higher expression of MCT1 and MCT4, as well as their molecular chaperones, under hypoxia. The molecular weights are 43kDa for both MCT1 and MCT4, 50 – 60 kDa for CD147 and around 90kDa for CD44. **(C)** Immunocytochemistry revealed increased plasma membrane expression for MCT1, CD147 and CD44, under hypoxia, while MCT4 expression was mainly encountered in cytoplasm.

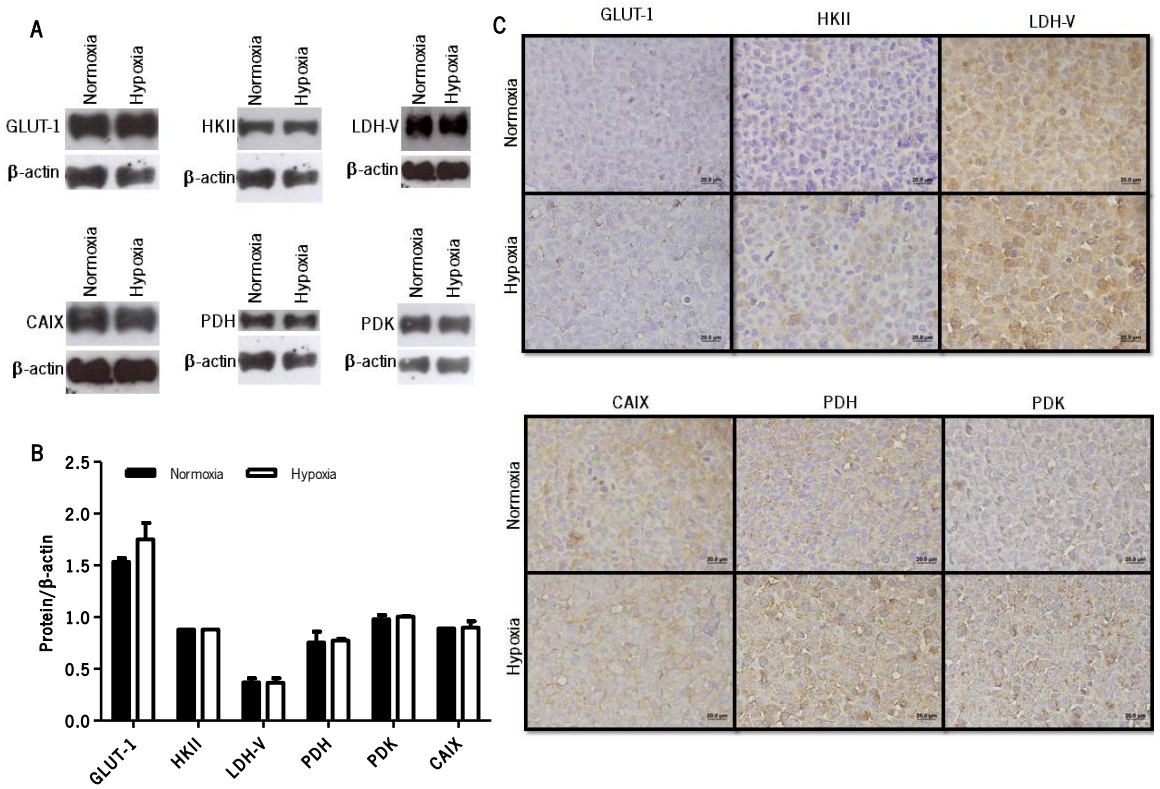


Figure 13|Expression levels of key glycolytic markers, under normoxia and hypoxia, in HBMEC cells. Western Blot analysis **(A)** and respective quantification **(B)** reveal higher expression of GLUT-1, under hypoxia, whereas the other glycolytic markers exhibited similar expression levels, under both conditions. The molecular weights are around 55kDa for both GLUT-1 and CAIX, 102 kDa for HKII, 36kDa for LDH-V, 43 kDa for both PDH and PDK. **(C)** Immunocytochemistry revealed slightly increased plasma membrane expression for GLUT-1, under hypoxia, while the expression of the other metabolic markers remained unchanged between normoxia and hypoxia.

3.1.3 Endothelial cell metabolism

The glycolytic rates of endothelial cells were evaluated through both glucose consumption and lactate production, during 24 hours, under normoxia and hypoxia.

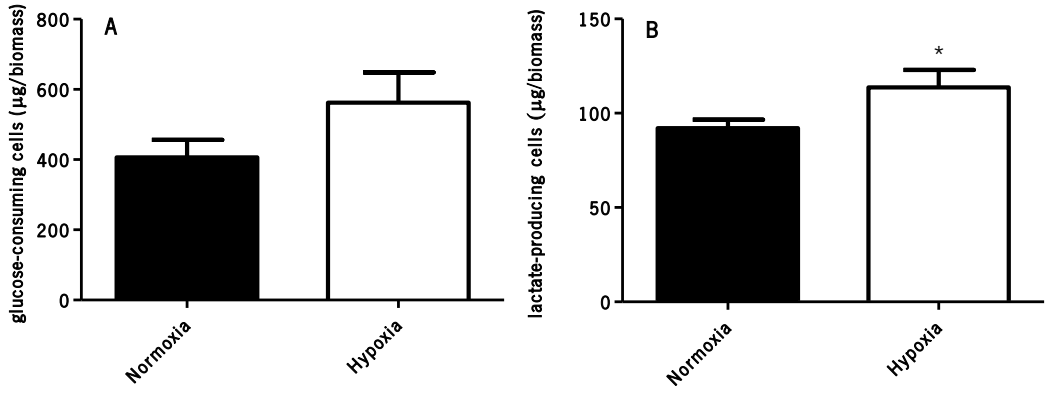


Figure 14|Endothelial cell metabolism at 48 hours, under normoxia and hypoxia. Glucose consumption **(A)** and lactate production **(B)** rates of HBMEC cell line up to 24 hours. *p<0.05 comparing normoxia and hypoxia.

As depicted in Figure 14A, there is a tendency for endothelial cells to increase their glucose consumption under a hypoxic environment, which is accompanied by a significant increase on lactate production (Figure 14B).

3.2 Effects of inhibition of MCT activity on endothelial cell function

3.2.1 Cell Survival

Firstly, in order to evaluate the sensitivity of brain endothelial cells, HBMEC, to MCT inhibition, cells were exposed 24 hours to different CHC concentrations, under both normoxia and hypoxia.

CHC significantly decreased endothelial cell viability in a dose-dependent manner, under both normoxia and hypoxia (Figure 15). However, hypoxia did not sensitize endothelial cells to CHC, such as demonstrated by similar IC_{50}-calculated values under both environmental conditions (Figure 15).

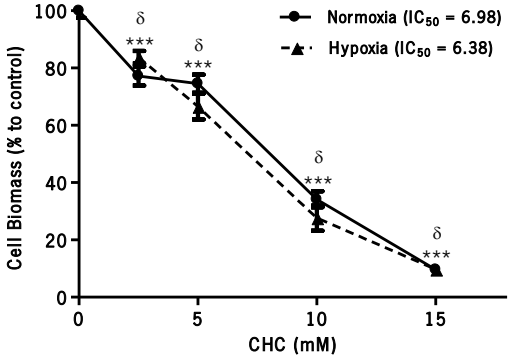


Figure 15 | Effects of MCT inhibition on endothelial cell growth at 24 hours, under normoxia and hypoxia. Values are depicted as normalized to the control (CTR) group (100%), DMSO-treated cells. CHC significantly reduced endothelial cell biomass in a dose-dependent manner, under normoxia and hypoxia. However, hypoxia did not affect endothelial cell sensitivity to CHC. ***p<0.001 comparing CTR group and CHC-treated cells under normoxia; δp<0.001 comparing CTR group and CHC-treated cells under hypoxia

Then, the potential cytotoxic effects of CHC were assessed in brain endothelial cells, under normoxia and hypoxia, through Annexin V/PI assay.

Despite lacking statistical significance, CHC tended to increase endothelial cell death in a dose-dependent manner, under both normoxia and hypoxia (Figure 16). Furthermore, endothelial cell death appeared to be increased, upon treatment with the highest CHC concentration, which was more effective under hypoxia than normoxia (Figure 16).

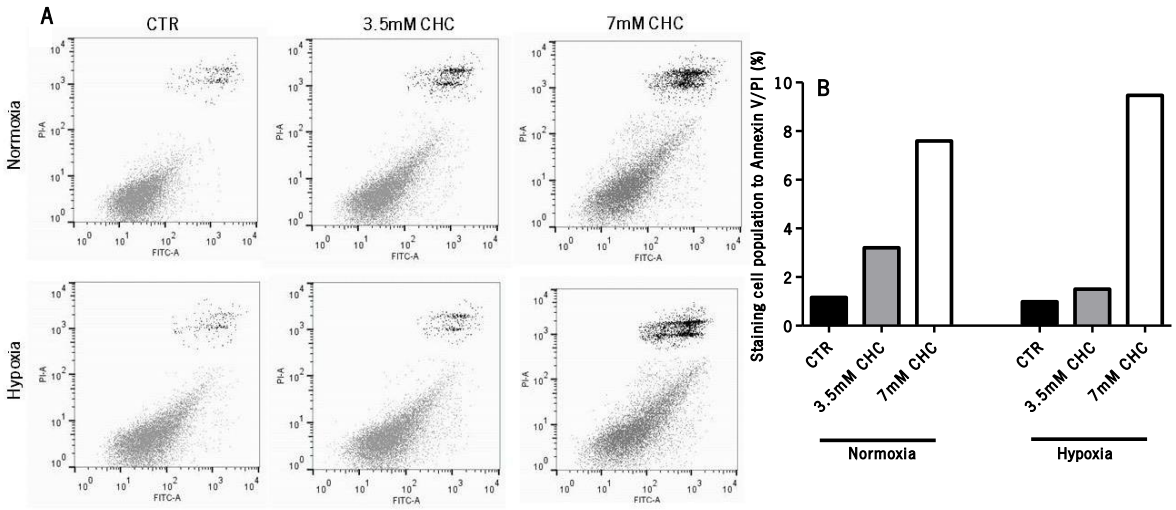


Figure 16| Influence of MCT inhibition on endothelial cell death at 24 hours, under normoxia and hypoxia. (A) Representative dotplot of cell population distribution stained for Annexin V and PI are depicted in the left panel (cell population in bottom/left = viable cells; cell population in upper/right = late apoptosis/necrosis). **(B)** In the right panel, barplot represents late apoptotic/necrotic endothelial cells, after 24 hours of treatment with CHC, under both normoxia and hypoxia. CHC seems to increase endothelial cell death, under both normoxia and hypoxia and, for the highest CHC concentration its effects tended to be more effective under hypoxia when compared to normoxia.

3.2.2 Cell Metabolism

The effects of MCT inhibition on endothelial cell metabolism were determined, under normoxia and hypoxia, through glucose consumption and lactate production measurements, after 24 hours of treatment with 3.5mM and 7mM of CHC.

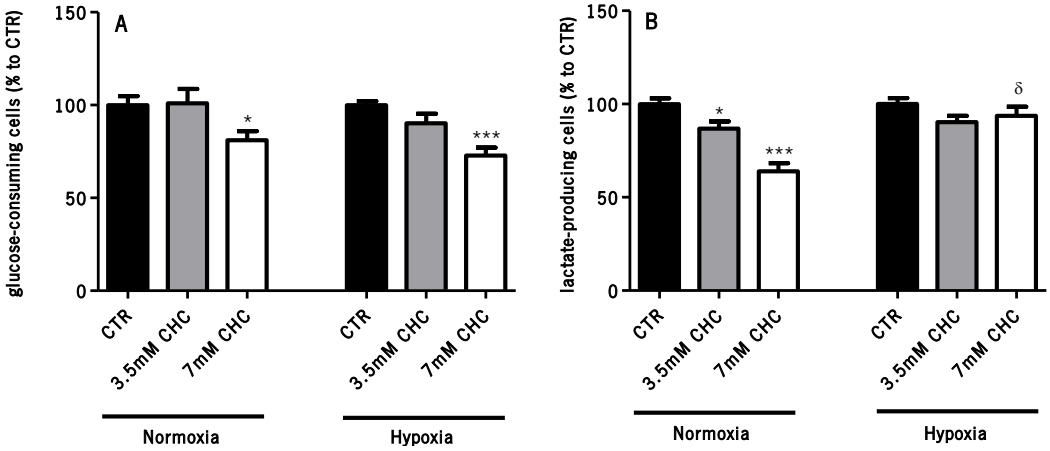


Figure 17|Effects of MCT inhibition on endothelial cell metabolism at 24 hours, under normoxia and hypoxia. Values are represented as normalized to the CTR group (100%). **(A)** The highest CHC concentration significantly reduced glucose uptake by HBMEC at 24 hours, mainly under hypoxia. **(B)** CHC significantly decreased lactate efflux in HBMEC cell line at 24 hours, under normoxia, while under hypoxia, no significant differences were found on lactate production, upon treatment with CHC. Comparatively to normoxia, under hypoxia CHC (7mM) effects on lactate production were lower. *p<0.05; ***p<0.001 comparing CTR and CHC-treat groups; δp<0.001 comparing normoxia and hypoxia.

The highest CHC concentration significantly decreased glucose consumption, mainly under hypoxia (Figure 17A). In addition, CHC significantly decreased lactate efflux, under normoxia, whereas no differences were found under hypoxia and, the effects of the highest concentration were more pronounced under normoxia than hypoxia (Figure 17B).

3.2.3 Cell Proliferation

Cell proliferation analysis was performed using a labeled DNA precursor - 5-bromo-2'-deoxyuridine (BrdU) - that was added to cells, and its incorporation into endothelial-proliferating cell's DNA was quantified after 24 hours of treatment with CHC (Figure 18).

CHC significantly impaired endothelial cell proliferation, under both normoxia and hypoxia (Figure 18B). Nevertheless, CHC treatment was less efficient under hypoxia than normoxia, mainly at the lowest concentration (Figure 18).

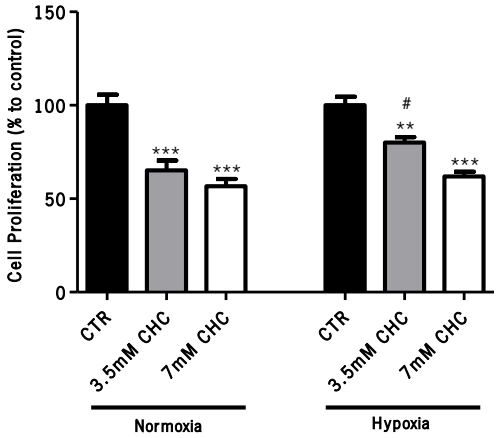


Figure 18 | Effect of MCT inhibition on endothelial cell proliferation at 24 hours, under normoxia and hypoxia. Values are depicted as normalized to the control group (100%). CHC significantly reduced proliferation rates in HBMEC cell line at 24 hours, under both normoxia and hypoxia. However, more pronounced effects of CHC in endothelial cell proliferation were found under normoxia, mainly at the lowest concentration. $^{**}p<0.01$; $^{***}p<0.001$ for comparisons between CHC-treated and CTR groups. $\#p<0.05$ comparing both normoxia and hypoxia.

Additionally, cell cycle analysis revealed a tendency to an increase of endothelial cell distribution in G2/M phase of cell cycle, upon HBMEC cells' treatment with CHC, under both normoxia and hypoxia, comparatively to CTR group (Figure 19A and 19B).

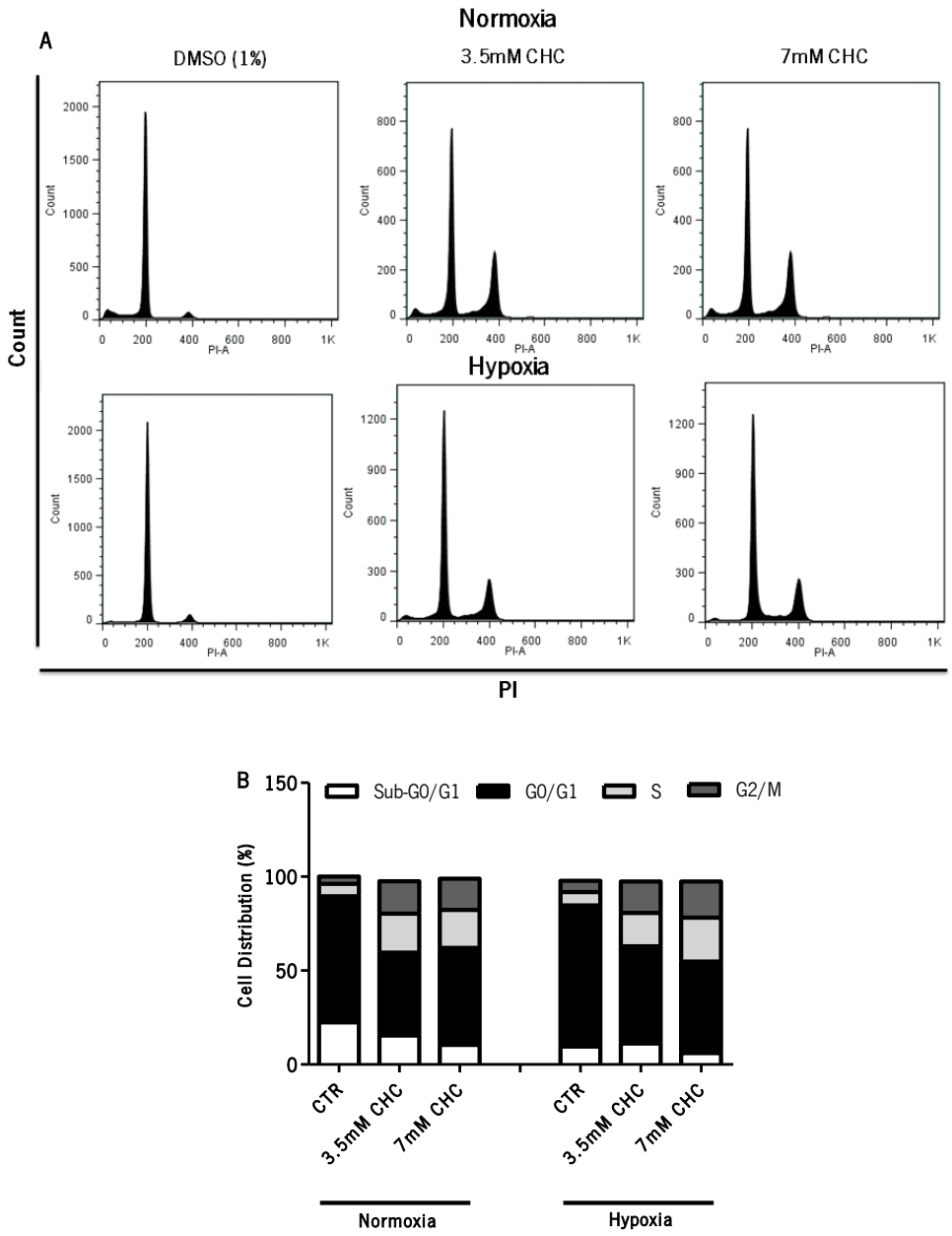


Figure 19 | Effect of MCT inhibition on cell cycle distribution at 24 hours, under normoxia and hypoxia. (A) Representative assay of cell cycle analysis for HBMEC cells, under normoxia and hypoxia, 24 hours upon treatment with CHC. Histograms of cell population distribution stained for PI are represented for both normoxia and hypoxia (cell population in the first peak = G0/G1 phase; cell population between two peaks = S phase; cell population in the second peak = G2/M phase). **(B)** Barplot represents endothelial cell distribution in each cell cycle phase, under both normoxia and hypoxia. CHC tended to increase HBMEC cells distribution in G2/M phase of the cell cycle, comparatively to CTR group.

3.2.4 Capillary-like structure assembling *in vitro*

The formation of capillary-like structures by HBMEC cells was assessed, under both normoxic and hypoxic conditions, throughout 24 hours, after inhibition of MCT activity, using CHC.

Under normoxia, CHC seems to disrupt capillary-like formation assembling *in vitro* by human brain endothelial cells, at 8 and 24 hours (Figure 20). Similarly, under hypoxia, CHC decreased capillary-like structures assembling, after 8 and 24 hours of treatment (Figure 21).

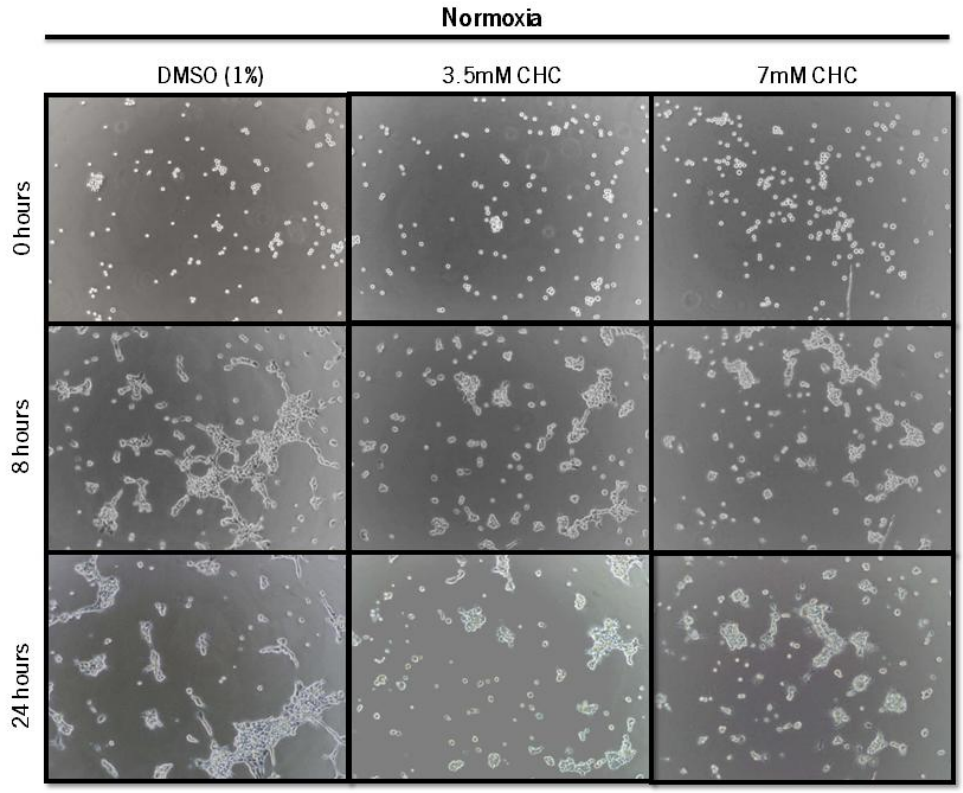


Figure 20 | The effects of CHC on the assembling of capillary-like structures by HBMEC cells up to 24 hours, under normoxia. Representative images of Matrigel-based tube formation assay (100X magnification). CHC decreased the formation of capillary-like structures, under normoxia.

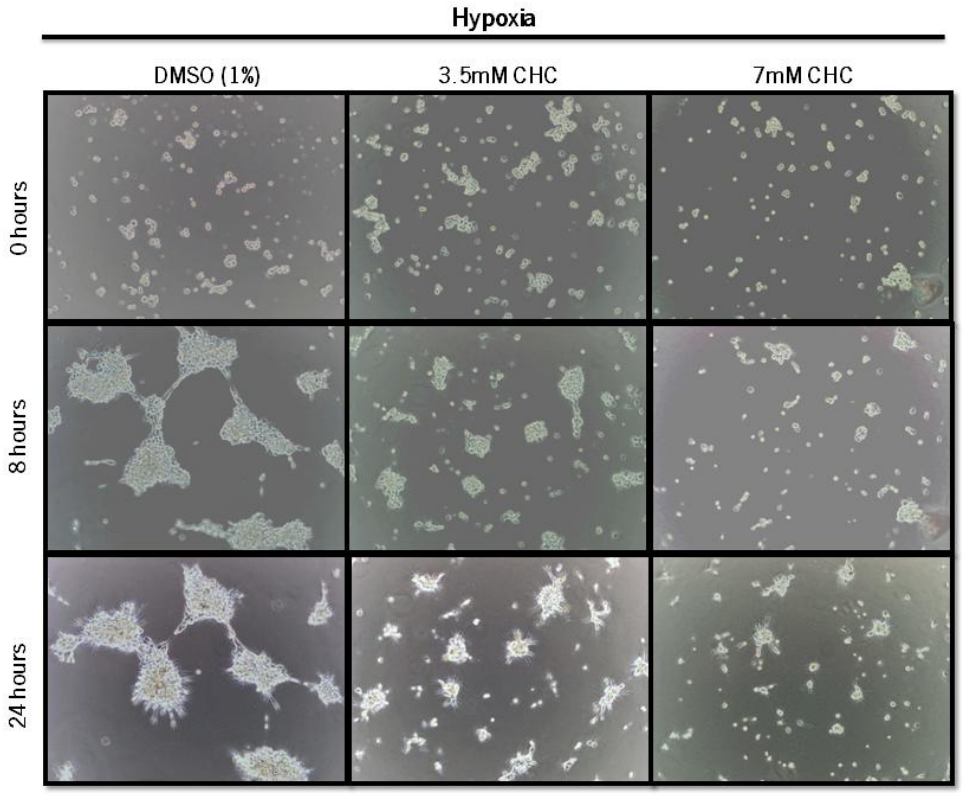


Figure 21 | The effects of inhibition of MCT activity on the formation of capillary-like structures by HBMEC cells up to 24 hours, under hypoxia. Representative images of Matrigel-based tube formation assay (100X magnification). CHC disrupted the assembling of capillary-like structures, under hypoxia.

3.3 Effect of MCT downregulation on endothelial cell function

Since MCT inhibitors may have other targets in the cell, which will limit their specificity, MCT downregulation using siRNA was also performed, to confirm the role of these transporters on the maintenance of endothelial cell function.

MCT downregulation, under both normoxia and hypoxia, was confirmed in brain endothelial cells by WB, as represented in Figure 22.

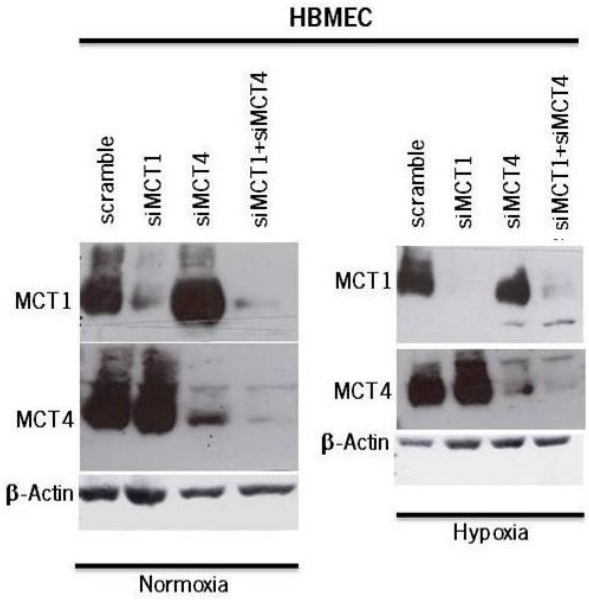


Figure 22 | Representative image of MCT expression, under normoxia and hypoxia, four days after transfection of HBMEC cells with siRNA. siRNA oligonucleotide sequences which were used in the transfection process successfully downregulated MCT1 and MCT4 isoforms, under normoxia and hypoxia.

3.3.1 Cell Survival

Downregulation of MCT1, alone or in combination with MCT4, significantly reduced endothelial cell viability at 24 hours, either in normoxia or hypoxia (Figure 23). Nevertheless, no significant differences were encountered between normoxia and hypoxia.

Furthermore, concerning Annexin V/PI staining, the knockdown of both MCT1 and MCT4 isoforms revealed a tendency to increase late apoptotic/necrotic cell population in HBMEC cells, under hypoxia (Figure 24A and 24B).

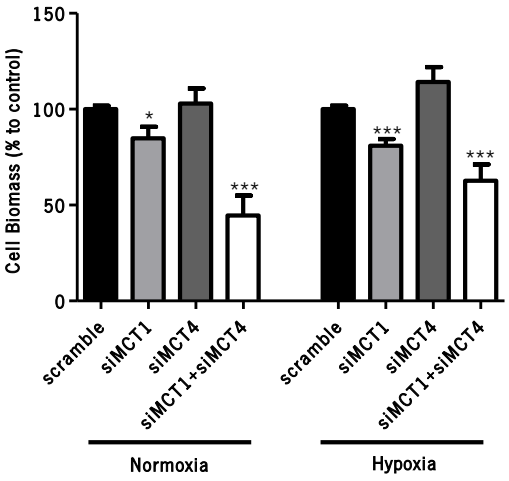


Figure 23|Influence of MCT knockdown on endothelial cell viability at 24 hours, under normoxia and hypoxia. Values are normalized to the CTR group (scramble, 100%). Under normoxia, MCT1 downregulation alone or in combination with MCT4 effectively reduced cell viability in HBMEC cell line at 24 hours. Similar effects were observed under hypoxia, however no significant differences were found between both conditions.

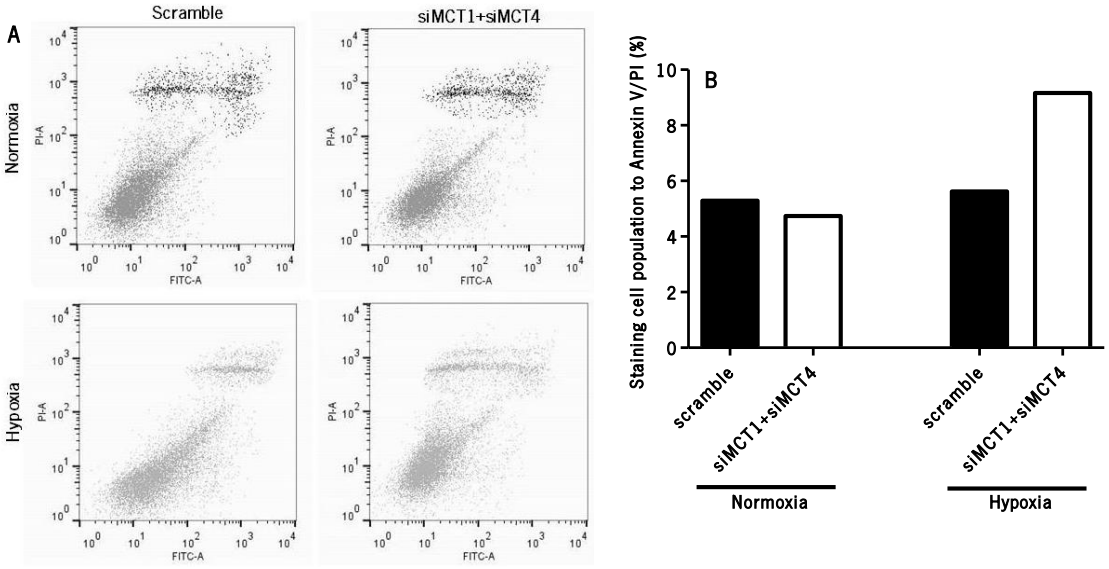


Figure 24|Influence of MCT knockdown on endothelial cell death at 24 hours, under normoxia and hypoxia. (A) Representative dotplot of cell population distribution stained for Annexin V and PI, 24 hours after MCT downregulation had been achieved (cell population in bottom/left = viable cells; cell population in upper/right = late apoptosis/necrosis). **(B)** Barplot representing late apoptotic/necrotic endothelial cells, under both normoxia and hypoxia. A tendency for increased cell death in HBMEC cells is observed upon downregulation of both MCT isoforms, under hypoxia. *p<0.05, ***p<0.001 comparing MCT-downregulated cells with the scramble group.

3.3.2 Cell Metabolism

Downregulation of MCT isoforms, alone or in combination, induced a significant decrease in glucose uptake by endothelial cells at 24 hours, under normoxia and hypoxia (Figure 25A). Furthermore, lactate production was significantly reduced upon MCT downregulation at 24 hours, under normoxia (Figure 25B). However, under hypoxia, only individual MCT knockdown induced a significant decrease in lactate efflux by endothelial cells (Figure 25B). Interestingly, under hypoxia, the impairment of the glycolytic metabolism through MCT downregulation seems to be less efficient. Indeed, simultaneous downregulation of both MCT1 and MCT4 induced a significantly higher reduction in glucose uptake by HBMEC cells, under normoxia than hypoxia. Additionally, MCT4 knockdown led to a higher decrease in lactate production, under normoxia than hypoxia, in endothelial cells (Figure 25B).

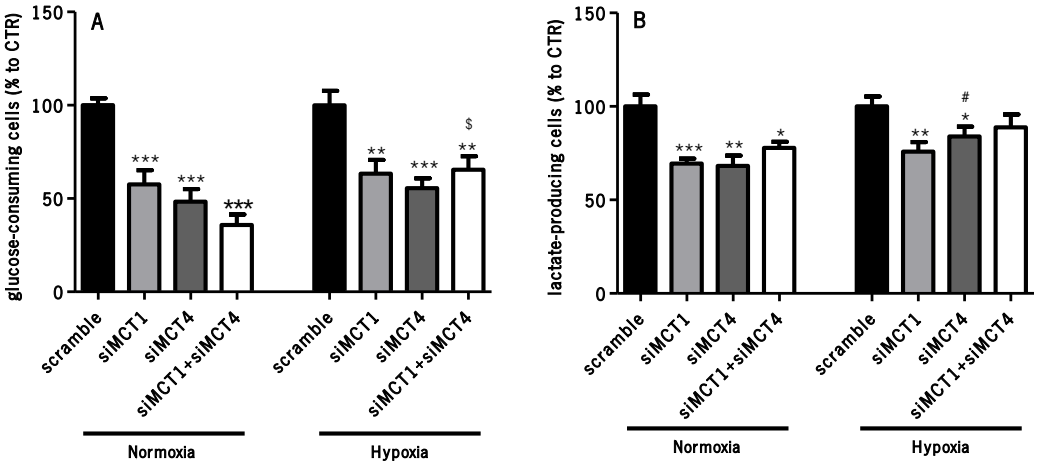


Figure 25 | Influence of MCT downregulation on endothelial cell metabolism at 24 hours, under normoxia and hypoxia. Values are depicted as normalized to the scramble group (100%). **(A)** MCT downregulation effectively reduced glucose uptake by HBMEC cell line at 24 hours, under both normoxia and hypoxia. Differences between both environmental conditions were only found when both MCT1 and MCT4 were simultaneously downregulated **(B)** Lactate efflux was also impaired upon MCT knockdown at 24 hours, under normoxia. However, only downregulation of each MCT isoform individually was able to decrease lactate production, under hypoxia. Additionally, differences between normoxic and hypoxic conditions were only encountered when MCT4 isoform was downregulated. *p<0.05, **p<0.01, ***p<0.001 comparing MCT-downregulated cells with scramble group; #p<0.05, \$$p$<0.01 comparing both normoxia and hypoxia conditions.

3.3.3 Cell Proliferation

MCT downregulation did not induce significant changes on endothelial cell proliferation at 24 hours, under normoxia (Figure 26). Interestingly, under hypoxia, downregulation of either

MCT1 or MCT4 significantly increased endothelial cell proliferation, while the knockdown of both MCT isoforms significantly reduced proliferation of HBMEC cell line (Figure 24C) and these observations were found to be significantly different comparatively to normoxia (Figure 24C).

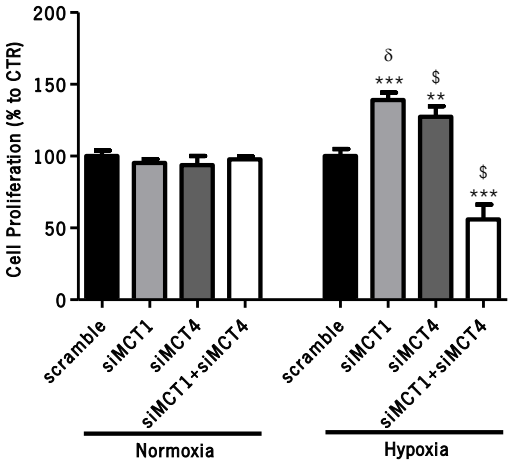
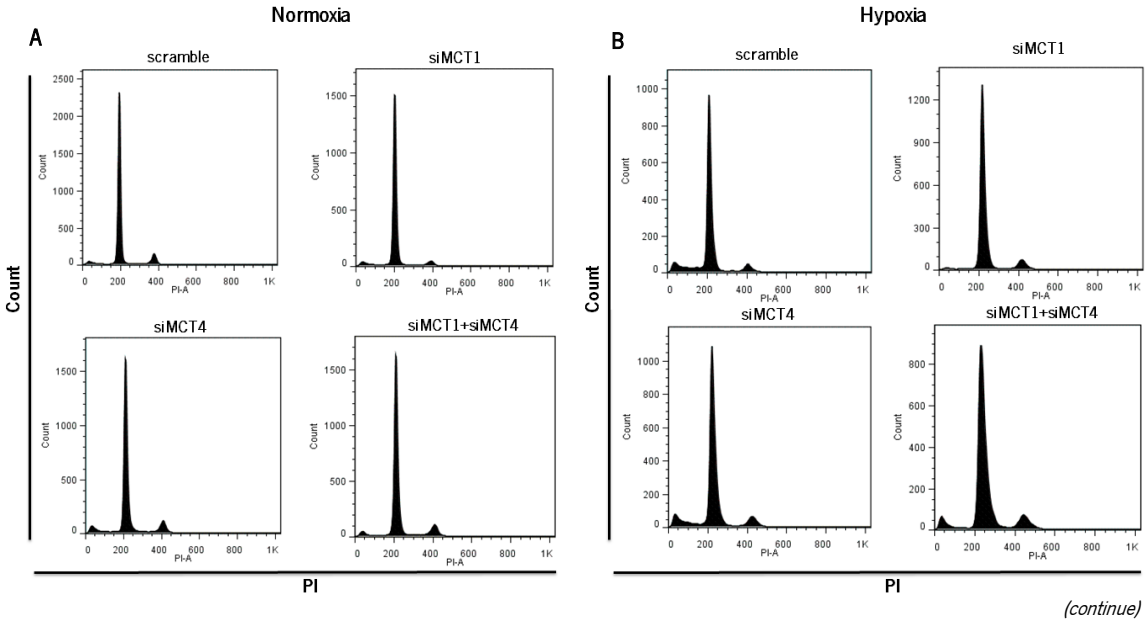


Figure 26|| Influence of MCT downregulation on endothelial cell proliferation at 24 hours, under normoxia and hypoxia. Values are normalized to scramble group (100%). At 24 hours, no significant effect on endothelial cell proliferation was found upon MCT knockdown, under normoxia. By contrast, under hypoxia, downregulation of each MCT isoform *per se* increased proliferation of HBMEC cells, while simultaneous knockdown of both MCT1 and MCT4 significantly decreased endothelial cell proliferation. Additionally, the effects observed under hypoxia were found to be statistically different when compared with normoxia. $^{**}p<0.01$, $^{***}p<0.001$ comparing MCT-downregulated cells with scramble group; $\$p<0.01$; $\delta p<0.001$ comparing both normoxia and hypoxia.

Additionally, the cell cycle of HBMEC cells was unaffected upon MCT downregulation, under both normoxia (Figure 27A and 27C) and hypoxia (Figure 27B and 27C).



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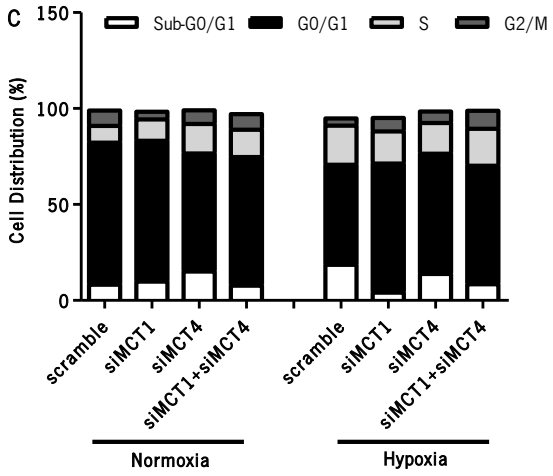


Figure 27 | Influence of MCT downregulation on cell cycle distribution at 24 hours, under normoxia and hypoxia. Representative assay of cell cycle analysis for HBMEC cells, under both normoxia and hypoxia, 24 hours after MCT downregulation had been achieved. Histograms of cell population distribution stained for PI are represented in the left panel for normoxic **(A)** and in the right panel for hypoxic **(B)** conditions (cell population in the first peak = G0/G1 phase; cell population between two peaks = S phase; cell population in the second peak = G2/M phase). **(C)** Barplot representing endothelial cell distribution in each cell cycle phase, under both normoxia and hypoxia. No differences in the HBMEC cells' cycle were found after MCT downregulation.

3.3.4 Capillary-like structure assembling *in vitro*

The formation of capillary-like structures by HBMEC cells was assessed, under normoxia and hypoxia, throughout 24 hours, after MCT downregulation.

The qualitative evaluation of images indicates that MCT1 downregulation did not affect vessel assembling *in vitro* neither at 8 or 24 hours (Figure 28). On the other hand, simultaneous downregulation of both MCT1 and MCT4 impaired the formation of capillary-like structures by brain endothelial cells at 8 and 24 hours (Figure 28).

Under hypoxia, MCT1 downregulation, alone or in combination with MCT4, did not affect the formation of capillary-like structures, at 8 hours (Figure 29). However, at 24 hours the number of capillary-like structures being assembled was found to be decreased in HBMEC cells (Figure 29). Importantly, the effects of MCT downregulation on the development of capillary-like structures were higher under normoxia than hypoxia (Figure 28 and 29).

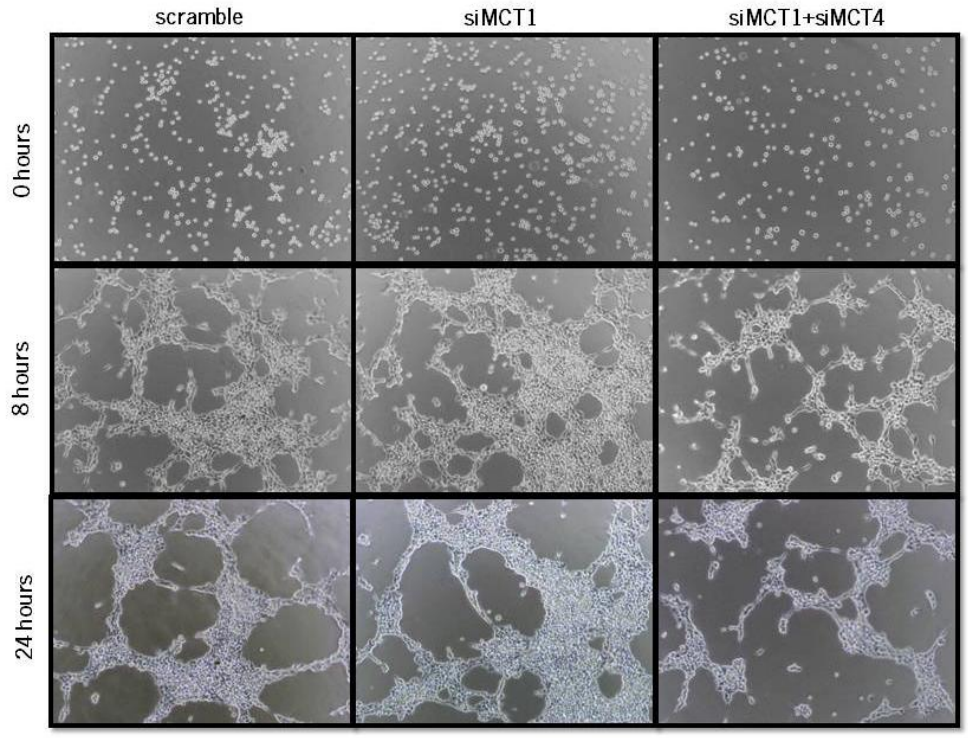


Figure 28| Influence of MCT downregulation on the assembling of capillary-like structures by HBMEC cells up to 24 hours, under normoxia. Representative images of Matrigel-based tube formation assay (100X magnification). Only the simultaneous downregulation of both MCT isoforms seems to lead to a decrease on vessel assembling *in vitro*.

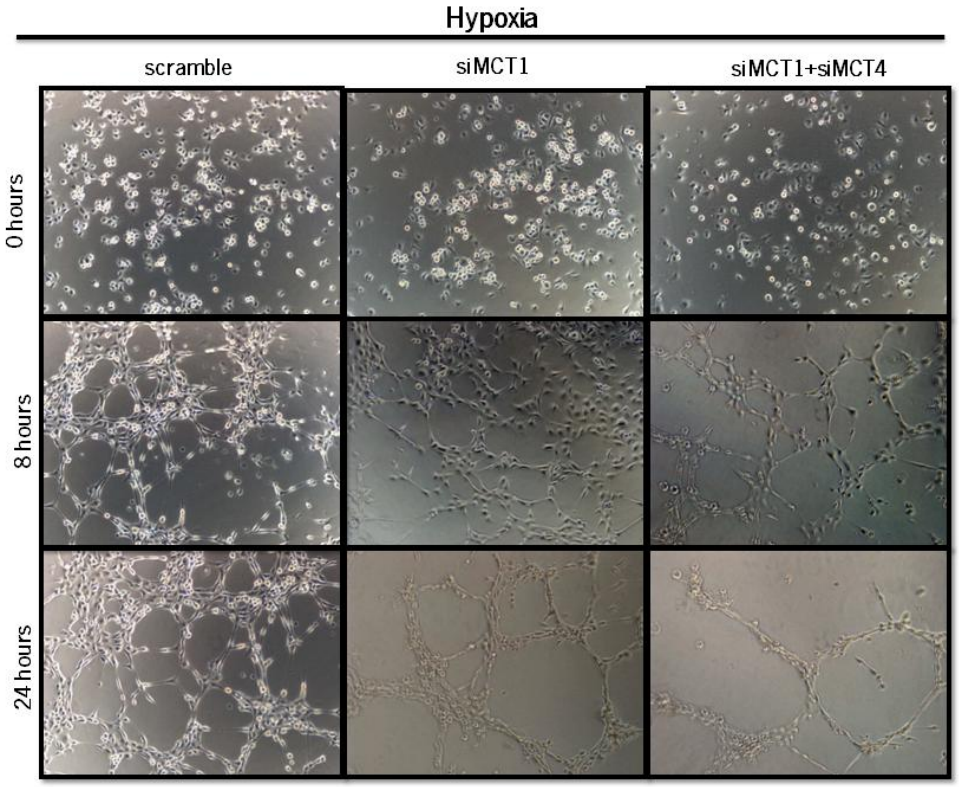


Figure 29|Effects of MCT downregulation on the development of capillary-like structures by HBMEC cells up to 24 hours, under hypoxia. Representative images of Matrigel-based tube formation assay (100X magnification). Either MCT1 or MCT1/4 knockdown decreased the number of capillary-like structures, mainly at 24 hours.

3.4 Effect of MCT downregulation on the angiogenic stimulation by glioma cells

MCT downregulation, alone or in combination, was also performed in glioma cells (U251), under normoxia and hypoxia. Then, glioma cells' conditioned media were harvested and transferred to endothelial cells to study the angiogenic capacity of tumor cells.

MCT downregulation in glioma cells, under both normoxia and hypoxia, was confirmed by WB, as represented in Figure 30.

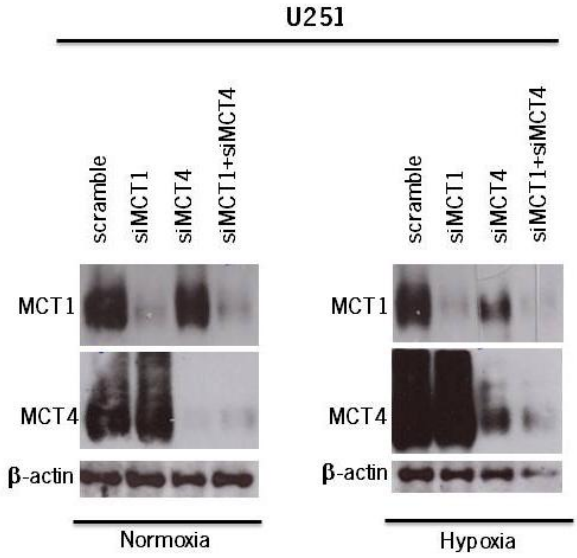


Figure 30 | Representative image of MCT expression, under normoxia and hypoxia, five days after transfection of U251 cells with siRNA. siRNA oligonucleotide sequences which were used in the transfection process effectively downregulated MCT1 and MCT4 isoforms, under normoxia and hypoxia.

3.4.1 Glioma cells' conditioned media: glucose and lactate contents

Figure 31A shows similar glucose amounts in scramble-derived glioma cells' CM from both normoxia and hypoxia, 48 hours after MCT downregulation. However, lactate levels in scramble-derived glioma cells' CM from hypoxia were significantly higher than scramble-derived CM from normoxia (Figure 31B)

Despite lacking statistical significance, MCT-silenced glioma cells' CM from both normoxia and hypoxia tended to increase glucose levels in the media, 48 hours after MCT

knockdown (Figure 31C). By contrast, there was a tendency to decrease lactate amounts in the conditioned media, under both normoxia and hypoxia, comparatively to scramble (Figure 31D).

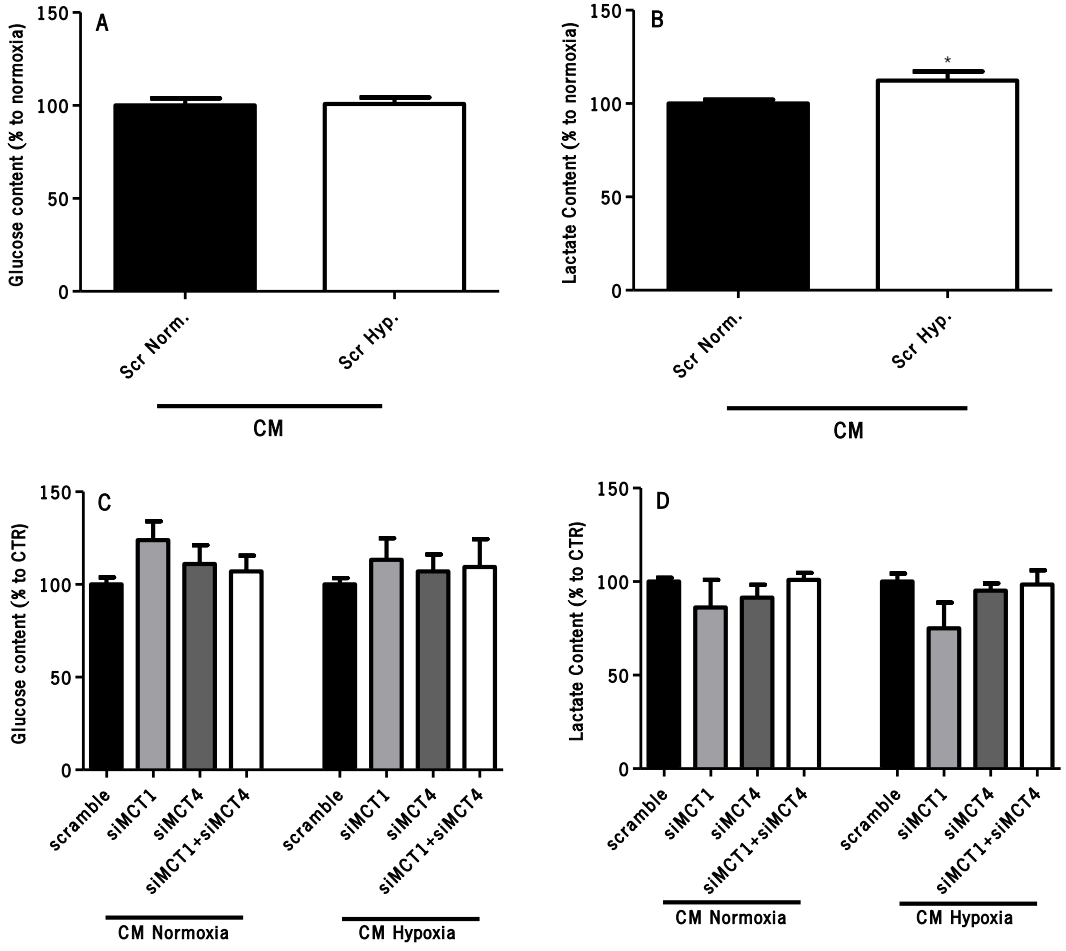


Figure 31| Glucose and lactate contents in glioma cells' conditioned media 48 hours after MCT downregulation, under normoxia and hypoxia. Glucose **(A)** and lactate **(B)** levels in scramble-derived glioma cells' CM from normoxia and hypoxia. Similar glucose amounts were found between both scramble-derived glioma cells' CM from normoxia and hypoxia **(A)**, while lactate levels were significantly increased in scramble-derived glioma cells' CM from hypoxia, comparatively to its normoxic counterpart **(B).** Glucose **(C)** and lactate **(D)** contents in glioma cells' CM from normoxia and hypoxia, 48 hours upon MCT downregulation. Comparatively to scramble, a tendency for glucose accumulation was observed in MCT-silenced glioma cells' CM, under either normoxia or hypoxia **(C)**. In opposition, lactate amounts tended to be decreased in CM, after MCT downregulation, relatively to scramble, under both normoxia and hypoxia **(D)**. *p<0.05 comparing scramble-derived CM from hypoxia and normoxia.

3.4.2 Influence of MCT downregulation on total VEGF-A gene expression

Total VEGF-A mRNA levels were evaluated, after MCT downregulation in glioma cells, under normoxia and hypoxia.

Qualitative RT-PCR revealed that total VEGF-A expression was increased, upon simultaneous downregulation of both MCT1 and MCT4, under both normoxia and hypoxia. Additionally, the induction of VEGF-A expression by hypoxia was higher, comparatively to normoxia, when comparing between scramble groups (Figure 32A).

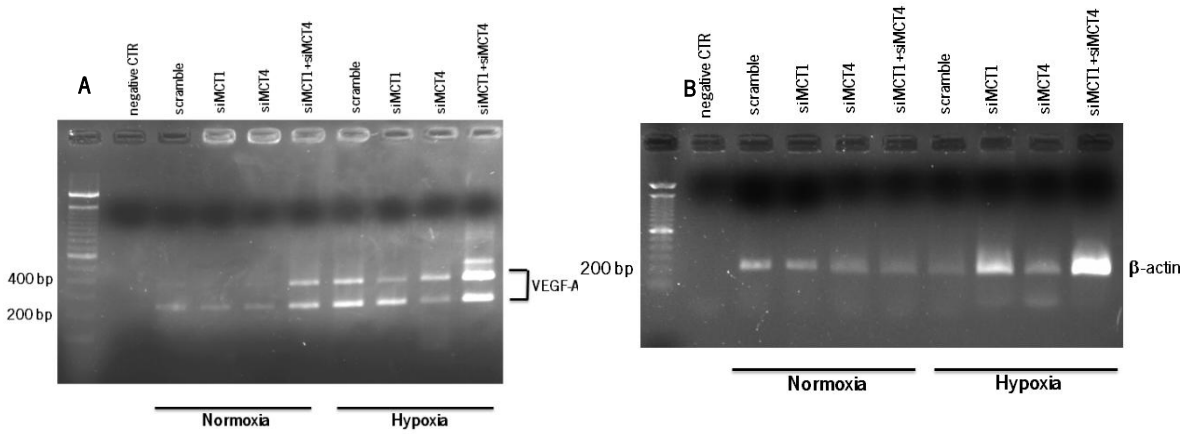


Figure 32| Qualitative evaluation of VEGF-A mRNA levels in glioma cells, upon MCT downregulation, under normoxia and hypoxia. **(A)** Simultaneous downregulation of both MCT isoforms increased total VEGF-A expression, under both normoxic and hypoxic conditions. Under hypoxia, the effects of MCT downregulation were higher. cDNA fragments were expected to be between 200 and 400 bp. **(B)** β-actin (200 bp) was used as housekeeping gene.

3.4.3 Endothelial cell survival

At 24 hours, no significant differences were found on endothelial cell viability, upon their growth in scramble-derived CM from both normoxia and hypoxia (Figure 33A).

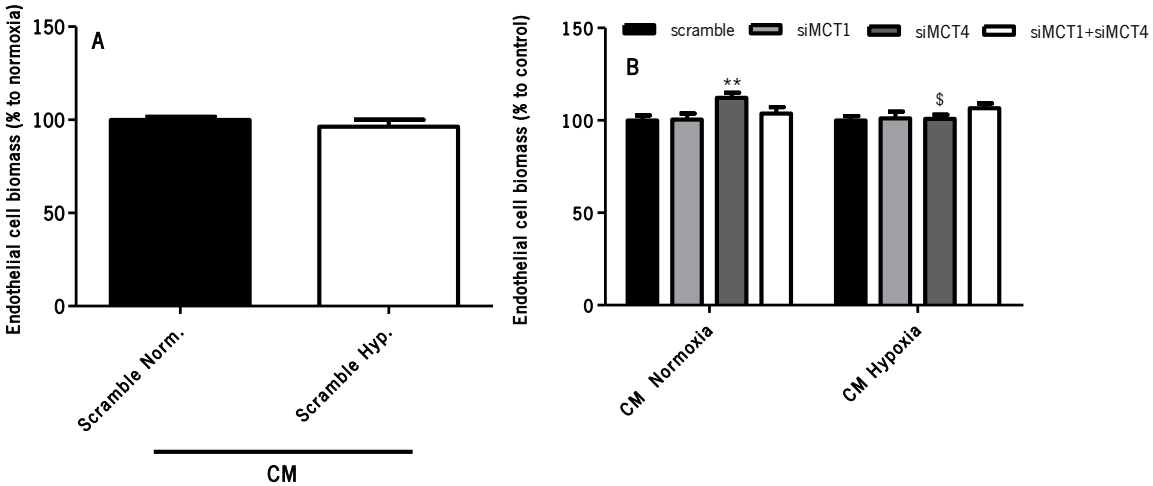


Figure 33|Effects of glioma cells' CM on endothelial cell biomass at 24 hours. Values are depicted as normalized to the control groups (100%). **(A)** Endothelial cell viability was unaffected, upon 24 hours of growth in scramble-derived CM from normoxia and hypoxia. **(B)** Only normoxia-derived CM upon MCT4 downregulation significantly improved endothelial cell survival, while its hypoxic counterparts did not have such effects. $^{**}p<0.01$ comparatively to CTR groups; $\$p<0.01$ comparatively to normoxia.

Furthermore, the effects of CM resultant from MCT downregulation in U251 cell line, under normoxia and hypoxia, were examined on endothelial cell biomass at 24 hours, under normoxia. In fact, only normoxia-derived CM upon MCT4 downregulation in glioma cells significantly improved survival of endothelial cells, comparatively with scramble group (Figure 33B). Interestingly, hypoxia-derived CM upon MCT4 downregulation in U251 cells did not induce such significant increase on endothelial cell biomass, when compared with its normoxic counterpart (Figure 33B)

3.4.4 Endothelial cell metabolism

Both glucose consumption (Figure 34A) and lactate production (Figure 34B) rates of HBMEC cells tended to be decreased after endothelial cell growth in scramble-derived CM from hypoxia in comparison to its normoxic counterparts.

In addition, MCT-silenced glioma cells' CM from normoxia tended to increase glucose uptake by brain endothelial cells, with significant effects observed in MCT4-silenced glioma cells' CM (Figure 34C) MCT-silenced glioma cells' CM from hypoxia revealed a tendency to a higher increase on glucose consumption rates of endothelial cells, comparatively to their normoxic-derived counterparts, with a significant result in the case of double-silenced glioma cells' CM (Figure 34C). However, endothelial cell growth in MCT1-downregulated glioma cells' CM significantly decreased glucose uptake rates in HBMEC cells.

Contrarily to glucose consumption rates, endothelial cells tended to decrease lactate production, when grown in MCT-silenced glioma cells' CM from normoxia, with significant effects observed in MCT1/4-downregulated glioma cells' CM (Figure 34D). In opposition, both MCT-4 and MCT1/4-downregulated glioma cells' CM from hypoxia had the opposite effect comparatively to their normoxia-derived counterparts, significantly increasing lactate production by HBMEC cells (Figure 34D).

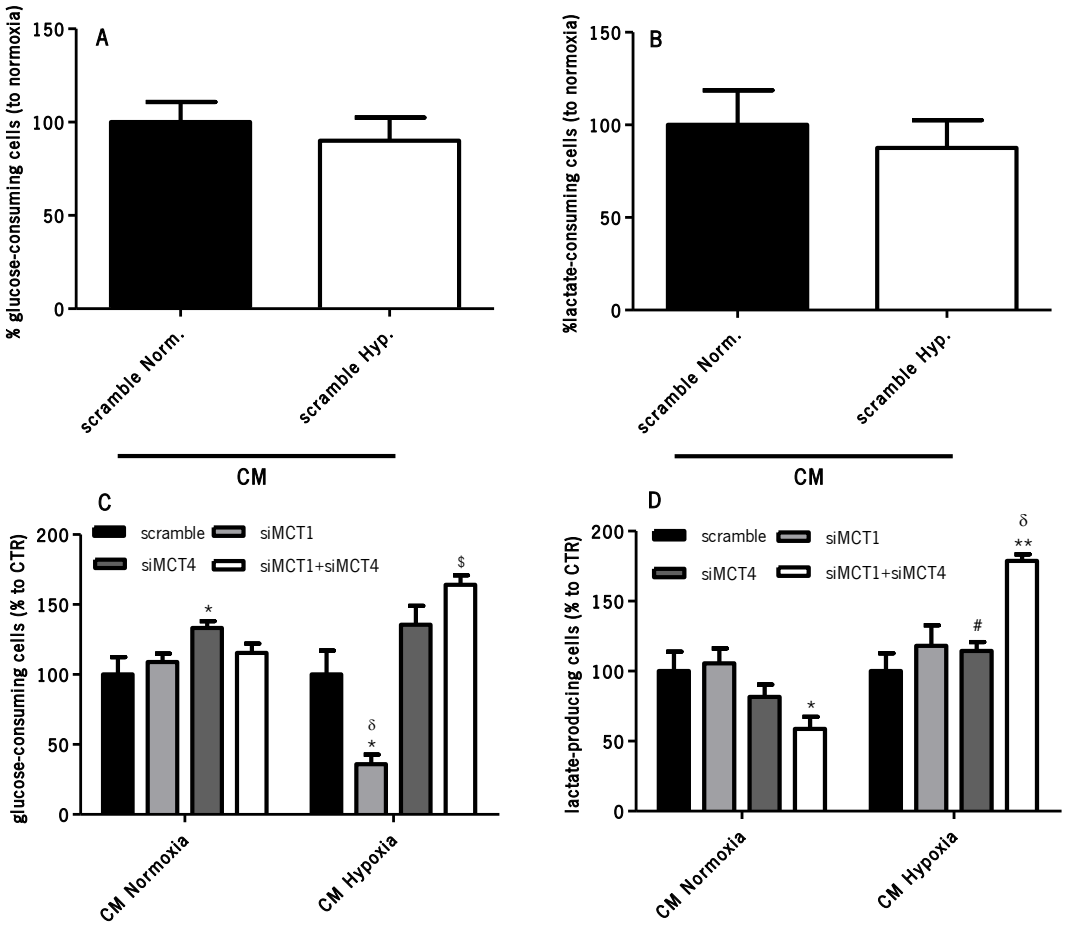


Figure 34|Effects of glioma cells' CM on endothelial cell metabolism, at 24 hours. Values are depicted as normalized to the CTR groups (100%). No significant differences were encountered on glucose uptake **(A)** and lactate efflux **(B)** by HBMEC cells, after 24 hours of growth in scramble-derived CM from hypoxia comparatively to normoxia. **(C)** MCT4-silenced glioma cells' CM from normoxia significantly increased glucose uptake by endothelial cells and, a similar tendency was observed, upon endothelial cell growth in glioma cells' CM from both normoxia and hypoxia, with the exception of MCT1-silenced glioma cells' CM from hypoxia. Interestingly, MCT1/4-downregulated CM from hypoxia induced a higher increase in glucose uptake rates of HBMEC cells, when compared to its normoxic counterparts. **(D)** Lactate production by HBMEC cells significantly decreased, upon their growth in MCT1/4-silenced glioma cells' CM from normoxia. By contrast, lactate efflux appeared to be increased, upon endothelial cell growth in MCT-downregulated glioma cells' CM from hypoxia, especially when both MCT isoforms were knockdown. *p<0.05, **p<0.01 comparatively to CTR groups; #p<0.05, δp<0.001 comparing normoxia and hypoxia.

3.4.5 Endothelial cell proliferation

There was a tendency for scramble-derived glioma cells' CM from hypoxia to increase endothelial cell proliferation, comparatively to scramble-derived CM from normoxia (Figure 35A).

The effects of MCT-silenced glioma cells' CM on endothelial cell proliferation were also evaluated (Figure 35B). Normoxia-derived CM resultant from MCT1 downregulation alone or in combination with MCT4 knockdown significantly decreased HBMEC proliferation. In addition,

MCT-silenced glioma cells' CM from hypoxia significantly decreased endothelial cell proliferation and, the effects of MCT4-silenced glioma cells' CM were higher comparatively to its normoxic counterpart (Figure 35B). In addition, no differences were found in endothelial cells' cycle, after HBMEC cells' growth in MCT-silenced glioma cells' CM from both normoxia (Figure 36A and 36C) and hypoxia (Figure 36B and 36C), comparatively to scramble groups.

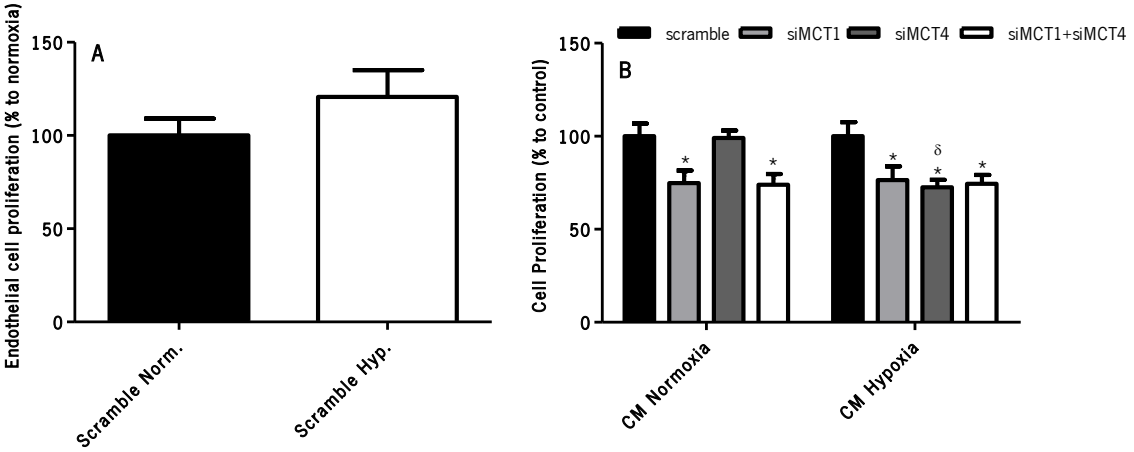
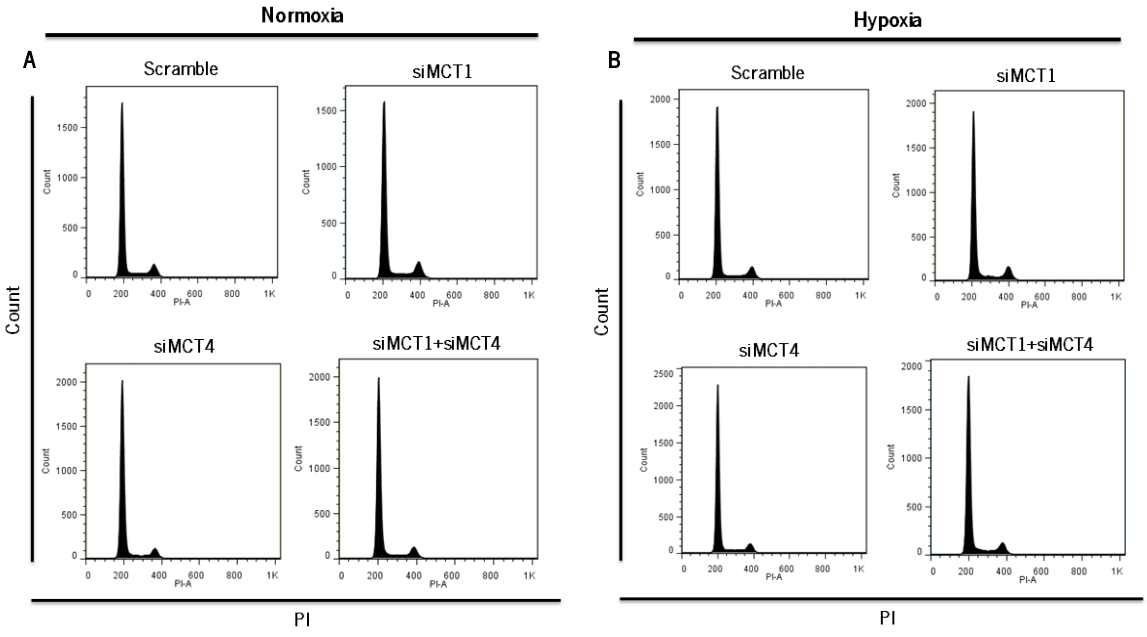


Figure 35 | Effects of glioma cells' CM on endothelial cell proliferation at 24 hours. Values are depicted as normalized to control groups (100%). **(A)** Scramble-derived CM from hypoxia tended to increase proliferation rates of HBMEC cells. **(B)** Endothelial cell proliferation was decreased, when cells were incubated with MCT1- and MCT/4-silenced glioma cells' CM from normoxia. Also, MCT-downregulated glioma cells' CM from hypoxia decreased HBMEC cells' proliferation and MCT4-downregulated CM induced a higher decrease than its normoxic-derived counterpart. *p<0.05 comparatively to control groups; δp<0.001 comparing normoxia and hypoxia.



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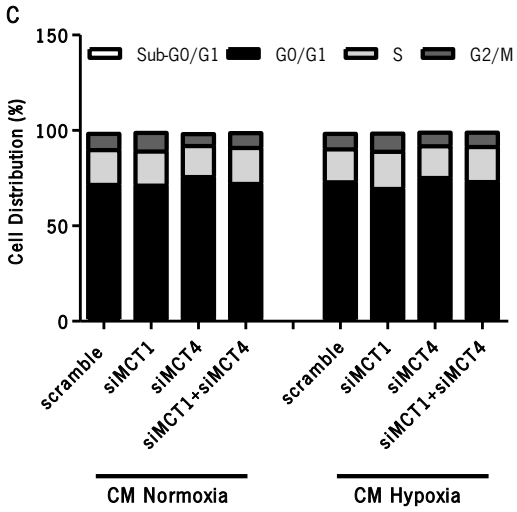

Figure 36| Representative assay of cell cycle analysis for HBMEC cells, after 24 hours of growth in MCT-silenced glioma cells' CM from normoxia and hypoxia. Histograms of cell population distribution stained for PI are represented in the upper panel for glioma cell' CM from normoxia **(A)** and in the lower panel from hypoxia **(B)** (cell population in the first peak = G0/G1 phase; cell population between two peaks = S phase; cell population in the second peak = G2/M phase). **(C)** Barplot representing endothelial cell distribution in each phase of the cell cycle, where no differences were found, upon endothelial cells' growth in MCT-silenced glioma cells' CM.

3.4.6 Capillary-like structure assembling *in vitro*

The qualitative assessment of the development of capillary-like structures, upon cell growth in MCT-downregulated glioma cells' CM, under both normoxia and hypoxia, was performed in HBMEC cells. As showed in Figure 37, MCT-downregulated glioma cells' CM from normoxia did not change the formation of capillary structures, at 8 hours, relatively to scramble-derived CM. At 24 hours, the previous assembled structures were disrupted, upon HBMEC cells' growth in MCT-downregulated glioma cells' CM (Figure 37).

In comparison with its normoxic counterparts, in hypoxia-derived glioma cells' CM, the assembling of capillary-like structures was enhanced, at 8 hours. However, no differences between scramble and MCT-downregulated glioma cells' CM were found. Although less than in normoxia-derived CM, the number of capillary-like structures were decreased at 24 hours in MCT1- and MCT1/4-downregulated glioma cells' CM (Figure 38).

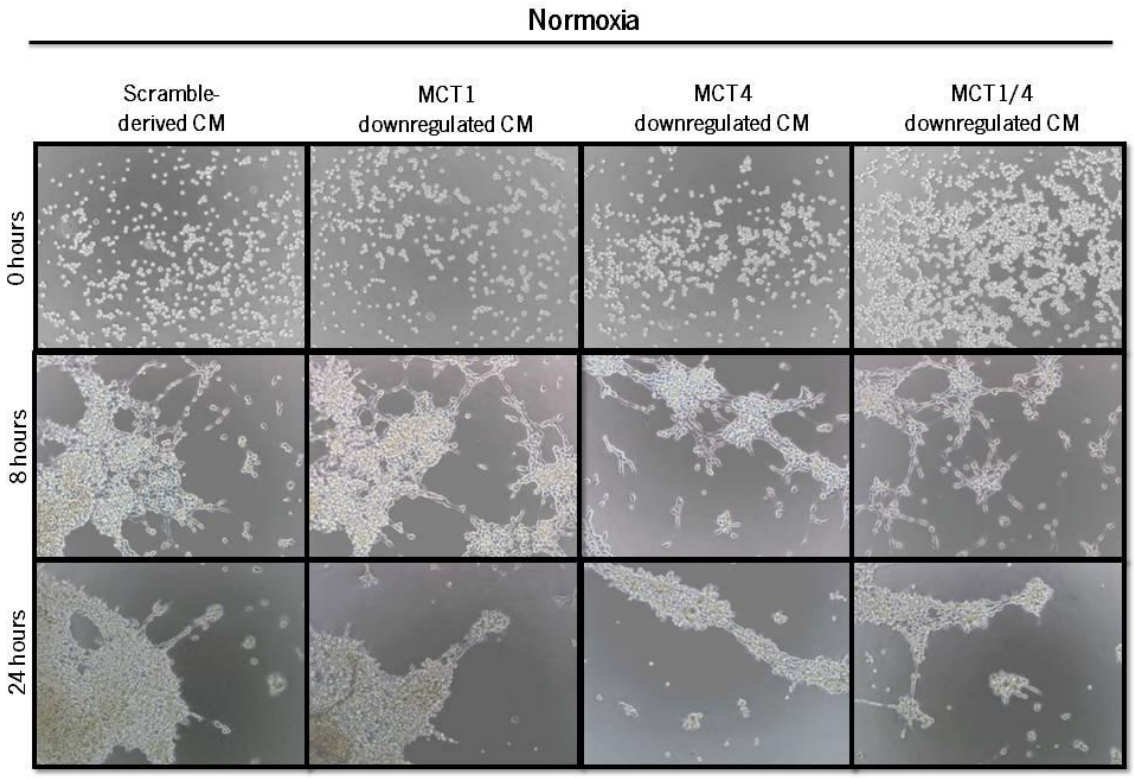

Figure 37 | Influence of MCT-silenced glioma cells' CM from normoxia in the development of capillary-like structures by HBMEC cell line. Representative images of Matrigel-based tube formation assay (100X magnification). At 8 hours, MCT-silenced glioma cells' CM did not interfere with the formation of capillary structures, comparatively to scramble-derived CM. By contrast, the previous effects were disrupted at 24 hours, upon endothelial cell growth in MCT-silenced glioma cells' CM.

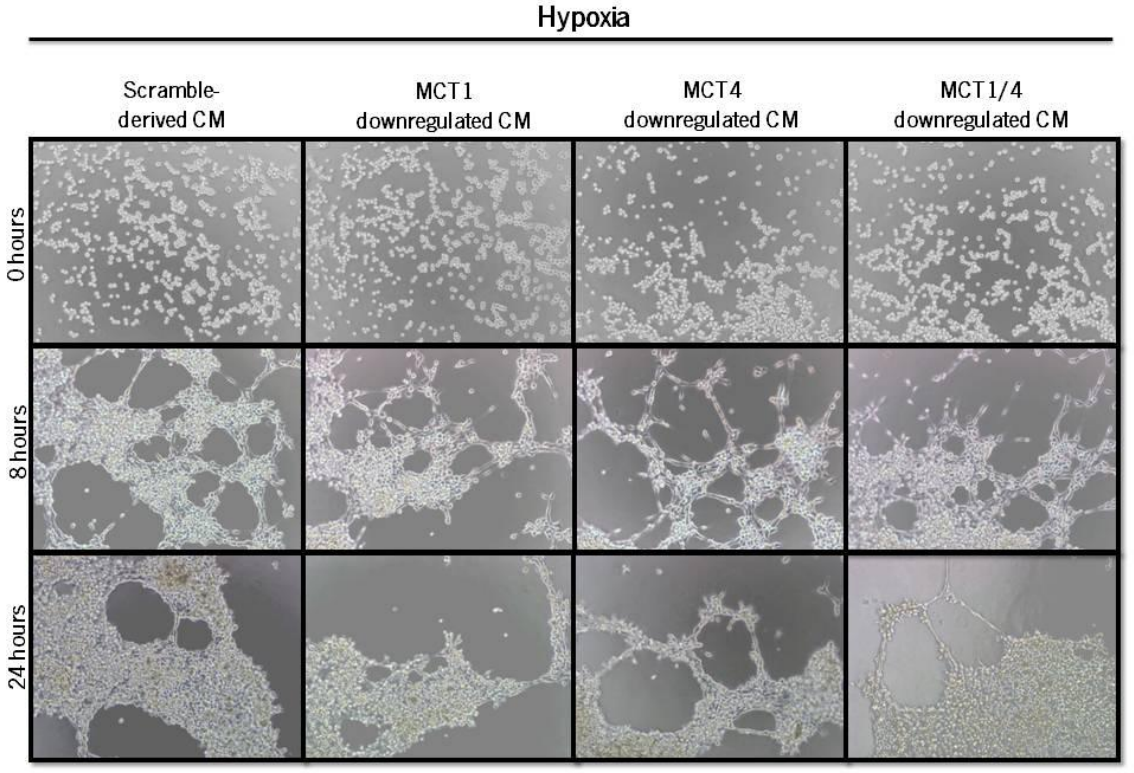

Figure 38 | Influence of MCT-silenced glioma cells' CM from hypoxia in the development of capillary-like structures by HBMEC cell line. Representative images of Matrigel-based tube formation assay (100X magnification). At 8 hours, all hypoxia-derived glioma cells' CM induced the development of capillary structures. However, the number of capillary-like structures was reduced, mainly in MCT1 and MCT1/4 glioma cells' CM, at 24 hours.

3.4.7 Influence of glioma cells' CM on CAM vascularization

In addition to the *in vitro* experiments, modulating the development of capillary-like structures, *in vivo* studies were further performed to evaluate the effects of glioma cells' CM, after MCT knockdown, under both normoxia and hypoxia, on CAM vascularization, 24 hours after injection.

Scramble-derived glioma cells' CM from both normoxia and hypoxia induced phenotypic alterations in the vascular network of chick chorioallantoic membrane, 24 hours after the injection (Figure 39). Additionally, MCT1-downregulated glioma cells' CM from both normoxia (Figure 40) and hypoxia (Figure 41) did not induce similar alterations on CAM vascularization, comparatively to the scramble group, while, the other MCT-downregulated glioma cells' CM triggered similar effects to scramble groups (Figure 40 and 41).

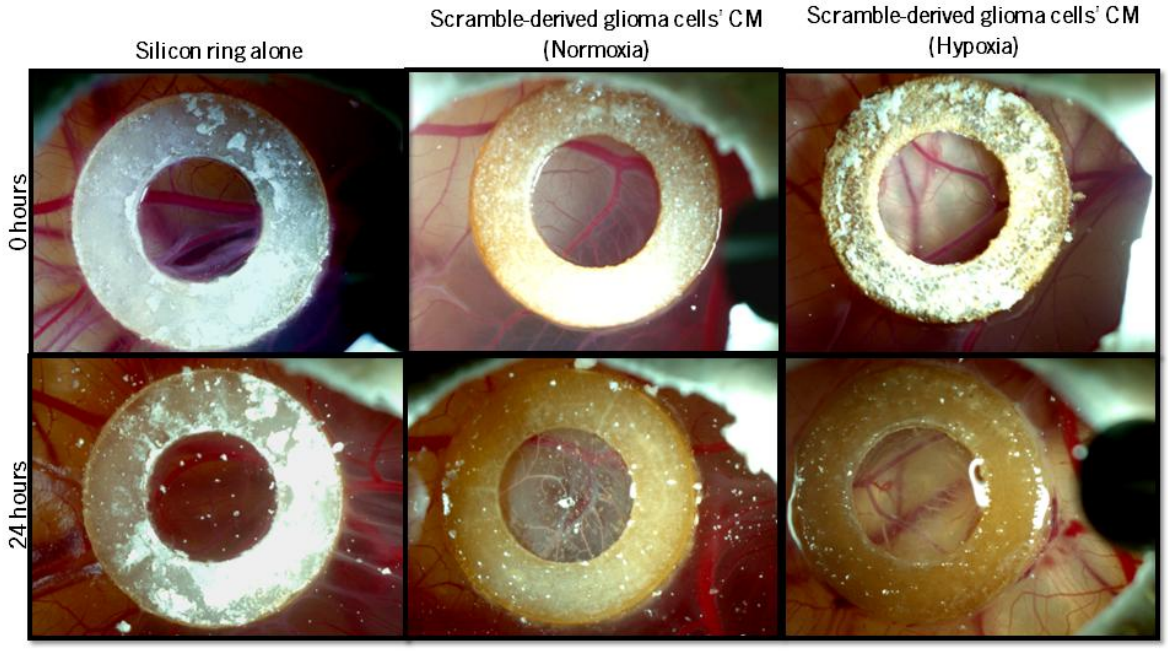

Figure 39| Effects of scramble-derived glioma cells' conditioned media from normoxia and hypoxia on CAM vascularization, *in vivo*. Representative images (10X magnification) of chick chorioallantoic membrane, 24 hours after CM injection (n=3 eggs *per* group). Conditioned media (CM) from both scramble groups induced phenotypic alteration on CAM vascularization, 24 hours after CM injection.

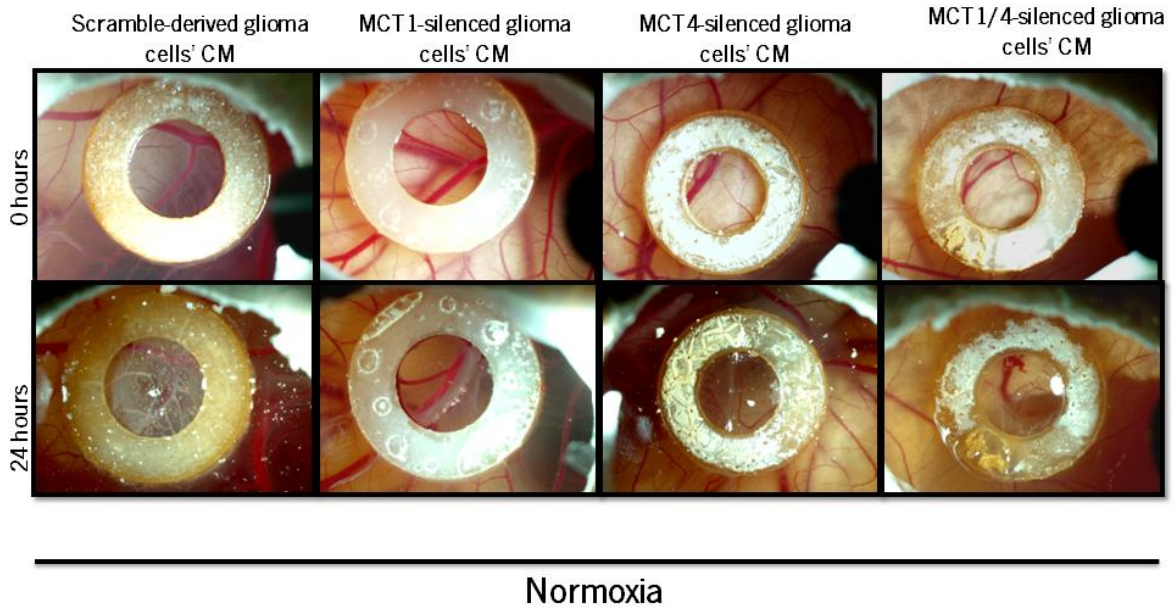

Figure 40 | Influence of MCT-downregulated glioma cells' CM from normoxia on CAM vascularization, in vivo. Representative images (10X magnification) of chick chorioallantoic membrane, 24 hours after CM injection (n=3 eggs *per* group). Comparatively to scramble group, MCT1-dowregulated glioma cells' CM from normoxia did not change the vascular network of chick chorioallantoic membrane, at 24 hours. Similarly to scramble group, both MCT4- and MCT1/4-silenced glioma cells' CM induced phenotypic alterations on CAM vascularization, 24 hours after CM injection.

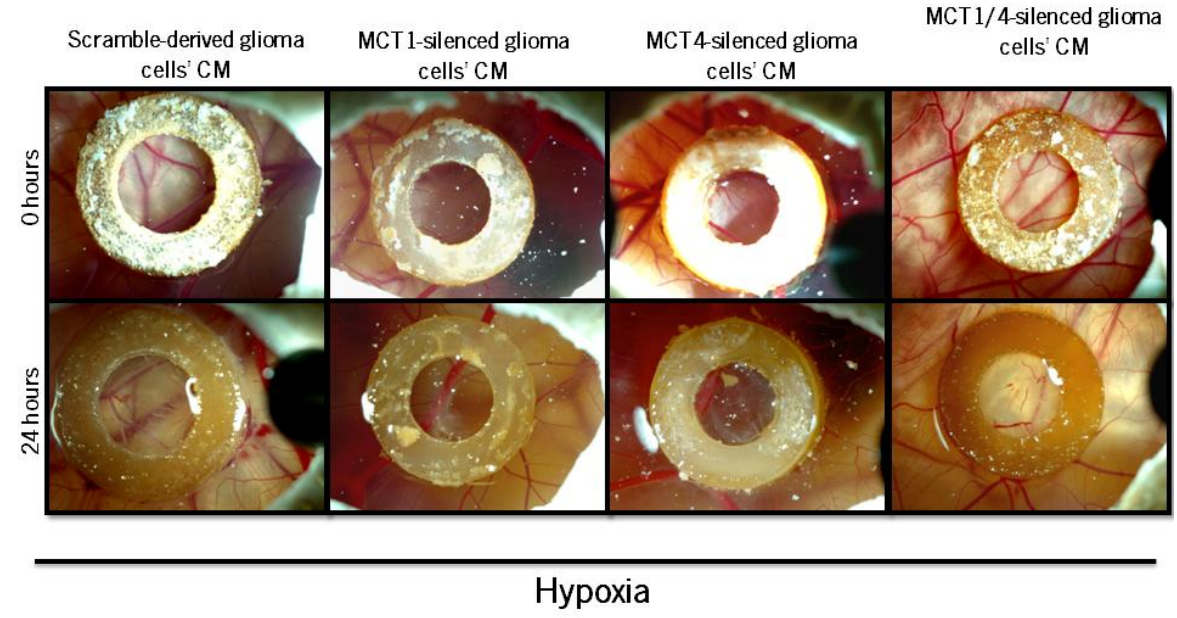

Figure 41 | Effects of MCT-downregulated glioma cells' CM from hypoxia on CAM vascularization, in vivo. Representative images (10X magnification) of chick chorioallantoic membrane, 24 hours after CM injection (n=3 eggs *per* group). Likewise glioma cells' CM from normoxia, also MCT1-dowregulated glioma cells' CM from hypoxia did not alter the vascular network of chick chorioallantoic membrane, at 24 hours, comparatively to scramble group. Similarly to scramble group, both MCT4- and MCT1/4-silenced glioma cells' CM induced phenotypic alterations on CAM vascularization, 24 hours after the CM injection.

3.5 Influence of hierarchical fibrous scaffolds on the maintenance of tumor and endothelial cells' function

3.5.1 Cell Viability

Hierarchical fibrous scaffolds, resembling the complexity and the hierarchical organization of the natural ECM, were used to assess the influence of a 3D environment on the maintenance of tumor and endothelial cells' functions. To achieve that propose, human glioma cells (U251 cell line) and human brain microvascular endothelial cells (HBMEC cell line) were individually seeded into those polymeric structures and the cell viability evaluated at 1, 3 and 7 days of culture, using the standard MTS assay.

Endothelial cells' viability was significantly increased upon their seeding into hierarchical fibrous scaffolds at day 7 (Figure 42A), while tumor cell viability started to be impaired at day 3 and remained decreased up to day 7 (Figure 42B), comparatively to day 1 (i.e. 24 hours after cell seeding and adhesion to the 3D polymeric structure) . Comparing both culture systems, significant differences in cell viability were only found in U251 cell line, at day 3, where glioma cells' viability appeared to be increased in the standard 2D system (Figure 42B).

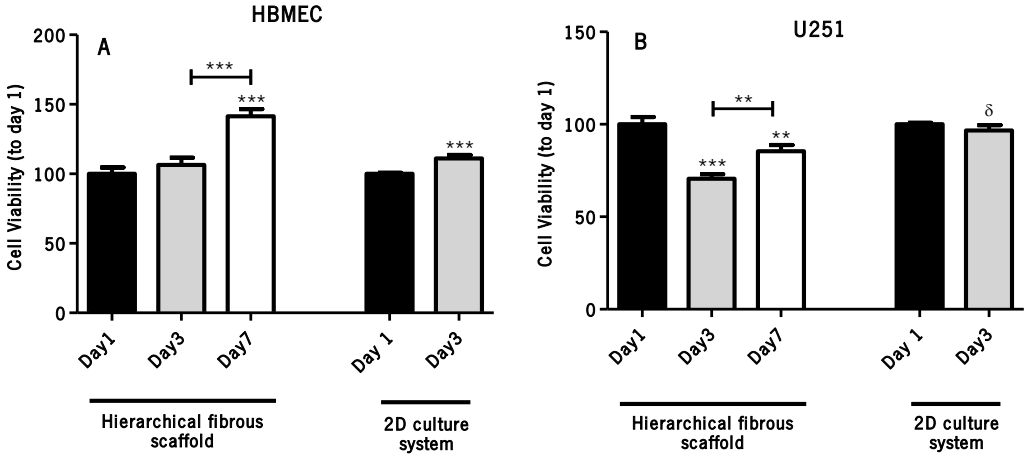

Figure 42 | Influence of hierarchical fibrous scaffolds on cell viability. (A) Endothelial cell viability was significantly increased at day 7, relatively to day 1. **(B)** Tumor cell viability was significantly impaired at day 3 and remained decreased at day 7, comparatively to day 1. At day 3, glioma cells' viability was enhanced in 2D culture system, comparatively to hierarchical fibrous scaffolds $^{**}p<0.01$, $^{***}p<0.001$ comparing days of culture; $\delta p<0.001$ comparing the hierarchical fibrous scaffolds with the standard 2D culture system.

3.5.2 Cell Metabolism

In addition to cell viability, cell metabolism was also evaluated through determination of glucose consumption and lactate production, upon cell seeding into the hierarchical fibrous scaffolds, up to 3 days.

At day 3, both glucose uptake (Figure 43A) and lactate production (Figure 43B) rates were significantly increased in endothelial cells, seeded into hierarchical fibrous scaffolds, comparatively to day 1.

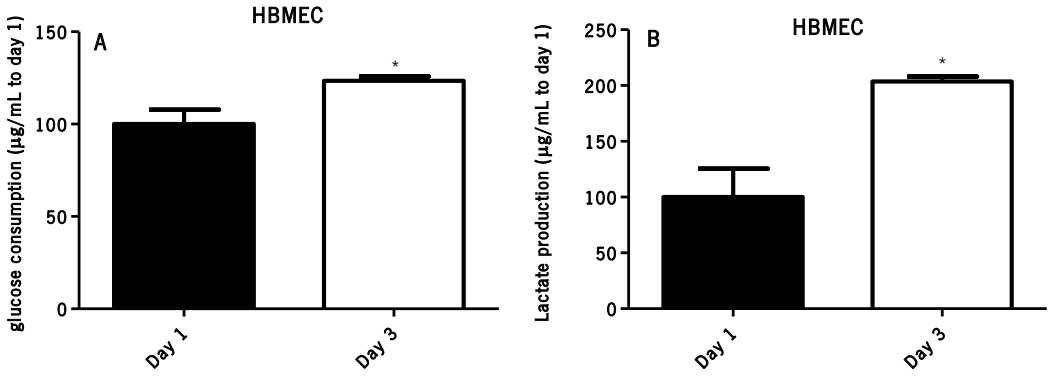

Figure 43 | Influence of hierarchical fibrous scaffolds in human endothelial cells' metabolism. Both glucose consumption **(A)** and lactate production **(B)** rates were significantly improved at day 3, in HBMEC cells, comparatively to day 1. *p<0.05 comparing both day 3 and 1.

Similarly, a significant increase in glucose consumption (Figure 44A) and lactate production (Figure 44B) were observed in glioma cells. Furthermore, these effects on cell metabolism were higher in tumor than in endothelial cells.

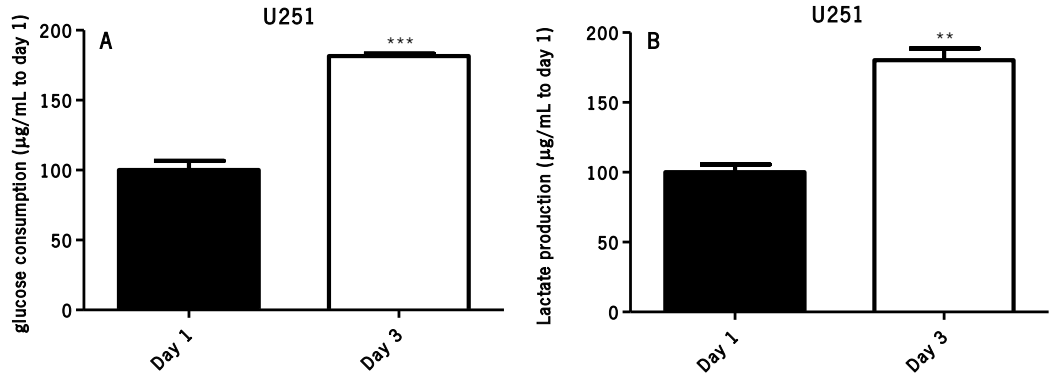

Figure 44 | Influence of hierarchical fibrous scaffolds in human glioma cells' metabolism. Glucose uptake **(A)** by U251 cells was significantly increased, accompanied by an increase in lactate production **(B)** at day 3, relatively to day 1. **p<0.01, ***p<0.001 comparing both day 3 and 1.

4 Discussion

During hyperplasic growth of tumors, there is an impairment in both nutrient and oxygen supply to the neoplastic cells located far away from blood vessels, which would limit tumor progression. However, cancer cells have acquired the ability to develop their own vasculature, commonly from the preexisting blood vessels – tumor angiogenesis. Besides its role in supporting tumor growth, angiogenesis contributes for tumor aggressiveness, since it provides a key mechanism through which cancer cells disseminate via the blood stream to distant organs, to develop metastasis [34].

Tumor blood vessels exhibit some structural and functional abnormalities [29], giving rise to hypoxic regions within tumors [45]. As a consequence, tumor cells are able to reprogram their energy metabolism from oxidative phosphorylation to increased glycolytic rates, regardless of oxygen availability - "Warburg effect". This upregulation of glycolysis leads to the production of large amounts of lactic acid, which is released to the tumor milieu through MCTs, contributing for malignant progression, invasion and metastasis [137]. The pro-angiogenic effects of lactate have been described over the time. Nevertheless, the influence of hypoxia, as well as the role of MCTs on the angiogenic capacity of tumor cells is still an unanswered question.

In the present study, hypoxia induced an increase in both MCT1 and MCT4 total expression, in brain endothelial cells, together with an increase in the plasma membrane expression for MCT1. However, comparatively with MCT1 levels, which were found mainly at the plasma membrane, immunocytochemical analysis revealed only a slight increase of MCT4 at the plasma membrane under hypoxia, remaining the greatest part of the protein in the cytoplasm. Such MCT4 expression in the cytoplasm may be related with its role in lactate-pyruvate shuttles located in intracellular organelles (e.g. mitochondria) [223, 224]. Accordingly, both GLUT-1 and CAIX expressions were slightly increased at the plasma membrane of endothelial cells, under hypoxia, whereas the expression levels of other glycolytic markers, including HKII, PDH and PDK, were not affected by hypoxic conditions. Importantly, endothelial cells maintained their high LDH-V levels, under hypoxia. It is important to highlight that our results from immunocytochemistry suggest a higher increase on the expression of certain metabolic markers than the one observed

in WB analysis. Importantly, immunocytochemical approach has the advantage of allowing the evaluation of protein localization, which is not possible using Western blotting. Consistent with these results, glucose consumption rates tended to be enhanced, along with a significant increase on lactate production, under hypoxia, by HBMEC cells, which, in turn, will prepare endothelial cells for rapid sprouting and migration [225].

There are still some controversies around MCT1 function in endothelial cells. Some literature points at MCT1 as an effector molecule, performing lactate uptake in endothelial cells [207, 219, 220]. In opposition, other studies report MCT1 as a mediator on lactate efflux from corneal endothelium to anterior chamber, due to the high lactate gradient from cornea (≅13mM) to anterior chamber (≅7mM) [212, 214]. However, studies addressing MCTs' contribution in endothelial cell response to hypoxia are inexistent. In the present work, similarly to what is already described in corneal endothelium, our data suggest the involvement of MCT1 and, in at lower extent MCT4, on lactate efflux from brain endothelial cells, mainly under hypoxia. These observations are also supported by increased expression of their chaperone proteins, CD147 and CD44, at plasma membrane of HBMEC cells, under these conditions.

In order to understand the biological role of MCTs on the maintenance of endothelial cell function, under hypoxia, several *in vitro* assays were performed, using both inhibition of MCT activity and MCT downregulation. CHC was used as an inhibitor of MCT activity, namely because it is the best described MCT inhibitor in the literature, having MCT1 as primary target [118]. However, in the recent years, CHC specificity has been questioned, since it is likely to have additional targets (e.g. chloride-bicarbonate exchanger AE1) [84] and, because of that, we also performed specific MCT downregulation using siRNA, to validate the results obtained with CHC.

Our results revealed sensitivity of brain endothelial cells to CHC, under both normoxia and hypoxia. Since MCT1 was found to be upregulated under hypoxic environments, we expected a higher sensitivity of HBMEC cells to CHC inhibition, under these conditions. However, hypoxia did not sensitize brain endothelial cells to CHC. Despite lacking higher sensitivity to CHC under hypoxia, there was a tendency for increased endothelial cell death, especially under those conditions, suggesting the potential cytotoxic effects of CHC in HBMEC cells.

In opposition to normoxia, where CHC effects on endothelial cell viability seem to be due to impairments on lactate transport activity, in hypoxic environments, such association is unlikely

to occur, since CHC-treated cells exhibited similar lactate production rates compared to CTR group, despite their lower glucose uptake rates. These similar levels of lactate production between both CTR and treated groups may be due to an increase on MCT4 expression at endothelial cell plasma membrane, as a compensatory mechanism of MCT1 inhibition, under hypoxia. Actually, Ullah *et al.* reported MCT4 overexpression under hypoxia, in a HIF-1α-mediated mechanism [112].

According to the previous results, CHC decreased endothelial cell proliferation, under both environmental conditions. The highest CHC concentration decreased endothelial cell proliferation around 50%, under both normoxia and hypoxia, which was accompanied by an increased tendency for endothelial cell death, suggesting the cytotoxic effects of CHC at higher concentrations. The lowest CHC concentration also decreased endothelial cell proliferation, more effectively under normoxia, together with a tendency to increase endothelial cell death, suggesting that, depending on the environmental condition, lower doses of CHC may also trigger cytotoxic or cytostatic effects in endothelial cells. These effects on endothelial cell proliferation under hypoxia are likely to occur, due to other mechanisms rather than lactate transport inhibition, as the lactate transport activity performed by HBMEC cells seems to be unaffected upon treatment. Since there was a decrease in endothelial cell proliferation rates, after CHC treatment, cell cycle analysis seems to reveal a tendency for increased endothelial cell distribution in G2/M phase, upon CHC treatment, suggesting a cell cycle arrest in that phase of the cell cycle. Accordingly, it seems that CHC may promote alterations in endothelial cells' DNA, avoiding the progression of cell cycle.

The development of capillary-like structures also tended to be disrupted upon endothelial cells' treatment with CHC. However, our results are somehow inconclusive, mainly under normoxia, since not even non-treated HBMEC cells were able to assemble well-defined capillary-like structures. Therefore, this approach need to be further optimized regarding, for instance, cellular density and fetal bovine serum content.

Altogether, our evidence demonstrate the importance of MCT1 on the maintenance of endothelial cell function, mainly under normoxia, decreasing both endothelial cell viability and proliferation, which seems to be related with reduced lactate transport activity, under these conditions. Under hypoxia, MCT1 inhibition did not affect endothelial cells in the same way,

which may be due to MCT4 upregulation, as a compensatory mechanism. However, this fact needs further investigation.

Thus, we intended to confirm the role of MCTs on endothelial cell function, under normal conditions and in response to hypoxia, performing MCT downregulation experiments using siRNA.

Similarly to CHC, MCT1 downregulation decreased endothelial cell biomass, mainly under hypoxia, whereas no significant differences in endothelial cell survival were found upon MCT4 knockdown. Importantly, when both isoforms were simultaneously downregulated, total endothelial cell biomass was decreased around to 50 and 60%, under normoxia and hypoxia, respectively. Accordingly to CHC experiments, this impairment on cell viability, observed under normoxia, may be partially due to a disruption on lactate transport activity, suggesting the role of MCTs on the maintenance of endothelial cell function, under these conditions.

A disruption in glucose consumption rates, without interference on lactate production suggests that endothelial cells are producing lactate from other sources (e.g. glutamine, through glutaminolysis). Indeed, endothelial cells effectively transport and utilize glutamine as fuel, mainly when glycolytic metabolism is decreased [225, 226]. In addition to MCT1 and MCT4, there are other MCT isoforms, such as MCT2, performing lactate transport activity [81, 86]. Accordingly, previous results from our group demonstrated MCT2 expression in the cytoplasm of HBMEC cells, under normoxic conditions (Freitas-Cunha, M.: unpublished data). Additionally, some authors have documented the expression of MCT2 in mammalian endothelial cells [215, 216, 227], and other have suggested its role in lactate efflux [214]. Hence, we may hypothesize that MCT2 is overexpressed in brain endothelial cells after simultaneous downregulation of MCT1 and MCT4, mainly under hypoxia. There are some controversies concerning MCT2 expression and hypoxia. Hypoxia increased MCT2 expression in human breast cancer cell lines, whereas MCT2 expression in brain cancer ones was decreased [228]. Also, in adipose tissue, MCT2 mRNA levels were found to be downregulated under hypoxia [114]. Additionally, in rat hippocampal samples, MCT2 expression was decreased, upon the induction of intermittent hypoxia [229]. However, studies addressing the influence of hypoxia on MCT2 expression in endothelial cells are inexistent.

Regarding endothelial cell proliferation, measured by BrdU incorporation, no significant differences were found upon MCT downregulation, under normoxia. In addition, under hypoxia,

MCT1 and MCT4 downregulation, *per se,* improved proliferation rates of HBMEC cells, whereas double-MCT1/4 knockdown decreased endothelial cell proliferation, similarly to the highest CHC concentration. In a hypoxic microenvironment, such decrease in endothelial cell proliferation rates, upon MCT1/4 downregulation is supported by increased cell death, under these conditions, as indicated by Annexin V/PI staining. However, the same approach should be performed upon individual knockdown of MCT1 and MCT4, in order to validate the results from BrdU. Additionally, cell cycle analysis did not support our results from BrdU obtained under hypoxic conditions, but the results presented are only from one assay. Thus, our conclusions regarding this matter are limited and need further investigation.

Altogether our results seem to indicate that, under normoxia, as expected, impairments in endothelial cell viability may be due to a disruption in lactate transport activity, when MCT1 was downregulated, alone or in combination with MCT4. Under these conditions, MCT2 seems to have no influence on lactate efflux, since its expression remained in the cytoplasm (Freitas-Cunha, M.: unpublished observations). Under hypoxic conditions, a decrease in endothelial cell viability may be explained through a decrease in HBMEC proliferation rates, which were accompanied by a tendency to increase endothelial cell death, after simultaneous downregulation of MCT1 and MCT4 isoforms, in spite of no differences being found in lactate transport activity. A reasonable explanation may be the upregulation of MCT2 at the plasma membrane of brain endothelial cells, under hypoxia.

In agreement with the previous results, the development of capillary-like structures were also found to be decreased 24 hours after MCT1/4 downregulation had been achieved, mainly under normoxia, which once again may be associated with the impairment of lactate transport activity, under these conditions. In contrast, under hypoxia, a decreased number of capillary-like structures may be due to decreased proliferation rates, which were observed upon simultaneous downregulation of MCT1 and MCT4. This impairment observed under hypoxia, without influence of lactate transport activity may be related with potential alterations in downstream signaling cascades which, in turn, would compromise endothelial cell function.

Accordingly to the previous results from CHC, results from MCT downregulation point out the importance of the glycolytic phenotype and MCT1/4 expression on the maintenance of endothelial cell function, mainly under normoxia. In contrast, under hypoxia, the absence of both

MCT1 and MCT4 seems to be counterbalanced, which may be due to the upregulation of MCT2 at the plasma membrane of brain endothelial cells, under these conditions.

After addressing the role of MCTs on endothelial cell response to hypoxia, we decided to evaluate their influence on tumor-endothelial cell interplay. For that, MCT downregulation was performed in U251 glioma cells, under normoxia and hypoxia. Then, the resultant conditioned media (CM) was transferred to endothelial cells, in order to understand its effects on endothelial cell function.

As expected, scramble-derived glioma cells' CM from hypoxia exhibited higher lactate levels relatively to its normoxic counterpart, despite the similarities in glucose amounts. In fact, tumor hypoxia has been associated with lactate overproduction, contributing for the acidification of tumor microenvironment and, consequently, for tumor aggressiveness [230]. Despite lacking statistical significance, either individual or simultaneous MCT downregulation tended to increase glucose amounts and reduce lactate production, under both normoxia and hypoxia. These data seem to support the expression of other transporters performing lactate efflux (e.g. SMCTs), especially when both MCT1 and MCT4 isoforms are simultaneous downregulated.

Upon growth in MCT4-silenced glioma cells' CM from normoxia, HBMEC cells' total endothelial cell biomass was significantly enhanced. However, the effects of the hypoxia counterpart were lower. The maintenance of endothelial cell viability, after their growth in MCT-downregulated glioma cells' CM may be due to metabolic adaptations occurring in endothelial cells under conditions of nutrient deprivation, which have been reported as mediated by VEGF-A signaling [231].

Endothelial cells' grown in MCT4- and MCT1/4-downregulated glioma cells' CM from hypoxia tended to improve its glucose consumption rates, consequently increasing lactate efflux. This may be related with VEGF-A mRNA levels, which were found to be upregulated, upon MCT knockdown in glioma cells, mainly, as expected, under hypoxia. In fact, in energy-depleted tumor cells, the stabilization of VEGF-A mRNA levels through AMPK activation has been reported [232] and, in brain endothelial cells VEGF-A increases GLUT-1 expression via Akt signaling, which may promote glycolytic energy production for angiogenic growth [233]. Furthermore, intracellular lactate accumulation has been demonstrated to decrease NAD^+ pool which, in turn, inhibits ADP-ribosylation processes, in macrophages, upregulating their angiogenic activity [234], via VEGF

synthesis [235]. ADP-ribosylation regulates several enzymes activities, as well as the function of many nuclear proteins, including proteins involved in DNA repair and gene expression [236]. Accordingly, it is suggested that ADP-ribosylation may regulate VEGF transcription, in a lactate-mediated mechanism [235]. Conversely, HBMEC cells' growth in MCT1-downregulated glioma cells' CM decreased its glucose consumption rates, without changing lactate production rates. Actually, the greatest metabolic impairment in glioma cells was observed upon MCT1 knockdown, which tended to decrease lactate amounts in the correspondent CM. In accordance, we may hypothesize that glutamine levels in the CM are high, comparatively to the other MCT-downregulated glioma cells' CM and, for that reason, it may constitute a preferential metabolic source for brain endothelial cells. In contrast, lactate production rates of endothelial cells tended to be decreased, after growth in MCT-downregulated glioma cells' CM from normoxia, with significant effects observed in MCT1/4-silenced glioma cells' CM. In opposition, glucose uptake rates tended to be enhanced upon endothelial cells' growth in MCT-silenced glioma cells' CM, with significant effects observed in MCT4-downregulated CM. Probably, under these conditions, endothelial cells drive glucose to biosynthetic pathways (e.g. nucleotide synthesis), while they take up lactate, through MCT1 and, then channelize it into TCA cycle and fuel OXPHOS to supply their energetic demands, since oxygen is available. Accordingly, some studies have recently shown that exogenous lactate and its subsequent uptake by endothelial cells through MCT1 stimulates autocrine NF-κB signaling pathway, promoting endothelial cell migration and the development of capillary-like structures [219]. Further experiments revealed that MCT1 inhibition on human umbilical vein endothelial cells (HUVEC) decreased lactate-induced angiogenesis [220]. Hence, increased amounts of exogenous lactate and its consumption by endothelial cells seem to contribute for survival in glucose-deprived environments [225].

Despite VEGF-A mRNA upregulation in glioma cells' after MCT downregulation, endothelial cell proliferation was found to be decreased, after HBMEC growth in both MCT1- and MCT1/4-downregulated glioma cells' CM from normoxia, while hypoxia-derived glioma cells' CM, upon MCT knockdown, significantly decreased endothelial cell proliferation. These observations were not supported by cell cycle analysis, probably due to the reduced number of experiments. However, a decrease in endothelial cell proliferation may be due to both post-transcriptional modifications in VEGF-A transcripts, preventing its complete translation into protein and post-translational modifications, which would influence protein expression levels. Secondly, some

VEGF-A spliced variants are not diffusible forms [61], thus the mitogenic effects of VEGF-A may not be observed, since we have used glioma cells' CM. Furthermore, the development of capillary-like structures was unaffected at early hours of incubation with MCT-silenced glioma cells' CM, mainly under hypoxia. However, a decrease on the capillary-like structures assembling was observed at 24 hours, especially under hypoxia. These facts suggest the necessity of high levels of VEGF-A, as well as a continuous VEGF-A signaling to maintain vascular integrity.

Interestingly, *in vivo* experiments revealed that exposure to MCT1-downregulated glioma cells' CM from normoxia and hypoxia did not affect the vascular network of chick chorioallantoic membrane, at 24 hours, while, the other MCT-silenced glioma cells' CM triggered similar effects to scramble groups, inducing phenotypic alterations on CAM vascularization. Curiously, the MCT1-downregulated glioma cells' CM from both normoxia and hypoxia exhibited lower lactate amounts, while, the other MCT-silenced glioma cells' CM tended to exhibit similar lactate levels to scramble groups. Thus, we may hypothesize that the pro-angiogenic effects of tumor-derived lactate may be so powerful, as to damage the developing chick chorioallantoic membrane. Despite the effects which were observed, the number of experiments should be increased to support these first *in vivo* results.

In addition to the 2D experiments, we intended to study the influence of an *in vitro* 3D culture system, on the maintenance of tumor and endothelial cell functions, using hierarchical fibrous scaffolds. Therefore, we started to assess, individually, whether tumor and endothelial cells would be able to maintain their functionalities in those systems.

Considering day 1 as reference, because it comprises alive and adhered cells to the 3D polymeric structure, human endothelial cells' viability was significantly improved when seeded into the hierarchical fibrous scaffolds, whereas human glioma cells' survival was found to be impaired, remaining decreased until day 7. Concerning cell metabolism, both endothelial and tumor cells significantly increased the amounts of glucose consumed and lactate produced at day 3, comparatively to day 1. Furthermore, those differences in glucose consumption and lactate production appear to be higher in glioma cells than in endothelial cells. Although glioma cells' viability is compromised along the culturing time, their glycolytic metabolism seems not to be affected, exhibiting increased values of glucose consumption and lactate production. Conversely, it seems there is a direct correlation between endothelial cells' viability and metabolism. Indeed, the high proliferative characteristic of tumor cells is in line with the need of an increased

glycolytic metabolism. However, in the case of normal endothelial cells, such accelerated glycolytic metabolism is not so demanding.

Taking together, our preliminary results from 3D culturing system open the window for a 3D co-culture system of both tumor and endothelial cells, to better study the cell-cell interactions occurring in tumors and, it can constitute, at short/middle term, a great platform for drug testing in whole *in vitro* system, dispensing the use of animals at early stages of investigation.

5 Concluding Remarks

The current work was based on the lack of studies concerning 1) the role of MCTs on the maintenance of endothelial cell function, especially under hypoxia and 2) the role of MCTs on the angiogenic capacity of tumor cells.

Hence, our experiments demonstrated that MCT1 and MCT4 isoforms and their molecular chaperones, CD147 and CD44 were expressed in brain endothelial cells, mainly under hypoxia. Furthermore, GLUT-1 and CAIX were also upregulated, and LDH-V levels were maintained increased, under hypoxia. The upregulation of these key metabolic markers, collectively, contributes to the increased glycolytic rates, observed under hypoxic conditions. Further, inhibition of MCT activity, using CHC, as well as MCT downregulation, using siRNA revealed impairment in endothelial cell viability and, a disruption on the development of capillary-like structures is suggested, under hypoxia, may be due to the increased cell death and/or decreased proliferation rates in HBMEC cells, independently of lactate transport activity, especially when MCT1 was inhibited, alone or in combination with MCT4.

Upon endothelial cell growth in glioma cells' CM, metabolic adaptations in HBMEC cells were observed, which may contribute to the maintenance of endothelial cell survival. However, impairment in endothelial cell proliferation and capillary-like structures assembling were observed *in vitro*. Additionally, exposure to MCT1-downregulated glioma cells' CM from both normoxia and hypoxia did not affect the vascular network of chick chorioallantoic membrane, comparatively to scramble groups, whereas the other MCT-silenced glioma cells' CM triggered similar effects to scramble groups.

In summary, besides their role in tumor cells, our data point out the importance of MCT1 and, in lower extension MCT4, on the maintenance of endothelial cell function, under normoxia. Under hypoxia, the absence of both these isoforms seems to be counterbalanced, may be due to the overexpression of other transporters at the plasma membrane of endothelial cells. In addition, MCTs seem to be players in tumor microenvironment, acting as essential mediators in tumor-endothelial cell interplay.

6 Future Perspectives

In spite of all the results pointing out the importance of MCTs on the maintenance of endothelial cell function and as essential molecules performing a role in tumor-endothelial cell crosstalk, this work should be further explored.

Since migration, along with proliferation, constitutes a key mechanism for the development of capillary-like structures, assays concerning this function, like the "wound-healing" assay should be performed, upon both inhibition of MCT activity and MCT downregulation, under normoxia and hypoxia, as well as upon endothelial cell growth in glioma cells' CM.

The influence of hypoxia, as well as the role of MCTs on the angiogenic capacity of glioma cells may also be further explored. For instance, the expression levels of VEGF-A- spliced variants, after MCT downregulation under both normoxia and hypoxia should be evaluated. Additionally, the protein levels of VEGF-A in glioma cells' CM should also be measured, through protein precipitation using trichloroacetic acid or ELISA assays. In this context, the expression levels of VEGFR-2, as well as phosphorylation at its specific tyrosine kynase domains must be evaluated in brain endothelial cells, through Western blotting.

Finally, regarding 3D *in vitro* culture systems, the quantification of both DNA and protein synthesis should be performed, in order to evaluate cell proliferation rates. At short/middle term, the implementation of a 3D co-culture system should be done in our glioma model, in order to better mimic the *in vivo* interactions between tumor and endothelial cells.

7 References

1. Semenza, G.L., *Angiogenesis in ischemic and neoplastic disorders.* Annu Rev Med, 2003. **54**: p. 17-28.
2. Junqueira, L.C., Carneiro, J., *Histologia Básica*. 10 ed. 2004: p. 250-260.
3. Karamysheva, A.F., *Mechanisms of angiogenesis.* Biochemistry (Mosc), 2008. **73**(7): p. 751-62.
4. Carmeliet, P., *Mechanisms of angiogenesis and arteriogenesis.* Nat Med, 2000. **6**(4): p. 389-95.
5. Chung, A.S. and N. Ferrara, *Developmental and pathological angiogenesis.* Annu Rev Cell Dev Biol, 2011. **27**: p. 563-84.
6. Patan, S., *Vasculogenesis and angiogenesis as mechanisms of vascular network formation, growth and remodeling.* J Neurooncol, 2000. **50**(1,2): p. 1-15.
7. Ema, M. and J. Rossant, *Cell fate decisions in early blood vessel formation.* Trends Cardiovasc Med, 2003. **13**(6): p. 254-9.
8. Choi, K., et al., *A common precursor for hematopoietic and endothelial cells.* Development, 1998. **125**(4): p. 725-732.
9. Vogeli, K.M., et al., *A common progenitor for haematopoietic and endothelial lineages in the zebrafish gastrula.* Nature, 2006. **443**(7109): p. 337-9.
10. Dvorak, H.F., *Angiogenesis: update 2005.* J Thromb Haemost, 2005. **3**(8): p. 1835-42.
11. Coultas, L., K. Chawengsaksophak, and J. Rossant, *Endothelial cells and VEGF in vascular development.* Nature, 2005. **438**(7070): p. 937-45.
12. Carmeliet, P., *Angiogenesis in health and disease.* Nat Med, 2003. **9**(6): p. 653-60.
13. Jain, R.K., *Molecular regulation of vessel maturation.* Nat Med, 2003. **9**(6): p. 685-93.
14. Folkman, J., *Angiogenesis and angiogenesis inhibition: an overview.* EXS, 1997. **79**: p. 1-8.
15. Robinson, C.J. and S.E. Stringer, *The splice variants of vascular endothelial growth factor (VEGF) and their receptors.* J Cell Sci, 2001. **114**(Pt 5): p. 853-65.
16. Ribatti, D., A. Vacca, and M. Presta, *The discovery of angiogenic factors: a historical review.* Gen Pharmacol, 2000. **35**(5): p. 227-31.
17. Roskoski, R., Jr., *Vascular endothelial growth factor (VEGF) signaling in tumor progression.* Crit Rev Oncol Hematol, 2007. **62**(3): p. 179-213.
18. Eklund, L. and B.R. Olsen, *Tie receptors and their angiopoietin ligands are context-dependent regulators of vascular remodeling.* Exp Cell Res, 2006. **312**(5): p. 630-41.

19. Heroult, M., F. Schaffner, and H.G. Augustin, *Eph receptor and ephrin ligand-mediated interactions during angiogenesis and tumor progression.* Exp Cell Res, 2006. **312**(5): p. 642-50.
20. Relf, M., et al., *Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis.* Cancer Res, 1997. **57**(5): p. 963-9.
21. O'Reilly, M.S., *Angiostatin: an endogenous inhibitor of angiogenesis and of tumor growth.* EXS, 1997. **79**: p. 273-94.
22. O'Reilly, M.S., et al., *Endostatin: an endogenous inhibitor of angiogenesis and tumor growth.* Cell, 1997. **88**(2): p. 277-85.
23. Dameron, K.M., et al., *Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1.* Science, 1994. **265**(5178): p. 1582-4.
24. Carmeliet, P. and R.K. Jain, *Molecular mechanisms and clinical applications of angiogenesis.* Nature, 2011. **473**(7347): p. 298-307.
25. Takano, S., *Glioblastoma angiogenesis: VEGF resistance solutions and new strategies based on molecular mechanisms of tumor vessel formation.* Brain Tumor Pathol, 2012. **29**(2): p. 73-86.
26. Caduff, J.H., L.C. Fischer, and P.H. Burri, *Scanning electron microscope study of the developing microvasculature in the postnatal rat lung.* Anat Rec, 1986. **216**(2): p. 154-64.
27. Hillen, F. and A.W. Griffioen, *Tumour vascularization: sprouting angiogenesis and beyond.* Cancer Metastasis Rev, 2007. **26**(3-4): p. 489-502.
28. Burri, P.H., R. Hlushchuk, and V. Djonov, *Intussusceptive angiogenesis: its emergence, its characteristics, and its significance.* Dev Dyn, 2004. **231**(3): p. 474-88.
29. Bergers, G. and L.E. Benjamin, *Tumorigenesis and the angiogenic switch.* Nat Rev Cancer, 2003. **3**(6): p. 401-10.
30. Timar, J., et al., *Angiogenesis-dependent diseases and angiogenesis therapy.* Pathol Oncol Res, 2001. **7**(2): p. 85-94.
31. Goldmann, E., *The Growth of Malignant Disease in Man and the Lower Animals, with special reference to the Vascular System.* Proc R Soc Med, 1908. **1**(Surg Sect): p. 1-13.
32. Folkman, J., et al., *Isolation of a tumor factor responsible for angiogenesis.* J Exp Med, 1971. **133**(2): p. 275-88.
33. Gullino, P.M., *Angiogenesis and oncogenesis.* J Natl Cancer Inst, 1978. **61**(3): p. 639-43.
34. Baeriswyl, V. and G. Christofori, *The angiogenic switch in carcinogenesis.* Semin Cancer Biol, 2009. **19**(5): p. 329-37.

35. Wesseling, P., et al., *Quantitative immunohistological analysis of the microvasculature in untreated human glioblastoma multiforme. Computer-assisted image analysis of whole-tumor sections.* J Neurosurg, 1994. **81**(6): p. 902-9.
36. Pezzella, F., et al., *Non-small-cell lung carcinoma tumor growth without morphological evidence of neo-angiogenesis.* Am J Pathol, 1997. **151**(5): p. 1417-23.
37. Holash, J., et al., *Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF.* Science, 1999. **284**(5422): p. 1994-8.
38. Mocellin, S., *Tumor Vasculature: the Achilles' heel of cancer?* International Journal of Cancer Research 2006. **2**(2): p. 176-187.
39. Maniotis, A.J., et al., *Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry.* Am J Pathol, 1999. **155**(3): p. 739-52.
40. Ricci-Vitiani, L., et al., *Tumour vascularization via endothelial differentiation of glioblastoma stem-like cells.* Nature, 2010. **468**(7325): p. 824-8.
41. Wang, R., et al., *Glioblastoma stem-like cells give rise to tumour endothelium.* Nature, 2010. **468**(7325): p. 829-33.
42. Soda, Y., et al., *Transdifferentiation of glioblastoma cells into vascular endothelial cells.* Proc Natl Acad Sci U S A, 2011. **108**(11): p. 4274-80.
43. Morikawa, S., et al., *Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors.* Am J Pathol, 2002. **160**(3): p. 985-1000.
44. Akino, T., et al., *Cytogenetic abnormalities of tumor-associated endothelial cells in human malignant tumors.* Am J Pathol, 2009. **175**(6): p. 2657-67.
45. Harris, A.L., *Hypoxia–a key regulatory factor in tumour growth.* Nat Rev Cancer, 2002. **2**(1): p. 38-47.
46. Giordano, F.J. and R.S. Johnson, *Angiogenesis: the role of the microenvironment in flipping the switch.* Curr Opin Genet Dev, 2001. **11**(1): p. 35-40.
47. Yang, Y., et al., *HIFs, angiogenesis and cancer.* J Cell Biochem, 2012.
48. Semenza, G.L., *Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy.* Trends Pharmacol Sci, 2012. **33**(4): p. 207-14.
49. Nurwidya, F., et al., *From tumor hypoxia to cancer progression: the implications of hypoxia-inducible factor-1 expression in cancers.* Anat Cell Biol, 2012. **45**(2): p. 73-8.
50. Rankin, E.B. and A.J. Giaccia, *The role of hypoxia-inducible factors in tumorigenesis.* Cell Death Differ, 2008. **15**(4): p. 678-85.
51. Semenza, G.L., *Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics.* Oncogene, 2010. **29**(5): p. 625-34.

52. Otrock, Z.K., et al., *Hypoxia-inducible factor in cancer angiogenesis: structure, regulation and clinical perspectives.* Crit Rev Oncol Hematol, 2009. **70**(2): p. 93-102.

53. Peng, J., et al., *The transcription factor EPAS-1/hypoxia-inducible factor 2alpha plays an important role in vascular remodeling.* Proc Natl Acad Sci U S A, 2000. **97**(15): p. 8386-91.

54. Liao, D. and R.S. Johnson, *Hypoxia: a key regulator of angiogenesis in cancer.* Cancer Metastasis Rev, 2007. **26**(2): p. 281-90.

55. Fraisl, P., et al., *Regulation of angiogenesis by oxygen and metabolism.* Dev Cell, 2009. **16**(2): p. 167-79.

56. Bos, R., et al., *Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis.* J Natl Cancer Inst, 2001. **93**(4): p. 309-14.

57. Jensen, R.L., et al., *Inhibition of hypoxia inducible factor-1alpha (HIF-1alpha) decreases vascular endothelial growth factor (VEGF) secretion and tumor growth in malignant gliomas.* J Neurooncol, 2006. **78**(3): p. 233-47.

58. Tang, N., et al., *Loss of HIF-1alpha in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis.* Cancer Cell, 2004. **6**(5): p. 485-95.

59. Ferrara, N., *Vascular endothelial growth factor: basic science and clinical progress.* Endocr Rev, 2004. **25**(4): p. 581-611.

60. Hoeben, A., et al., *Vascular endothelial growth factor and angiogenesis.* Pharmacol Rev, 2004. **56**(4): p. 549-80.

61. Ferrara, N., H.P. Gerber, and J. LeCouter, *The biology of VEGF and its receptors.* Nat Med, 2003. **9**(6): p. 669-76.

62. Delli Carpini, J., A.K. Karam, and L. Montgomery, *Vascular endothelial growth factor and its relationship to the prognosis and treatment of breast, ovarian, and cervical cancer.* Angiogenesis, 2010. **13**(1): p. 43-58.

63. Dvorak, H.F., *Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing.* N Engl J Med, 1986. **315**(26): p. 1650-9.

64. Ferrara, N. and T. Davis-Smyth, *The biology of vascular endothelial growth factor.* Endocr Rev, 1997. **18**(1): p. 4-25.

65. Nagy, J.A., A.M. Dvorak, and H.F. Dvorak, *VEGF-A and the induction of pathological angiogenesis.* Annu Rev Pathol, 2007. **2**: p. 251-75.

66. Qiu, Y., et al., *The anti-angiogenic isoforms of VEGF in health and disease.* Biochem Soc Trans, 2009. **37**(Pt 6): p. 1207-13.

67. Keyt, B.A., et al., *The carboxyl-terminal domain (111-165) of vascular endothelial growth factor is critical for its mitogenic potency.* J Biol Chem, 1996. **271**(13): p. 7788-95.

68. Shibuya, M., *Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis: A Crucial Target for Anti- and Pro-Angiogenic Therapies.* Genes Cancer, 2011. **2**(12): p. 1097-105.

69. Maes, C., et al., *Impaired angiogenesis and endochondral bone formation in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188.* Mech Dev, 2002. **111**(1-2): p. 61-73.

70. Bellamy, W.T., *Vascular endothelial growth factor as a target opportunity in hematological malignancies.* Curr Opin Oncol, 2002. **14**(6): p. 649-56.

71. Ishida, A., et al., *Expression of vascular endothelial growth factor receptors in smooth muscle cells.* J Cell Physiol, 2001. **188**(3): p. 359-68.

72. Shibuya, M., et al., *Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family.* Oncogene, 1990. **5**(4): p. 519-24.

73. Terman, B.I., et al., *Identification of a new endothelial cell growth factor receptor tyrosine kinase.* Oncogene, 1991. **6**(9): p. 1677-83.

74. Eming, S.A. and T. Krieg, *Molecular mechanisms of VEGF-A action during tissue repair.* J Investig Dermatol Symp Proc, 2006. **11**(1): p. 79-86.

75. Plate, K.H., et al., *Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo.* Nature, 1992. **359**(6398): p. 845-8.

76. Toi, M., et al., *Association of vascular endothelial growth factor expression with tumor angiogenesis and with early relapse in primary breast cancer.* Jpn J Cancer Res, 1994. **85**(10): p. 1045-9.

77. Maeda, K., et al., *Prognostic value of vascular endothelial growth factor expression in gastric carcinoma.* Cancer, 1996. **77**(5): p. 858-63.

78. Folkman, J., *Tumor angiogenesis: therapeutic implications.* N Engl J Med, 1971. **285**(21): p. 1182-6.

79. Kubota, Y., *Tumor angiogenesis and anti-angiogenic therapy.* Keio J Med, 2012. **61**(2): p. 47-56.

80. Abdollahi, A. and J. Folkman, *Evading tumor evasion: current concepts and perspectives of anti-angiogenic cancer therapy.* Drug Resist Updat, 2010. **13**(1-2): p. 16-28.

81. Halestrap, A.P. and N.T. Price, *The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation.* Biochem J, 1999. **343 Pt 2**: p. 281-99.

82. Poole, R.C. and A.P. Halestrap, *Transport of lactate and other monocarboxylates across mammalian plasma membranes.* Am J Physiol, 1993. **264**(4 Pt 1): p. C761-82.

83. Juel, C. and A.P. Halestrap, *Lactate transport in skeletal muscle - role and regulation of the monocarboxylate transporter.* J Physiol, 1999. **517 (Pt 3)**: p. 633-42.

84. Halestrap, A.P. and D. Meredith, *The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond.* Pflugers Arch, 2004. **447**(5): p. 619-28.

85. Enerson, B.E. and L.R. Drewes, *Molecular features, regulation, and function of monocarboxylate transporters: implications for drug delivery.* J Pharm Sci, 2003. **92**(8): p. 1531-44.

86. Morris, M.E. and M.A. Felmlee, *Overview of the proton-coupled MCT (SLC16A) family of transporters: characterization, function and role in the transport of the drug of abuse gamma-hydroxybutyric acid.* AAPS J, 2008. **10**(2): p. 311-21.

87. Merezhinskaya, N. and W.N. Fishbein, *Monocarboxylate transporters: past, present, and future.* Histol Histopathol, 2009. **24**(2): p. 243-64.

88. Pinheiro, C., et al., *Role of monocarboxylate transporters in human cancers: state of the art.* J Bioenerg Biomembr, 2012. **44**(1): p. 127-39.

89. Halestrap, A.P., *The monocarboxylate transporter family–Structure and functional characterization.* IUBMB Life, 2012. **64**(1): p. 1-9.

90. Halestrap, A.P. and M.C. Wilson, *The monocarboxylate transporter family–role and regulation.* IUBMB Life, 2012. **64**(2): p. 109-19.

91. Poole, R.C., C.E. Sansom, and A.P. Halestrap, *Studies of the membrane topology of the rat erythrocyte H+/lactate cotransporter (MCT1).* Biochem J, 1996. **320 (Pt 3)**: p. 817-24.

92. Carpenter, L. and A.P. Halestrap, *The kinetics, substrate and inhibitor specificity of the lactate transporter of Ehrlich-Lettre tumour cells studied with the intracellular pH indicator BCECF.* Biochem J, 1994. **304 (Pt 3)**: p. 751-60.

93. Broer, S., et al., *Characterization of the monocarboxylate transporter 1 expressed in Xenopus laevis oocytes by changes in cytosolic pH.* Biochem J, 1998. **333 (Pt 1)**: p. 167-74.

94. Brooks, G.A., *Cell-cell and intracellular lactate shuttles.* J Physiol, 2009. **587**(Pt 23): p. 5591-600.

95. Garcia, C.K., et al., *cDNA cloning of MCT2, a second monocarboxylate transporter expressed in different cells than MCT1.* J Biol Chem, 1995. **270**(4): p. 1843-9.

96. Lin, R.Y., et al., *Human monocarboxylate transporter 2 (MCT2) is a high affinity pyruvate transporter.* J Biol Chem, 1998. **273**(44): p. 28959-65.

97. Zhu, S., P.J. Goldschmidt-Clermont, and C. Dong, *Inactivation of monocarboxylate transporter MCT3 by DNA methylation in atherosclerosis.* Circulation, 2005. **112**(9): p. 1353-61.

98. Wang, Q., et al., *Characterization of monocarboxylate transport in human kidney HK-2 cells.* Mol Pharm, 2006. **3**(6): p. 675-85.

99. Bonen, A., *The expression of lactate transporters (MCT1 and MCT4) in heart and muscle.* Eur J Appl Physiol, 2001. **86**(1): p. 6-11.

100. Landolt-Marticorena, C. and R.A. Reithmeier, *Asparagine-linked oligosaccharides are localized to single extracytosolic segments in multi-span membrane glycoproteins.* Biochem J, 1994. **302 (Pt 1)**: p. 253-60.

101. Iacono, K.T., et al., *CD147 immunoglobulin superfamily receptor function and role in pathology.* Exp Mol Pathol, 2007. **83**(3): p. 283-95.

102. Kirk, P., et al., *CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression.* EMBO J, 2000. **19**(15): p. 3896-904.

103. Poole, R.C. and A.P. Halestrap, *Interaction of the erythrocyte lactate transporter (monocarboxylate transporter 1) with an integral 70-kDa membrane glycoprotein of the immunoglobulin superfamily.* J Biol Chem, 1997. **272**(23): p. 14624-8.

104. Philp, N.J., et al., *Loss of MCT1, MCT3, and MCT4 expression in the retinal pigment epithelium and neural retina of the 5A11/basigin-null mouse.* Invest Ophthalmol Vis Sci, 2003. **44**(3): p. 1305-11.

105. Ovens, M.J., et al., *The inhibition of monocarboxylate transporter 2 (MCT2) by AR-C155858 is modulated by the associated ancillary protein.* Biochem J, 2010. **431**(2): p. 217-25.

106. Wilson, M.C., et al., *Basigin (CD147) is the target for organomercurial inhibition of monocarboxylate transporter isoforms 1 and 4: the ancillary protein for the insensitive MCT2 is EMBIGIN (gp70).* J Biol Chem, 2005. **280**(29): p. 27213-21.

107. Slomiany, M.G., et al., *Hyaluronan, CD44, and emmprin regulate lactate efflux and membrane localization of monocarboxylate transporters in human breast carcinoma cells.* Cancer Res, 2009. **69**(4): p. 1293-301.

108. Pinheiro, C., et al., *Expression of monocarboxylate transporters 1, 2, and 4 in human tumours and their association with CD147 and CD44.* J Biomed Biotechnol, 2010. **2010**: p. 427694.

109. Hao, J., et al., *Co-expression of CD147 (EMMPRIN), CD44v3-10, MDR1 and monocarboxylate transporters is associated with prostate cancer drug resistance and progression.* Br J Cancer, 2010. **103**(7): p. 1008-18.

110. Cuff, M.A. and S.P. Shirazi-Beechey, *The human monocarboxylate transporter, MCT1: genomic organization and promoter analysis.* Biochem Biophys Res Commun, 2002. **292**(4): p. 1048-56.

111. Gallagher-Colombo, S., et al., *Modulation of MCT3 expression during wound healing of the retinal pigment epithelium.* Invest Ophthalmol Vis Sci, 2010. **51**(10): p. 5343-50.

112. Ullah, M.S., A.J. Davies, and A.P. Halestrap, *The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism.* J Biol Chem, 2006. **281**(14): p. 9030-7.

113. Thomas, C., et al., *Effects of high-intensity training on MCT1, MCT4, and NBC expressions in rat skeletal muscles: influence of chronic metabolic alkalosis.* Am J Physiol Endocrinol Metab, 2007. **293**(4): p. E916-22.

114. Perez de Heredia, F., I.S. Wood, and P. Trayhurn, *Hypoxia stimulates lactate release and modulates monocarboxylate transporter (MCT1, MCT2, and MCT4) expression in human adipocytes.* Pflugers Arch, 2010. **459**(3): p. 509-18.

115. Macdonald, P.M., *Translational control: a cup half full.* Curr Biol, 2004. **14**(7): p. R282-3.

116. Merezhinskaya, N., S.A. Ogunwuyi, and W.N. Fishbein, *Expression of monocarboxylate transporter 4 in human platelets, leukocytes, and tissues assessed by antibodies raised against terminal versus pre-terminal peptides.* Mol Genet Metab, 2006. **87**(2): p. 152-61.

117. Porporato, P.E., et al., *Anticancer targets in the glycolytic metabolism of tumors: a comprehensive review.* Front Pharmacol, 2011. **2**: p. 49.

118. Granchi, C. and F. Minutolo, *Anticancer agents that counteract tumor glycolysis.* ChemMedChem, 2012. **7**(8): p. 1318-50.

119. Kondoh, H., et al., *Glycolytic enzymes can modulate cellular life span.* Cancer Res, 2005. **65**(1): p. 177-85.

120. Spencer, T.L. and A.L. Lehninger, *L-lactate transport in Ehrlich ascites-tumour cells.* Biochem J, 1976. **154**(2): p. 405-14.

121. Manning Fox, J.E., D. Meredith, and A.P. Halestrap, *Characterisation of human monocarboxylate transporter 4 substantiates its role in lactic acid efflux from skeletal muscle.* J Physiol, 2000. **529 Pt 2**: p. 285-93.

122. Sonveaux, P., et al., *Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice.* J Clin Invest, 2008. **118**(12): p. 3930-42.

123. Wahl, M.L., et al., *Regulation of intracellular pH in human melanoma: potential therapeutic implications.* Mol Cancer Ther, 2002. **1**(8): p. 617-28.

124. Colen, C.B., et al., *Metabolic remodeling of malignant gliomas for enhanced sensitization during radiotherapy: an in vitro study.* Neurosurgery, 2006. **59**(6): p. 1313-23; discussion 1323-4.

125. Poole, R.C. and A.P. Halestrap, *Reversible and irreversible inhibition, by stilbenedisulphonates, of lactate transport into rat erythrocytes. Identification of some new high-affinity inhibitors.* Biochem J, 1991. **275 (Pt 2)**: p. 307-12.

126. Jackson, V.N. and A.P. Halestrap, *The kinetics, substrate, and inhibitor specificity of the monocarboxylate (lactate) transporter of rat liver cells determined using the fluorescent intracellular pH indicator, 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein.* J Biol Chem, 1996. **271**(2): p. 861-8.

127. Volk, C., B. Kempski, and O.S. Kempski, *Inhibition of lactate export by quercetin acidifies rat glial cells in vitro.* Neurosci Lett, 1997. **223**(2): p. 121-4.

128. Murray, C.M., et al., *Monocarboxylate transporter MCT1 is a target for immunosuppression.* Nat Chem Biol, 2005. **1**(7): p. 371-6.

129. Guile, S.D., et al., *Potent blockers of the monocarboxylate transporter MCT1: novel immunomodulatory compounds.* Bioorg Med Chem Lett, 2006. **16**(8): p. 2260-5.

130. Ovens, M.J., et al., *AR-C155858 is a potent inhibitor of monocarboxylate transporters MCT1 and MCT2 that binds to an intracellular site involving transmembrane helices 7-10.* Biochem J, 2010. **425**(3): p. 523-30.

131. WHO. *Cancer*. 2013 20-02-2013]; Available from: http://www.who.int.

132. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.

133. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.

134. Allavena, P., et al., *The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages.* Crit Rev Oncol Hematol, 2008. **66**(1): p. 1-9.

135. Wu, R. and E. Racker, *Regulatory mechanisms in carbohydrate metabolism. IV. Pasteur effect and Crabtree effect in ascites tumor cells.* J Biol Chem, 1959. **234**(5): p. 1036-41.

136. Porter, J.R., *Louis PASTEUR; achievements and disappointments, 1861.* Bacteriol Rev, 1961. **25**: p. 389-403.

137. Vander Heiden, M.G., L.C. Cantley, and C.B. Thompson, *Understanding the Warburg effect: the metabolic requirements of cell proliferation.* Science, 2009. **324**(5930): p. 1029-33.

138. Gatenby, R.A. and R.J. Gillies, *Why do cancers have high aerobic glycolysis?* Nat Rev Cancer, 2004. **4**(11): p. 891-9.

139. Bristow, R.G. and R.P. Hill, *Hypoxia and metabolism. Hypoxia, DNA repair and genetic instability.* Nat Rev Cancer, 2008. **8**(3): p. 180-92.

140. Warburg, O., *On the origin of cancer cells.* Science, 1956. **123**(3191): p. 309-14.

141. Warburg, O., *On respiratory impairment in cancer cells.* Science, 1956. **124**(3215): p. 269-70.

142. Warburg, O., F. Wind, and E. Negelein, *The Metabolism of Tumors in the Body.* J Gen Physiol, 1927. **8**(6): p. 519-30.

143. Semenza, G.L., *Hypoxia, clonal selection, and the role of HIF-1 in tumor progression.* Crit Rev Biochem Mol Biol, 2000. **35**(2): p. 71-103.

144. Semenza, G.L., *HIF-1: mediator of physiological and pathophysiological responses to hypoxia.* J Appl Physiol, 2000. **88**(4): p. 1474-80.

145. Semenza, G.L., *HIF-1: upstream and downstream of cancer metabolism.* Curr Opin Genet Dev, 2010. **20**(1): p. 51-6.

146. Kim, J.W., et al., *HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia.* Cell Metab, 2006. **3**(3): p. 177-85.

147. Holness, M.J. and M.C. Sugden, *Regulation of pyruvate dehydrogenase complex activity by reversible phosphorylation.* Biochem Soc Trans, 2003. **31**(Pt 6): p. 1143-51.

148. Ebert, B.L., J.D. Firth, and P.J. Ratcliffe, *Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter-1 via distinct Cis-acting sequences.* J Biol Chem, 1995. **270**(49): p. 29083-9.

149. Ebert, B.L., et al., *Isoenzyme-specific regulation of genes involved in energy metabolism by hypoxia: similarities with the regulation of erythropoietin.* Biochem J, 1996. **313 (Pt 3)**: p. 809-14.

150. Mathupala, S.P., A. Rempel, and P.L. Pedersen, *Glucose catabolism in cancer cells: identification and characterization of a marked activation response of the type II hexokinase gene to hypoxic conditions.* J Biol Chem, 2001. **276**(46): p. 43407-12.

151. Semenza, G.L., et al., *Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1.* J Biol Chem, 1996. **271**(51): p. 32529-37.

152. Wykoff, C.C., et al., *Hypoxia-inducible expression of tumor-associated carbonic anhydrases.* Cancer Res, 2000. **60**(24): p. 7075-83.

153. Chiche, J., et al., *Hypoxia-inducible carbonic anhydrase IX and XII promote tumor cell growth by counteracting acidosis through the regulation of the intracellular pH.* Cancer Res, 2009. **69**(1): p. 358-68.

154. Ruch, R.J., et al., *Modification of gap junctional intercellular communication by changes in extracellular pH in Syrian hamster embryo cells.* Carcinogenesis, 1990. **11**(6): p. 909-13.

155. Martinez-Zaguilan, R., et al., *Acidic pH enhances the invasive behavior of human melanoma cells.* Clin Exp Metastasis, 1996. **14**(2): p. 176-86.

156. Schlappack, O.K., A. Zimmermann, and R.P. Hill, *Glucose starvation and acidosis: effect on experimental metastatic potential, DNA content and MTX resistance of murine tumour cells.* Br J Cancer, 1991. **64**(4): p. 663-70.

157. Kennedy, K.M. and M.W. Dewhirst, *Tumor metabolism of lactate: the influence and therapeutic potential for MCT and CD147 regulation.* Future Oncol, 2010. **6**(1): p. 127-48.

158. Izumi, H., et al., *Cellular pH regulators: potentially promising molecular targets for cancer chemotherapy.* Cancer Treat Rev, 2003. **29**(6): p. 541-9.

159. Sattler, U.G. and W. Mueller-Klieser, *The anti-oxidant capacity of tumour glycolysis.* Int J Radiat Biol, 2009. **85**(11): p. 963-71.

160. Frauwirth, K.A. and C.B. Thompson, *Regulation of T lymphocyte metabolism.* J Immunol, 2004. **172**(8): p. 4661-5.

161. Fischer, K., et al., *Inhibitory effect of tumor cell-derived lactic acid on human T cells.* Blood, 2007. **109**(9): p. 3812-9.

162. Lu, H., R.A. Forbes, and A. Verma, *Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis.* J Biol Chem, 2002. **277**(26): p. 23111-5.

163. Walenta, S., T. Schroeder, and W. Mueller-Klieser, *Metabolic mapping with bioluminescence: basic and clinical relevance.* Biomol Eng, 2002. **18**(6): p. 249-62.

164. Spector, J.A., et al., *Osteoblast expression of vascular endothelial growth factor is modulated by the extracellular microenvironment.* Am J Physiol Cell Physiol, 2001. **280**(1): p. C72-80.

165. Kumar, V.B., et al., *Endothelial cell response to lactate: implication of PAR modification of VEGF.* J Cell Physiol, 2007. **211**(2): p. 477-85.

166. Hunt, T.K., et al., *Aerobically derived lactate stimulates revascularization and tissue repair via redox mechanisms.* Antioxid Redox Signal, 2007. **9**(8): p. 1115-24.

167. Stern, R., et al., *Lactate stimulates fibroblast expression of hyaluronan and CD44: the Warburg effect revisited.* Exp Cell Res, 2002. **276**(1): p. 24-31.

168. Rudrabhatla, S.R., C.L. Mahaffey, and M.E. Mummert, *Tumor microenvironment modulates hyaluronan expression: the lactate effect.* J Invest Dermatol, 2006. **126**(6): p. 1378-87.

169. Schwickert, G., et al., *Correlation of high lactate levels in human cervical cancer with incidence of metastasis.* Cancer Res, 1995. **55**(21): p. 4757-9.

170. Walenta, S., et al., *Correlation of high lactate levels in head and neck tumors with incidence of metastasis.* Am J Pathol, 1997. **150**(2): p. 409-15.

171. Walenta, S., et al., *Metabolic classification of human rectal adenocarcinomas: a novel guideline for clinical oncologists?* J Cancer Res Clin Oncol, 2003. **129**(6): p. 321-6.

172. Walenta, S., et al., *High lactate levels predict likelihood of metastases, tumor recurrence, and restricted patient survival in human cervical cancers.* Cancer Res, 2000. **60**(4): p. 916-21.

173. Brizel, D.M., et al., *Elevated tumor lactate concentrations predict for an increased risk of metastases in head-and-neck cancer.* Int J Radiat Oncol Biol Phys, 2001. **51**(2): p. 349-53.

174. Fulham, M.J., et al., *Mapping of brain tumor metabolites with proton MR spectroscopic imaging: clinical relevance.* Radiology, 1992. **185**(3): p. 675-86.

175. Yokota, H., et al., *Lactate, choline, and creatine levels measured by vitro 1H-MRS as prognostic parameters in patients with non-small-cell lung cancer.* J Magn Reson Imaging, 2007. **25**(5): p. 992-9.

176. Mazurek, S., et al., *Tumor M2-PK and glutaminolytic enzymes in the metabolic shift of tumor cells.* Anticancer Res, 2000. **20**(6D): p. 5151-4.

177. Gladden, L.B., *Muscle as a consumer of lactate.* Med Sci Sports Exerc, 2000. **32**(4): p. 764-71.

178. Gertz, E.W., et al., *Myocardial substrate utilization during exercise in humans. Dual carbon-labeled carbohydrate isotope experiments.* J Clin Invest, 1988. **82**(6): p. 2017-25.

179. Stanley, W.C., *Myocardial lactate metabolism during exercise.* Med Sci Sports Exerc, 1991. **23**(8): p. 920-4.

180. Ide, K. and N.H. Secher, *Cerebral blood flow and metabolism during exercise.* Prog Neurobiol, 2000. **61**(4): p. 397-414.

181. van Hall, G., et al., *Blood lactate is an important energy source for the human brain.* J Cereb Blood Flow Metab, 2009. **29**(6): p. 1121-9.

182. Ritzhaupt, A., et al., *Identification of a monocarboxylate transporter isoform type 1 (MCT1) on the luminal membrane of human and pig colon.* Biochem Soc Trans, 1998. **26**(2): p. S120.

183. Lambert, D.W., et al., *Molecular changes in the expression of human colonic nutrient transporters during the transition from normality to malignancy.* Br J Cancer, 2002. **86**(8): p. 1262-9.

184. Koukourakis, M.I., et al., *Comparison of metabolic pathways between cancer cells and stromal cells in colorectal carcinomas: a metabolic survival role for tumor-associated stroma.* Cancer Res, 2006. **66**(2): p. 632-7.

185. Pinheiro, C., et al., *Increased expression of monocarboxylate transporters 1, 2, and 4 in colorectal carcinomas.* Virchows Arch, 2008. **452**(2): p. 139-46.

186. Mathupala, S.P., P. Parajuli, and A.E. Sloan, *Silencing of monocarboxylate transporters via small interfering ribonucleic acid inhibits glycolysis and induces cell death in malignant glioma: an in vitro study.* Neurosurgery, 2004. **55**(6): p. 1410-9; discussion 1419.

187. Froberg, M.K., et al., *Expression of monocarboxylate transporter MCT1 in normal and neoplastic human CNS tissues.* Neuroreport, 2001. **12**(4): p. 761-5.

188. Miranda-Goncalves, V., et al., *Monocarboxylate transporters (MCTs) in gliomas: expression and exploitation as therapeutic targets.* Neuro Oncol, 2013. **15**(2): p. 172-88.

189. Asada, K., et al., *Reduced expression of GNA11 and silencing of MCT1 in human breast cancers.* Oncology, 2003. **64**(4): p. 380-8.

190. Pinheiro, C., et al., *Monocarboxylate transporter 1 is up-regulated in basal-like breast carcinoma.* Histopathology, 2010. **56**(7): p. 860-7.

191. Koukourakis, M.I., et al., *Lung cancer: a comparative study of metabolism related protein expression in cancer cells and tumor associated stroma.* Cancer Biol Ther, 2007. **6**(9): p. 1476-9.

192. Ladanyi, M., et al., *The precrystalline cytoplasmic granules of alveolar soft part sarcoma contain monocarboxylate transporter 1 and CD147.* Am J Pathol, 2002. **160**(4): p. 1215-21.

193. Pinheiro, C., et al., *Increasing expression of monocarboxylate transporters 1 and 4 along progression to invasive cervical carcinoma.* Int J Gynecol Pathol, 2008. **27**(4): p. 568-74.

194. Pinheiro, C., et al., *Monocarboxylate transporters 1 and 4 are associated with CD147 in cervical carcinoma.* Dis Markers, 2009. **26**(3): p. 97-103.

195. Chen, H., et al., *Co-expression of CD147/EMMPRIN with monocarboxylate transporters and multiple drug resistance proteins is associated with epithelial ovarian cancer progression.* Clin Exp Metastasis, 2010. **27**(8): p. 557-69.

196. Pertega-Gomes, N., et al., *Monocarboxylate transporter 4 (MCT4) and CD147 overexpression is associated with poor prognosis in prostate cancer.* BMC Cancer, 2011. **11**: p. 312.

197. Pertega-Gomes, N., et al., *Monocarboxylate transporter 2 (MCT2) as putative biomarker in prostate cancer.* Prostate, 2012.

198. Pinheiro, C., et al., *The prognostic value of CD147/EMMPRIN is associated with monocarboxylate transporter 1 co-expression in gastric cancer.* Eur J Cancer, 2009. **45**(13): p. 2418-24.

199. Gallagher, S.M., J.J. Castorino, and N.J. Philp, *Interaction of monocarboxylate transporter 4 with beta1-integrin and its role in cell migration.* Am J Physiol Cell Physiol, 2009. **296**(3): p. C414-21.

200. Izumi, H., et al., *Monocarboxylate transporters 1 and 4 are involved in the invasion activity of human lung cancer cells.* Cancer Sci, 2011. **102**(5): p. 1007-13.

201. Colen, C.B., et al., *Metabolic targeting of lactate efflux by malignant glioma inhibits invasiveness and induces necrosis: an in vivo study.* Neoplasia, 2011. **13**(7): p. 620-32.

202. Le Floch, R., et al., *CD147 subunit of lactate/H+ symporters MCT1 and hypoxia-inducible MCT4 is critical for energetics and growth of glycolytic tumors.* Proceedings of the National Academy of Sciences, 2011. **108**(40): p. 16663-16668.

203. Polet, F. and O. Feron, *Endothelial cell metabolism and tumour angiogenesis: glucose and glutamine as essential fuels and lactate as the driving force.* J Intern Med, 2013. **273**(2): p. 156-65.

204. Oldendorf, W.H., *Carrier-mediated blood-brain barrier transport of short-chain monocarboxylic organic acids.* Am J Physiol, 1973. **224**(6): p. 1450-3.

205. Nemoto, E.M. and J.W. Severinghaus, *Stereospecific permeability of rat blood-brain barrier to lactic acid.* Stroke, 1974. **5**(1): p. 81-4.

206. Gerhart, D.Z., et al., *Expression of monocarboxylate transporter MCT1 by brain endothelium and glia in adult and suckling rats.* Am J Physiol, 1997. **273**(1 Pt 1): p. E207-13.

207. Kido, Y., et al., *Functional clarification of MCT1-mediated transport of monocarboxylic acids at the blood-brain barrier using in vitro cultured cells and in vivo BUI studies.* Pharm Res, 2000. **17**(1): p. 55-62.

208. Uhernik, A.L., C. Tucker, and J.P. Smith, *Control of MCT1 function in cerebrovascular endothelial cells by intracellular pH.* Brain Res, 2011. **1376**: p. 10-22.

209. Smith, J.P., et al., *Regulation of Mct1 by cAMP-dependent internalization in rat brain endothelial cells.* Brain Res, 2012. **1480**: p. 1-11.

210. Mac, M. and K.A. Nalecz, *Expression of monocarboxylic acid transporters (MCT) in brain cells. Implication for branched chain alpha-ketoacids transport in neurons.* Neurochem Int, 2003. **43**(4-5): p. 305-9.

211. Vannucci, S.J. and I.A. Simpson, *Developmental switch in brain nutrient transporter expression in the rat.* Am J Physiol Endocrinol Metab, 2003. **285**(5): p. E1127-34.

212. Klyce, S.D., *Stromal lactate accumulation can account for corneal oedema osmotically following epithelial hypoxia in the rabbit.* J Physiol, 1981. **321**: p. 49-64.

213. Riley, M.V. and B.S. Winkler, *Strong Pasteur effect in rabbit corneal endothelium preserves fluid transport under anaerobic conditions.* J Physiol, 1990. **426**: p. 81-93.

214. Adijanto, J. and N.J. Philp, *The SLC16A family of monocarboxylate transporters (MCTs)–physiology and function in cellular metabolism, pH homeostasis, and fluid transport.* Curr Top Membr, 2012. **70**: p. 275-311.

215. Chidlow, G., et al., *Expression of monocarboxylate transporters in rat ocular tissues.* Am J Physiol Cell Physiol, 2005. **288**(2): p. C416-28.

216. Nguyen, T.T. and J.A. Bonanno, *Bicarbonate, NBCe1, NHE, and carbonic anhydrase activity enhance lactate-H+ transport in bovine corneal endothelium.* Invest Ophthalmol Vis Sci, 2011. **52**(11): p. 8086-93.

217. Albini, A. and M.B. Sporn, *The tumour microenvironment as a target for chemoprevention.* Nat Rev Cancer, 2007. **7**(2): p. 139-47.

218. Dhup, S., et al., *Multiple biological activities of lactic acid in cancer: influences on tumor growth, angiogenesis and metastasis.* Curr Pharm Des, 2012. **18**(10): p. 1319-30.

219. Vegran, F., et al., *Lactate influx through the endothelial cell monocarboxylate transporter MCT1 supports an NF-kappaB/IL-8 pathway that drives tumor angiogenesis.* Cancer Res, 2011. **71**(7): p. 2550-60.

220. Sonveaux, P., et al., *Targeting the lactate transporter MCT1 in endothelial cells inhibits lactate-induced HIF-1 activation and tumor angiogenesis.* PLoS One, 2012. **7**(3): p. e33418.

221. Polyak, K., I. Haviv, and I.G. Campbell, *Co-evolution of tumor cells and their microenvironment.* Trends Genet, 2009. **25**(1): p. 30-8.

222. Kim, J.B., *Three-dimensional tissue culture models in cancer biology.* Semin Cancer Biol, 2005. **15**(5): p. 365-77.

223. Dubouchaud, H., et al., *Endurance training, expression, and physiology of LDH, MCT1, and MCT4 in human skeletal muscle.* Am J Physiol Endocrinol Metab, 2000. **278**(4): p. E571-9.

224. Benton, C.R., et al., *Monocarboxylate transporters in subsarcolemmal and intermyofibrillar mitochondria.* Biochem Biophys Res Commun, 2004. **323**(1): p. 249-53.

225. Harjes, U., K. Bensaad, and A.L. Harris, *Endothelial cell metabolism and implications for cancer therapy.* Br J Cancer, 2012. **107**(8): p. 1207-12.

226. Hinshaw, D.B. and J.M. Burger, *Protective effect of glutamine on endothelial cell ATP in oxidant injury.* J Surg Res, 1990. **49**(3): p. 222-7.

227. Balmaceda-Aguilera, C., et al., *Glucose transporter 1 and monocarboxylate transporters 1, 2, and 4 localization within the glial cells of shark blood-brain-barriers.* PLoS One, 2012. **7**(2): p. e32409.

228. Cheng, C., et al., *Alterations of monocarboxylate transporter densities during hypoxia in brain and breast tumour cells.* Cell Oncol (Dordr), 2012. **35**(3): p. 217-27.

229. Wang, Y., et al., *Monocarboxylate transporter 2 and stroke severity in a rodent model of sleep apnea.* J Neurosci, 2011. **31**(28): p. 10241-8.

230. DeBerardinis, R.J., *Is cancer a disease of abnormal cellular metabolism? New angles on an old idea.* Genet Med, 2008. **10**(11): p. 767-77.

231. Helmlinger, G., et al., *Formation of endothelial cell networks.* Nature, 2000. **405**(6783): p. 139-41.

232. Yun, H., et al., *Glucose deprivation increases mRNA stability of vascular endothelial growth factor through activation of AMP-activated protein kinase in DU145 prostate carcinoma.* J Biol Chem, 2005. **280**(11): p. 9963-72.

233. Yeh, W.L., C.J. Lin, and W.M. Fu, *Enhancement of glucose transporter expression of brain endothelial cells by vascular endothelial growth factor derived from glioma exposed to hypoxia.* Mol Pharmacol, 2008. **73**(1): p. 170-7.

234. Zabel, D.D., et al., *Lactate stimulation of macrophage-derived angiogenic activity is associated with inhibition of Poly(ADP-ribose) synthesis.* Lab Invest, 1996. **74**(3): p. 644-9.

235. Constant, J.S., et al., *Lactate elicits vascular endothelial growth factor from macrophages: a possible alternative to hypoxia.* Wound Repair Regen, 2000. **8**(5): p. 353-60.

236. Althaus, F.R. and C. Richter, *ADP-ribosylation of proteins. Enzymology and biological significance.* Mol Biol Biochem Biophys, 1987. **37**: p. 1-237.