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

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

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

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

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

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

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transport. Inducible knockdown of CD147/Basigin was sufficient to phenocopy these antitumor effects. Finally, by a genetic approach, we succeeded in uncoupling MCT1/MCT4 inhibition from CD147/Basigin expression. We propose that the protumoral action of CD147/Basigin is due to its major action on energy metabolism by controlling lactic acid export. Taken together, these studies suggest that the hypoxia-regulated lactate transporter MCT4 and its ancillary protein CD147/Basigin are important anticancer targets for hypoxic and rapidly growing tumors.

Results

Pharmacological Inhibition of MCT1/2 Suppressed Glycolysis and Tumor Growth of Ras-Transformed Fibroblasts Not Expressing MCT4. We first analyzed the expression of MCT1–MCT4 isoforms in the Ras-transformed fibroblast CCL39 cell line for which we possess mutants impaired either in glycolysis (CCL39-gly[−]) (29) or in respiration (CCL39-res[−]) (30). By quantitative RT-PCR and immunoblotting, we found that the CCL39 fibroblast series expressed only MCT1 (Fig. 1A) and MCT2 (Fig. S1A). Uptake of ¹⁴C-lactic acid is totally suppressed in CCL39 cells treated with the MCT1/2 inhibitor AR-C155858 (28) but not if they express MCT4 (CCL39-mct4) (Fig. S1A–C). This experiment, as well as the intracellular lactate accumulation (Fig. 1F), confirmed the specificity of this inhibitor for MCT1 and MCT2 (31). The growth and survival (colony size and number, Fig. 1B, Left) of CCL39-ev cells were not affected by the MCT1/2 inhibitor in normoxia (N), but it was severely reduced in hypoxia 1% O₂ (Hx) and abolished when cells are strictly relying on glycolysis in response to oligomycin treatment (N + oligo). Fig. 1B, Right shows that ectopic expression of MCT4 (CCL39-mct4) eliminated all effects of the MCT1/2 inhibitor in all of the conditions analyzed (hypoxia and oligomycin treatment). By inhibiting lactic acid export, AR-C155858 strongly reduced the rate of glycolysis in the three conditions tested (Fig. 1C, Left). This inhibition of glycolysis did not impact on cell growth in normoxia. In contrast, in hypoxia or in oligomycin-treated cells, inhibiting lactic acid export disrupted pH_i control (Fig. S1D). As expected, CCL39 cells expressing MCT4 (CCL39-mct4; Fig. 1C, Right) became fully resistant to the MCT1/2 inhibitor and, interestingly, their rate of glycolysis increased, particularly in cells

incubated with oligomycin or in hypoxia 1% O₂. These findings on the MCT1/2 inhibitor action were fully supported by the growth features of the CCL39-derived metabolic mutants. In vitro growth of glycolysis-defective cells (CCL39-gly[−]) is not affected by MCT1/2 inhibition in normoxia and hypoxia conditions, whereas the respiratory-deficient cells (CCL39-res[−]), which rely only on glycolysis, do not grow and die in the presence of the MCT1/2 inhibitor (Fig. S2B).

The impact of MCT1/2 inhibition on tumor growth in vivo was assessed by using the Ras-transformed CCL39 fibroblasts (CCL39-ev) implanted in nude mice (Fig. 1D). In contrast to tumor control mice injected with PBS-Tween (PBS-T), tumors from mice receiving 30 mg/kg AR-C155858 (iMCT1/2) twice daily (b.i.d.) did not develop during the 6 d of treatment. At the end of treatment, tumors reestablished a similar growth rate to the untreated controls. Ectopic expression of MCT4 in the same cells (CCL39-mct4) had two major consequences: (i) increased tumorigenicity (shorter tumor latency/increased growth rate), and (ii) total insensitivity to MCT1/2 inhibition (Fig. 1E). The same experiment conducted with the glycolytic-deficient mutant expressing only MCT1/2 showed full insensitivity to the MCT1/2 inhibitor (Fig. S2C), highlighting that the antitumor effects seen with the MCT1/2 inhibitor (Fig. 1D) were not due to side non-specific toxic effects. We conclude that tumors, from Ras-transformed fibroblasts, experienced a hypoxic microenvironment in vivo, rendering these cells glycolytic and sensitive to the lactic acid export inhibitor.

Export of Lactic Acid via MCT1 and MCT4 Is Crucial for Tumor Growth.

As we have seen above, tumor growth from Ras-transformed CCL39 fibroblasts that express only MCT1/2 was extremely sensitive to blockade of lactic acid export. The respiration-defective mutant (CCL39-res[−]) that relies only on glycolysis for energy supply is poorly tumorigenic (low tumor incidence and elongated tumor latency; Fig. 2A). Ectopic expression of MCT4 in these cells (see immunohistochemistry in circle inserts; Fig. 2A) restored full tumorigenicity (100% tumor incidence and reduced latency from ≈62 to 20 d; Fig. 2A). Moreover the res[−] cells cultivated in the presence of 10 nM MCT1/2 inhibitor (iMCT1) died in 24 h, a result

Fig. 1. Pharmacological inhibition of MCT1/2 of Ras-transformed fibroblasts expressing or not MCT4. Impact on glycolysis and tumor growth. (A) Total extracts from Ras-transformed fibroblasts CCL39-ev and CCL39-mct4 exposed to normoxia were analyzed by immunoblotting with antibodies against MCT1, MCT4, and Hsp90 used as a loading control. (B) Clonal growth of CCL39-ev and CCL39-mct4 cells in the presence or absence of oligomycin (1 μg/mL) or iMCT1/2 (100 nM) or both compounds either in normoxia (N) or hypoxia 1% O₂ (Hx) for 10 d before staining and visualization of the colonies. (C) The rate of glycolysis (mmol lactate · 10⁶ cells^{−1} · h^{−1}) was determined in the supernatant of CCL39-ev and CCL39-mct4 cells incubated either in normoxia (N) or normoxia in the presence of 1 μg/mL oligomycin (N oligo) or in hypoxia 1% O₂ (Hx) in pyruvate-free DMEM containing (+) or not (−) iMCT1/2 (100 nM). (D and E) Xenograft tumor growth of Ras-transformed control fibroblasts CCL39-ev (D) and MCT4-expressing CCL39-mct4 (E) s.c. injected into the back of athymic nude mice. When the size of the tumor reached 5 mm in diameter, mice were injected s.c. twice per day with either vehicle PBS-5% Tween (PBS-T) or with the AstraZeneca AR-C155858 (28) inhibitor referred to as iMCT1/2 (30 mg/kg) during 6 d (arrows). Five mice were studied per condition. In vivo experiments were repeated twice. (F) Time-course of intracellular lactate concentration in response to glucose (25 mM) addition in the presence of DMSO or iMCT1/2 (100 nM, dotted line).

Fig. 2. Key role of hypoxia-induced MCT4 in glycolysis and tumor growth. (A) Tumorigenicity in nude mice of Ras-transformed fibroblasts CCL39-*mct4* and mutant impaired in respiration and expressing with (*res*⁻-*mct4*) or without (*res*⁻) MCT4. Cells (10^6) were s.c. injected into the back of nude mice. Expression of MCT4 is shown by circle insets: Immunohistological confirmation. (Magnification: 20 $\times$). (B) Single and double knockdown of MCT1 and MCT4 in colon carcinoma cell line LS174T. The LS174T cell line that expresses the tetracycline repressor (LS174T cell line) were stably transfected with the pTER-*mct1* plasmid that allows expression of shRNA targeting MCT1 under tetracycline treatment (LS174T *shmct1*ⁱⁿ). Second, the LS174T *shmct1*ⁱⁿ cells were infected by a lentivirus expressing a shRNA nontarget (*shctl*) or a shRNA targeting MCT4 (*shmct4*). The cells were cultivated in the absence (-Tet) or presence (+Tet) tetracycline for 5 d and maintained in hypoxia 1% O₂ for the last 48 h before lysis. The whole cell expression of MCT1 and MCT4 was analyzed by immunoblotting. ERK1/2 level was displayed for loading normalization. (C) Tumorigenicity in nude mice. LS174T *shmct1*ⁱⁿ/*shctl* cells (1×10^6) or LS174T *shmct1*ⁱⁿ/*shmct4* cells where s.c. injected in nude mice drinking (+Dox) or not drinking (-Dox) doxycycline in water 4 d before cell injection and during the tumorigenicity assay. Five mice were used per condition. Mice were killed when tumor size reached the mean value of 600 mm³ (% animal survival). This experiment was reproduced twice. (D) Cells were cultivated in the absence (-) or presence (+) tetracycline for 5 d and maintained either in normoxia or in hypoxia 1% O₂ during the last 48 h before lysis. The whole cell expression of HIF1 α , MCT1, MCT4, and CD147/Bsg was analyzed by immunoblotting. ERK1/2 was used for loading normalization. (E) The cell surface expression of CD147/Bsg was analyzed by flow cytometry on LS174T *shmct1*ⁱⁿ/*shctl* cells and LS174T *shmct1*ⁱⁿ/*shmct4* cells cultivated in normoxia in the presence (+Tet) or absence of tetracycline (-Tet). For each condition, the CD147/Bsg fluorescence intensity was compared with the one obtained with the LS174T *shmct1*ⁱⁿ/*shctl* cultivated in the absence of tetracycline (*shctl* -Tet, dark curve, 100%); *shctl* + Tet (blue curve, 64%); *shmct4* - Tet (green curve, 74%); and *shmct4* + Tet (red curve, 53%). Peaks on the left correspond to control incubation with the anti-mouse antibody alone.

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

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

Next, we analyzed the human colon adenocarcinoma cell line LS174T that expresses both MCT1 and MCT4 in normoxia. This cell line exhibits a three- to fivefold increase in MCT4 mRNA and protein expression in hypoxia 1% O₂ (Fig. 2D, compare lanes 1 and 5). Introduction of a tetracycline (Tet)-inducible shRNA targeting MCT1 (LS174T *shmct1*ⁱⁿ) reduced MCT1 expression by 90% in the presence of Tet (compare Fig. 2B,

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

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

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

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

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

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

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

These are key experiments demonstrating that we can disable tumor growth of the colon adenocarcinoma cell line LS174T simply by directly blocking lactic acid export while maintaining a high level of CD147/Basigin. Conversely, as long as MCT activity is sustained, Basigin ablation does not suppress the tumoral potential. We therefore conclude that a major protumoral action of CD147/Basigin is mediated by its direct action in energy metabolism by controlling the export of lactic acid in glycolytic tumors.

Our previous findings on the disruption of the Na^+/H^+ exchanger (16, 36), a universal pH_i-regulating system from bacteria to man, lead us to propose that disabling pH_i control mechanisms could represent an alternative anticancer approach, particularly to highly proliferating, hypoxic, and glycolytic tumors. We proposed that HIF-1-mediated pH_i control is a key determinant for cellular adaptation, tumor survival, and progression in a hypoxic microenvironment. We hypothesized that carbonic anhydrase IX (CAIX) and the $\text{H}^+/\text{lactate}$ symporter MCT4, induced by HIF-1, should be considered as two prominent candidates for pH_i regulation and promising targets for anticancer strategies (8). The first target, CAIX, characterized by a very restricted pattern of tissue expression, is hijacked in many malignancies (37), and recent work from several groups has validated the hypothesis whereby CAIX controls pH_i by accelerating H^+ extrusion and promoting tumor growth (19, 38) and metastasis (39). For the second target, we recently demonstrated by monitoring tumor pH_i in vivo by ^{31}P -NMR spectrometry that tumors expressing MCT4 had a more alkaline pH_i and lower external pH (pH_e) than tumors expressing MCT1/2 only (40). We concluded that MCT4, which is an efficient lactate exporter, should contribute to increased glycolysis and tumor growth rate in a hostile acidic microenvironment.

for pyruvate, are mostly appropriate for both lactate efflux/influx (MCT1) or lactate/pyruvate recapture only (MCT2) (41).

As yet no pharmacological inhibitor exists for MCT4 despite its close sequence homology to MCT1 and MCT2. We therefore investigated their respective roles by specific knockouts or shRNA silencing of MCT4, MCT1, and CD147/Basigin in the colon adenocarcinoma LS174T. Silencing of CD147/Basigin, which induced a concomitant down-regulation of MCT1 and MCT4 expression, had a profound effect in reducing tumorigenicity of LS174T cells. This study confirmed and extended the profound effect of CD147 silencing on the lactate transporters MCT1 and MCT4 and tumor growth recently reported for pancreatic cancer (32). The reciprocal loss of CD147/Basigin by cosilencing MCT1 and MCT4 would suggest that the major role of CD147/Basigin in cells is the cochaperoning of MCT1 and MCT4, an action going in both directions as suggested (43).

Before being recognized as a MCT subunit, CD147/Basigin has been reported as a key protumoral cell surface glycoprotein. Forced expression of CD147 in breast cancer cells resulted in increased tumor growth and metastasis, an action correlated with its capacity to promote extracellular matrix metalloproteinase induction (alternative name EMMPRIN) and to modify the tumor microenvironment (26). This tumor invasive capacity was also associated more recently to increased angiogenesis via Basigin-mediated VEGF induction. The profound antitumoral effects induced by CD147/Basigin silencing raises the question of whether the effects seen are reflecting the impairment of energy metabolism by inhibition of lactate export or of the protumoral action initially reported for Basigin or both. Because of the interdependency of CD147/MCTs for plasma membrane expression, it is not easy to uncouple the expression/function of both proteins. However, we could provide an answer to this question with the two tumor models we reported in this study. The Ras-transformed CCL39 cells that express only MCT1 and MCT2 are extremely sensitive to the lactic acid transporter inhibitor (iMCT1/2) that fully blocks lactic transport (Fig. S1B), glycolysis, and xenograft tumor (Fig. 1 C and D), yet the level of the CD147/Basigin remained unchanged (Fig. 1D *Inset*). In the colon adenocarcinoma cell line, the knockout of the *mct4* gene reduced cell surface expression of CD147/Basigin by only 29%. This mutant cell line, with a fully functional MCT1, was not affected for in vitro growth in normoxia or hypoxia 1% O₂ (Fig. S6 C and D). However, the pharmacological inhibition of MCT1/2 in these *mct4*-null cells abolished lactic acid export and proliferation under conditions where the expression level of the glycosylated CD147/Basigin remained unchanged (Fig. S6D, *Right*). Finally, forced expression of a catalytically dead MCT4 (K40R) that ensured full maintenance of Basigin surface expression did not affect the reduced tumorigenicity when MCT activity was silenced (Fig. 3 B and C). Conversely, we succeeded in embigin-LS174T cells to maintain MCT activity and full tumorigenicity despite the genetic deletion of Basigin (Fig. 3 E and F). We therefore conclude that the major protumoral action of CD147/Basigin is mediated by its cardinal function of chaperoning MCTs, an action central in energy metabolism particularly in highly glycolytic tissues and aggressive malignancies. The question whether CD147/Basigin has additional protumoral functions, particularly through its interaction with signaling integrins, CD98/LAT1 complex or through promotion of MMPs in the acidic tumor microenvironment, remains an open question.

Increased glycolysis and cell migration go together in embryonic development, wound healing, and metastasis. In this context, it is interesting that the Basigin/MCT4 complex has been found to associate with integrin $\beta 1$ and localize at the leading edge of migrating cells, an area very active for invasive growth and also very demanding in metabolic energy (44). More importantly Philp et al. (45) reported that silencing MCT4 but not MCT1 in retinal pigment epithelial cells slowed down their migration, a process highly

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

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

Materials and Methods

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

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

See *SI Materials and Methods* for additional details.

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