Chapter 13
Carbonic Anhydrase IX (CAIX) as a Mediator of Hypoxia-Induced Stress Response in Cancer Cells

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Abstract The development of hypoxic microenvironments within many types of solid tumors imposes a significant stress on cancer cells to which they must respond appropriately in order to survive and grow. Tumor-specific, hypoxia-induced upregulation of Carbonic Anhydrase IX (CAIX) is a component of the complex response of cancer cells to the evolving low oxygen environment. Here, we discuss evidence from in vivo tumor models employing inhibition or enhancement of CAIX expression, using gene depletion or overexpression strategies, respectively, or inhibition of its catalytic activity, using CAIX-specific small molecules or antibodies, to demonstrate that CAIX is a functional mediator of tumor growth and metastasis. We also discuss the functional contribution of CAIX to several specific biological processes critical for cancer progression, including pH regulation and cell survival, adhesion, migration and invasion, the maintenance of cancer stem cell function, and the acquisition of chemo and radioresistant properties. The demonstration of CAIX as a functional mediator of cancer progression provides a biological rationale for its use as a cancer-specific, clinically relevant therapeutic target.

Keywords Hypoxia • Therapeutic target • Enzymatic activity • Metastasis • Tumor growth

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1 Introduction

Hypoxia is a central environmental feature common to many types of solid cancers and stems from the inability of the tumor vasculature, which is composed of structurally abnormal and functionally unstable vessels, to adequately respond to the increased oxygen demands of the growing tumor [1, 2]. The evolution of an intratumoral hypoxic microenvironment imposes a significant stress on cancer cells and they must adapt if they are to survive and grow in this hostile environment. To cope effectively with reduced oxygen availability, cancer cells engage a highly conserved hypoxia-induced intracellular signaling cascade regulated by the transcription factors hypoxia-inducible factor 1 and 2 (HIF-1/2) [2]. The activation of the HIF pathway results in the modulation of many genes that regulate several critical processes involved in tumor progression, including cell survival and proliferation, metabolic reprogramming [3, 4], stem cell maintenance, growth factor signaling, epithelial-mesenchymal transition (EMT), angiogenesis, invasion, metastasis, and resistance to radiation therapy and chemotherapy.

Amongst the genes targeted for upregulation by the HIF pathway in cancer cells, Carbonic Anhydrase IX (CAIX) often shows the most dramatic transcriptional activation [5, 6]. The robust and systematic overexpression of CAIX by many types of cancer cells in response to hypoxia underscores its importance in mediating the response of these cells to aberrant oxygen status within tumors. Indeed, CAIX is now well-recognized as having a critical role in primary cancer development and progression, as well as in metastatic disease (Fig. 13.1) [6, 7] (also see Chaps. 10, 11, and 12). Furthermore, its membrane-bound, extracellular location and its tumor-specific overexpression, together with its highly restricted expression profile in normal tissue, have made CAIX a very attractive target for cancer therapy [6, 8–10]. However, while its striking upregulation in hypoxic regions and the well-established demonstration that high CAIX expression is poorly prognostic in many types of solid cancers [6] suggest that its expression is critically important and functionally relevant for tumor growth, the use of CAIX as a therapeutic target in cancer, particularly for therapies designed to interfere with its enzymatic activity, hinges on robust demonstration of CAIX as a functional mediator for the growth and spread of cancer.

In this Chapter, we will first critically review the data demonstrating that perturbation of CAIX expression using genetic strategies or inhibition of enzymatic function using small molecule inhibitors or antibodies results in the inhibition of tumor growth and metastasis in vivo, a key measure of the ability of CAIX to function as a mediator of cancer progression. We will then focus on data showing that CAIX contributes to several specific biological processes critical for cancer progression, including pH regulation and cell survival, adhesion, migration and invasion, the maintenance of cancer stem cell function, and the acquisition of chemo and radioresistant properties.

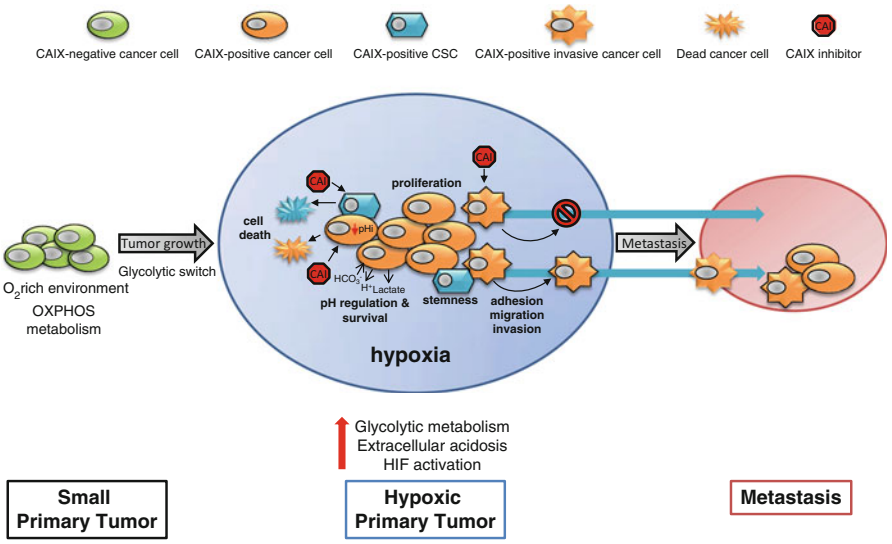

Fig. 13.1 Carbonic Anhydrase IX (*CAIX*) is a functional effector of several biological processes important for tumor growth and metastasis. The development of hypoxia by a growing tumor imposes a significant stress on cancer cells and results in HIF-mediated regulation of many genes, including the dramatic upregulation of CAIX. CAIX contributes to the maintenance of an intracellular pH favorable for tumor cell survival and growth, while also participating in the generation of an increasingly acidic extracellular environment, promoting survival, growth, migration and invasion of cancer cells. CAIX is also an important mediator of the maintenance and stemness properties of cancer stem cells. Inhibition of CAIX enzymatic activity results in reduced survival, migration and invasion in hypoxia. The stemness properties and survival of CSCs are also negatively impacted. Abbreviations. CAIX, Carbonic Anhydrase IX; CAI, Carbonic Anhydrase IX Inhibitor; CSC, cancer stem cell; H^+, proton; HCO_3^-, bicarbonate; HIF, hypoxia-inducible factor; O_2, oxygen; OXPHOS, oxidative phosphorylation; pHi, intracellular pH

2 Inhibition of CAIX Expression and/or Activity Impacts Tumor Growth and Metastasis in Preclinical Cancer Models

The recognition that CAIX is selectively and systematically upregulated by many types of cancer cells in response to tumor hypoxia, together with its cell surface localization and highly restricted normal tissue expression pattern, has solidified CAIX as a robust cancer-specific target [6] and has driven the development of antibodies and highly selective small molecules based on several chemical scaffolds designed to inhibit CAIX catalytic activity [8, 11]. However, the rationale that targeting CAIX enzymatic activity in the context of hypoxia-induced expression will lead to therapeutic benefit is valid only if CAIX is an effective functional mediator of tumor progression and metastasis. A critical measure of the capacity of CAIX

to function as a mediator of malignant disease progression is to determine whether inhibition or enhancement of its expression, using gene depletion or overexpression strategies, respectively, or inhibition of its catalytic activity, using CAIX-specific small molecules or antibodies, results in altered tumor progression in vivo. In the following sections we will examine the evidence suggesting that hypoxia-induced CAIX is a critical mediator of tumor growth and metastasis, and that interference with its catalytic activity is a valid strategy for the development of targeted therapies.

2.1 Effect of Perturbation of CAIX Expression by Cancer Cells on the Growth of Tumors In Vivo

Data from several studies evaluating the effects of silencing hypoxia-induced CAIX expression in vivo have shown that stable depletion of CAIX in an appropriate microenvironmental context results in attenuation of tumor growth across multiple tumor types. Reduced tumor growth has been observed in mouse and human models of breast cancer [12], as well as in human models of colorectal cancer [13, 14] and glioblastoma [14]. Interestingly, data from these studies suggest that some tumor types, such as breast cancer, may be particularly dependent on CAIX expression for continued growth as regions of hypoxia become established [12], while tumors derived from other tissue types, notably colorectal cancer, may exhibit compensatory regulation related to the expression of extracellular CAs, particularly CAXII, in the event that CAIX function is compromised [13–15]. However, the degree to which tumors compensate for the loss of CAIX with the induction of CAXII expression is variable, as are the functional consequences. In studies of HT-29 colon cancer xenografts, induction of CAXII expression in the absence of CAIX was modest and the functional consequences were not specifically investigated [14]. In contrast, stable downregulation of CAIX together with CAXII in the LS174Tr colon carcinoma model resulted in an 85 % reduction in tumor growth, significantly more than the 40 % reduction in tumor growth attributed to CAIX silencing alone in this model system [13]. Therefore, while the extent to which the growth of hypoxic tumors is reduced in the absence of CAIX expression may be dependent on tumor type, the published data clearly demonstrate that the presence of CAIX in these tumors is of functional importance for their growth.

In addition to depletion of gene expression, the role of CAIX in mediating the growth of tumors has been interrogated using exogenous expression of human CAIX. In this context, it is important to recognize the inherent challenges associated with constitutive overexpression of proteins in situations where the production and functional relevance of the endogenous protein is dictated by intratumoral environmental conditions. Overexpression studies often employ tumors derived from cell lines that do not express endogenous CAIX in normoxia or hypoxia. However, the fact that these cells do not upregulate CAIX in response to appropriate environmental cues means that these cells may not require CAIX in vivo to

gain a competitive advantage and often complicates the interpretation of data derived using these models. Despite these caveats, studies have demonstrated an impact of forced CAIX expression on tumor growth. For example, introduction of constitutive CAIX expression in HCT-116 colorectal cancer xenografts, a model that only weakly induces CAIX in vivo, resulted in an increased rate of tumor growth [14], suggesting that the introduction of CAIX expression may enhance cancer progression. Experiments have also been conducted recently wherein shRNA has been used to silence endogenous hypoxia-induced CAIX expression in 4T1 mouse breast cancer cells, a cell type that is dependent on CAIX upregulation for continued tumor growth in the presence of hypoxia in vivo [12]. Introduction of human CAIX into this CAIX-depleted system resulted in the rescue of growth of tumors established from these cells, compared to CAIX silenced controls [12], demonstrating that CAIX acts as a mediator of hypoxic tumor progression. Thus, based on data demonstrating that silencing CAIX gene expression reduces tumor growth, together with findings that CAIX overexpression results in enhancement of tumor growth or rescue of growth in a hypoxia-specific context, it is clear that the expression of CAIX plays a role in mediating tumor progression.

However, while manipulation of gene expression provides important insights into the functional consequences of protein depletion and demonstrates proof-of-principle for the importance of CAIX as a mediator of cancer growth, such strategies do not allow investigation of individual aspects (e.g. catalytic activity) of a multifunctional protein, nor are they currently viable as options for cancer therapy. Observations using specific small molecule inhibitors are equally important for addressing questions related to whether targeted inhibition of CAIX catalytic activity results in a reduction in tumor growth, thereby demonstrating the functional importance of CAIX enzymatic activity and providing an avenue for cancer treatment.

2.2 The Effect of Treatment with CAIX-Specific Small Molecule Inhibitors on Tumor Growth In Vivo

Similar to studies that demonstrate that altering CAIX gene expression has a measurable impact on tumor growth, the development of specific small molecule inhibitors allows the effect of selectively inhibiting CAIX catalytic activity on the growth of tumors to be ascertained. An important consideration for the use of targeted therapeutic agents against CAIX is the ability of the drug to reach regions of high CAIX expression, which are defined by poor vascular networks and diffusion characteristics [9]. Several studies have now demonstrated tumor-specific accumulation of CAIX-specific inhibitors in a number of tumor models [16–18], demonstrating that these agents can indeed access CAIX-expressing cancer cells in vivo.

CA inhibitors have been available for some time, but only recently have inhibitors that are selective for cancer associated extracellular CAs such as CAIX (and CAXII) over other, closely related "off-target" CA isoforms become available [8, 10] (also see Chap. 15). Initial strategies included efforts to reduce the membrane permeability of the pan CA inhibitor, acetazolamide, thereby making it more selective for CAIX [16]. Treatment of SK-RC-52 human renal cell carcinoma xenografts consitutively expressing CAIX with this inhibitor resulted in decreased tumor growth, but the effect was not reproduced in LS174tr colorectal xenograft tumors, a model of hypoxia-induced CAIX expression [13, 16]. Treatment of human breast tumors derived from MDA-MB-231 cells with a sulfamate inhibitor of CAIX has also been reported, but whereas this inhibitor reduced metastatic burden in the lung (see Sect. 3, below), no effect on primary tumor growth was observed [19]. Recently, however, several CAIX-selective sulfonamide inhibitors have been developed that directly and specifically inhibit the growth of hypoxic, CAIX-positive tumors in vivo. Administration of a fluorescently labelled sulfonamide inhibitor to mice harboring syngeneic breast tumors derived from highly metastatic 4T1 cells that express CAIX in hypoxia resulted in significant inhibition of tumor growth [12]. In contrast, similar treatment of syngeneic breast tumors derived from isogenic non-metastatic, CAIX-negative 67NR cells had no effect, demonstrating that CAIX inhibitors are effective specifically against tumors expressing the target in a biologically relevant context [12]. Furthermore, novel ureido sulfonamides [20] and indanesulfonamides [21] have been used successfully to treat preclinical models of hypoxic, CAIX-positive breast cancer [12] and colorectal cancer [21]. Novel glycosyl coumarin inhibitors of CAIX, which function by a mechanism distinct from that of the sulfonamides, have also been described recently and treatment of human and mouse breast cancer models with this class of inhibitors have resulted in significant inhibition of tumor growth [22, 23]. Taken together, these studies demonstrate, using specific inhibitors of CAIX enzymatic activity, that CAIX functions as an important mediator of the growth of hypoxic tumors and indicate that the anti-tumor effect observed with depletion of CAIX expression involves, at least in part, its enzymatic function.

3 CAIX as a Mediator of Cancer Metastasis

Given that the vast majority of cancer deaths are attributable to metastatic disease, understanding whether CAIX functions as a mediator in cancer metastasis is an important question. Indeed, several studies have evaluated CAIX as a target for treatment of metastatic disease [7]. A review of CAIX as a target of cancer metastasis can be found in Chap. 11 and 12. Here we will briefly discuss the evidence showing that gene depletion or inhibition with small molecules defines CAIX as a mediator in this process. Silencing of CAIX expression by 4T1 breast tumor cells resulted in inhibition of both spontaneous and experimental lung metastases [12]. Treatment of experimental models of breast cancer metastasis with novel ureido sulfonamide

and glycosyl coumarin CAIX inhibitors resulted in a significant decrease in lung metastases [12, 20], suggesting that this new generation of selective CAIX inhibitors have the capacity to work as targeted therapeutics for the treatment of both primary tumor growth and metastasis. CAIX-specific inhibitors have also been used to assess spontaneous metastasis. It has been reported that a sulfamate CAIX inhibitor, while not affecting the growth of primary human breast tumors derived from MDA-MB-231 cells, did reduce the metastatic tumor burden in the lung, further demonstrating the capacity of CAIX inhibitors as anti-metastatic agents [19]. Recent data have also demonstrated that an ureido sulfonamide CAIX inhibitor, alone and especially when used in conjunction with conventional chemotherapy, effectively attenuated spontaneous lung metastases in a model of highly metastatic human breast cancer [22]. Moreover, tumor cells obtained from pleural effusions of breast cancer patients, a source of patient-derived metastatic cancer cells, demonstrated high CAIX expression and when these cells were cultured as tumorspheres in hypoxia in the presence of CAIX inhibitors, a decrease in the number of viable cells and impaired growth as aggregates was observed [22], suggesting that inhibition of CAIX is effective against human primary metastatic breast cancer cells.

Thus, from the discussion above, CAIX is not only overexpressed in aggressive, hypoxic tumors, but also is an effector of cancer growth and metastasis, and targeted inhibition of its expression using gene depletion strategies or its enzymatic function using CAIX specific inhibitors clearly and significantly reduces disease burden, demonstrating that its inhibition is a rational strategy for cancer therapy. However, while these in vivo data clearly show that CAIX is functionally important for tumor growth and metastasis, the mechanisms by which CAIX acts to effect these changes are equally important and evoke a number of questions. For example, do the data in cancer cells support the idea that regulation of pH to influence survival are at work in these models? Are alterations in proliferation, migration and invasion involved? Is CAIX expression and activity relevant to the maintenance of cancer stem cells? Does CAIX mediate functions related to chemo and radioresistance? Data supporting these tenants of CAIX function are reviewed and discussed in the following sections.

4 CAIX as a Mediator of Cell Survival and Growth Through Regulation of pH

It is now well-recognized that CAIX is an important component of a complex cellular system involving several proteins and buffer systems dedicated to ensuring the stability of intracellular pH in the presence of increasing extracellular acidosis resulting from the accumulation of byproducts produced by highly metabolic cancer cells engaged in glycolytic metabolism [3, 24]. The inability to control the intracellular pH has adverse consequences for critical cellular processes including ATP synthesis, proliferation, and survival (Fig. 13.1) [24, 25]. It is generally

accepted that, through the conversion of CO_2 to bicarbonate and protons, CAIX contributes to the maintenance of a pHi favorable for tumor cell survival and growth, while also participating in the generation of an increasingly acidic extracellular environment, fueling survival and growth as well as breakdown of the extracellular matrix and consequent tumor cell invasion [24, 26]. The finding that depletion of CAIX expression or inhibition of its catalytic activity results in a reduction in tumor growth across multiple types of cancer suggests that CAIX is important for cancer cell survival and growth in hypoxic environments, and may be acting through this mechanism, as discussed below.

Using depletion of CAIX gene expression or inhibition of its catalytic activity in the context of hypoxia to dysregulate pH homeostasis, several studies have established a link between pH regulation by CAIX and the viability of several types of cancer cells in vitro. Silencing of hypoxia-induced CAIX expression in 4T1 breast cancer cells results in the inhibition of acidification of culture medium [12], together with reduced survival of CAIX-depleted cells in hypoxia [12]. Reductions in clonogenic survival and cell growth have also been reported for human breast cancer cells depleted of CAIX [27]. Studies using human tumor cell lines that constitutively express CAIX or induce the enzyme in hypoxia have demonstrated that CAIX-selective inhibitors reduce CAIX-mediated acidification of the extracellular environment [12, 21, 28, 29] or induce acidification of the intracellular pH [30], while negatively impacting cell survival [21, 30]. Recent studies have shown that U251 glioblastoma cells depleted of CAIX expression and grown in hypoxic and glycolytic conditions exhibited decreased ATP levels and reduced cell viability [31]. It should be noted that in some cancer cell types, for example human hepatocellular carcinoma (HCC) cells, neither transient depletion of CAIX expression using siRNA nor treatment with a CAIX inhibitor alone affected cell growth or induced apoptosis in hypoxia [32], indicating that some types of cancer cells may have evolved compensatory mechanisms designed to counter the effects of interference with CAIX function.

Interestingly, recent data derived from cells cultured as monolayers and as spheroids in three dimensions suggest that the impact of CAIX expression on pH homeostasis may be somewhat more complex and intimately links cell survival and cell proliferation in the hypoxic environment. There is also now accumulating data suggesting that CAIX, through its ability to maintain cancer cell survival in hypoxia, may allow cancer cells to sustain a robust proliferative capacity, providing continued tumor growth, but depleting available resources due to increased metabolic demand, ultimately leading to an increase in cell death and necrosis [14]. Silencing of CAIX in HT-29 cells grown in three dimensions reduced cell proliferation, but also reduced the amounts of apoptosis and necrosis [14]. Silencing hypoxia-induced CAIX or CAIX together with CAXII in LS174Tr colorectal cancer cells resulted in an increase in the proportion of cells in the radiosensitive G1/G2/M phases of the cell cycle, together with an increase in markers associated with reduced proliferation [15]. Furthermore, growth of CAIX-depleted and CAIX/CAXII-depleted LS174Tr cells as spheroids also resulted in decreased proliferation and, in the case of dual knockdown, an increase in cell death [15].

Examination of HT-29 xenografts grown in the presence or absence of CAIX has revealed that, while CAIX-depleted tumors grow more slowly, they also exhibit lower levels of necrosis, apoptosis and proliferation. Paradoxically, CAIX expression promotes necrosis, apoptosis and proliferation in spheroid cultures and in tumors in vivo [14], suggesting that the maintenance of cellular pH by CAIX overexpression allows cells to attain a high level of proliferation, thereby increasing the metabolic demand and depleting available resources in the hypoxic environment. However, CAIX expression ultimately results in larger numbers of viable cells, suggesting that the pro-survival and proliferative capacity imparted by CAIX outweighs the increase in apoptosis and necrosis.

5 CAIX as a Mediator of Adhesion, Migration and Invasion

The observation that depletion of CAIX gene expression or inhibition of its catalytic activity results in inhibition of metastasis in vivo suggests that CAIX may function as a mediator of cancer progression through an effect on the adhesive, migratory or invasive properties of cancer cells (Fig. 13.1). Indeed, the role of CAIX in maintaining pH homeostasis in cancer cells serves to increase the acid load in the extracellular space. The development of acidosis within the extracellular environment is thought to then drive local invasion through disruption of the extracellular matrix, activation of metalloproteases and increased cell invasiveness [33]. However, in addition to modulation of the extracellular environment, there is evidence that CAIX mediates adhesion, migration and invasion by directly modulating cancer cell behaviour. Differential gene expression profiling of cervical carcinoma cells transfected with CAIX or fibrosarcoma cells stably depleted of CAIX expression in normoxia have revealed perturbation of pathways related to cell-matrix adhesion [34], as well as cell growth and cytoskeletal organization [34, 35]. Recently, CAIX has been described as a "hypoxia-controlled catalyst of cell migration" [36] and several studies using models of constitutive CAIX expression have demonstrated that CAIX actively contributes to migration and invasion.

Studies using MDCK cells constitutively overexpressing CAIX in normoxia have suggested that CAIX may modulate E-cadherin-mediated cell adhesion through interactions with β-catenin, resulting in reduced adhesion [37]. Furthermore, this cell line exhibited increased scattering and migration properties in the presence of full length CAIX, but not when a CAIX variant lacking the catalytic domain was introduced, linking CAIX enzymatic activity with migration [38]. These studies further showed that CAIX controls cell migration by facilitating ion transport and pH control in the lamellopodia of motile cells, linking migration to CAIX mediated intracellular pH regulation [38]. Cytoskeletal remodelling, weakened cell-cell and cell-matrix adhesion, and increased migration were observed in cervical carcinoma cells overexpressing CAIX in normoxia, and these processes were shown to involve Rho-GTPase signaling in this cell model [34]. Combined knockdown of COX-2 and CAIX in colorectal cancer cells in normoxia resulted in blunted expression of

active metalloproteinase-2 (MMP-2) and reduced ECM invasion [39], and a mild hypoxic environment induced by high cell density increased the expression of these genes and induced invasive potential [39]. However, these studies have employed constitutive expression of CAIX in normoxia, leaving open the question of whether CAIX modulates these processes in the context of hypoxia.

Findings from several recent studies now provide evidence for a role of CAIX in mediating cancer cell adhesion, migration and invasion in the context of hypoxia. Knockdown of CAIX expression in hypoxic glioblastoma cells results in reduced cell attachment and invasion in vitro [31]. Stable depletion of CAIX expression by highly metastatic MDA-231 LM2 human breast cancer cells in hypoxia reduced invasion of these cells through matrigel [22]. Hypoxia-mediated upregulation of CAIX expression by HeLa cells and subsequent inhibition of its activity by treatment with CA inhibitors or forced expression of a dominant negative isoform of CAIX resulted in reduced migration [38], suggesting the importance of catalytically active CAIX for migration of cancer cells. In agreement with these data, treatment of highly metastatic human breast cancer cells with ureido sulfonamide inhibitors of CAIX in hypoxia significantly reduced migration through Matrigel in 3 dimensions. Furthermore, a series of sulfamate CAIX inhibitors was evaluated and shown to inhibit the migration of human breast cancer cells cultured in conditions of very low oxygen [19] and re-expression of CAIX by gastric cancer cells in the context of 5-azadeoxycytidine treatment resulted in increased invasion. Thus, CAIX is important in the processes of migration and invasion, a functional phenotype typical of cancer cells having undergone EMT [22]. It should be noted that early studies in a few cancer cell types did not demonstrate a reduction in cell invasion with siRNA-mediated depletion of hypoxia-induced CAIX expression or overexpression of CAIX [27], possibly indicating certain cell type differences.

6 CAIX as a Mediator of CSC Stemness

The hypoxic niche is an environment that drives aggressive tumor cell behaviour and is a well-known source of cancer stem cells (CSCs), a subpopulation of tumor cells capable of self-renewal and important for continued growth and progression of solid malignancies. Furthermore, analysis of CSC markers in patients with invasive breast carcinoma showed that cells positive for stem cell markers, including CD133 and $CD24^{+}/CD24^{-/low}$, are associated with hypoxic, CAIX-positive regions of the tumor [40]. Recently published data now suggest that, in hypoxia, CAIX plays a critical role in the maintenance and stemness properties of CSCs (Fig. 13.1). Stable depletion of CAIX in the 4T1 mouse breast cancer cell model inhibited the hypoxia-driven expansion of the CSC population in tumorspheres, as well as attenuation of EMT and stemness markers, a hallmark of the EMT-mediated mesenchymal phenotype typical of CSCs in hypoxia [22]. Similarly, treatment with CAIX-specific inhibitors blocked the EMT/mesenchymal phenotype, suggesting a critical requirement of hypoxia-induced CAIX in the maintenance of stemness in

the breast CSC population [22]. Interestingly, activation of mTORC1 signaling, a pathway known to be regulated by intracellular pH, in hypoxia was dependent on the expression of CAIX, both in cultured cells and in vivo [22], suggesting that CAIX may be an upstream regulator of this pathway. There is also evidence for a functional role of CAIX in maintenance of the CSC population in vivo. Treatment of orthotopic MDA-231 LM2-4 human breast cancer xenografts with either ureido sulfonamide or glycosyl coumarin inhibitors of CAIX significantly reduced the population of EpCAM + CSCs in these tumors, as determined by FACS [22]. Furthermore, immunostaining for ALDH1A3, a complementary marker of CSCs in this tumor type, was reduced in the inhibitor-treated animals [22]. These results suggest the functional maintenance of CSCs by CAIX.

7 Role of CAIX in Radioresistance and Chemoresistance

It is well-recognized that CAIX is most robustly expressed in regions of hypoxia, intratumoral areas often considered privileged microenvironments resistant to conventional chemo- and radiotherapy, and the site of residence of cancer stem cells (CSCs). Both conventional chemotherapeutic drugs and radiotherapy target rapidly dividing cells in well-oxygenated environments. However, these therapies are much less effective at killing tumor cells and CSCs in the hypoxic niche, allowing these cells to evade therapy and ultimately spur tumor growth, often resulting in more aggressive disease. Therefore, development of targeted therapeutics capable of killing cells in regions of hypoxia is critical if the ideal of treatment with curative intent is to be realized. Based on the premise that targeting CAIX will eliminate tumor cells resistant to conventional therapies, thereby producing a greater therapeutic benefit relative to either intervention alone, several studies have now evaluated the use of CAIX-targeted therapies in combination with radio- and chemotherapy. Importantly, these studies serve to demonstrate the ability of CAIX to mediate cancer progression in intratumoral regions protected from the effects of conventional cancer therapy.

The effects of silencing CAIX gene expression or inhibiting its catalytic activity using CAIX-specific small molecule inhibitors in conjunction with ionizing radiation in vivo have been reported in models of colorectal cancer. Silencing of CAIX gene expression, or tandem depletion of CAIX and CAXII expression in LS174Tr colorectal cancer xenografts compromised tumor growth when combined with ionizing radiation, an effect attributed to inhibition of the regulation of intracellular pH by CAIX [15]. Treatment of HT-29 colorectal cancer xenografts with radiotherapy in combination with an indanesulfonamide inhibitor of CAIX improved the therapeutic efficacy compared to single agents alone [21], indicating that CAIX enzymatic activity is important for the radioresistance of these tumors. In addition to these in vivo findings, depletion of hypoxia-induced CAIX expression by glioblastoma cells rendered them sensitive to radiation treatment and resulted in a higher rate of apoptotic cell death [31]. These studies suggest that CAIX is

functionally important for imparting radioresistance on hypoxic tumor cells, thus helping to perpetuate tumor growth, and show that inhibition of CAIX can be used to augment the effects of radiotherapy.

While targeted agents are entering the clinic in ever increasing numbers, conventional chemotherapy remains the mainstay for systemic therapy. In fact, targeted therapies are often administered in combination with chemotherapy to increase therapeutic response. Studies have used this paradigm to evaluate CAIX inhibitors in combination with conventional chemotherapy or antiangiogenic therapy. For example, depletion of hypoxia-induced CAIX expression by glioblastoma cells enhanced the cytotoxic effect of temozoloide [31] and the inhibition of CA activity with acetazolamide in cancer cells highly expressing CAIX has been reported to modulate the toxicity of chemotherapeutic agents such as doxorubicin and melphalan [41], indicating that targeting CAIX function in combination with other agents may be effective in reducing tumor growth. Indeed, in vivo administration of CAIX inhibitors in combination with clinically available systemic therapeutic agents has demonstrated greater efficacy compared to single agent treatment. Specifically, combination of an ureido sulfonamide inhibitor of CAIX with paclitaxel provided benefit greater than that seen with either treatment alone in mouse and human orthotopic models of breast cancer [22], demonstrating the functional importance of CAIX catalytic activity in this hypoxic tumor setting. Moreover, it has been suggested recently that the use of antiangiogenic agents such as sunitinib and Bevacizumab result in increased hypoxia, and treatment of human breast cancer xenografts with these antiangiogenic agents was found to increase the population of CSCs through the generation a hypoxic environment [42]. These findings indicate the necessity of targeting additional tumor components concomitant with the use of angiogenic inhibitors. As part of the response to hypoxia triggered by these agents, CAIX is upregulated, offering an additional therapeutic target for inhibiting tumor growth in this context. While initial studies using the CAIX-selective albumin-binding derivative of acetazolamide described above in combination with sunitinib did not significantly outperform sunitinib alone [16], likely because of the high efficacy of sunitinib in the tumor model used, combination of CAIX inhibitors with anti-angiogenic agents effectively limited tumor growth in the HT-29 colorectal cancer model [14], demonstrating that CAIX-specific inhibitors can be used in conjunction with inhibitors of angiogenesis to exact a therapeutic advantage. Taken together, these findings suggest that CAIX is functionally important for the development of hypoxic tumor cell populations resistant to conventional therapies, thereby contributing to continued tumor growth, and that effective inhibition of its activity using CAIX-selective inhibitors can increase treatment efficacy.

8 Summary

As solid malignancies progress, they often become hypoxic, triggering a core, HIF-1-mediated signaling cascade that results in the activation of a plethora of genes vital for the adaptation of tumor cells to an increasingly stressful environment. One

facet of this adaptive response is the marked upregulation of CAIX. Importantly, stable depletion of CAIX expression or inhibition of its enzymatic activity using selective small molecule inhibitors in the context of tumor hypoxia in vivo results in inhibition of primary tumor growth and metastasis, demonstrating a functionally active role of CAIX in mediating cancer progression. The regulation of extracellular and intracellular pH by CAIX is central to its ability to modulate a host of tumorigenic and metastatic processes, including cell survival, proliferation and invasion. Furthermore, hypoxia-induced CAIX plays an important role in the maintenance and stemness properties of CSCs, a population of tumor cells critical for continued growth and progression of solid malignancies as well as for resistance of cancers to chemotherapy and radiotherapy. The recognition that CAIX, especially in the biologically relevant context of hypoxia, is an important functional mediator of tumor growth and metastasis provides a solid rationale with which to specifically inhibit this cancer-specific target for therapy in patients, both as a monotherapy and in combination with conventional chemotherapy and radiotherapy.

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