TUMOR HYPOXIA: IMPACT IN TUMORIGENESIS, DIAGNOSIS, PROGNOSIS AND THERAPEUTICS

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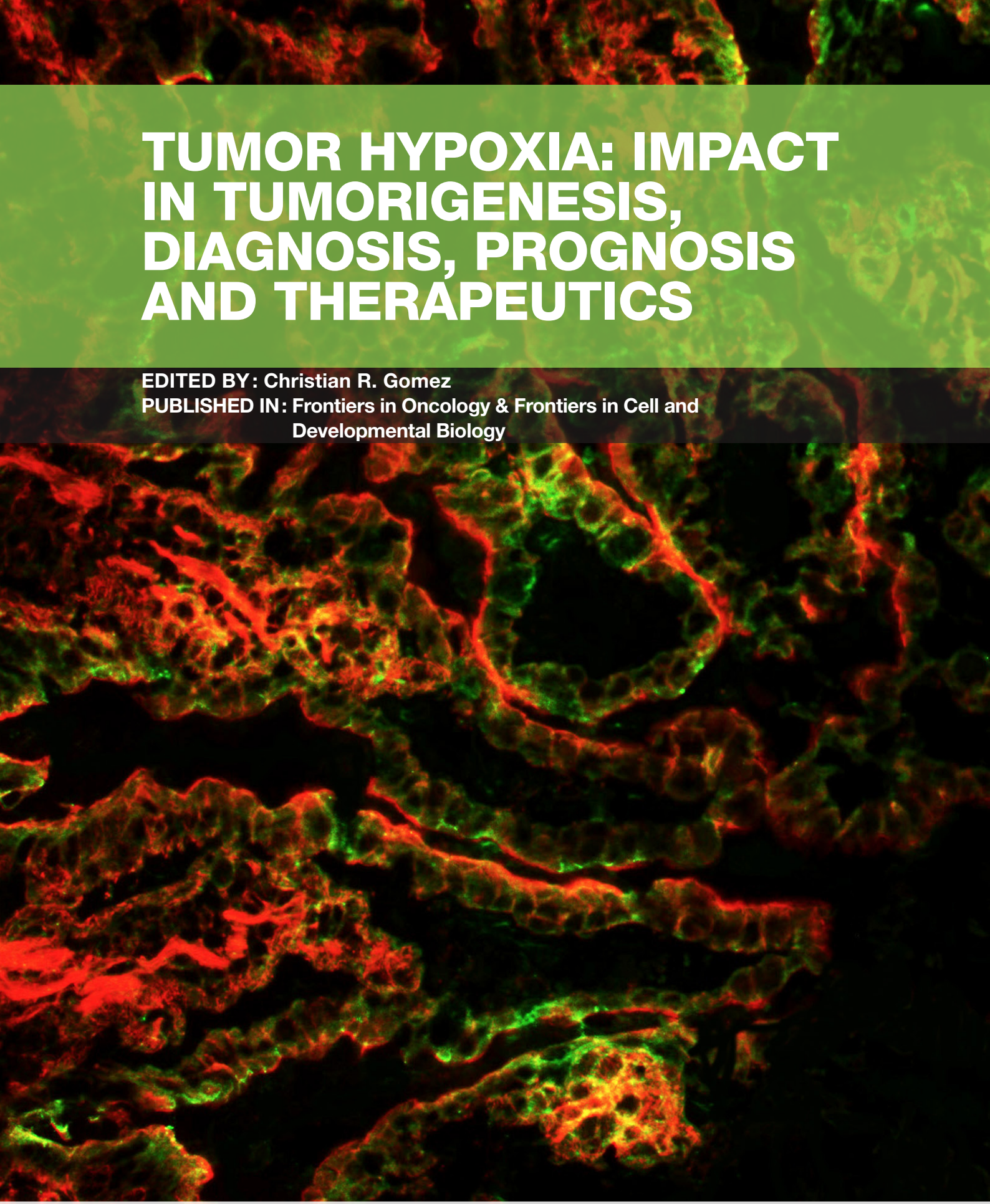

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TUMOR HYPOXIA: IMPACT IN TUMORIGENESIS, DIAGNOSIS, PROGNOSIS AND THERAPEUTICS

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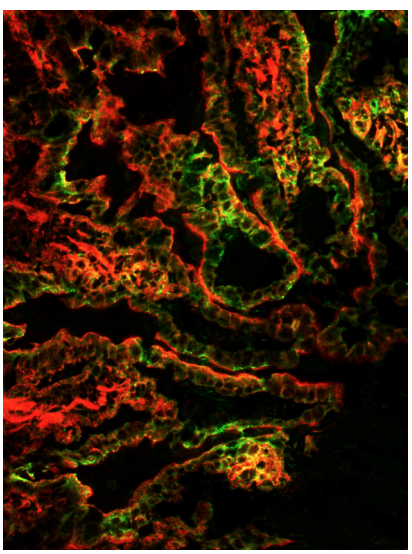

The cover picture shows an immunofluorescence depicting a tissue with intricate histology, complex cell-to-cell interaction and a big dark zone on the right-bottom corner. The cover represents the spirit of this special topic aiming to unravel the not yet understood involvement of hypoxia (what is beyond the dark zone?) on cancer origin, progression, and therapeutics.

Picture taken by Christian Gomez, edited by Marcelo J. Sakiyama.

Hypoxic regions have been identified within tumors and its presence has been linked to malignant progression, metastasis, resistance to therapy, and poor clinical outcomes following treatment. Acute and chronic hypoxia are integral components of tumor microenvironment and conduce to metabolic adaptations of tumor cells leading to genetic instability, intratumor heterogeneity and malignant progression.

On the success of our fight against cancer, the continued adaptability of tumors to their microenvironmental stresses, such as hypoxia, must be considered. Tumor cells are endowed with a very high plasticity and capacity to adapt. It is our challenge to find populations and conditions of the tumor microenvironment germane for target success. Interdisciplinary work will be the key for achievement of these goals.

This e-book is a compendium of original reports and review articles contributed by world-class experts in the field of tumor hypoxia. This material will be useful to foster discussion and increase understanding of the involvement of hypoxia in tumorigenesis, biomarker development, and therapeutics.

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EDITORIAL
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Editorial: Tumor Hypoxia: Impact in Tumorigenesis, Diagnosis, Prognosis, and Therapeutics

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The Editorial on the Research Topic

Tumor Hypoxia: Impact in Tumorigenesis, Diagnosis, Prognosis, and Therapeutics

A key to advance the rational design of better tools for cancer detection, prognosis, and therapeutics is to increase our knowledge of the tumor microenvironment. This concept is supported by a continuously growing volume of literature reviewed by Horsman and Vaupel. The stroma and tumor parenchyma coexist in a network of nutrients, growth factors, metabolites, acids, interstitial pressure, and other physiological agents (Elkhattouti et al.). The temporal and spatial dynamics of those complex interactions, considered "abnormal and adverse" relative to conditions of normal tissues (Horsman and Vaupel), affect individual cancer cells, contribute to the natural selection of distinct clones, and establish aggressive tumors.

Among the multiple components of the tumor microenvironment with strong effects on tumor aggressiveness, tumor-associated hypoxia is a particularly relevant one (Horsman and Vaupel). The abnormal and chaotic vasculature of growing solid tumors cannot provide proper delivery of oxygen to all tumor cells. Not only observed in solid tumors, but also in aggressive non-solid tumors as indicated by the Mazurier group (Deynoux et al.), irregular oxygen supply is a crucial component of tumorigenesis. A yet unexplored field related to the involvement of the hypoxia pathway in tumorigenesis is its association with heritable cancer syndromes. Thus, the inactivating germline changes in tumor-suppressor genes belonging to DNA repair pathways are associated with an increased risk of carcinogenesis. Henegan and Gomez propose the hypoxia pathway can be linked to heritable cancer syndromes. Further exploration of this hypothesis may provide emergent concepts relevant for personalized stratification and treatment of tumors associated with germline mutations of genes associated with the hypoxia pathway.

Oxygen levels affect the cells largely by regulating activity of the transcription factor hypoxia-inducible factor (HIF). Regulation of HIFα subunits is complex. As noted by Kietzmann et al., an array of kinases are key regulators of HIFα's protein stability, subcellular localization, and transactivatory properties. It is reasonable to propose that modulation of HIFα by phosphorylation may be cell type- and cellular context-dependent. This becomes relevant when kinases can be exploited as upstream therapeutic targets of HIFα subunits in cancer therapy. HIF is also controlled by other factors. Schober and Berra pinpoint deubiquitinating enzymes, known regulators of cellular protein stability, as counterbalancing factors of ubiquitin E3 ligases (Schober and Berra). Aberrant in cancer, the ubiquitination cycle of HIFα is associated with disease progression and poor prognosis (Schober and Berra).

Other intracellular signaling mechanisms such as stress or redox response mechanisms constitute the hypoxia-activated gene expression program in tumors. For instance, the review from

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Mazure highlights the involvement of the voltage-dependent anion channel in cancer under the context of hypoxia. This channel, present in mitochondria, under hypoxia acts as a survival factor by promoting survival pathways in tumor cells and increased resistance to therapy agents (Mazure). These and other signaling molecules define a core for the response of cancer cells to varying oxygen conditions. Components of these networks adapt to low oxygen, play a critical role in tumor development, and offer a yet unexplored therapeutic potential.

Recently, tumor-associated hypoxia molecules have been identified as markers of poor prognosis. One example is carbonic anhydrase IX (CAIX), an enzyme involved in maintaining the cellular pH balance. Most of the studies, limited by low numbers of patients, were inconclusive. van Kuijk et al. provide the first comprehensive meta-analysis of the association between CAIX expression and treatment outcome in tumors. Patients having tumors with high CAIX expression have higher risk of locoregional failure and disease progression (van Kuijk et al.). The results of this study suggest that measuring CAIX expression can improve disease management, which in turn may decrease overtreatment and clinical relapse.

Due to the adaptation mechanisms of cancer cells to low oxygen, hypoxic tumors have reduced sensitivity to cytotoxic therapeutic agents. Hepatoma upregulated protein (HURP), a hypoxia-related molecule (1), conferred radiation resistance to prostate cancer cells (2). Noted by Espinoza et al. in their original research article, HURP expression in prostate tumors was associated with the increased expression of HIF-1α, vascular endothelial growth factor, and heat-shock protein 60, as well as increasing tumor grade. The data reinforce the relevance of hypoxia-related molecules as predictors of tumor aggressiveness, and propose a basis to further advance our understanding of the mechanistic role of hypoxia responsive molecules in therapy resistance.

On the establishment of novel approaches targeting hypoxia-related targets, the Dedhar group proposes exploiting pH regulation in the hypoxic niche (McDonald et al.). Since pH regulation is particularly relevant for cancer stem cells in hostile microenvironments, inhibition of pH regulatory enzymes, such as CAIX and monocarboxylate transporters, may result in a reduced response to hypoxia (McDonald et al.). Remarkably hypoxia promotes various mechanisms of immunoevasion (McDonald et al.). A hypothesis that will need clinical validation can be proposed: use of checkpoint inhibitors to restore antitumor immunity in combination with inhibitors of pH regulation, in the context of hypoxia, may effectively target aggressive tumors.

Equally relevant for therapeutical development is the application of novel models to study the adaptation of cancer cells to low oxygen. Chronic myeloid leukemia and hepatoblastoma are suggested by Cipolleschi et al. as tools to study low-oxygen-sensitive, -resistant, and -adapted cancer stem cells. On the basis of new findings revealed by the use of these models, application of metabolic inhibitors to target cancer stem cells may have an unexplored therapeutic potential for treatment of highly refractory disease (Cipolleschi et al.).

On the success of any experimental drug targeting tumor hypoxia, the continued adaptability of tumors to their microenvironmental stresses must be considered. Tumor cells are endowed with a very high plasticity and capacity to adapt. It is our challenge to find populations and conditions of the tumor microenvironment germane for target success. Interdisciplinary work will be the key for achievement of these goals. Benefits include; increased understanding of the involvement of hypoxia in tumorigenesis, development of better biomarker development, and more effective therapeutics.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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Pathophysiological Basis for the Formation of the Tumor Microenvironment

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Poor microenvironmental conditions are a characteristic feature of solid tumors. Such conditions occur because the tumor vascular supply, which develops from the normal host vasculature by the process of angiogenesis, is generally inadequate in meeting the oxygen and nutrient demands of the growing tumor mass. Regions of low oxygenation (hypoxia) is believed to be the most critical deficiency, since it has been well documented to play a significant role in influencing the response to conventional radiation and chemotherapy treatments, as well as influencing malignant progression in terms of aggressive growth and recurrence of the primary tumor and its metastatic spread. As a result, significant emphasis has been placed on finding clinically applicable approaches to identify those tumors that contain hypoxia and realistic methods to target this hypoxia. However, most studies consider hypoxia as a single entity, yet we now know that it is multifactorial. Furthermore, hypoxia is often associated with other microenvironmental parameters, such as elevated interstitial fluid pressure, glycolysis, low pH, and reduced bioenergetic status, and these can also influence the effects of hypoxia. Here, we review the various aspects of hypoxia, but also discuss the role of the other microenvironmental parameters associated with hypoxia.

Keywords: hypoxia, tumor microenvironment, radiotherapy, chemotherapy, malignant progression

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INTRODUCTION

Most solid tumors are just like normal tissues in that they need a regular supply of oxygen and nutrients to be able to exist, as well as processes for the elimination of the waste products of cellular metabolism. When tumors first appear, this function is provided by the normal blood supply of the host organ in which the tumor arises. However, unlike normal tissues, tumors continually expand in size and a point is reached where the host vascular supply becomes inadequate in supplying these needs. To compensate, tumors will actually develop their own functional vascular supply. This they do from the normal host vessels by the process of angiogenesis. Unfortunately, the tumor neo-vasculature that is formed is not only primitive and chaotic when compared to the normal tissue vascular supply from which it develops, but it also suffers from numerous structural and functional abnormalities. As a result, it is still unable to meet all the demands of the growing tumor mass (with sizes larger than 2–3 mm). Consequently, a hostile microenvironment develops within the tumor and this can be summarized by the so-called "crucial Ps." These are listed in **Table 1** and

basically reflect conditions of poor perfusion, oxygen deprivation, nutrient deficiency, severe acidity, and elevated interstitial fluid pressure (IFP).

The tumor cells that exist in this hostile microenvironment are actually still viable. But, as a result of being in these adverse microenvironmental conditions, those same tumor cells can exhibit resistance to conventional cancer therapies, including radiation and certain types of chemotherapy. The poor tumor microenvironment also causes these cells to upregulate the expression of various genes and biosynthesis of proteins, an effect that not only increases their survival potential but can also increase tumor aggressiveness and metastatic spread. Numerous attempts have, and are being made, to identify the microenvironmental conditions within tumors so as to select appropriate therapies to target those cancer cells that thrive in the hostile microenvironment.

Of these poor microenvironmental conditions within tumors, low oxygenation (hypoxia) is the one that has been the focus of most studies and is often considered as the only factor of importance. While hypoxia is clearly critical for outcome of cancer patients, it is generally associated with the other crucial Ps and as such any discussion of the role of hypoxia in tumors must be made in connection with the other hostile microenvironmental parameters. Thus, we will review the general pathophysiological characteristics of the tumor microenvironment, how that microenvironment develops, and what significance that has for cancer.

FACTORS INFLUENCING THE TUMOR MICROENVIRONMENT

Importance of the Tumor Vascular Supply

Angiogenesis is clearly an essential requirement for the growth and development of solid tumors (2–4). This process begins with the release of angiogenic factors, primarily vascular endothelial growth factor (VEGF), by the tumor cells (5). The actual triggers that initiate this process are not fully established. Loss of suppressor gene function and oncogene activation certainly play a role (5, 6), but the development of hypoxia as a result of tumor growth is also a major factor (6). Additional studies with tumor cells grown in culture have shown that the secretion rate of the VEGF protein increases as soon as the oxygen concentration is lowered from 21 to only 5% and that this secretion rate increases as the oxygen concentration decreases reaching maximal levels at around 0.5% and below (7). Release of VEGF and other growth factors set in motion a number of biochemical and physical steps that include enzymatic destruction of the basal membrane of the endothelial cells of the host vasculature, migration of endothelial cells into the extracellular matrix to form sprouts, and endothelial cell division away from the sprout tip (8). Solid strands of endothelial cells are then formed in the extracellular matrix, a lumen develops within those strands, neighboring sprouts fuse to form loops, and from the primary loops new buds and sprouts emerge (8). Finally, functional vessels are established.

Although a functional tumor vascular supply is necessary, the neo-vasculature that develops is actually inadequate to meet all the demands of the growing tumor mass. Endothelial cells divide at a slower rate than tumor cells (9) and as a consequence, the developing tumor vasculature is unable to keep pace with the expanding tumor population. The tumor vasculature that is formed is also very different from that of normal tissues (see **Figure 1**). Structurally, it is very chaotic. Vascular density is abnormal with increased intervessel distances and the existence of avascular areas. There are contour irregularities reflected by vessels that are elongated, tortuous, and large, and have aberrant

TABLE 1 | The crucial Ps characterizing the hostile tumor microenvironment.

Pathophysiological characteristics showing a reduced level
• Partial pressure of oxygen
• Production of high-energy compounds
• pH of the extracellular compartment
• Paucity of nutrients
• Paucity of bicarbonate
Pathophysiological characteristics showing an enhanced level
• Perfusion inadequacies/vascular chaos
• Perfusion heterogeneities
• Permeability of tumor microvessels
• Pressure of interstitial fluid
• Production of lactate
• Production of protons
• Production of adenosine
• Partial pressure of carbon dioxide

Modified from Ref. (1).

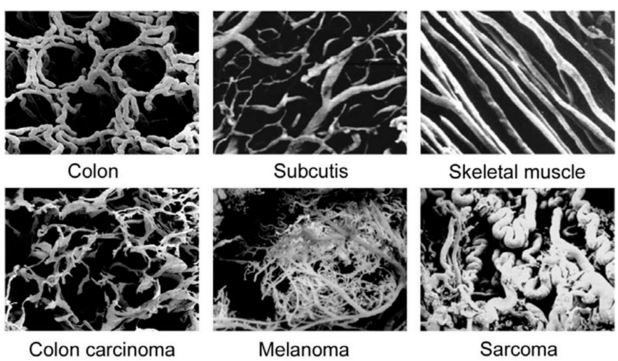

FIGURE 1 | Vascular casting images showing differences in microcirculation between normal tissues (top three panels) and malignant tumors (lower three panels). Specific details of the corrosion casting technique used to produce these images can be found in Konerding et al. (14). Images shown were obtained courtesy of Prof. Konerding, Dept. Functional and Clinical Anatomy, University Medical Center, Mainz, Germany and are from Vaupel (10). Bottom text lists the major structural and functional abnormalities of tumor vessels when compared to normal tissues; composite information based on work by Kimura et al. (12), Reinhold and van der Berg-Blok (15), Vaupel et al. (10, 16), and Baronzio et al. (17).

branching and blind ends. The pattern of vessel interconnection is also haphazard. Unlike normal vessels, there is a loss of hierarchy. The vessels are also very primitive in nature, having incomplete or missing basement membranes and endothelial lining, and lacking pericytes, smooth muscle, pharmacological receptors, and even innervation. Tumor vessels are often highly permeable allowing significant plasma leakage. Due to an absence of vasomotion and flow regulation, blood flow velocities through tumor vessels can be unstable as can the direction of flow [for review, see Ref. (10)]. It has been estimated that 1–8% of vessels can experience flow stasis (11, 12) and that around 8% of all microvessels show plasma flow only (12). Some of these changes will be mediated through effects of the various blood-borne cells. These include erythrocyte sludging, leukocyte sticking, and blockage of vessels by circulating white blood cells or tumor cells. High IFP can also play a role here. In normal tissues, there exists a perfusion pressure difference of about 20 mmHg between the arterial and venous ends of microvessels and this drives blood flow through capillaries. However, in tumors, the increased leakiness of vessels and the lacking of functional lymphatics result in an increased IFP. Transmural coupling between this high IFP and microvascular pressures can result in abolished perfusion pressure differences between the arterial and venous ends, thus, causing flow stasis and hypoxia (13). However, this is more likely to result in chronic rather than acute hypoxia (13). Finally, the hematocrit within tumor microvessels can be increased by 5–14% and this will also influence flow.

Other Parameters Affecting the Micromilieu

Apart from the inadequacies of the tumor vascular supply, there are a number of other important factors that can influence the microenvironment within tumors. Chief among these is the oxygen carrying capacity of the blood. This can be substantially reduced under specific conditions, thus making less oxygen available. Such an effect is seen with anemia where the normal hemoglobin levels of 7.5–9.5 mmol/L in females and 8–10 mmol/L in males can be reduced by 50% in anemic patients. Additional studies in which tumor oxygenation status was directly measured with polarographic needle electrodes have reported a correlation between the level of tumor hypoxia and hemoglobin concentration (18). Reduced oxygen availability is also observed in patients who smoke. Smoking impairs the delivery of oxygen to tumors due to the presence of carboxyhemoglobin (HbCO) that is formed by the binding of carbon monoxide (CO) to hemoglobin (19). Heavy smokers can have up to 16–18% HbCO in their blood. This reaction not only decreases the amount of hemoglobin available for oxygen transport but will also shift the oxygen-dissociation curve to the left making it more difficult for the hemoglobin to release oxygen to the cells. Since the affinity of hemoglobin for CO is approximately 250 times the affinity for oxygen, even low concentrations of CO can result in significant levels of HbCO in the blood.

The microenvironment of tumors will also depend on the cellular consumption of oxygen and essential nutrients. As a result of tumor cells close to the vascular supply consuming what they need for growth and survival, less will be available for those cells further away. Consequently, radial oxygen, nutrient, and pH gradients are established (16). The extent of those gradients will depend not only on the rate of consumption but also on what is actually delivered to the cells by the blood supply. Indeed, it has been reported that in the case of oxygen the cells next to the blood vessel can have oxygen concentrations as low as 2% [approximately 15 mmHg; (20)] and this would certainly reduce the oxygen diffusion distance. As it is the case in normal physiology, there is also an intravascular (longitudinal) oxygen partial pressure gradient when the blood moves from the arterial to venous end of the microvessels (21). All these factors, coupled with the structural and functional aberrations described in the previous section, will result in the development of areas within the tumor that can be considered "abnormal and adverse" when compared to those conditions found in normal tissues (10, 16).

MICROENVIRONMENTAL CHARACTERISTICS OF TUMORS

Hypoxia

The microenvironmental parameter that has been the most extensively investigated is hypoxia. By definition, hypoxia is a state of reduced oxygenation that influences biological functions (22). The first indirect indication that hypoxia existed in tumors was made by Thomlinson and Gray (23). From histological sections of carcinomas of the bronchus, they typically found viable tumor regions surrounded by vascular stroma, with regions of necrosis evolving in the center of the tumor mass. The thickness of the resulting shell of viable tissue was found to be between 100 and 180 μm. They suggested that as oxygen diffused from the stroma, it was consumed by the cells, and although those beyond the diffusion distance were unable to survive, the cells immediately bordering the necrosis might be viable yet hypoxic; unfortunately, in this concept, the diffusion of glucose and other nutrients is completely ignored. Later, an inverted version of the Thomlinson and Gray model was described, with functional blood vessels surrounded by cords of viable tumor cells outside of which were areas of necrosis [Krogh model of oxygen diffusion; (24)]. This corded structure is the more typical picture found in most solid tumors and is illustrated in **Figure 2**. As with the Thomlinson and Gray model, an oxygen gradient is created as the oxygen diffuses from the blood vessel, resulting in a region of cells at the edge of the cord that are oxygen deprived and are commonly referred to as diffusion-limited chronic hypoxia. This type of hypoxia has been seen in both animal and human tumors (25). It has been suggested that such hypoxic cells can survive under these adverse conditions for several days (26). Death will also occur as the hypoxic cells move further away from the blood supply as the tumor grows, although this is more likely to result from a glucose deficit rather than just a lack of oxygen.

Diffusion-limited chronic hypoxia was the working model for hypoxia from the 1950s until around the 1980s when it was then suggested that a second type of hypoxia could exist in tumors and one that was acute in nature (28). This was later confirmed and shown to be the result of the transient stoppages in tumor blood flow described earlier (29). The hypoxia that results was originally called perfusion limited acute hypoxia, although other terms are often used, including cyclic, intermittent,

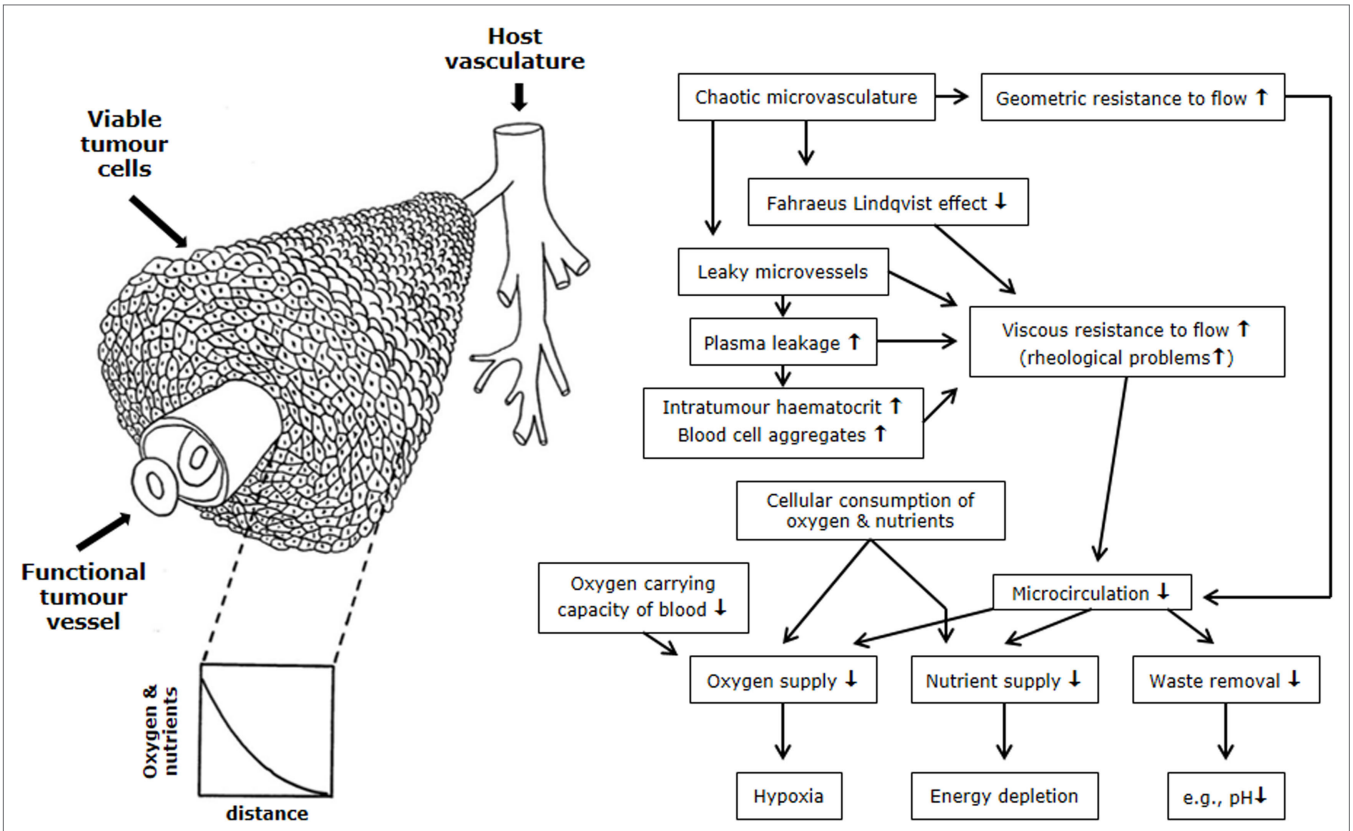

FIGURE 2 | Schematic illustration of the relationship between the tumor vasculature and microenvironment. The left side shows tumor cells growing in a corded structure around a functional vessel from which the cells receive their oxygen and nutrient supply, but as these substances diffuse out from the vessel they are utilized by the cells so that gradients are established. On the right side is a flow chart showing the relationship between the hostile microenvironment of tumors and the factors that give rise to its development. Figure is modified from Ref. (1, 27).

transient, repetitive, or fluctuating, and it is probably the latter term that is the most appropriate description. Evidence for fluctuating hypoxia has been reported in murine tumors (30), human tumor xeongrafts (31), and even in tumors in cancer patients (32–34).

Today, hypoxia is often considered as a single entity even though we know there are at least two types. However, even the concept of chronic and acute/fluctuating hypoxia is an oversimplification of the real picture (13). Acute hypoxia can result from a total or partial shut-down in perfusion (12); a complete shut-down would starve cells of oxygen and nutrients and result in ischemic hypoxia, which would not be the case for a partial shut-down where plasma flow, thus nutrient supply, can occur leading to hypoxemic hypoxia. For chronic hypoxia, the picture is even more complicated. It can result from a diffusion limitation under "normal" conditions (diffusional hypoxia), or be due to reduced oxygen availability as with high HbCO or anemia (anemic hypoxia). But, it will also be dependent on the oxygenation level; cells close to the vessel could be slightly hypoxic, while cells next to necrosis could even be anoxic (a situation where no oxygen can be detected) but still viable if they have sufficient nutrient supply (i.e., glucose). Whatever the description, hypoxia is now known to be a characteristic feature of most solid animal tumor models (35) and numerous human cancers (16, 36).

Interstitial Fluid Pressure

Unlike normal tissues, tumors often contain vessels that are abnormally leaky and also lack a functional lymphatic system (37). These, coupled with a large hydraulic conductivity, results in a significant bulk flow of free fluid in the interstitial space. In normal tissues, water influx into the interstitial compartment has been estimated to be between 0.5 and 1.0% of plasma flow, yet in human tumors values up to 15% can be reached (38). As a result of fluid accumulating in the tumor matrix, there is a build-up of interstitial pressure (39–41). Interstitial fibrosis, contraction of the interstitial space mediated by stromal fibroblasts, and high oncotic pressures within the interstitium may also contribute to the development of interstitial hypertension (42). In most normal tissues, IFP is just above or below atmospheric values (43), but in tumors it can reach 50 or even 100 mmHg (1). IFP is generally uniformally high throughout the center of tumors, but drops steeply in the tumor periphery (44). However, since vascular permeability varies from tumor to tumor and can be heterogeneous within the same tumor, IFP is not constant (1). It can also fluctuate with changes in microvascular pressures (45).

Glycolysis and pH

Warburg's classic work in the 1920s showed that cancer cells intensively converted glucose to lactate (glycolysis) even in

the presence of oxygen [for a review see Ref. (46)]. Today, it is believed that there is no clear evidence that cancer cells are inherently glycolytic, but that some tumors might be glycolytic *in vivo* as a result of hypoxic response mechanisms (47). Hypoxia will shift the balance of cellular energy production toward glycolysis with the generation and subsequent accumulation of lactate (48). Indeed, several studies have found high median lactate levels of around 7 mM in head and neck cancers (49) and up to 14 mM in uterine cervix (50). Although lactate is generally considered a waste product, there is evidence that the lactate produced by hypoxic cells can be taken up in normoxic cancer cells via the monocarboxylate transporter-1 and can then be utilized for oxidative phosphorylation instead of glucose as a substrate (51). However, cellular lactate production and release will lead to tumor acidosis. What is clear is that like normal cells, tumor cells have efficient mechanisms for exporting protons into the extracellular space (52, 53), thus a pH gradient exists across the tumor cell membrane so that intracellular pH (pHi) remains higher than the extracellular pH (pHe). In normal tissues, this gradient is reversed such that pHi is actually lower than pHe (16, 54–56). The production and release of lactate alone does not fully account for the acidosis found in the extracellular compartment of solid tumors. Other key mechanisms may play an important role, especially ATP hydrolysis, glutaminolysis, carbon dioxide production, and bicarbonate depletion (48).

Bioenergetic Status

Various techniques have been used to monitor the bioenergetic status within tumors. These include *ex vivo* quantitative bioluminescence (57) and high-performance liquid chromatography [HPLC; (38, 58)], and non-invasive ^{31}P-nuclear magnetic resonance/spectroscopy [NMR/MRS; (59)]. The global concentrations of ATP measured in experimental tumors using HPLC were found to be typically between 0.4 and 2.0 mM (38, 58). These global ATP concentrations and adenylate energy charge only changed marginally provided tumors did not exceed biologically relevant tumor sizes (i.e., 1% of the body weight). With increasing tumor mass, ATP hydrolysis increased. As a result of this increased ATP degradation, an accumulation of purine catabolites, and the final degradation product uric acid, has been observed (38). Using quantitative bioluminescence, the microregional distribution of ATP has been assessed in cryobiopsies of cervix tumors and found to be heterogeneous and comparable to high flow experimental tumors (38). This ATP distribution profile was similar to those seen for both glucose and lactate, but there was no clear-cut correlation between tumor oxygenation and regional ATP levels (38). Bioluminescence measurements of regional ATP distributions in experimental brain tumors reported ATP levels that were similar to normal brain, whereas glucose was slightly lower and lactate substantially higher (38, 60), with these metabolites showing marked tumor heterogeneity (60). Additional studies using NMR have shown that in many human malignancies, high concentrations of phosphomonoesters, phosphodiesters, and inorganic phosphate, as well as low phosphocreatine, are often found. The exception is again in human brain tumors, where no significant differences in ^{31}P-NMR spectra were seen when compared to normal brain tissue (38).

Hypoxia-Driven Adenosine Accumulation

The development of tumor hypoxia is accompanied by a substantial accumulation of the nucleoside adenosine (ADO) in the range of 50–100 μM (61). By contrast, ADO levels in normal tissues have been found to be in the range of 10–100 nM (62, 63). ADO accumulation is preferentially caused by an ATP release from cancer cells into the extracellular space upon hypoxic stress. After transport out of cancer cells, extracellular ATP is converted into ADO by hypoxia/hypoxia-inducible factor (HIF)-sensitive, membrane-bound ectoenzymes CD39 and CD73. Intracellular ADO-formation from AMP by a cytosolic AMP-nucleotidase with subsequent ADO-export into the extracellular space through a nucleoside transporter seems to play a subordinate role. ADO-actions (adenosinergic effects) are mediated upon binding to surface receptors, mainly A2A-receptors on tumor and immune cells. Receptor activation leads to a broad spectrum of strong immune-suppressive properties through modulation of the innate and adaptive immune system, thus facilitating tumor escape from immune control (62, 64–66). Mechanisms include (a) an impaired activity of $CD4^+$ T and $CD8^+$ T, NK cells, and dendritic cells (DCs), a decreased production of immune-stimulatory lymphokines, and (b) an activation of Treg cells, expansion of myeloid-derived suppressor cells (MDSCs), promotion of pro-tumor M2-macrophages, and increased activity of major immune-suppressive cytokines. In addition, ADO can directly stimulate tumor cell proliferation and angiogenesis. Taken together, there is clear evidence that ADO-mechanisms described can thwart anti-tumor immune responses elecited by radiotherapy and fever-range hyperthermia (67).

SIGNIFICANCE OF THE TUMOR MICROENVIRONMENT FOR CANCER

Radiation

The potential of microenvironmental parameters to influence outcome to radiotherapy was first suggested from experiments in which the radiation response of skin was markedly decreased if the blood flow to the irradiated area was reduced by compression (68). This was followed by a report that tissues in which blood flow was stimulated by diathermia showed a more prominent response to radiation (69). Further experimental observations led Gray and co-workers to finally postulate the role of oxygen deficiency as a major source of radiation resistance (70). This occurs because oxygen is critical for the response of cells to radiation. The mechanism responsible is generally referred to as the "oxygen-fixation hypothesis" (71). When radiation interacts with the cellular target, which is usually DNA, it results in the production of free radicals. These are produced either directly by the radiation itself or indirectly from other molecules that are affected by radiation and then diffuse sufficiently to reach and damage the DNA target. Since water constitutes around 70% of all mammalian cells, most of the indirect radicals are probably produced from water molecules. In the absence of oxygen or in the presence of hydrogen-donating species (i.e., thiols), the free radicals formed in the DNA can react with hydrogen ions and the target is then chemically restored to its original form. However,

if oxygen is present, it will react with the free radical to form a product that undergoes further reaction, ultimately producing a chemical change in the target. The damage is now fixed and can only be removed by enzymatic repair processes.

It has been demonstrated from rapid-mix studies that the oxygen effect occurs only if oxygen is present either during irradiation or within a few milliseconds thereafter (72, 73). The amount of oxygen is also critical. An almost maximum enhancement of radiation is seen with oxygen partial pressures above around 20 mmHg (approximately 3%). Below this partial pressure radiation sensitivity decreases in an oxygen-dependent fashion (71); in the absence of oxygen roughly three times as much radiation is required to kill the same number of cells as seen under normoxic conditions. This effect is generally referred to as the "oxygen enhancement ratio" (OER; ratio of the radiation dose in hypoxia/anoxia to that in air, to give the same biological effect). For radiation of higher energy than X-rays produced by modern radiotherapy units the OER actually decreases (74).

Numerous animal studies have demonstrated that hypoxia in tumors can influence radiation response. Three classical assays have been used (35, 75). They are (a) the clamped clonogenic survival assay, in which tumors are excised after treatment and cell survival measured in culture; (b) the clamped tumor growth delay assay, where measurements are made of the time taken for tumors to reach a specific size after treatment; and (c) the clamped tumor control assay, whereafter the percentage of animals showing local tumor control at a certain time after treatment is recorded. For each technique, it is necessary to produce full radiation dose–response curves under air breathing and fully anoxic (clamped tumor) conditions. The results of such assays not only demonstrate that hypoxia in tumors influences radiation response, but the degree of displacement of the dose–response curves also allows us to calculate the actual percentage of radiobiological cells in the tumor. Using these assays, the hypoxic fractions have been estimated to range from 1% to well over 50% of the viable tumor cells in animal tumors and human tumor xenografts (35).

Demonstrating that hypoxia can influence the radiation response of human tumors is more difficult, since none of the above approaches are applicable to humans. Although numerous other methods have been developed to try and identify hypoxia in human tumors (25, 76, 77), not all have been used to demonstrate the relationship between hypoxia and radiation response. The earliest attempts to do the latter were based on indirect approaches (78), and involved estimates of tumor vascularization, using such endpoints as intercapillary distance, vascular density and the distance from tumor cells to the nearest blood vessel (79–81). All showed that patients with less well vascularized tumors, and presumably more hypoxic, had a poorer outcome to radiation therapy. More direct approaches have used exogenous markers that are injected into the host and bind to regions of tumor hypoxia, or endogenous markers that are genes/proteins upregulated under hypoxia. The former include nitroimidazole or copper-based derivatives, which can be identified immunohistochemically from histological sections or non-invasively using positron emission tomography, SPECT, or magnetic resonance spectroscopy (25). Although such exogenous markers can be used to identify tumor hypoxia and even associated with outcome following radiation therapy in head-and-neck carcinoma patients (82), there has never been a proper radomized trial. The endogenous markers include such factors as carbonic anhydrase IX, GLUT-1, HIF-1, and osteopontin (83–86). These can be measured from biopsy material using protein immunohistochemistry or as gene expression, or proteins identified from blood samples. Although endogenous markers have been correlated with outcome to radiation therapy in some studies, it is not a universal finding (82), which probably reflects the fact that many of these endogenous markers are not hypoxia-specific rather than any indication that hypoxia does not play a role in influencing radiation response.

Probably the most direct method for estimating tumor hypoxia and one that has certainly been used to show the negative influence of such hypoxia on radiation response is the measurement of oxygen partial pressure (pO_2) distributions with polarographic electrodes. Early attempts to achieve this used "home-made" glass electrodes which were cumbersome, fragile, and only a few pO_2 values 3–4 mm below the surface of the tumor were possible. Nevertheless, clinical data were obtained in cervix (79) and head and neck (87) that clearly demonstrated a relationship between such oxygenation measurements and outcome to radiation therapy, in that those patients with tumors that were better oxygenated had a significantly superior local response to irradiation.

This whole area was revolutionized with the development of the Eppendorf histography system, which had two distinct improvements. The first was having the oxygen microsensor inside a metal needle and the second the attachment of this needle to a stepping motor that allowed for multiple measurements along the needle track through the tumor. Numerous clinical studies were, thus, undertaken in a variety of human tumor types. The results clearly showed that hypoxia was to be found in virtually all human tumors investigated, although the degree of hypoxia could be variable (16, 36, 88, 89). Probably the most significant finding from these studies was the confirmation that hypoxia influenced outcome to therapy, especially where radiation was given. This has been reported for cervix (90–95), head and neck (96–100), soft tissue sarcomas (101, 102), and prostate (103, 104). Results for all four tumor types are summarized in **Table 2** and clearly illustrate that the patients with more hypoxic tumors had a poorer outcome response.

One major focus of current cancer research is the role of cancer stem cells in tumorigenesis and therapy. Such cells amount to around 1–25% of the total viable tumor cell population (105), but they are believed to be the cells that must be completely eliminated to obtain tumor control (106). Significant effort is currently being made to identify these cells and specifically target them. However, recent evidence suggests a possible link between hypoxia and cancer stem cells (106). Hypoxia may affect cancer stem cell generation and maintenance through the upregulation of hypoxia-induced factors (105, 106). Pre-clinical studies have also shown an inverse correlation between hypoxia and local tumor control after irradiation (107, 108), suggesting that hypoxia may also actually protect the cancer stem cells from the lethal effects of radiation. If the link between cancer stem cells and hypoxia is proven, then hypoxia may be an even more important issue for radiation response than we currently believe.

TABLE 2 | Relationship between tumor oxygenation estimated prior to therapy using the Eppendorf histograph and outcome to therapy.

Tumor type	Patient treatments[a]	No. of patients	Response endpoints[b]	Less hypoxic (%)	More hypoxic (%)	Oxygenation endpoint[c]	Reference
Cervix	RT/CT/SG	31	OS at 22 months	80	32	Median pO_2 above or below 10 mmHg	(90)
	RT/CT/SG	89	OS at 5 years	69	37	Median pO_2 above or below 10 mmHg	(91)
	RT	51	DFS at 3 years	69	33	Median pO_2 above or below 10 mmHg	(92)
	RT	74	DFS at 3 years	69	34	HF5 above or below 50%	(93)
	RT	106	DFS at 5 years	58	42	HF5 above or below 50%	(94)
Head and neck	RT	35	LTC at 2 years	77	33	Median pO_2 above or below 2.5 mmHg	(96)
	RT/SG	28	DFS at 12 months	78	22	Median pO_2 above or below 10 mmHg	(97)
	RT/CT	59	OS at 12 months	63	31	Hypoxic subvolume	(98)
	RT/CT	134	OS at 3 years	22	7	Median pO_2 above or below 2.5 mmHg	(99)
	RT/CT/SG	310	OS at 3 years	38	28	Median pO_2 above or below 2.5 mmHg	(100)
Sarcoma	RT/HT/SG	22	DFS at 18 months	70	35	Median pO_2 above or below 10 mmHg	(101)
	RT/SG	28	OS at 5 years	77	28	Median pO_2 above or below 19 mmHg	(102)
Prostate	RT	57	FFBF at 8 years	78	46	P/M ratio above or below 0.10	(103)

[a]*Patient treatments consisted of various combinations of RT (radiotherapy), CT (chemotherapy), SG (surgery), or HT (hyperthermia).*
[b]*Response endpoints were either OS (overall survival), DFS (disease free survival), LTC (local tumor control), or FFBF (freedom from biochemical failure).*
[c]*HF5 (percentage pO_2 values below 5 mmHg), hypoxic subvolume (percentage pO_2 below 5 mmHg × total tumor volume), or P/M (prostate/muscle).*

Chemotherapy

The pathophysiological characteristics of tumors play a significant role in influencing the response of the tumor cells to chemotherapy (109). An inadequate vascular supply will naturally be expected to hinder blood-borne drug delivery. A decrease in drug availability will certainly be seen in areas where flow fluctuates, especially where complete cessations in flow occur (110). In addition, the mean vascular density in tumors is lower than that found in normal tissues and, thus, diffusion distances are enlarged (1). Thus, transport of drugs from tumor microvessels to tumor cells that are distant from them will be compromised. The high IFP within solid tumors will also decrease extravasation (111). IFP at the tumor to normal tissue interface is low and as a result interstitial fluid oozes out of the tumor into the surrounding normal tissue. At the same time, it will also carry away anti-cancer drugs (37).

Cells most distant from the vascular supply will also be cycling at a reduced rate and this can act as a protective mechanism against a number of chemotherapeutic agents that work by interacting with cellular DNA and only kill the cell when it divides. Such cells are also exposed to hypoxia and acidic conditions, factors which are known to influence chemotherapeutic agent activity (111). However, these adverse microenvironmental parameters do not always have a negative effect of drug activity; some drugs are actually more effective under such conditions as illustrated in **Table 3**.

TABLE 3 | Influence of the hostile tumor microenvironment on the activity of chemotherapeutic drugs.

Hypoxia dependency		pH (below 6.8) dependency	
Decreased effect	**Increased effect**	**Decreased effect**	**Increased effect**
Doxorubicin	Etoposide	Doxorubicin	Chlorambucil
Actinomycin D	BCNU/CCNU (?)	Daunorubicin	Melphalan
Bleomycin	Alkylating agents (?)	Bleomycin	Cyclophospamide
Vincristine	Mitomycin C	Vinblastin	Mitomycin C
Methotrexate (?)	EO9	Paclitaxol	Tiophosphamide
Cisplatin (?)	PR-104	Methotrexate	Cisplatin
5-Flurouracil (?)	TH-302	Mitoxantrone	5-Flurouracil
Procarbazine	Tirapazamine	Topotekan	Camptothecin
Streptonigrin	Banoxantrone		

Drugs marked with (?) indicate those agents that are included in the relative categories due to their in vitro response, but in which in vivo studies suggest may not be correct. Classification is based on information from Ref. (111–114).

Other Tumor Therapies

Hyperthermia is a less conventional therapy, but is one example where the more deficient the tumor vasculature and the more deprived the tumors cells, the better the tumor response. Blood flow, being one of the major means by which heat is dissipated from tissues, will affect the ability to heat tumors (115). Thus, the poorer the blood supply, the easier it should be to heat. This has been demonstrated *in vivo* in which blood flow was compromised using agents that could reduce tumor blood flow (116, 117). *In vitro* studies have also reported that cells under hypoxic conditions were more sensitive to killing by hyperthermia than the same cells in a well-oxygenated environment (118, 119). However, this is not a consequence of hypoxia *per se* because under well-defined nutrient conditions, acute hypoxia does not significantly alter cellular response to heat (119). However, cells under prolonged oxygen deprivation will show an increased sensitivity to heat, an effect that is the result of chronically hypoxic cells becoming acidic (118).

Another treatment modality in which the tumor microenvironment influences response is *photodynamic therapy* (PDT). It involves the administration of a photosensitizing agent and its subsequent activation by light. This reaction is strongly dependent on oxygen concentration (120–123). Cell killing by PDT appears to be complete at normal oxygen levels and above, but decreases as the oxygen concentration drops below 5% (123). This is perhaps not surprising since the mechanism of action of PDT involves the generation of singlet oxygen (124).

Malignant Progression

One of the most striking results found with the Eppendorf electrode measurements made in cervix cancers and soft tissue sarcomas was that hypoxia influenced outcome in patients in which surgery was the primary or only treatment (90, 91, 102): this suggested that hypoxia could also influence malignant progression, especially metastatic spread. In fact, one other study in cervix was able to show that the primary tumors of patients with metastases were indeed less oxygenated than those of patients without metastases (125).

Pre-clinical studies also support the importance of hypoxia in inducing metastatic spread. The earliest studies involved exposing murine fibrosarcoma cells in culture to different oxygen concentrations for up to 24 h and then intravenously injecting these cells and observing the number of lung metastasis that develop (126). That study clearly showed that the more hypoxic the exposure, the greater the number of lung metastases. *In vivo* attempts to investigate this issue have involved either correlating the constitutive level of hypoxia in primary tumors or exposing the mice to different oxygen environments to change the level of tumor hypoxia, and then determining the number of metastases formed (127–130). The results generally show that the greater the degree of hypoxia, the more metastases observed and that acute hypoxia was better at inducing metastases than chronic. These hypoxia-induced effects on malignant progression can be the result of changes at the transcriptional level in which a range of different genes are over-expressed or at the translational level with various proteins being upregulated. Such effects can be mediated via activation of various oxygen-sensitive signaling pathways (130, 131).

Other microenvironment factors found in tumors have also been shown to influence malignant progression. Tumor cells exposed to glucose deprivation and acidosis prior to intravenous injection into mice resulted in more lung metastases than cells not exposed to these conditions (132, 133). The deleterious effects of glucose deprivation *in vivo* have made it impossible to investigate its potential effects on malignancy in animals, but there have been attempts to relate pHe *in vivo* with metastases formation. Those studies involved either experimentally increasing tumor acidity (134) or simply making probe measurements of pHe in untreated tumors (135). But in both situations, no correlations were found between pH and metastases. This is perhaps somewhat surprising because one of the major factors causing acidity is lactate production and clinical studies have shown high tumor lactate concentrations to be associated with an increased risk of nodal and distant metastases (49), and a shorter overall and disease-free survival (50).

The relationship between IFP and metastatic dissemination is also controversial. Using melanoma xenografts, an association was found between high IFP and the development of pulmonary and lymph node metastases (136). Another study from the same research group reported that IFP was significantly higher in intramuscularly implanted TS-415 cervix carcinoma xenografts that metastasized than those that did not (135). However, that same study showed this not to be the case for CK-160 cervical xenografts. Furthermore, a lack of any correlation between IFP and metastatic spread was also reported using two other cervical xenograft models (ME180 and SiHa) regardless of whether they were grown intramuscularly or orthotopically (137). Clinical studies have been less controversial, with IFP measurements in cervix cancer patients being shown to predict for survival (41) and for both nodal and distant metastases (94). A possible relationship between IFP and metastatic disease in other tumor sites is not yet known.

CONCLUSION

The pathophysiological characteristics of the tumor microenvironment are very different from those conditions found in normal tissues. In many respects, the tumor microenvironment can be considered abnormal and hostile. These adverse pathophysiological conditions, especially hypoxia, are now known to play a significant role in determining the tumor response to therapy and influencing the metastatic potential of tumors. Clearly, the future requirement is the application of methods by which one can accurately and reliably image the various important microenvironmental parameters, especially using techniques that are routinely available in the clinic. With such information, it should be possible to identify those patients, on an individual basis, that would be expected to have a poor prognosis and, thus, select appropriate additional treatments to dramatically improve that prognosis.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to the concept, development, and the writing of this manuscript.

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Heritable Cancer Syndromes Related to the Hypoxia Pathway

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Families of tumor-suppressor genes, such as those involved in homologous recombination or mismatch repair, contain individual genes implicated in hereditary cancer syndromes. Collectively, such groupings establish that inactivating germline changes in genes within pathways related to genomic repair can promote carcinogenesis. The hypoxia pathway, whose activation is associated with aggressive and resistant sporadic tumors, is another pathway in which tumor-suppressor genes have been identified. von Hippel–Lindau disease, some of the hereditary paraganglioma–pheochromocytoma (PGL/PCC) syndromes, and the syndrome of hereditary leiomyomatosis and renal cell carcinoma are heritable conditions associated with genes involved or associated with the hypoxia pathway. This review links these heritable cancer syndromes to the hypoxia pathway while also comparing the relative aggression and treatment resistance of syndrome-associated tumors to similar, sporadic tumors. The reader will become aware of shared phenotypes (e.g., PGL/PCC, renal cell carcinoma) among these three hypoxia-pathway-associated heritable cancer syndromes as well as the known associations of tumor aggressiveness and treatment resistance within these pathways.

Keywords: von Hippel–Lindau disease, hereditary leiomyomatosis and renal cell cancer, SDHx hereditary paraganglioma–pheochromocytoma syndromes, hypoxia-inducible factor, pseudo-hypoxia

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INTRODUCTION

Heritable cancer syndromes provide important clinical and research avenues. Clinically, diagnosing a heritable cancer syndrome allows a patient and his/her family to receive appropriate, targeted cancer screenings or preventive interventions. From a research standpoint, discovery and investigation of heritable cancer syndromes allows for better understanding of mechanisms of carcinogenesis and tumor behavior.

Families of tumor-suppressor genes consist of individual genes implicated in hereditary cancer syndromes that share common molecular pathways, such as the homologous recombination (e.g., *BRCA1*, *BRCA2*, *PALB2*) or mismatch repair (e.g., *MLH1*, *MSH2*, *MSH6*) pathways. Collectively, such groupings establish that inactivating germline changes in genes within pathways related to genomic repair can promote carcinogenesis. Insights related to these pathways led to the

Abbreviations: FH, fumarate hydratase; HIF1, hypoxia-inducible factor 1; HLRCC, hereditary leiomyomatosis and renal cell cancer, PGL/PCC, paraganglioma–pheochromocytoma; RCC, renal cell carcinoma; SDH, succinate dehydrogenase; VEGF, vascular endothelial growth factor; VHL, von Hippel–Lindau.

development of pathway-related therapy (e.g., poly ADP ribose polymerase inhibitors) (1) and promising hypotheses regarding personalized, targeted therapy (e.g., PD-1 blockade in mismatch repair-deficient tumors) (2).

Families of tumor-suppressor genes have also been identified within or affecting pathways related to the tumor microenvironment – in particular, the hypoxia pathway (**Figure 1**). Under normal cellular conditions, the transcription factor hypoxia-inducible factor 1 (HIF1) (3) regulates the cellular response to variations in oxygen tension. This transcription factor is a heterodimer formed by an alpha and a beta subunit. Degradation of the alpha subunit (HIF1-α) is regulated by oxygenation – when cellular oxygenation is low HIF1-α degradation is decreased, allowing HIF1 to promote cellular survival and growth (3). In malignancies, this "hypoxia driver" phenotype utilizes the hypoxia pathway to produce an aggressive and/or resistant tumor (4). Pseudo-hypoxic states are ones that display similar hypoxia-pathway gene expression but under normoxic conditions. Pseudo-hypoxia may be achieved through inactivation of tumor-suppressor genes, such as the von Hippel–Lindau (VHL) tumor suppressor, E3 ubiquitin ligase gene (*VHL*); the genes associated with the succinate dehydrogenase (SDH) complex (the SDHx genes); and the fumarate hydratase (*FH*) gene.

The purpose of this review is to highlight the grouping of heritable cancer syndromes associated with genes (i.e., *VHL,* the SDHx genes, and *FH*) in or related to the hypoxia pathway. Since these syndromes involve germline mutations associated with activation of the hypoxia pathway, and activation of this pathway may lead to aggressive and resistant sporadic tumors, this review will also compare clinical aspects of carcinogenesis, tumor growth, local/distant spread, and treatment resistance between syndrome-associated tumors and similar sporadic tumors.

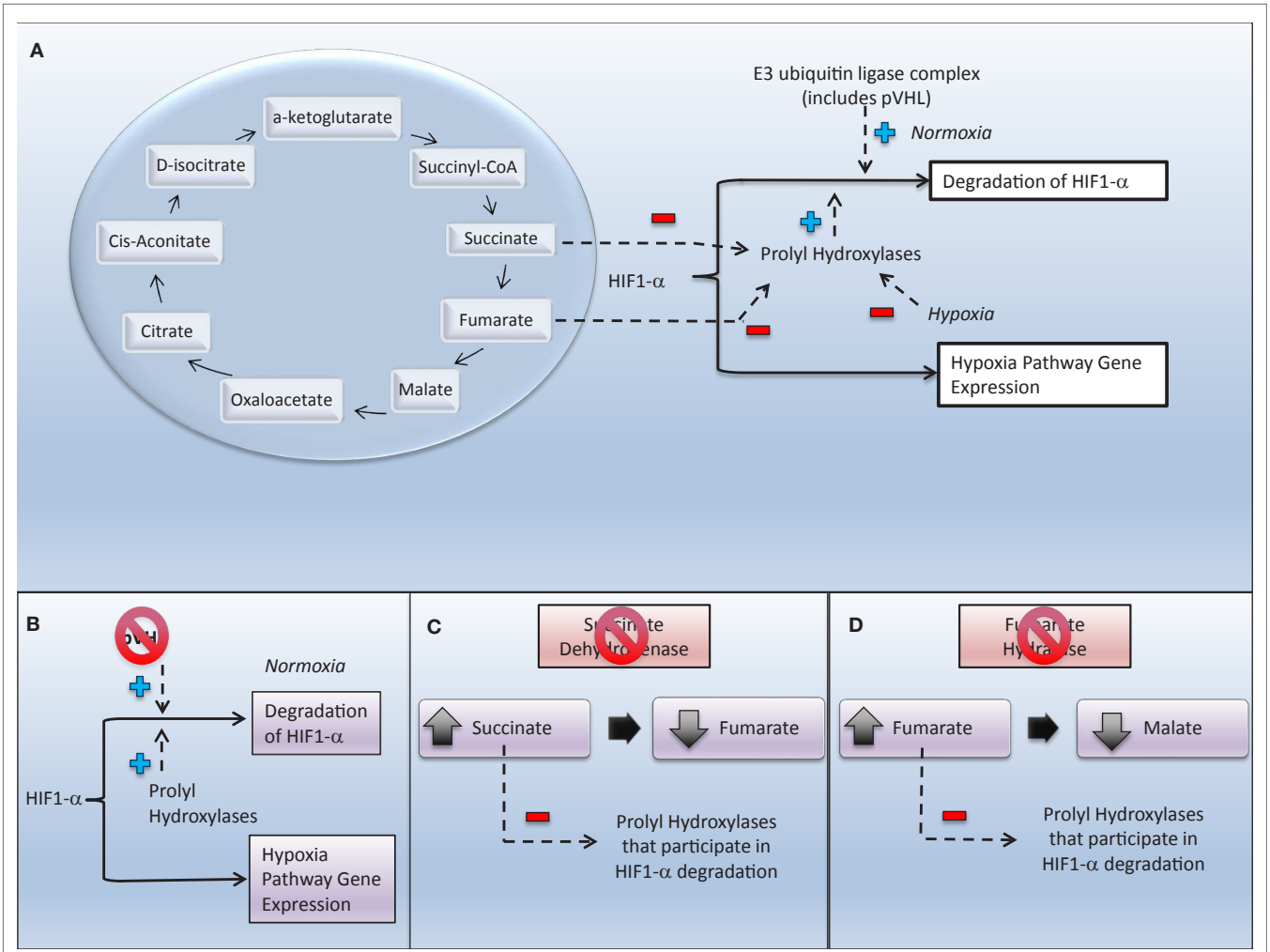

FIGURE 1 | Relationships between select heritable cancers and the hypoxia pathway. Inactivation of von Hippel–Lindau protein (pVHL), succinate dehydrogenase (SDH), or fumarate hydratase (FH) leads to increased expression of genes in the hypoxia pathway. **(A)** Tricarboxylic acid cycle and its relationship to regulation of hypoxia-inducible factor. **(B)** Inactivation of pVHL in von Hippel–Lindau disease causes upregulation of genes expressed in the hypoxia pathway through decreased degradation of HIF1-α. **(C)** Inactivation of SDH in the SDHx hereditary paraganglioma–pheochromocytoma syndromes causes increase in succinate, which inhibits prolyl hydroxylases that would assist in the degradation of HIF. **(D)** Inactivation of FH in HLRCC causes increase in fumarate, which inhibits prolyl hydroxylases that would assist in the degradation of HIF.

VON HIPPEL–LINDAU DISEASE

von Hippel–Lindau disease is an autosomal-dominant hereditary cancer syndrome involving a germline mutation in *VHL* (5). In a VHL disease registry (6), tumors with a frequency of more than 10% in VHL disease included retinal angiomas (41%), cerebellar hemangioblastomas (60%), spinal hemangioblastomas (15%), renal cell carcinomas (RCCs) (25%), and PCCs (15%). Pancreatic carcinomas, pituitary hemangioblastomas, and duodenal carcinoid tumors are described in 5% or less of patients. These frequencies are in line with other VHL disease reviews (7).

von Hippel–Lindau disease is diagnosed (6) in a patient who fulfills any one of the following four conditions: (1) two or more CNS hemangioblastomas; (2) one CNS hemangioblastoma and a disease-associated visceral tumor (i.e., RCC, PCC, pancreatic tumor or cysts, or broad ligament cystadenomas); (3) a family history of VHL disease and one of the following: (a) retinal angioma, (b) spinal or cerebellar hemangioblastoma, (c) PCC, (d) RCC, (e) or multiple renal and pancreatic cysts; or (4) a pathogenic *VHL* variant.

Clinically, VHL disease is associated with high penetrance and a shortened lifespan. VHL disease penetrance is an estimated 97% by 60 years of age (8). The three most common disease-related causes of death in VHL disease include cerebellar hemangioblastoma (48%), RCC (27%), and pancreatic carcinoma (7%) with a mean age of death of 40.9 years (6). In a review of a heritable cancer registry review, patients with VHL disease had a significantly shorter life expectancy than patients with four other heritable cancer syndromes – neurofibromatosis 1, neurofibromatosis 2, familial adenomatous polyposis, and Gorlin syndrome (9).

VHL is translated into von Hippel–Lindau tumor suppressor (pVHL), a hypoxia-associated protein. pVHL is a component of an intracellular multi-protein complex that also includes elongin C, elongin B, and cullin-2. This complex is an E3 ubiquitin protein ligase that, under conditions of adequate cellular oxygenation, targets HIF1-α for destruction (10) (**Figure 1**). VHL disease requires a mutation or in-frame deletion/insertion (11) of *VHL* that leads to loss of a functional protein. Loss of functional pVHL leads to upregulation of HIF that increases expression of various proteins (e.g., vascular endothelial growth factor (VEGF), platelet-derived growth factor, matrix metalloproteinases, and transforming growth factor-alpha) involved in cancer growth and development.

Despite VHL disease-associated tumors manifesting earlier in life than comparable sporadic ones (8), the VHL disease-associated malignancies are less aggressive in their risk of local recurrence and distant spread. Reviews of registry data indicate that patients with VHL-associated RCC have a higher primary tumor size threshold for metastatic disease, a significantly higher overall survival (12), and an increased cancer-specific survival when compared to patients with similarly sized sporadic RCC (13). Other tumors associated with VHL disease also have less relative aggressiveness in regard to disease progression or recurrence. For example, when compared to similar sporadic tumors, VHL-associated endolymphatic sac tumors are less likely to invade surrounding structures (14), VHL-associated spinal hemangioblastomas are less likely to be clinically symptomatic (15), and resected VHL-associated pancreatic neuroendocrine tumors have a significantly lower rate of recurrence than similar sporadic tumors (16).

Malignancies associated with VHL disease seem to be as responsive, if not more so, than sporadic tumors to pharmacologic interventions. In a small, single institution retrospective review of patients with VHL disease treated with first-line sunitinib for either multifocal (29%) or metastatic (71%) RCC, there was a median progression-free survival of approximately 3.5 years with 9 of 14 patients obtaining a partial response on therapy (17). For comparison, the phase 3 trial which led to sunitinib's approval in metastatic RCC reported a median progression-free survival of 11 months and an objective response rate of 42% (18). Perhaps the potential higher response rate in VHL disease is not surprising, as a study of sporadic metastatic clear cell RCC indicated that patients with *VHL* inactivation have a higher, albeit not statistically significant different, response rate (41 versus 31%) to VEGF targeted therapy than did sporadic tumors with wild-type *VHL* (19).

In summary, VHL disease is highly penetrant and has a relatively early age of onset for its manifestations. However, VHL disease-associated tumors are less aggressive in regard to local invasion and to potential for metastatic spread as well as more responsive to therapy when compared to similar tumors.

SDHx HEREDITARY PARAGANGLIOMA–PHEOCHROMOCYTOMA SYNDROMES

The hereditary paraganglioma–pheochromocytoma (PGL/PCC) syndromes are a collection of autosomal-dominant hereditary cancer syndromes. Germline mutations associated with PGL/PCC are clustered into two groups: those involved with the pseudo-hypoxic pathway and those involved in kinase signaling pathways. The former cluster includes mutations in genes related to SDH, known as the SDHx genes (20).

The SDHx hereditary PGL/PCC syndromes are relatively newly described entities that involve a mutation in *SDHA*, *SDHB*, *SDHC*, *SDHD*, or *SDHAF2*. In 2000, the first report was published of an association of one of the SDHx genes with hereditary PGL/PCC syndromes (21). Since that time, in addition to PGL/PCC, the recognized tumor spectrum among patients with a mutation in one of the SDHx genes has been expanded to also include RCC, pituitary tumors, gastrointestinal stromal tumors, and pancreatic neuroendocrine tumors (22, 23). A meta-analysis of prevalence studies found the pooled risk for malignant PGL to be 13 and 4% for *SDHB* and *SDHD* mutations, respectively (24). Penetrance may be affected by environmental oxygenation factors as patients with *SDHD* mutations who lived at lower (as opposed to higher) altitudes have less disease penetrance, have more findings of single (as opposed to multiple) tumors, and do not typically develop PCCs (25).

The diagnosis of a SDHx hereditary PGL/PCC syndrome requires finding a germline mutation in one of the SDHx genes. In clinical practice, germline genetic testing may be considered in all patients with a PGL or PCC. However, some providers may consider factors related to the probability of detecting a mutation, such as tumor location, presence of multiple tumors, age of onset,

and pathological characteristics of the tumors in their decision to recommend germline molecular testing (26, 27).

The SDHx genes are involved in the structure and/or function of SDH. SDH catalyzes the conversion of succinate to fumarate in the tricarboxylic acid cycle by removing one hydrogen atom from each of the two methylene carbons of succinate and placing them in the respiratory chain (28) (**Figure 1**). The four subunits of SDH include two anchorage proteins (SDHD and SDHC) that are part of the mitochondrial membrane and two catalytic proteins (SDHA and SDHB) that transfer an electron to coenzyme Q. *SDHAF2* encodes a protein needed for flavination of SDHA.

Succinate's contribution to pseudo-hypoxia has been attributed to competitive inhibition of enzymes involved in HIF1-α degradation, changes in oxidative stress, changes in energy utilization, and alterations in gene expression. The relative increase in the succinate-to-fumarate ratio is associated with succinate competitively inhibiting alpha-ketoglutarate in its binding to HIF1/2-α prolyl hydroxylases, thus preventing these enzymes from aiding in the degradation of HIF (**Figure 1**) (29) and leading to pseudo-hypoxia (30). PCCs with *SDHB* knockdown, like those in familial PGL/PCC, demonstrate HIF1-α stabilization despite normoxic conditions, consistent with pseudo-hypoxia (31). This has been recapitulated in tumor specimens where dysfunction of SDH due to mutations in SDHx genes leads to events consistent with pseudo-hypoxia, including mitochondrial dysfunction (32); increased expression of HIF1-α by immunohistochemistry (33); increased expression of miR-210, a key regulator of response to hypoxia (34); and increased VEGF expression (35). Other factors that may be involved in the malignant transformation, proliferation, and survival of SDHx-related tumors include an increase in reactive oxygen species, augmentation of the Warburg effect by HIF1-α, and utilization of glutamine as an energy source (29). Alterations in epigenetic regulation (36) and differential expressions of stemness may also impact the malignant potential of SDHx-mutated PGL/PCC (37).

Paragangliomas associated with *SDHB* mutations are more aggressive and resistant to treatment than sporadic PGLs. Malignant PGLs more frequently have *SDHB* mutations than do sporadic tumors (38). In a retrospective study of 34 patients undergoing primary carotid body PGL resections, there was significantly worse disease-free survival among patients with a *SDHB* mutation than among patients without a *SDHB* mutation (39). In a cohort of patients with malignant PCC/PGL, there was an association of decreased survival for those patients with a *SDHB* mutation compared to others within this cohort (40). Clinical trials (e.g., NCT02495103) are underway to explore targeted therapies for RCC associated with SDHx gene mutations.

The relatively recently discovered SDHx hereditary PGL/PCC syndromes highlight a method of carcinogenesis involving the hypoxia pathway. Pseudo-hypoxia in SDHx hereditary PGL/PCC syndrome tumors is achieved after substrate accumulation leads to competitive inhibition of an enzyme involved in degradation of HIF1-α. In contrast to VHL-associated tumors, tumors in hereditary PGL/PCC syndromes (especially those associated with *SDHB* germline mutations) behave more aggressively and are more resistant to therapy than their sporadic counterparts.

HEREDITARY LEIOMYOMATOSIS AND RENAL CELL CANCER

Hereditary leiomyomatosis and renal cell cancer (HLRCC) is an autosomal-dominant hereditary cancer syndrome first associated with mutations in *FH* in 2002 (41). Clinically, patients with HLRCC may present with single or multiple cutaneous leiomyomata; uterine leiomyomata; and/or a RCC, which may be tubolo-papillary, collecting-duct, or papillary type 2 (42). The risk of RCC associated with HLRCC appears variable based on geography as kindreds in the United States of America and Finland, when compared to other countries, more often have multiple HLRCC-associated RCC cases (43).

Like the SDHx hereditary PGL/PCC syndromes, the diagnosis of HLRCC is made by molecular testing. Evaluation of *FH* should be considered if either there is (a) histologically confirmed multiple cutaneous leiomyomata or (b) at least two of the following: surgery required for symptomatic uterine leiomyomata before 40 years of age, type 2 papillary RCC before 40 years of age, or a first-degree relative who meets one of the above criteria (44).

There is variable expression in HLRCC, with one study reporting 87% of patients with *FH* mutations having skin leiomyomata, 96% of females having uterine leiomyomata (typically younger in age than those with sporadic tumors) (45), and 42% having RCC (46) – although a separate reviews put the risk of RCC between 15 and 20% (47). A rare manifestation of germline *FH* mutations is PCC (48).

FH encodes FH, the tricarboxylic acid cycle enzyme that catalyzes the conversion of fumarate to malate (49) (**Figure 1**). The identification of *FH* as a tumor suppressor was the second description, following the identification of the SDHx genes in hereditary PGL/PCC syndromes, of a gene translated into an intermediary metabolism enzyme also being a tumor-suppressor gene (50). HLRCC is associated with *FH* germline changes that lead to a significant reduction in FH enzyme activity (51) and an accumulation of fumarate. Like succinate, fumarate acts as a competitive inhibitor of HIF prolyl hydroxylases, causing HIF upregulation (52).

Tumor specimens from patients with HLRCC demonstrate changes consistent with *FH* inactivation and pseudo-hypoxia. Leiomyomata associated with HLRCC have large increases in fumarate consistent with levels needed to impair HIF degradation (53). Leiomyomata associated with HLRCC, compared to sporadic leiomyomata, also demonstrate higher microvessel density and increased expression of anaerobic-associated or hypoxia responsive genes (54, 55). Other mechanisms of carcinogenesis may contribute to HLRCC tumor development as cellular models and cell lines of HLRCC-associated tumors demonstrate a dependence on glycolysis (56); alterations in expression of antioxidant-response element genes (57); changes in expression of genes involved in lipid metabolism, apoptosis, and energy production/glycolysis (58); and aberrant succination (59).

Hereditary leiomyomatosis and renal cell cancer-associated RCC is aggressive in its regional and distant spread but its relative resistance or susceptibility to therapy has yet to be demonstrated. Up to 47% of HLRCC patients with RCC present with nodal or

TABLE 1 | Aggressiveness and treatment resistance of tumors associated with heritable cancer syndromes that lead to pseudo-hypoxia compared to similar sporadic tumors.

	von Hippel–Lindau Disease	Reference	SDHx hereditary paraganglioma/pheochromocytoma syndrome	Reference	Hereditary leiomyomatosis and renal cell cancer	Reference
Risk of local invasion	Lower	(14)	No data	NA	No data	NA
Risk of regional or distant spread	Lower	(8)	Higher	(38)	Higher	(60)
Risk of recurrence	Lower	(8, 16)	Higher	(39)	No data	NA
Resistance to standard treatment	Less resistant	(17)	No data	NA	No data	NA
Risk of death	Lower	(12, 13)	Higher	(40)	No data	NA

distant metastases (60), as opposed to the 33% of patients with sporadic RCC (61). Some metastatic RCC lesions in HLRCC occur despite the primary tumor being <3 cm in size, leading to the recommendation that renal masses <3 cm cannot be observed in HLRCC – a departure from the recommendation for observation of small tumors in other RCC hereditary cancer syndromes, including VHL disease (60). There is a lack of evidence to date regarding HLRCC-associated tumors' responsiveness to therapy although clinical trials are underway to evaluate therapeutic options for patients with HLRCC-associated RCC (e.g., NCT01130519 and NCT02495103).

Hereditary leiomyomatosis and renal cell cancer shares many similarities with the SDHx hereditary PGL/PCC syndromes. Both are relatively newly discovered heritable cancer syndromes that involve a germline mutation in a tumor-suppressor gene that is translated into a tricarboxylic acid cycle enzyme. Both lead to competitive inhibition of an enzyme that in turn decreases the degradation of HIF1-α. Clinically, both are highly penetrant and can be associated with aggressive tumors.

DISCUSSION

Like germline mutations in genes in the homologous recombination pathway and their association with hereditary breast and ovarian cancer; or germline mutations in mismatch repair genes and their association with colorectal cancer; germline mutations in genes associated with the hypoxia pathway (e.g., *VHL*, the SDHx genes, *FH*) appear to be associated with RCC and PGL/PCC. We suggest that future research should investigate the association of these germline mutations and these clinical phenotypes to assess, for instance, if perturbations within the hypoxia pathway drive a proportion of these tumor types.

A difference in aggressiveness and resistance to therapy was seen among these three heritable cancer syndromes associated with the hypoxia pathway (**Table 1**). VHL disease-associated tumors seem less aggressive and more responsive to therapy compared to similar sporadic tumors. However, tumors associated with the SDHx hereditary PGL/PCC syndromes as well as with HLRCC are more aggressive and there is ongoing research into potentially effective, personalized therapies for these syndromes. More research is needed to determine if the differential aggressiveness and resistance to therapy across these three syndromes is due to other effects associated with the germline mutations, such as alterations in energy metabolism or mitochondrial function.

AUTHOR CONTRIBUTIONS

JH and CG both made substantial contributions to the conception and design of the work; drafted the work and revised it critically; provided final approval of the version to be published; and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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REVIEW
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Hypoxia-Inducible Factors (HIFs) and Phosphorylation: Impact on Stability, Localization, and Transactivity

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The hypoxia-inducible factor α-subunits (HIFα) are key transcription factors in the mammalian response to oxygen deficiency. The HIFα regulation in response to hypoxia occurs primarily on the level of protein stability due to posttranslational hydroxylation and proteasomal degradation. However, HIF α-subunits also respond to various growth factors, hormones, or cytokines under normoxia indicating involvement of different kinase pathways in their regulation. Because these proteins participate in angiogenesis, glycolysis, programmed cell death, cancer, and ischemia, HIFα regulating kinases are attractive therapeutic targets. Although numerous kinases were reported to regulate HIFα indirectly, direct phosphorylation of HIFα affects HIFα stability, nuclear localization, and transactivity. Herein, we review the role of phosphorylation-dependent HIFα regulation with emphasis on protein stability, subcellular localization, and transactivation.

Keywords: phosphorylation, HIF-1α, hypoxia, kinase, MAPK pathway, PI3K/PKB pathway

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INTRODUCTION

An adaequate supply of oxygen is mandatory for aerobic life. To cope with an inadequate O_2 supply, commonly termed hypoxia, mammals have developed response mechanisms which are crucial for their survival.

To achieve responsiveness toward hypoxia on the molecular level, cells integrate a complex biochemical system involving short-term reactions/modifications with no changes in gene expression and a long-term programme including changes in gene expression. Both processes can be interlinked; in particular, when the short-term response includes changes in the activity of enzymes which initiate a series of posttranslational signaling events that often regulate the activity of transcription factors and thus gene expression. On the level of gene expression the response to hypoxia is crucially dependent on the α-subunits of hypoxia-inducible transcription factors (HIFα) (Semenza, 2003; Kaelin, 2011; Masson and Ratcliffe, 2014).

As such, HIF α-subunit proteins contribute to proper embryonic development and to the pathology of many diseases associated with hypoxia like anemia, myocardial infarction, thrombosis, atherosclerosis, diabetes mellitus, or cancer (Semenza, 2003; Kaelin, 2011; Masson and Ratcliffe, 2014).

To achieve adaequate function, HIFα levels, subcellular distribution and activity need to be tightly regulated. Although regulation at the transcriptional and translational level was shown to play a role, posttranslational stabilization of HIFα proteins in response to hypoxia appears to be of major importance (Wenger, 2002; Gross et al., 2003; Gorlach, 2009; Kietzmann, 2009).

Interestingly, the HIFα proteins are not only regulated by hypoxia, but also in response to various stresses, growth and coagulation factors, hormones, or cytokines under normoxic

conditions (reviewed by Dimova et al., 2009). These "normoxic" HIFα stimuli often use different protein kinase regulated pathways for signal transduction indicating an important role of different kinases in HIFα regulation. Indeed, different kinases have been identified to regulate HIFα in a direct or indirect fashion (**Figure 1**). This review will primarily discuss the role of the kinases using HIFα proteins as a direct substrate and the impact of these modifications on HIFα stabilization, nuclear translocation, and transactivation.

HYPOXIA-INDUCIBLE TRANSCRIPTION FACTORS: α- AND β-SUBUNITS

Three O_2-sensitive HIFα proteins (HIF-1α, HIF-2α -also known as EPAS (Tian et al., 1997), HLF (Ema et al., 1997), HRF (Flamme et al., 1997), or MOP2 (Hogenesch et al., 1998)—and HIF-3α) are known today. Together with HIF β-subunits, primarily represented by the stable nuclear and ubiquitously found ARNT (arylhydrocarbon receptor-nuclear translocator) protein, they form heterodimeric transcription factors binding to hypoxia response elements (HRE) with the core DNA sequence 5′-RCGTG-3′ (Wenger et al., 2005).

The best studied HIFα isoforms are HIF-1α and HIF-2α which share a number of structural and functional similarities but also show some differences with respect to cell type expression pattern, embryonic deletion phenotypes, target genes, and effects during carcinogenesis (Hu et al., 2003; Scortegagna et al., 2003; Sowter et al., 2003). Not much is known about HIF-3α from which several splice variants exist in humans (Pasanen et al., 2010) and where some variants as well as a mouse splice variant termed inhibitory PAS protein (IPAS) appear to act as negative regulators of the hypoxic response (Makino et al., 2007; Heikkila et al., 2011) while others appear to act as an oxygen-regulated transcription activator (for review see Duan, 2015).

Like the ARNT proteins, the HIF α-proteins belong to the basic helix-loop-helix (bHLH) PAS (Per-ARNT-Sim) protein family (Wang et al., 1995a); HIF-1α and HIF-2α show the highest degree of sequence identity in the bHLH (∼85%), PAS-A (∼68%), and PAS-B (∼73%) domains. Both contain also two conserved nuclear localization sequences (NLS) responsible for translocation to the nucleus; they are localized in the N-terminus (aa 17–33 in HIF-1α; aa 1–50 in HIF-2α) and in the C-terminus (aa 718–721 in HIF-1α; aa 689–870 in HIF-2α) (Kallio et al., 1998). Except for the full length HIF-3α which does not contain a C-terminal transactivation domain but a unique LZIP (leucine zipper) C-terminal domain (Hara et al., 2001; Kietzmann et al., 2001), HIF α-subunits contain also a N-terminal transactivation domain (N-TAD) and a C-terminal transactivation domain (C-TAD). An oxygen-dependent degradation domain (ODDD, aa 401–603 in HIF-1α; aa 517–682 in HIF-2α) is overlapping the N-TAD and is important for the oxygen-dependent regulation of all vertebrate HIFα proteins (Huang et al., 1998; Duan, 2015). The residues between the N-TAD and C-TAD constitute an inhibitory domain (ID) (Jiang et al., 1997) (**Figure 2**).

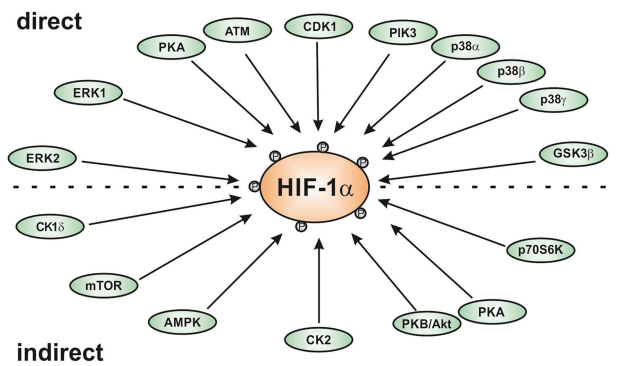

FIGURE 1 | Scheme of kinases involved in regulating HIF-1α either direcly or indirectly. AMPK, AMP-activated kinase; ATM, ataxia and teleangiectasia mutated; CK1, casein kinase1; CDK1, cyclin-dependent kinase-1; ERK; extracellular regulated kinase; GSK3β, glycogen synthase kinase-3β; PKA, protein kinase A; PKB/Akt, protein kinase B or Akt kinase; p38, p38 mitogen activated protein kinase; Plk3, polo-like kinase-3.

HYPOXIA-INDUCIBLE REGULATION OF α-SUBUNITS

The levels of the HIF α-subunits increase exponentially with declining O_2 concentration as a result of reduced hydroxylation, ubiquitylation and proteasomal degradation (Semenza, 2003; Kaelin, 2011; Masson and Ratcliffe, 2014). To date, four HIF specific prolyl 4-hydroxylase domain containing enzymes (PHDs) have been identified from which PHD2 appears to be of major importance for HIFα degradation (Berra et al., 2003). All HIF hydroxylases belong to a family of dioxygenases which depend on the presence of O_2 for their action. Thus, in the presence of O_2, i.e., normoxia, PHDs are able to hydroxylate crucial proline residues in the HIFα ODDDs (P402/P564 in HIF-1α; P405/P531 in HIF-2α; P492 in HIF-3α). This event recruits the von Hippel-Lindau tumor suppressor protein (pVHL) which together with Elongin C, Elongin B, RBX1, Cullin 2, and an E2 ubiquitin-conjugating enzyme forms an ubiquitin E3 ligase complex. As a consequence, HIFα proteins become ubiquitylated and degraded by the proteosome (Semenza, 2003; Kaelin, 2011; Masson and Ratcliffe, 2014).

Another hydroxylase called factor-inhibiting HIF (FIH-1) hydroxylates an asparagine in the C-TADs of HIF-1α and HIF-2α (N803 in HIF-1α and N847 in HIF-2α) with the result that the interaction of the HIFα proteins with the co-activators CBP/p300 is inhibited (Mahon et al., 2001; Hewitson et al., 2002; Lando et al., 2002). Thus, a limited O_2 supply decreases the activities of HIF hydroxylases and allows HIFα stabilization, followed by nuclear translocation, dimerization, and transactivation (for review see Kaelin, 2005).

REGULATION OF HIF α-SUBUNITS BY PHOSPHORYLATION

Phosphorylation is a crucial posttranslational modification which regulates the activity and stability of various proteins including transcription factors. However, the extent to which transcription

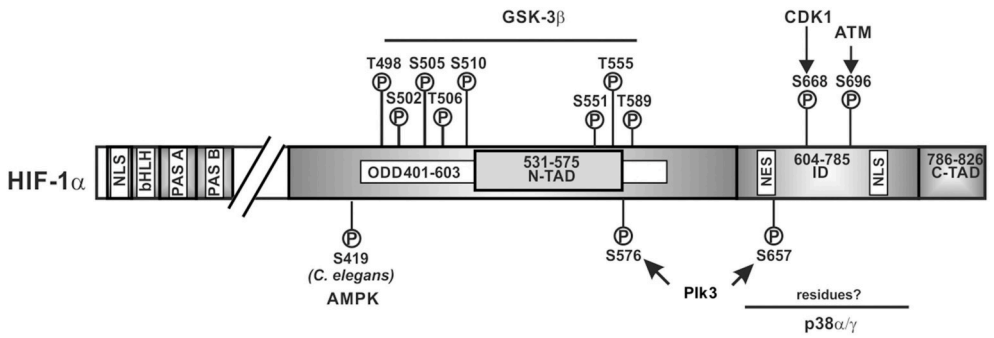

FIGURE 2 | Kinases and amino acid residues in HIF-1α involved in regulation of HIF-1α stability. Scheme of HIF-1α and its domain organization with specific amino acid residues which can be phosphorylated; all have been shown to contribute to the regulation of HIF-1α protein stability. HIF-1α is phosphorylated on specific residues (T498, S502, S505, T506, and S510 or S551, T555, and S589) by GSK3β; Plk3 can phosphorylate S576 and S657; ATM can phosphorylate S696 and CDK1 can phosphorylate S668. ATM, ataxia and teleangiectasia mutated; CDK1, cyclin-dependent kinase-1; GSK3β, glycogen synthase kinase-3b; Plk3, polo-like kinase 3; bHLH, basic helix loop helix domain; NLS, nuclear localization sequence; PAS, Per-ARNT-Sim domain; N-TAD, N-terminal transactivation domain; ID, inhibitory domain; C-TAD, C-terminal transactivation domain; numbers indicate the amino acid residue range of the respective domain.

factors including HIFα proteins are phosphorylated may vary according to the signal, cell-type, or tissue. Thus, it is plausible that a modulation of HIFα action due to phosphorylation may be a cell type specific event which could be explained by different layers of regulations where kinases are affected depending on the cellular context.

The first evidence indicating that phosphorylation plays a role in HIFα regulation came from electrophoretic mobility shift assay experiments where addition of calf intestinal alkaline phosphatase to hypoxic nuclear extracts led to a loss of HIF-1 DNA-binding activity (Wang et al., 1995b). In the meantime a panel of protein kinases was reported to affect HIFα regulation, mainly HIF-1α, indirectly or directly (for review see Dimova et al., 2009). Thereby it appeared that direct phosphorylation of HIFα has an immediate impact on HIFα stability, nuclear localization, transactivity, and protein-protein interactions.

Phosphorylation of HIFα Proteins: Role for Subunit Stabilization

A number of findings indicated that the PI3K/PKB(Akt) pathway can induce HIFα, transcription, stabilization (Mazure et al., 1997; Zhong et al., 2000; Zundel et al., 2000; Hirota and Semenza, 2001), translation (Koritzinsky et al., 2006), and coactivator recruitment (Kallio et al., 1998). So far, no evidence has been presented showing that HIFα proteins are directly phosphorylated by PKB(Akt); rather its action is indirect involving other PKB/Akt targets. Although a number of PKB/Akt targets are known, so far only the human homolog of mouse double minute-2 (HDM2) (Bardos et al., 2004; Skinner et al., 2004), mammalian target of rapamycin (mTOR) (Treins et al., 2002), and glycogen synthase kinase-3 (GSK3) (Flügel et al., 2007, 2012) were shown to affect HIF-1α levels with most evidence indicating that only GSK3 acts directly on HIF-1α.

Although the name GSK3 implies that this is a specific kinase acting only on glycogen synthase, it is rather pleiotropic with a number of substrates through which GSK3 may affect various signaling pathways often associated with hypoxia like developmental processes, stem cell renewal, cell proliferation, and apoptosis (reviewed in Cohen and Frame, 2001; Grimes and Jope, 2001; Force and Woodgett, 2009).

Mammals possess two GSK3 isoforms, GSK3α (51 kDa) and GSK3β (47 kDa) which are structurally similar, but not entirely functionally overlapping (reviewed in Force and Woodgett, 2009). This became evident from the different phenotypes of GSK3 knockout mice. GSK3$\beta^{-/-}$ mice are embryonically lethal and die around day 16 because of hepatic apoptosis and a cardiac pattern defect (Hoeflich et al., 2000; Kerkela et al., 2008). By contrast, GSK3$\alpha^{-/-}$ mice are viable, and fertile (MacAulay et al., 2007). Interestingly, it exists also a minor spliced GSK3β variant called GSK3β2 that contains a 13-amino acid residue insert within the kinase domain. This isoform was shown to be neuron-specific and has reduced kinase activity toward the microtubule-associated protein, tau, compared to GSK3β (Mukai et al., 2002; Saeki et al., 2011).

GSK3 is a target of the PKB/Akt pathway and it is unusual that its protein kinase activity tends to be high in resting cells. Furthermore, its inhibition is mediated by various stimuli, such as growth factors, cytokines, and hormones. PKB/Akt can phosphorylate both GSK3 isoforms (S21 of GSK3α and S9 of GSK3β), leading to an inhibition of GSK3 activity (Cross et al., 1995). Several other kinases are also able to phosphorylate these serine residues like ERK1/2, a downstream kinase of the MAPK pathway (Brady et al., 1998), p70 ribosomal S6 kinase-1 (Armstrong et al., 2001), cAMP-dependent protein kinase A (PKA) (Li et al., 2000), and PKC (Ballou et al., 2001).

Findings showing that inhibition of GSK3, siRNA-mediated depletion of GSK3β and absence of GSK3β in MEFs induced HIF-1α protein levels (Schnitzer et al., 2005; Flügel et al., 2007, 2012) were in line with the notion that GSK3 can phosphorylate at least HIF-1α. Indeed, GSK3β was found to directly phosphorylate HIF-1α in the ODDD and N-TAD (Sodhi et al., 2001; Flügel et al., 2007, 2012; Cassavaugh et al., 2011). The residues phosphorylated in HIF-1α by GSK3β were reported to be S551, T555, and S589 in one study (Flügel et al., 2007) whereas another study showed involvement of T498, S502, S505, T506, and S510

(Cassavaugh et al., 2011) (**Figure 2**). The difference between studies may have resulted from different oxygen concentrations (8% O_2 compared to 2% O_2) and the different cell types (HepG2 compared to SK-OV-3) used. Despite the different phosphorylation sites, both studies show that regulation of HIF-1α by GSK3β is independent of O_2, hydroxylation, and VHL-mediated proteasomal degradation (Flügel et al., 2007, 2012; Cassavaugh et al., 2011). Thereby, phosphorylation of HIF-1α by GSK3β recruits the F-box and WD protein Fbw7 (also known as hCdc4 in yeast, hSel10 in *Caenorhabditis elegans*, or Ago in Drosophila) as the substrate-recognition component of a multi-subunit E3 ubiquitin ligase and forms together with SKP1 (S-phase kinase-associated protein 1), CUL1 (cullin 1), and RBX1 (RING box 1, also called ROC1 or HRT1) the so called SCF complex which then contributes to HIF-1α degradation (Cassavaugh et al., 2011; Flügel et al., 2012).

Similar to pVHL, Fbw7 is also a tumor suppressor; 6% from 1500 investigated human tumors showed mutations in the Fbw7 coding region. Strikingly, nearly half (43%) of these were missense mutations within the WD40 domain (Arg465 and Arg479), shared by all three alternatively spliced Fbw7 isoforms. In line, all three Fbw7 isoforms could target HIF-1α for proteasomal degradation and loss of the Fbw7 WD domain abolished GSK3β initiated HIF-1α degradation (Flügel et al., 2012).

Together, the findings showing that two different E3 substrate recognition proteins which both are tumor suppressors can contribute to HIFα degradation indicates the importance of the highly dynamic HIF system for carcinogenesis.

Ubiquitylation of proteins is reversible and the reversion is mediated by a family of deubiquitylating enzymes (DUBs). About 100 DUBs encoded by the human genome are supposed to counteract the action of around 600 E3 ligases (Nijman et al., 2005; Scheel and Hofmann, 2005). DUBs can be divided into five groups: ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), Josephins, and JAMMs. The USP, UCH, OUT, and Josephins are papain-like cysteine proteases, whereas the JAMM members are zinc metalloproteases (reviewed in Love et al., 2007).

Based on this, it appears plausible that the normoxia and pVHL-mediated ubiquitylation as well as the GSK3β and Fbw7-mediated ubiquitylation can be opposed by DUBs. Indeed, two VHL-interacting deubiquitylating enzymes, VDU1 (USP33) and VDU2 (USP20) were identified (Li et al., 2002a,b). However, by using *in vitro* pull down assays with GST-HIF-1α (amino acid 530–826) and co-immunoprecipitation experiments in COS-7 cells it was shown that only VDU2 but not VDU1 could interact with HIF-1α (Li et al., 2005). In addition, it was shown that VDU2 but not VDU1 can deubiquitylate HIF-1α and increase it's half-life (Li et al., 2005).

Experiments with GSK3β and Fbw7-deficient cells revealed that the GSK3β and Fbw7-dependent HIF-1α degradation can be antagonized by the ubiquitin specific protease 28 (USP28) (Flügel et al., 2012). In contrast to VDU2 which directly interacts with HIF-1α, USP28 forms a ternary complex with HIF-1α via its association with HIF-1α bound Fbw7 (Flügel et al., 2012).

Together, degradation of HIF-1α by the GSK3/Fbw7/USP28 system appears to be an additional mode to regulate HIF-1α function in response to various physiologic and non-physiologic signals affecting cell division, cell growth, differentiation, and apoptosis independent of the O_2 tension.

While GSK3 provides a metabolic link to cell growth and differentiation, p38 MAP kinases link different stress stimuli, such as ultraviolet irradiation, heat shock, and osmotic shock with cell differentiation, apoptosis, and autophagy (Olson and Hallahan, 2004; Raman et al., 2007; Tormos et al., 2013; Sabio and Davis, 2014). Indeed, p38 was supposed to regulate HIF-1α stability during ischemic stress and in line, the p38 inhibitors SKF86002 and SB203580 decreased HIF-1 dependent gene expression (Sodhi et al., 2001). Further, treatment of the MiaPaca2 pancreatic cancer cell line with the p38 inhibitor SB203580 caused an increase in VHL-HIF-1α binding (Kwon et al., 2005) suggesting that p38 contributes to HIF-1α stabilization, though no half-life measurements were performed. Two members of the p38 MAPK family, p38α and p38γ, were then shown to possess the ability to phosphorylate HIF-1α (Sodhi et al., 2000). Altogether, this implies that p38 can contribute to HIF-1α stabilization, the phosphorylation by p38 occurred in the inhibitory domain (aa 576–785) (Sodhi et al., 2001) which has not yet been shown to be involved in VHL-dependent degradation. Moreover, the exact localization of the eight serine residues which could serve as putative p38 phosphorylation sites in the HIF-1α inhibitory domain as well as their contribution to HIFα degradation remains still to be determined (**Figure 2**).

Another kinase linking HIFα function with regulation of cell division is cyclin-dependent kinase 1 (CDK1). Although about 20 CDKs known to date can contribute to cell cycle control, CDK1 was found to be the only one essential for the cell cycle in all eukaryotic cells (Malumbres et al., 2009). CDK1 belongs to a highly conserved family of heterodimeric serine/threonine kinases which require a regulatory cyclin subunit for their activity. As such, the CDK1-cyclin B complex constitutes a serine/threonine protein kinase composed of the catalytic subunit CDK1 and its positive regulatory subunit cyclin B (B1 isoform) (Malumbres et al., 2009).

Activation of CDK1 promotes entry into the M phase of the cell cycle. This is achieved in the late G2 phase by phosphorylation mediated by the CDK activating kinase (CAK) phosphorylating T161 in its kinase-activation loop (Russo et al., 1996) as well as Cdc25C phosphatase mediated dephosphorylation of T14 and Y15 within CDK1. The inactive state of CDK1 throughout the S and G2 phases of the cell cycle is achieved by phosphorylation at two negative regulatory sites, T14 and Y15, by the CDK1 inhibitory protein kinases, Myt1 and Wee1 respectively (Watanabe et al., 2005) for review see (Malumbres, 2014, 2015).

A recent report showed that siRNA-mediated knockdown or Ro-3306-mediated inhibition of CDK1 reduced HIF-1α half-life whereas overexpression of CDK1 enhanced HIF-1α levels. *In vitro* kinase assays revealed that S668 in HIF-1α is the CDK1 target site (**Figure 2**). Accordingly, a construct of HIF-1α with a phospho-site mimicking mutation (S668E) was more stable under both normoxia and hypoxia. Moreover, phosphorylation of HIF-1α at S668 lead to an expression of HIF-1 target genes and promoted tumor angiogenesis, proliferation, and tumor growth (Warfel et al., 2013). Together, these findings underlie

the importance of HIF-1α for the M-phase of the cell cycle since it can be stabilized by CDK1-mediated phosphorylation already under normoxia.

Genotoxic stress represents a burden under which cell cycle progression and cell cycle checkpoints need to be tightly controlled. A kinase participating in the response to genotoxic stresses is Polo-like kinase 3 (Plk3) (Barr et al., 2004). Plk3 is a member of a family consisting of four proteins (Plk1, Plk2, Plk3, and Plk4) not only involved in the stress response, but also strongly involved in tumorigenesis with an abnormal expression found in multiple tumors (Archambault and Glover, 2009; Degenhardt and Lampkin, 2010). The role of Plk3 in the development of tumors remains controversial. While one study showed a non-tumorigenic phenotype in Plk3 deficient mice (Myer et al., 2011), another study reported that mice deficient in Plk3 develop highly vascularized tumors in multiple organs suggesting a tumor-suppressing activity in particular via HIF driven angiogenesis (Yang et al., 2008). The latter finding is in line with the finding that Plk3 can regulate HIF-1α stability (Xu et al., 2010). Plk3 immunoprecipitation and pulldown analyses revealed interaction between HIF-1α and Plk3 which was able to phosphorylate S576 and S657 of HIF-1α (Xu et al., 2010) (**Figure 2**). Further, $Plk3^{-/-}$ murine embryonic fibroblasts contained increased HIF-1α levels. In line with that, half-life measurements demonstrated that the half-life of wild-type HIF-1α was <10 min, whereas the half-lives of the HIF-1α-S576A, HIF-1α-S657A, and HIF-1α-S576A/S657A mutants were about 37, 49, and 51 min, respectively (Xu et al., 2010). Together, these studies indicate that Plk3-mediated phosphorylation destabilizes HIF-1α.

In contrast to the above mentioned kinases, the knowledge about the involvement of the Jun N-terminal kinases (c-JNK) in regulating HIF-1α is quite limited and inconsistent. One study reported that c-JNK contributes to the activation of HIF-1α (Comerford et al., 2004) whereas other studies showed that HIF-1α is not phosphorylated by c-JNK (Richard et al., 1999; Sodhi et al., 2001).

Links between Hypoxia and Kinases in the Regulation of HIFα Stabilization

In addition to hormones or growth factors, hypoxia may also have an impact on the activity of certain kinases and thus activation of the hypoxia signal chain and a kinase pathway at the same time may lead to interference at the level of HIFα.

It has been shown that hypoxia is capable to induce GSK3β phosphorylation and thus its inactivation in different cell types such as PC-12 cells (Beitner-Johnson et al., 2001), HT1080 cells (Chen et al., 2001), and HepG2 cells (Mottet et al., 2003; Flügel et al., 2007) as well as *in vivo* (Roh et al., 2005). Further, early/acute hypoxia also enhanced PI3K/Akt activity, inhibited GSK3, and increased HIF-1α protein levels whereas prolonged/chronic hypoxia increased GSK3β activity which led to decreased HIF-1α protein levels in HepG2 cells (Mottet et al., 2003; Flügel et al., 2007). This indicates that hypoxia can also be a signal for the PI3K/Akt/GSK3 pathway and depending on the duration of hypoxia it is possible to induce a biphasic HIF-1α response. This would imply that GSK3β inhibition could reverse the negative effect of prolonged hypoxia on HIF-1α accumulation; however, these effects may be cell type specific since the hypoxia effects on GSK3β phosphorylation were not observed in other cell types including some different breast cancer cell lines (Blancher et al., 2000), PC-3 prostate cancer cells (Zhong et al., 2000), and 3T3 cells (Laughner et al., 2001).

GSK3 appears not to be the only kinase which may regulate HIFα stability by phosphorylation under normoxia and hypoxia. Recently it was found that the protein kinase ataxia-telangiectasia mutated (ATM) may be involved in the hypoxia-dependent modulation of HIF-1α function. Although ATM is best known for its role as an upstream activator of the DNA damage response due to DNA double-strand breaks (DSBs) (Shiloh and Ziv, 2013), it was described that ATM-deficient cells failed to accumulate HIF-1α under hypoxic conditions. In addition, ATM activity—but not protein—was found to be increased by about two-fold when NHFB cells were exposed to 0.2% oxygen; an increase in activity similar to that seen after ionizing irradiation. ATM was also able to phosphorylate HIF-1α at S696 in the ID and a HIF-1α S696A mutant was found to be less stable than wild-type HIF-1α under hypoxic conditions suggesting that S696 phosphorylation stabilizes HIF-1α (Cam et al., 2010) (**Figure 2**). However, not only stability but also activity of the HIF-1α S696A mutant was reduced with the consequence of reduced DNA-damage-inducible transcript 4 (DDIT4; also known as Dig2, HIF-1-responsive RTP801, REDD-1) expression (Shoshani et al., 2002). These features integrate the ATM DNA damage response pathway with the hypoxia signaling pathway.

In addition to hypoxia, reactive oxygen species (ROS) are also an important trigger of the DNA damage response and have been shown to be involved in the regulation of HIFα levels (Kietzmann and Gorlach, 2005; Gorlach and Kietzmann, 2007; Kietzmann, 2010). Although their major effects on HIFα stabilization are exerted via regulation of the proline hydroxylation- and VHL-dependent degradation pathway (Kietzmann and Gorlach, 2005; Gorlach and Kietzmann, 2007), also the PI3K/Akt and ERK1/2 pathway contributed to the ROS mediated HIFα regulation (Gorlach et al., 2001, 2003; Diebold et al., 2010). Recent studies in the roundworm *C. elegans* indicated that another kinase, namely AMP-activated protein kinase (AMPK) couples ROS and HIF-1α regulation in a direct manner. AMPK is a key sensor of the cellular energy status (Hardie et al., 2015) and considered to act downstream of reduced mitochondrial respiration. In their studies the authors demonstrated that mutations in the AMPK ortholog of *C. elegans* led to increased levels of HIF-1α indicating that AMPK is required for reducing HIF-1α. Further analyses revealed that AMPK regulates HIF-1α post-transcriptionally and by combining *in vitro* kinase assays with LC-MS analyses it was shown that AMPK phosphorylates S419 in *C. elegans* HIF-1α (Hwang et al., 2014) (**Figure 2**). Although, these data raise the possibility that AMPK down-regulates HIF-1α via direct phosphorylation, that study did not address to which extent this phosphorylation involves or requires VHL.

Although the *C. elegans* study also left open whether the direct regulation of HIFα is conserved among other species, it is known from studies with cancer cells that ROS-dependent HIF-1α activation requires AMPK (Jung et al., 2008). Interestingly and

opposite to the regulation of HIF-1α by AMPK in *C. elegans* a recent study showed a link between AMPK function and HIF-1α regulation in the human hepatic cancer cell line Hep3B (Irigoyen et al., 1999; Chen et al., 2015). In these cells, the link between AMPK and HIF-1α appeared to be rather indirect involving histone deacetylase 5 (HDAC5) activity which can be phosphorylated by AMPK at S259 and S498. Since this phosphorylation of HDAC5 by AMPK promotes its shuttling from the nucleus to the cytosol (McKinsey et al., 2001) the authors examined whether cytosolic HDAC5 activity is involved in HIF-1α stabilization. They found that activation of AMPK by AICAR enhanced cytosolic presence of HDAC5 and levels of HIF-1α whereas the AMPK inhibitor compound C blocked HDAC5 nuclear export and HIF-1α accumulation (Irigoyen et al., 1999; Chen et al., 2015). Compound C, has also been shown to prevent hypoxia-dependent HIF-1α activation in DU145 cells (Lee et al., 2003; Hwang et al., 2004); however, this could be an independent effect since the inhibition of HIF-1α by compound C was also seen in AMPK$^{-/-}$ cells (Emerling et al., 2007). Together, it appears that AMPK can be involved in regulation of HIF-1α in a direct and indirect manner where the extent may be also depending on the species.

Altogether, these findings indicate that the HIFα system displays an enormous plasticity since its protein stabilization can be induced by hydroxylation and phosphorylation events either alone or in combination.

Regulation of HIF α-Subunit Nuclear Localization and Transactivity by Phosphorylation

Activation of multiple oncogenic pathways including growth factor signaling coupled with enhanced MAPK signaling is a common event in tumors (Raman et al., 2007). From the conventional MAP kinases the extracellular regulated kinases, ERK1 and ERK2 (p44/p42), c-Jun NH2-terminal kinase (JNK1/2/3), p38 MAPK (p38α/β/γ/δ) are known to be of importance for regulating cellular processes like proliferation, differentiation, development, stress responses, and apoptosis (Morrison and Davis, 2003; Olson and Hallahan, 2004; Coulombe and Meloche, 2007; Raman et al., 2007; Rincon and Davis, 2009; Gaestel, 2013; Serviddio et al., 2013; Tormos et al., 2013). Therefore, up-regulation of HIFα activity by MAPK signaling may play an essential role during tumor growth and metastasis.

To be able to act as transcription factor, stabilized HIFα proteins need to be translocated to the nucleus. This nuclear translocation was shown to be independent of ARNT and to be a dynamic process where nuclear import is commonly counterbalanced by nuclear export. Thus, the degree of nuclear HIFα accumulation depends on the relative nuclear import and export rates. In the case of HIFα, nuclear translocation was shown to involve its N-terminal and C-terminal NLS, respectively, as well as its interaction with importin 4 and 7 (Depping et al., 2015). The nuclear presence was then further shown to be regulated by ERK1 (p44)—and ERK2 (p42)-dependent phosphorylation. Thereby, mass spectroscopy with *in vitro* phosphorylated recombinant HIF-1α revealed that HIF-1α S641 and S643 (within the ID) served as p42/p44 MAPK targets (Mylonis et al., 2006; Triantafyllou et al., 2006) (**Figure 3**). Intriguingly, inhibition of these phosphorylation sites impaired HIF-1α nuclear accumulation and transcriptional activity by favoring nuclear export (Mylonis et al., 2006; Triantafyllou et al., 2006). This implies that ERK1/2 regulates rather the ability of HIFα to exit the nucleus rather than the import. Indeed, an atypical but CRM1 (exportin 1 or chromosome region maintenance)-dependent nuclear export signal (NES) (within aa 616–658 in HIF-1α) (Mylonis et al., 2008) was found to be phosphorylation-sensitive. Phosphorylation of S641 and S643 within the NES by ERK1/2 inhibited interaction between HIF-1α and the exporting CRM1 and facilitated nuclear accumulation.

In line with the nuclear accumulation are findings reporting that enhanced transcriptional activity of both HIF-1α and HIF-2α can be observed after direct phosphorylation of the HIFα isoforms by ERK1/2 *in vitro* and *in vivo* (Richard et al., 1999; Conrad et al., 2000; Minet et al., 2000; Sang et al., 2003; Mylonis et al., 2008). In line, the MEK1 inhibitor PD98059 and MKK inhibitor U0126 decreased HIF-target gene expression (Hur et al., 2001; Sodhi et al., 2001; Comerford et al., 2004; Dimova et al., 2005; Kaluz et al., 2006).

While the C-TAD of HIF-1α and HIF-2α is important for recruitment of the coactivator CBP/p300, phosphorylation sites within the C-TAD (Minet et al., 2000; Sodhi et al., 2001; Lee et al., 2002) and within the ID (Sodhi et al., 2001; Lee et al., 2002; Sang et al., 2003) of HIFα may also contribute to induction of transactivity. Indeed, the first functionally relevant phosphorylation sites were reported to be T796 in HIF-1α and T844 in HIF-2α (Gradin et al., 2002). Although the kinases phosphorylating these sites were not defined, phosphorylation of these residues increased interaction between the HIFα-C-TAD and CBP/p300. Moreover, it was shown that the MEK1 inhibitor PD98059 affected the transactivity of CBP/p300 and that ERK1 could also phosphorylate the transactivation domain of p300 (aa 1751–2414) which subsequently facilitated interaction between the HIF-1α C-TAD and p300 (Sang et al., 2003). Together with the finding that phosphorylated HIF-1α is the major form binding to ARNT (Suzuki et al., 2001), it appears plausible that HIF-1 transcriptional activity increases in response to induction of the MAPK pathway.

All together, these reports indicate that direct phosphorylation of HIF-1α and HIF-2α by ERK1/2 can affect their nuclear localization and transactivity.

Links between Hypoxia and Kinases in the Regulation of HIF-1α Transactivity

A number of findings have indicated that ERK1/2 can also serve as additional transmitter of the hypoxic signal since hypoxia has been shown to moderately activate ERK1/2 in different cell lines (Salceda et al., 1997; Conrad et al., 1999; Minet et al., 2000). Thereby cell type specific variations may appear as shown for HMEC-1 cells where involvement of ERK1 but not ERK2 in hypoxia-mediated HIF-1 transactivation was reported (Minet et al., 2000). In addition, by using PD98059 and by employing a mammalian two-hybrid assay, it was shown that the ERK pathway is also involved in hypoxia-dependent HIF-1α transactivation (Lee et al., 2002; Sang et al., 2003). By contrast,

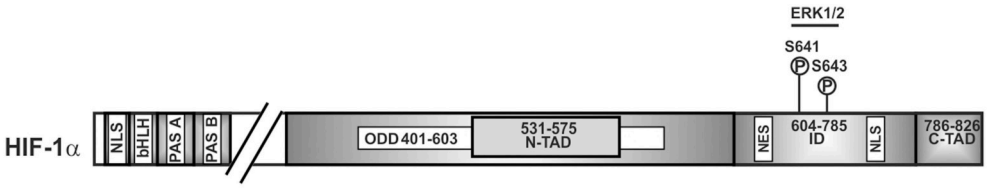

FIGURE 3 | Kinases involved in nuclear accumulation of HIF-1α. Scheme of HIF-1α and its domain organization with specific amino acid residues phosphorylation of which affects nuclear translocation. ERK2, extracellular regulated kinase2; bHLH, basic helix loop helix domain; NLS, nuclear localization sequence; PAS, Per-ARNT-Sim domain; N-TAD, N-terminal transactivation domain; ID, inhibitory domain; C-TAD, C-terminal transactivation domain; numbers indicate the amino acid residue range of the respective domain.

ERK1/2 activity was not increased in hypoxic growth-arrested Chinese hamster fibroblast CCL39 cells (Richard et al., 1999) implying that an activation of either ERK1 or ERK2 in response to hypoxia as well as their involvement in HIFα regulation may be cell type specific. Accordingly, the MEK1 inhibitor PD98059 suppressed hypoxia-mediated HIF-1α transcriptional activity in Hep3B and HMEC-1 cells (Salceda et al., 1997; Minet et al., 2000) whereas the same inhibitor was ineffective in fibroblasts exposed to hypoxia (Agani and Semenza, 1998). However, in all these studies direct mapping of the involved residues within HIFα proteins were not performed; thus only an approximate localization can be given (**Figure 4**).

In addition to ERK1/2, protein kinase CK2 (formerly known as casein kinase II) has important functions in the regulation of various cellular processes (Niefind et al., 2009; St-Denis and Litchfield, 2009; Montenarh, 2010). CK2 was shown to affect HIF-1α transcriptional activity (Mottet et al., 2005; Hubert et al., 2006); however, the exact mechanisms and CK2 phosphorylation sites in HIF-1α were not determined; likely CK2-mediated HIF-1α phosphorylation prevents recruitment of cofactors like CBP/p300 or stimulates HIF-1α degradation in an indirect manner (see below).

Together, these findings indicate an interrelation between hypoxia, ERK1/2, and CK2 signaling pathways in particular for the regulation of HIF-1α transactivity.

KINASES REGULATING HIFα ABUNDANCE IN AN INDIRECT MANNER

In addition to being a direct substrate for kinases, HIFα appears to be regulated via phosphorylation of HIFα regulating proteins in an indirect manner.

The protein kinase A (PKA) is among the best characterized kinases and was suggested to be involved in HIF-1α phosphorylation under intermittent hypoxia in EAhy926 endothelial cells (Toffoli et al., 2007). However, from that study it remained open whether or not HIFα proteins can be direct substrates for that kinase since no functional phosphorylation site(s) was identified yet.

As mentioned above, protein kinase CK2, a constitutive serine/threonine kinase which interestingly shows high CK2 activity in most human cancers can indirectly contribute to HIF-1α degradation. Thereby, CK2 phosphorylates S33, S38, and S43 within VHL. Mutation of the CK2 sites within VHL or inhibition of VHL phosphorylation with CK2 inhibitors increased VHL protein half-life and promoted degradation of HIF-1α (Ampofo et al., 2010). At the same time inhibited CK2 could also sequester p53 and reduce the transcriptional activity of p53. Together, this indicates that the indirect action of CK2 on HIF-1α and p53 can contribute to the survival of tumor cells (**Figure 5**).

One of the best known kinase pathways affecting HIFα in an indirect manner by regulating HIFα protein synthesis involves the mammalian target of rapamycin (mTOR). The mTOR is a serine/threonine protein kinase [also known as FK506 binding protein 12-rapamycin associated protein 1 (FRAP1)] (Brown et al., 1994; Moore et al., 1996) that apart from cell growth, cell proliferation, cell motility, cell survival, and transcription contributes to the regulation of protein synthesis in response to nutrients, hormones, growth factors, cytokines, and stress (for review see Hay and Sonenberg, 2004; Beevers et al., 2006; Dunlop and Tee, 2009). Thereby mTOR regulates translation primarily via phosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and ribosomal S6 kinase (S6K) (reviewed by Hay and Sonenberg, 2004; Inoki et al., 2005). By binding to translation initiation factor 4E (eIF4E) 4E-BP1 prevents interaction of eIF4E with other members of the translation initiation complex and inhibits ribosomal complex formation at the 5′-cap mRNAs. The phosphorylation of 4E-BP1 by mTOR results in its dissociation from eIF4E and in activation of mRNA translation (reviewed by Hay and Sonenberg, 2004; Inoki et al., 2005). In addition, phosphorylation of ribosomal S6K promotes translation of mRNAs containing a terminal oligopyrimidine tract (5′TOP) in their 5′-UTR (**Figure 5**).

Two major multiprotein complexes can be distinguished in which mTOR contributes to signaling; (i) the rapamycin-sensitive mTOR complex 1 (mTORC1) and (ii) the rapamycin-insensitive mTOR complex 2 (mTORC2) (Wullschleger et al., 2006). Several excellent reviews discussing in detail the composition of the TOR complexes and the impact of the participating proteins for signaling are available (see Hay and Sonenberg, 2004; Dunlop and Tee, 2009) and therefore we limit ourselves to the issue of HIFα regulation. While mTORC1 appears to be involved in nutrient/energy/redox sensing, mTORC2 seems to be mainly regulated by insulin, growth factors, serum and nutrients (Kim et al., 2003; Sarbassov et al., 2004; Frias et al., 2006).

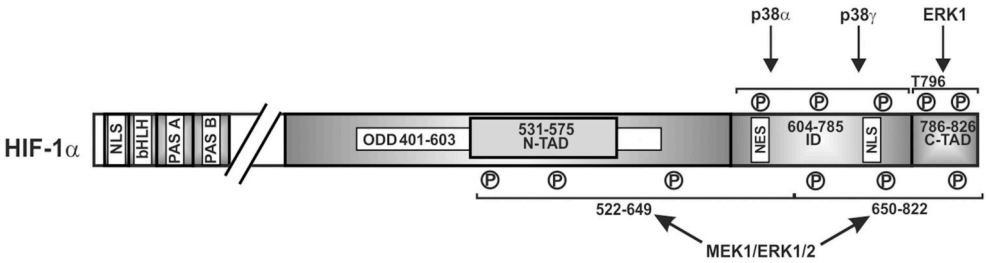

FIGURE 4 | Kinases involved in regulating HIF-1α transactivity. Scheme of HIF-1α and its domains in which phosphorylation has been shown to affect transactivity. P, represents a phosphorylated amino acid, no specific single residues sites have been mapped. ERK1/2, extracellular regulated kinase1/2; bHLH, basic helix loop helix domain; NLS, nuclear localization sequence; PAS, Per-ARNT-Sim domain; N-TAD, N-terminal transactivation domain; ID, inhibitory domain; C-TAD, C-terminal transactivation domain; numbers indicate the amino acid residue range of the respective domain.

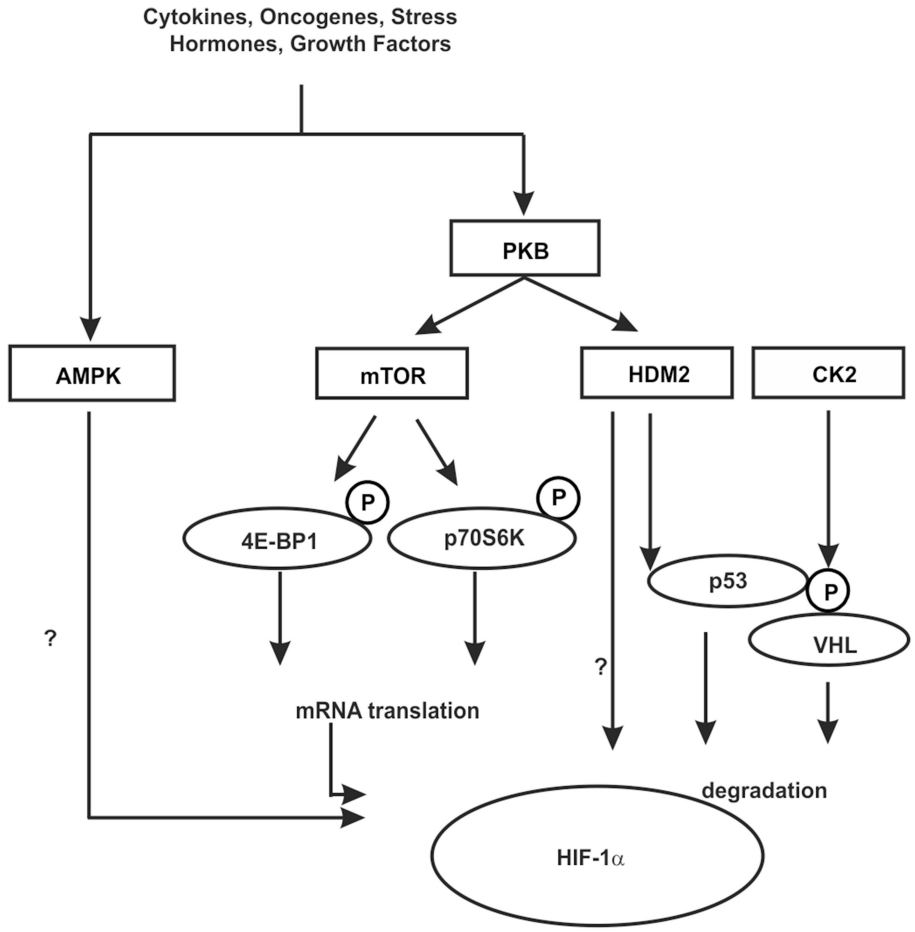

FIGURE 5 | Kinases contributing to HIF-1α regulation in an indirect manner. In response to various hormones, growth factors, cytokines, oncogenes, and stress phosphorylation events can be initiated which contribute to the regulation of HIF-1α in an indirect manner. These can influence HIF-1α mRNA translation, the interaction with cofactors or components of the protein degradation machinery like HDM2 and VHL. Some kinases may act in both ways, however, the knowledge about the exact mechanisms is limited. See text for more details.

The participation of mTOR in the regulation of HIF-1α protein translation was first shown in a study with breast cancer cells where stimulation with heregulin and HER2 signaling increased the rate of HIF-1α synthesis in a rapamycin-dependent manner (Laughner et al., 2001). Other studies in HUVEC and HeLa cells (Kim et al., 2009) supported that view and also showed that not only HIF-1α but also HIF-2α was found to be regulated by mTOR signaling, though HIF-1α expression seems to be regulatable by TORC1 and TORC2 whereas HIF-2α expression is primarily dependent on TORC2 (Toschi et al., 2008).

Hypoxia has been reported to inhibit mTOR (Arsham et al., 2003) via induction of the hypoxia-responsive gene DDIT4 (Dig2/RTP801/REDD1) and subsequent formation of a complex consisting of the tuberous sclerosis tumor suppressor proteins TSC1 (hamartin) and TSC2 (tuberin) (Brugarolas et al., 2004). The TSC1/TSC2 complex inhibits primarily mTORC1 signaling; destruction/inhibition of the TSC1/2 complex due to growth factors leads to activation of mTORC1 signaling (Hay and Sonenberg, 2004). For removal of TSC2 different kinase pathways, including PI3K/AKT, and ERK1/2 appear to be important (Hay and Sonenberg, 2004). Once phosphorylated, TSC2 can be captured by 14-3-3 proteins, thus leaving the complex with TSC1 and rendering mTORC1 active (Li et al., 2003).

Reciprocally, the hypoxia mediated inhibition of mTORC1 signaling (Brugarolas et al., 2004) appeared to be the result of a dissociation of TSC2 from the growth factor stimulated TSC2/14-3-3 complex. Thereby, hypoxic induction of DDIT4 seemed to be critical. Due to the ability of DDIT4 to bind 14-3-3 proteins this resulted in a release of TSC2 with formation of TSC1/2 complexes which subsequently inhibited of mTORC1 (DeYoung et al., 2008).

Thus, DDIT4 and TSC1/TSC2 formation could decrease mTOR activity and would reduce HIF-1α translation under hypoxia. However, under hypoxia when the cellular protein translation is generally suppressed, HIF-1α is still translated. This occurs likely by a mechanism involving the 5′-UTR of the HIF-1α mRNA which contains a terminal oligopyrimidine tract that enables HIF-1α translation even when mTOR is inhibited (Laughner et al., 2001; Thomas et al., 2006). Like with mTOR, ATR (for ataxia telangiectasia and Rad3 related kinase) appeared also to regulate HIF-1α translation in a region located within the HIF-1α ORF (Fallone et al., 2013).

The involvement of mTOR in HIF-1α translation was challenged in studies showing that rapamycin decreased hypoxia-induced HIF-1α stability at the ODD in PC-3 cells (Hudson et al., 2002; Dayan et al., 2009). Further, mTORC1 appeared to act also directly on HIF-1α since an mTOR signaling motif (FVMVL) modulating recruitment of CBP/p300 was found immediately C-terminal of the PAS-A domain in HIF-1α (Land and Tee, 2007). Thus, although mTOR signaling appears to affect HIFα abundance in a more indirect manner, it appears that also direct interactions are possible which may depend on the stimulus.

Growth factor stimulation and hence kinase signaling is not only important for mTOR signaling but also for crosstalk between the HIF-1α and the p53 network (Fukuda et al., 2002; Bardos et al., 2004). The murine double minute-2 (mdm2) and its human ortholog HDM2 protein are negative regulators of the p53 tumor suppressor protein (for review see Eischen and Lozano, 2009; Kruiswijk et al., 2015). In addition to p53, HDM2, which is a direct target of PKB/Akt (Ashcroft et al., 2002), was shown to regulate HIF-1α expression in response to IGF-1 in p53-null mouse embryo fibroblasts ($p53^{-/-}$ MEFs) (Bardos et al., 2004). Moreover, this appeared to involve protein synthesis and HDM2 phosphorylation at S166 by PKB/Akt (Bardos et al., 2004) suggesting that the PKB/Akt pathway also affects HIF-1α synthesis via HDM2 in a p53 independent manner (**Figure 5**).

Altogether, kinases regulating HIFα synthesis or degradation by acting on critical regulators of these processes are important mediators which interlink growth factor controlled pathways with hypoxia signaling.

EXPLOITING KINASES AS UPSTREAM REGULATORS OF HIF-1α IN CANCER THERAPY

An impaired regulation of kinase signaling is associated with a number of systemic diseases including cardiovascular diseases, pulmonary diseases, Alzheimer's disease, type 2 diabetes mellitus, and last but not least cancer. In particular, intermittent hypoxia in pre-malignant lesions and HIF-1α were proposed to contribute to the reprogramming of metabolism toward permanent conversion of glucose to lactate even in aerobic conditions (Gatenby and Gillies, 2004) known as the "Warburg effect," mitochondrial suppression as well as to acidosis. This provides a growth advantage, and an altered response to growth factors which are major actors on kinase signaling pathways. Thus, the interconnection of kinase signaling pathways and hypoxia signaling, i.e., HIFα regulation, is of high therapeutic interest. This is most obvious in cancer therapy where different kinase inhibitors are in clinical use and where severe hypoxic tumors are more resistant to chemotherapy and radiation. Interestingly the most successful kinase inhibitors currently used in cancer therapy are tyrosine kinase inhibitors like imatinib, gefitinib, and erlotinib. Tyrosine kinases are often found to act as receptors for hormones and growth factors and therefore they appear often to have an effect also on HIF-1α which is either direct or indirect (**Figure 5**). In addition to the tyrosine kinase inhibitors, other small molecules with the potential to act on MAPK, mTOR, or Akt pathways are under heavy investigation. Interestingly, the inhibitor of pyruvate dehydrogenase kinase II, dichloroacetate, has been shown to reactivate mitochondria via inhibition of HIF1α involving a PHD-dependent mechanism and a PHD-independent mechanism, involving activation of p53 and GSK3β (Sutendra et al., 2013).

However, these inhibitors are often not entirely specific but rather selective which explains their variety of actions as well as their effectiveness also in other disorders, including immunological, neurological, metabolic, and infectious diseases. Although this is already an advantage, it is difficult to predict to which extent kinase inhibitors could be made selective or even specific to target the HIF pathway. This is not only complicated by the fact that the respective kinase having a dominant role in HIFα regulation needs to have a role in the particular tumor entity. Thus, significant challenges remain. In addition to quick evolvement of tumors resistant to kinases inhibitors, appropriate multi-targeted inhibitors or combinations appear currently to be of advance in clinical therapy. Further, more understanding of the kinase inhibitor specificities toward HIF-1α, metabolic and toxic side effects would be needed to optimize cancer therapy.

CONCLUSION

Detailed knowledge about the kinase pathways and their effect on HIFα regulation is essential to optimize and to develop highly efficient cancer therapies. It is now especially necessary to gather more knowledge about the involvement of kinase pathways for the regulation of HIF-2α and HIF-3α since most of the data so far, with respect to kinases and HIFα regulation, have been gained from studies on HIF-1α. Given that certain aspects between HIF-1α and HIF-2α as well as the occurrence of several splice variants of HIF-3α point to more different roles of each HIFα protein in a number of processes, it is obvious that this knowledge would be beneficial for therapeutic purposes.

Overall, the HIFα system appears to be a central integrator of various signals coming from different pathways. Thereby it displays an enormous plasticity being regulated by a number of post-translational modifications, among them phosphorylation, either alone or in combination.

AUTHOR CONTRIBUTIONS

TK, DM, and ED, have written the manuscript and generated the data and figures.

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DUBs, New Members in the Hypoxia Signaling clUb

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Cellular protein homeostasis is tightly regulated by ubiquitination. Responsible for target protein ubiquitination is a class of enzymes, the so-called ubiquitin E3 ligases. They are opposed to a second class of enzymes, called deubiquitinating enzymes (DUBs), which can remove polyubiquitin chains from their specific target proteins. The coaction of the two sets of enzymes allows the cell to adapt its overall protein content and the abundance of particular proteins to a variety of cellular and environmental stresses, including hypoxia. In recent years, DUBs have been highlighted to play major roles in many diseases, including cancer, both as tumor suppressors and oncogenes. Therefore, DUBs are emerging as promising targets for cancer-cell specific treatment. Here, we will review the current understanding of DUBs implicated in the control of hypoxia-inducible factor, the regulation of DUBs by hypoxia, and the use of DUB-specific drugs to target tumor hypoxia-signaling.

Keywords: DUBs, ubiquitination, HIF, protein homeostasis, cancer

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INTRODUCTION

Like most other posttranslational modifications (PTMs), ubiquitin (Ub) conjugation is a reversible modification (1). Ub E3 ligases covalently attach monomers of Ub to lysine (and also cysteine) residues of their target proteins. Furthermore, ligases also convert monoubiquitination into polyubiquitin chains by attaching one by one further Ub monomers to one of the seven internal lysine residues (K6/K11/K27/K29/K33/K48/K63) of the preceding Ub molecule. In contrast, the family of DeUBiquitinating enzymes (DUBs) breaks down those mono- and polyubiquitin chains from the target protein. Besides counteracting the action of the Ub E3 ligases, DUBs are proteases that process Ub precursors.

Of the nearly 100 DUBs encoded by the human genome, 79 are predicted to be active and mostly cleave particular types of Ub chain linkages from their respective target proteins. DUBs can be grouped into six families based on sequence and structure similarity: ubiquitin-specific proteases (USPs) that comprise the largest and most diverse subfamily, ubiquitin carboxyl-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), Josephins, JAB1/MPN/MOV34 (JAMMs), and the more recently discovered monocyte chemotactic protein-induced proteins (MCPIP). With the exception of JAMMs, which belong to the Zn^{2+}-dependent metalloproteases, all the rest use the classical cysteine protease triad in the catalytical side (2).

Classically, the reversal of the polyubiquitination protects the target protein from being degraded by the proteasome, but ubiquitination has also been shown to have a broad range of non-catabolic functions (3). Thus, it is not surprising that DUB activity or inappropriate expression impacts on the regulation of multiple biological processes and several signaling pathways that are frequently altered

in many disorders from cancer over neurodegenerative pathologies to inflammatory diseases [for more details, please refer to Ref. (4)]. Because of their direct or indirect implications in those diseases and because of their potential druggability, DUBs have become of increasing interest in recent years.

Hypoxia is a feature of most human cancers (5). The cancer cells and their environment adapt to and survive under low oxygen availability. The activation of the hypoxia-inducible factor (HIF) that orchestrates the hypoxia-signaling pathway is instrumental to this adaptation. HIF is a heterodimeric transcription factor that consists of a constitutively expressed β-subunit (HIF-β) and HIF-α, whose expression is tightly regulated through the ubiquitin-proteasome system (UPS) (6–8). HIF triggers the expression of hundreds of direct target genes, indirect transcription factors, and non-coding RNAs that enable cancer cell survival and tumor progression by promoting, among others, angiogenesis, metabolic rewiring, genomic instability, drug resistance, and the self-renewal capacity of cancer stem cells. HIFs are also activated by genetic alterations in human cancers, such as the von Hippel–Lindau protein (pVHL) loss of function in clear-cell renal carcinoma (9). Accordingly, sustained expression of HIF-α in tumors has been associated with higher aggressiveness, migratory, and metastasis-initiating potential and therefore worse prognosis (10, 11).

In this review, we will summarize the current knowledge about the action of DUBs on HIF-α and the regulation of those enzymes by hypoxia. We will also discuss the potential of exploiting DUBs to target tumor hypoxia signaling.

THE CANONICAL HIF SIGNALING PATHWAY

The adaptive cellular program in response to low oxygen availability is mainly triggered by two HIF-α subunits (HIF-1α and HIF-2α), which share several common targets but also exhibit non-redundant functions (12). Anyhow, the levels of both HIF-α subunits result from the dynamic interplay between their ubiquitination and deubiquitination. In well-oxygenated cells, HIF-α is very unstable, as it is degraded by the proteasome within approximately <5 min after translation, whereas HIF-α's half-life is greatly increased in hypoxia (7, 8, 13). Proteasomal degradation is triggered by the continuous polyubiquitination of HIF-α by pVHL (9). pVHL is part of an E3 ligase complex and binds to HIF-α after the hydroxylation of two designated proline residues in HIF-α's oxygen-dependent degradation domain (ODDD), the central regulatory domain that confers its oxygen sensibility (8). This binding can be stabilized by SSAT2, therefore enhancing HIF-α ubiquitination (14). The family of prolyl hydroxylase domain-containing proteins (PHDs), the oxygen sensors also referred to as EGLNs or HPHs, catalyze the hydroxylation of HIF-α (Pro^{402} and Pro^{564}, in the case of HIF-1α) (15–19). HIF-α also harbors an N-terminal basic helix-loop-helix (bHLH) domain that mediates HIF-binding to the target DNA after heterodimerization with HIF-β/ARNT via the adjacent PAS domain. Of the two transactivation domains (TAD), the N-terminal TAD (N-TAD) lies within the ODDD, while the C-terminal TAD (C-TAD) is responsible for the recruitment of CBP/p300 needed to successfully induce the transcription of the HIF target genes that are characterized by having one or more HREs (hypoxia response elements) (20, 21). This C-TAD contains an asparagine residue (Asn^{803}, in the case of HIF-1α) that upon oxygen-dependent hydroxylation by FIH (factor inhibiting HIF) hinders the successful interaction of HIF with CBP/p300 and therefore, HIF's transactivation activity is reduced (22). Interestingly, HIF induces the expression of two of its negative regulators, PHD2 and PHD3, in order to ensure its own rapid degradation upon reoxygenation (19, 23). However, in conditions of chronic hypoxia, once the transcriptional adaptive program has been triggered, HIF-α levels drop again to avoid sustained HIF signaling and assure cell survival (24).

In the context of the canonical HIF signaling pathway, so far there are relatively few DUBs reported in the literature, and reports are mostly focused on the impact on HIF-1α (**Figure 1** upper part). *USP20* (also called pVHL interacting DUB2, VDU2) was the first DUB to be described to reverse pVHL-mediated HIF-1α ubiquitination (25). In turn, USP20 is a pVHL target (26). *MCPIP1* also deubiquitinates HIF-1α to promote angiogenesis (27). In the context of ciliogenesis, *USP8* has been found to bind to HIF-1α's PAS domain and to partially protect HIF-1α from degradation (28). More recently, *UCHL1* has been shown to be a positive regulator of HIF-1α protein stability acting on HIF-1α's ODDD (29).

THE NON-CANONICAL HIF SIGNALING

Not surprisingly because of HIF's crucial role in cell fate, many more proteins have been described to be involved in the control of its stability (**Figure 1** lower part). The heat-shock protein 90 (HSP90) that interacts with the PAS domain of HIF-α regulates its degradation in an O_2/PHD/pVHL-independent manner (30). HSP90 competes with RACK1 for binding to HIF-α and prevents the recruitment of the elongin C/B Ub E3 ligase complex (31). A similar mechanism has been proposed for HIF-α activation by ErbB4 (32). As for other HSP90 client proteins, Cullin5 also regulates HIF-α degradation independently of elongin C/B function (33). The tumor suppressors p53, TAp73, and pTEN promote the Ub-mediated degradation of HIF-1α via recruitment of the Ub E3 ligase Mdm2 (34–36). Furthermore, Fbw7 ubiquitinates and induces HIF-1α degradation following phosphorylation by GSK3β (37, 38). Interestingly, this degradation can be antagonized by the Ub-specific protease (*USP28*) (38). Until now, this is the only non-canonical Ub E3 ligase–DUB pair identified for proteasomal degradation of HIF-α. HAF, the hypoxia-associated factor, seems to play a dual role in the control of HIF-α stability and/or activity. While HAF acts as an Ub E3 ligase targeting HIF-1α for degradation independently of oxygen availability, hypoxia-induced SUMOylated HAF promotes HIF-2α transactivation without affecting its stability (39, 40). Furthermore, RNF4 controls the levels of SUMOylated HIF-2α (41). *USP19* seems to be required for the hypoxic accumulation of HIF-1α, though the effect is not dependent on its deubiquitinase activity (42). USP19 is further substrate of Siah-1 and Siah-2 Ub E3 ligases, which also control the stability of PHD1, PHD3, and FIH (43–45). Thus, further studies are necessary to clarify the direct impact of USP19 in HIF-1α ubiquitination.

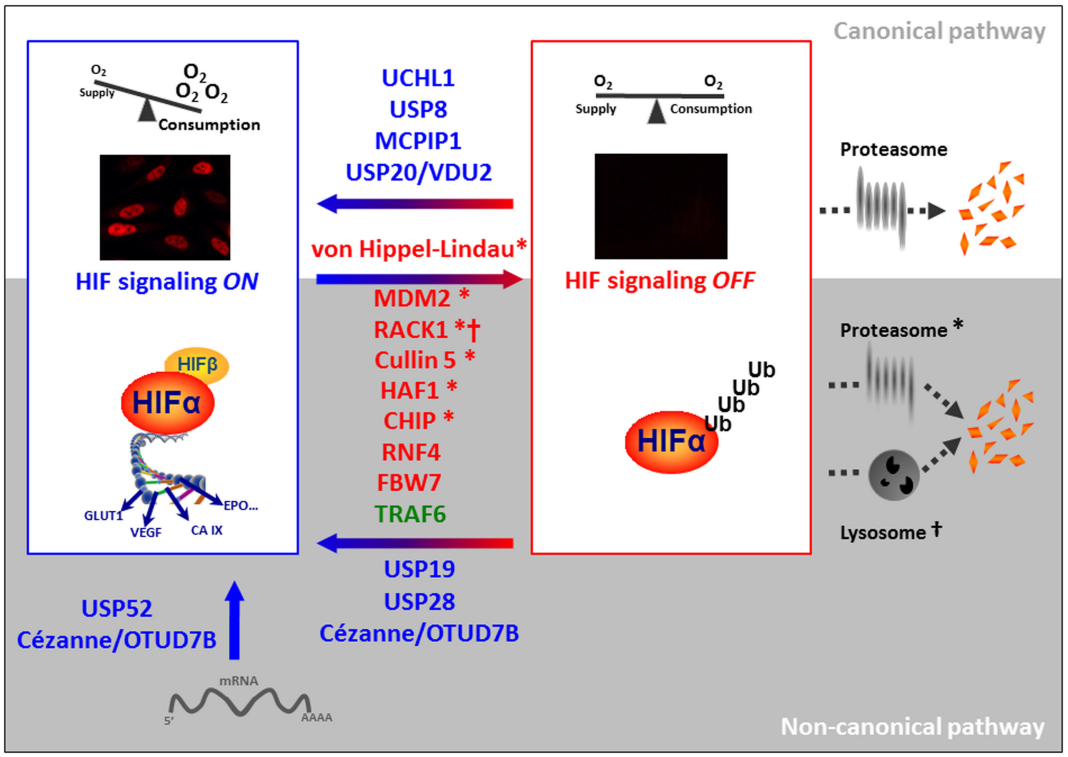

FIGURE 1 | Involvement of ubiquitinating and deubiquitinating enzymes in the regulation of HIF-α. Destabilizing and stabilizing E3 ligases targeting HIF-α are pictured in red and green, respectively and DUBs are depicted in blue. E3 ligases that have been described to target HIF-α for proteasomal degradation are marked with a *; † refers to lysosomal HIF-α degradation.

The chaperone-dependent Ub ligase CHIP targets HIF-1α but not HIF-2α for degradation either by the proteasome or by the autophagic machinery, the second big protein degradation and recycling pathway that has been implicated in the elimination of ubiquitinated HIF-α (46–49). In this regard, *Cezanne* (OTUD7B), a deubiquitinase targeting K11 Ub chains (50), has been reported to protect HIF-1α from lysosomal degradation. While this process is independent of HIF-1α prolyl hydroxylation, it depends on the presence of pVHL (51).

Calpain and the activation of the forkhead transcription factor FOXO4 destabilize HIF-α, although the underlying molecular mechanisms are unknown (52, 53). Further studies are also needed to characterize the role of Parkin in the regulation of HIF-α, based on its identification within the Parkin-dependent ubiquitinome by a proteomic approach (54). In contrast with all the previous reports, it is worth mentioning the role played by the Ub E3 ligase TRAF6. TRAF6 increases HIF-1α but not HIF-2α polyK-63 ubiquitination and protects the protein from proteasomal degradation (55).

In addition to HIF-α stability, mRNA expression and activity of the transcriptional complex fine-tune HIF regulation. In this regard, *USP52* is required for the protection of HIF-1α (but not HIF-2α) mRNA from premature degradation and therefore, allows the normal hypoxic induction of HIF-1α (56). The case of USP52 is somewhat special as this protein, although structurally related to the family of USPs, lacks the catalytic cysteine (57). Besides protecting HIF-1α protein from its degradation, *Cezanne*'s catalytical activity is also required for maintaining basal levels of the E2F1 transcription factor. Moniz et al. demonstrated that E2F1 controls the expression of HIF-2α mRNA and therefore, established an indirect role of the DUB Cezanne in HIF-2α expression (58).

Finally, a number of DUBs have been shown to regulate transcription factors and signaling pathways that cross talk with HIFs, likely contributing to the complexity and specificity of the cellular hypoxic response, even though they go beyond the scope of this review (59–61).

REGULATION OF DUBs BY HYPOXIA

As for other enzymes, there are several possible layers of regulation of DUB activity. Next to the transcriptional regulation, the stability and translation of the mRNA can be regulated by mRNA-processing enzymes. The turnover and therefore, the availability of the mature protein can be set by a variety of PTMs. PTMs can also interfere with the binding of the DUBs to their target proteins or other interactors, as well as modulate reversibly and irreversibly the (auto) catalytical activity of the DUB. Hypoxia, being an extreme cellular stress condition, should be able to regulate deubiquitinating activity on all the possible different layers in order to adapt DUB functions to the cell's needs. However, the literature about the regulation of specific DUBs by hypoxia (1% O_2, if not specified differently) is still scarce and

almost exclusively restricted to transcriptional regulation. For instance, the expression of *USP13* is reduced upon treatment with as little as 6 h of 2% O_2 in melanoma cell lines (59). The reduction of the mRNA also translates to the protein level and causes the loss of Siah-2 stabilization. Similarly, in colon cancer cells hypoxia reduces *USP46* mRNA and protein levels and, therefore, diminishes USP46's stabilizing effect on the tumor suppressors PHLPP1 and PHLPP2, conferring to the colon cancer cells an increased paclitaxel resistance (62, 63). Guo et al. provide more detailed information about the hypoxia-mediated transcriptional regulation of the UCH *CYLD*. They suggest that the decrease of CYLD mRNA and protein seen in glioblastoma cells is due to the hypoxia-induced increase of the transcriptional repressors Snail and Hes1 (64). In contrast, hypoxia has been shown to increase *Cezanne* via p38 MAPK (65).

An et al. claimed that CYLD is targeted for proteasomal degradation after interaction with the HPV E6 protein in hypoxia (66). This is to date the only report of a posttranslational regulation of DUB activity by hypoxia. However, Lee et al. present evidence that the activity of many, if not most, DUBs depends on the redox state of the cell. They show that the catalytically active cysteine residue can be oxidized, for instance, by intracellular hydrogen peroxide, leading to the abolishment of the deubiquitinating activity. The inactivating oxidation can be reversed in the presence of reducing agents, such as DTT, or prevented by antioxidants (67). As hypoxia and mitochondrial ROS production are intrinsically linked it might not be too far-fetched to propose that hypoxia directly modulates DUB activity via ROS.

DYSREGULATION OF HYPOXIA-RELATED DUBs IN CANCER

Given the importance of Ub-mediated changes in protein function and homeostasis, it is not by chance that the entire process is highly regulated. Disruption of the ubiquitination cycle by mutations or altered expression of specific components within the cascade has been associated with several disorders. In particular, more than 30 DUBs have been associated with cancer directly or indirectly. Both, the loss of a specific DUB activity or its hyperactivity are non-desired events if the targets are tumor suppressors or oncogenes, respectively. Recurrent mutations of DUBs are rare in cancer with only few exceptions. Gene fusions with RUNX are reported for USP42 and USP16 in hematologic diseases, such as chronic myelomonocytic leukemia and acute myeloid leukemia. However, dysregulated mRNA levels of DUBs are implicated in many malignancies. Here, we will focus only on a few examples of hypoxia-related DUBs, for a more extensive overview please refer to the very comprehensive review by D'Arcy et al. (68).

Germline mutations of the tumor-suppressor gene *CYLD* are prevalent in familial cylindromatosis, a genetic condition that leads to predisposition for developing multiple skin tumors (69, 70). In addition, CYLD deubiquitinating activity has been seen to be abolished in different cancers on the protein level by inactivating phosphorylations or destabilizing polyubiquitination (71). More recently, it has been reported that *USP8* is frequently mutated in adenomas causing Cushing's disease (72).

USP28 is a DUB whose overexpression has been reported in breast and colon cancer and glioblastoma (73, 74). A recent publication has proposed USP28 to be a potential predictive marker in bladder cancer, as they found correlation of USP28 with tumor histological grade, clinical stage, recurrence, and survival (75). Similar to USP28, *UCHL1* has also been proposed to be a useful biomarker, being overexpressed in gastric cancer (76) and in myeloma (77), and epigenetically down-regulated in colorectal cancer (78). As mentioned above, downregulation of *USP46* may serve as a biomarker of resistance to chemotherapy in colon cancer (63). Finally, despite being inconsistent to its role in the regulation of HIF-1α and HIF-2α, decreased *Cezanne* expression is associated with the progression and poor prognosis in hepatocellular carcinoma (79).

DUBs AS DRUGGABLE TARGETS FOR THERAPY

Modulators of individual UPS components are emerging as a novel class of anticancer drugs. The initial research focus had been directed toward targeting the proteasome, with activity described for many compounds with proteasome inhibitory activity, including bortezomib. Because Ub E3 ligases provide substrate specificity, their direct targeting may avoid the deleterious side effects associated with the global inhibition of the proteasome, making them interesting candidates as drug targets. Nutlin-3 and JNJ-26854165 are classic examples directed against the Ub E3 ligase MDM2 and are currently undergoing clinical evaluation as anticancer therapy.

Newly arising, DUBs may serve as equally or more useful targets. Indeed, DUBs are highly specialized and evolutionary linked to proteases, a typified pharmaceutical target class for drug discovery, thanks to their well-characterized catalytical domain. Several partial and specific inhibitors against a small number of DUBs have been developed, have proved active in preclinical studies as reviewed recently by D'Arcy and Linder (80), and have provided feasibility for targeting these enzymes for anticancer purposes. Among them, HBX 41,108 is a partially selective USP inhibitor because it inhibits USP5, USP7, USP8, and UCHL3 in addition to caspase 3 (81). This is to our knowledge the only DUB inhibitor so far described as targeting one of the DUBs linked to the HIF signaling pathway. Interestingly, the inhibition of USP8 suppresses growth of gefitinib-resistant non-small cell lung cancer cells, though no link to the potential impact on HIF-1α is reported (82). It is tempting to speculate about new drugs directed against hypoxia-related DUBs that succeed to fight intratumoral hypoxia-signaling in the coming years.

CONCLUSION

HIF-α protein homeostasis is tightly controlled in healthy cells in order to avoid inappropriate activation of HIF signaling. A variety of E3 ligases and DUBs are involved in this task by triggering and protecting HIF-α from its degradation, respectively. Permanent

activation of the HIF signaling pathway has been found in many tumors and seems to be beneficial for tumor growth and cancer progression. In most cases, the reason for sustained HIF-α protein levels in the tumor cells are still not revealed, but a possible mechanism is the pathological increase of HIF-α specific DUB activity. In recent years, the dysregulation of deubiquinating enzymes in cancer (and other diseases) has become of increasing interest, and alterations of their expression and activities have been shown to have diagnostic value. Whether cancer-related events that lead to the upregulation of DUB activity are the primary cause of uncontrolled HIF signaling, or whether initial hypoxia upregulates DUB expression as a positive feed-back-loop is not determined. But in the light of DUBs being druggable enzymes, it is important to understand their implications in HIF and tumor hypoxia-signaling.

AUTHOR CONTRIBUTIONS

ASS and EB contributed with review writing, editing, and final approval of the manuscript.

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MINI REVIEW
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News about VDAC1 in Hypoxia

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The voltage-dependent anion channel (VDAC) is the main interface between the cytosol and mitochondria of cells. It plays a crucial role in both mitochondrial metabolism and cell death. The main basic function of this channel is to mediate and gate the flux of small ions, metabolites, and adenosine triphosphate. Changes in its structure, and thus conformation, are expected to affect its activity and modulate the ability of cancer cells to expand. In this review, we describe a novel mechanism by which mitochondria of cells in hypoxia, a low level of oxygen, protects from apoptosis. In hypoxia, some mitochondria become enlarged due to hyperfusion. These mitochondria possess a truncated form of VDAC1 (VDAC1-ΔC), which is linked to the higher metabolic capacity and the greater resistance to cell death of hypoxic cells. However, not all of the VDAC1 protein is truncated, but the amount of the full-length form is diminished compared to the amount in normoxic cells. First, we describe how such a decrease effects cell proliferation, respiration, glycolysis, and other processes. Second, we report on a novel mitochondrial-endolysosomal crosstalk that leads to VDAC1 truncation. By pharmacological targeting of VDAC1-ΔC, the production of energy could be turned off and the sensitivity to cell death restored. This could counteract the favorable microenvironment that gives cancer cells a growth advantage and thereby disrupts the balance between life and death, which is controlled by VDAC1.

Keywords: cancer, mitochondria dysfunction, VDAC1, hypoxia-inducible factor 1, resistance to cell death

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INTRODUCTION

Mitochondria have evolved over time to take on a symbiotic relationship within eukaryotic cells to produce adenosine triphosphate (ATP) through activation of the electron transport chain (1). The production of ATP is probably the most important function of mitochondria, together with the regulation of apoptosis. Thereby, they are involved in different processes essential to the maintenance of cellular homeostasis. Modifications in metabolism and the redox status, critical steps in tumor cell transformation and progression, make mitochondria attractive targets for therapeutic treatment. Therefore, any modification to cancer cell metabolism and more specifically to mitochondrial metabolism, by increasing reactive oxygen species (ROS) production, or stimulating mitochondrial permeability transition to induce cell death, could be promising new therapeutic strategies (2).

How do mitochondria behave in hypoxia? Hypoxia is a decrease in the oxygen concentration compared to the normal physiological concentration and is a characteristic of the tumor microenvironment. Far from being a disadvantage, hypoxia is an undeniable force for the tumor cell. Functional benefits of hypoxia include epigenetic modifications, tumor vascularization, modified metabolism, signaling of metastasis, invasion and extravasation, cancer stemness, and innate immune activation,

all of which are under the control of complex molecular pathways driven by the transcription factor the hypoxia-inducible factor (HIF) (3, 4). These changes help cells to proliferate and resist cell death induced by chemotherapy or radiation. Among the processes activated during tumor growth, metabolism, and more specifically glycolysis, is the one that is the most exacerbated by hypoxia (5, 6). To counterbalance mitochondrial generation of ROS that interfere with cell survival, HIF-1 activates pyruvate dehydrogenase kinase (PDK1) to block the conversion of pyruvate to acetyl CoA resulting in decreased flux through the tricarboxylic acid (TCA) cycle (7, 8). Moreover, HIF-1 upregulates the expression of *COX4-2*, present in complex IV, and the mitochondrial protease *LONP1*, which in turn degrades COX4-1. COX4-2 is then more efficient at facilitating electron transfer to O_2 and thereby protects the cell from oxidative damage in hypoxia (9). HIF-1-mediated inhibition of MYC (10) and PGC-1 also results in reduced mitochondrial biogenesis (11). However, cancer cells have selectively found new mechanisms that promote their survival.

In this mini review, we highlight a new hypoxic mechanism that modulates the amount (decrease) and structure (a cleaved form lacking its C-terminus) of the most abundant protein of the outer mitochondrial membrane (12), the voltage-dependent anion channel (VDAC), which profoundly impacts cancer cell proliferation and survival.

THE VOLTAGE-DEPENDENT ANION CHANNEL: FUNCTION, ORGANIZATION, AND STRUCTURE

Voltage-dependent anion channel plays a key role in both mitochondrial metabolism and cell death, acting as a convergent point of control (13, 14). The function of VDACs as a pore seems quite clear-cut, but other suspected more complex functions are yet to be elucidated. VDACs play a crucial role as a gatekeeper for the entry and exit of many metabolites. They mediate and gate the flux of small ions (Cl^-, K^+, Na^+) with a preference for anions and metabolites (NADH, citrate, succinate, glutamate, pyruvate, and glucose) and act as channels that constitute the main pathway for passage of ATP/ADP (15–18). In addition to their role in bioenergetics, VDACs act as a scaffold through interactions with numerous proteins. They are anchors for pro- and anti-apoptotic proteins, respectively, of the hexokinase (HK) (19, 20) and Bcl-2 families (21–24) of proteins, which contribute to the balance between survival and cell death.

Voltage-dependent anion channels exist as three isoforms: VDAC1, VDAC2, and VDAC3, encoded by three different genes. The three human *VDAC* genes share the same number of exons for each gene isoform (25). The human *VDAC1* gene spans about 30 kb localized on the chromosome 5q31-32 (26), the human *VDAC2* has been mapped to chromosome 10q22 and is 16.4 kb in length whereas the human *VDAC3* is localized on chromosome 8p11.21 with a length of 14.3 kb. The *VDAC2* gene uses several polyadenylation sites, thus giving rise to multiple mRNA, whereas the *VDAC3* gene presents an alternative splicing event that corresponds to an additional ATG. At the protein level and in mammals, VDACs share ~70% identity with a very similar molecular mass of 30–35 kDa. They are known to be expressed ubiquitously in mammalian mitochondria, where VDAC1 remains the most abundantly expressed of the three isoforms (27). VDAC1 has also been detected in the plasma membrane of human lymphocytes (27, 28) and in the sarcoplasmic reticulum (29).

Analysis of the structure of VDACs revealed a 19-stranded β-barrel fold (13), yet only 13 of these strands form the wall of the channel. The N-terminal region of VDACs is very dynamic and exposed to the cytoplasm but located inside the pore. It acts as the voltage sensor and maintains the channel in an open or closed status (25). In an open-state configuration, VDACs are capable of passing millions of ATP molecules per second *in vitro* (17) and up to 100,000 ATP molecules per second under physiological conditions (16, 17), using at least five different trajectories (30). By contrast, very little is known about the function of the C-terminus of VDACs. It possesses NAD^+-binding sites, considered essential for glycolysis (31). Finally, VDAC1 can oligomerize and assemble into a dynamic equilibrium of dimers, trimers, tetramers, and higher oligomers (32). These conformational changes could occur upon induction of apoptosis (33). However, the function of VDAC1 oligomers is not known. They may contribute to the stabilization of the protein (34) and may offer a more stable platform to anchor HKs I and II (32).

THE VOLTAGE-DEPENDENT ANION CHANNEL: MODIFICATIONS, SILENCING, AND OVER-EXPRESSION

Post-translational modifications, changes in expression, or even mutation in VDACs profoundly disrupt metabolism and, thus, the balance between cell survival and cell death. The three isoforms of VDAC can be post-translationally modified by phosphorylation and acetylation at multiple sites (35). The role of VDAC1 phosphorylation remains unclear, as it is difficult to study these modifications on highly hydrophobic integral mitochondrial outer membrane proteins. The impact of these modifications has been studied mostly in the context of apoptosis. However, no direct relationship to VDAC function or activity has been demonstrated. The relevance of acetylation remains to be determined. Recently, our studies showed a new form of post-translational modification of VDAC1; C-terminal truncation of the protein to give VDAC1-ΔC (discussed in Section "The Hypoxic Mitochondrial Phenotype and VDAC1-ΔC") (**Figure 1**). This modification occurred specifically under hypoxic conditions. This hypoxic form was associated in some cancer cell lines with resistance to chemotherapy-induced apoptosis, a higher output of ATP and was found in late stage tumors of patients with lung cancer (36). A mutation in VDAC1 that resulted in the removal of 60% of the length of the C-terminal region has been described in colorectal and gastric cancers, but the consequence on metabolism and apoptosis is still to be determined (37).

Deletion of genes coding *Vdac* in mice models has provided information concerning the functions of VDACs. *Vdac1* and

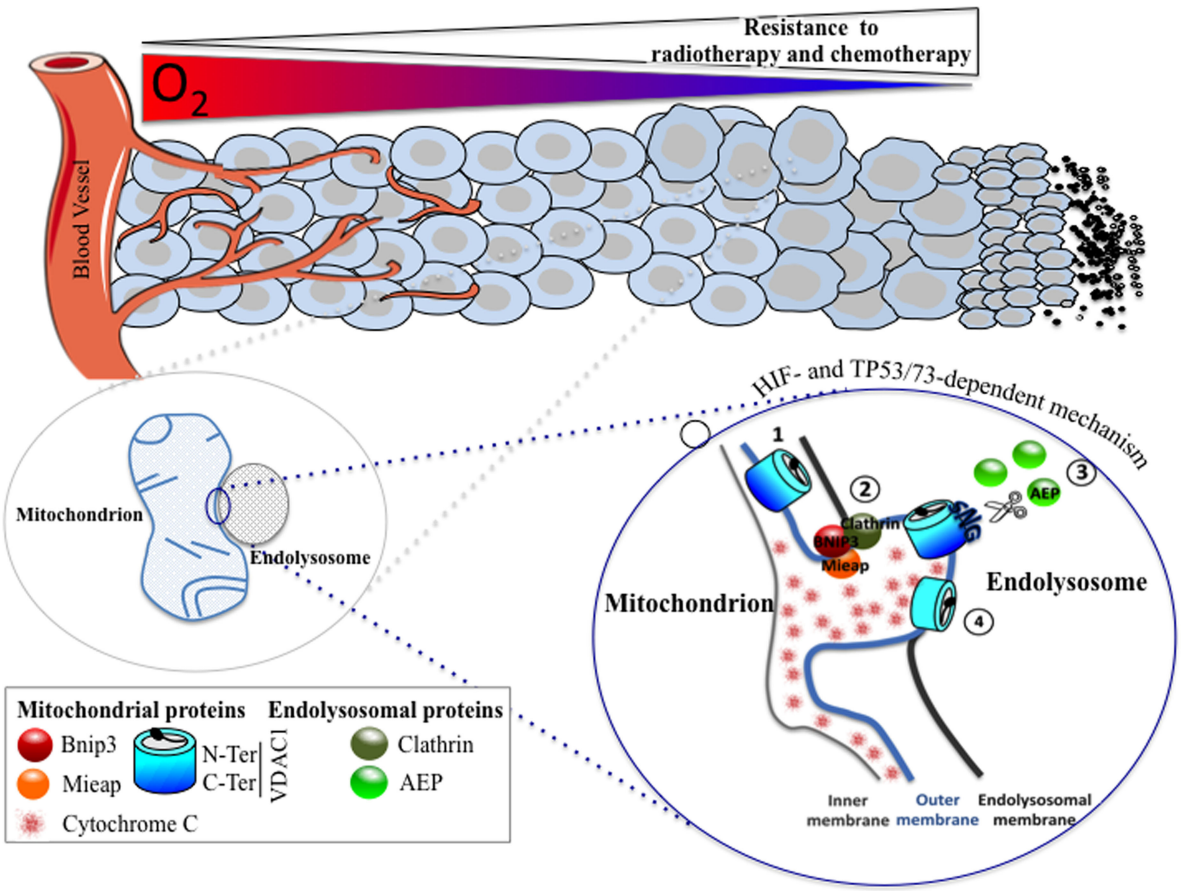

FIGURE 1 | A HIF- and TP53/73-dependent model that potentiates tumor cell survival in hypoxia through the formation of enlarged mitochondria that interact with endolysosomes to modify mitochondrial VDAC1, an ATP channel-regulating metabolism and apoptosis. As oxygen diffuses from a vessel, a decreasing gradient in the oxygen concentration occurs in the adjacent tissue (top). As the level in oxygen decreases, resistance to radiotherapy and chemotherapy increases since the former requires oxygen for DNA damage, and the latter depends on the limits of tissue diffusion of the drug. Hypoxic cells with resistance to chemotherapy show the presence of enlarged hypoxic mitochondria (magnified on the bottom left). Microfusion between the mitochondrial outer membrane and an endolysosomal membrane takes place (magnified on the bottom right), and a cleaved form of VDAC1 is produced, according to the following steps: (1) VDAC1 is exposed to an endolysosome, (2) a complex formed of BNIP3/Mieap (mitochondrial proteins) and clathrin (endolysosomal protein) maintained microfusion of both membranes, (3) the endolysosomal asparagine endopeptidase (AEP), in contact with the mitochondrial outer membrane, specifically cleaves VDAC1, and (4) VDAC1-ΔC promotes resistance to apoptosis and a blockade in cytochrome *c* release.

Vdac3 heterozygote embryonic stem cells have been generated to obtain hetero- and homozygote knockout mice (38, 39). *Vdac1*$^{-/-}$ mice did not meet the normal Mendelian pattern, suggesting partial embryonic lethality at days 10.5–11.5. For the mice that survived, multiple respiratory defects appeared in skeletal and cardiac muscles. The mice were fertile but were retarded in growth. Mitochondria from muscle fibers of skin contained enlarged mitochondria with very compact cristea. *Vdac3*$^{-/-}$ mice survived with defects in mitochondrial respiration in heart tissue. Males were also infertile with alterations to their sperm. A combination of both *Vdac1*$^{-/-}$ and *Vdac3*$^{-/-}$ deletions was not lethal. Finally, the homozygote mice lacking *Vdac2* died during development (40). Although it seems surprising not to find VDACs in a tissue, we recently reported, using the Cancer Genome Atlas (TCGA) data sets from 89 cancer studies, that deletion of the *VDAC1* gene is found in some cancer types (41). Even if the *VDAC1* gene was mainly heterologously lost, some homologous loss was found. Clear cell renal cell carcinoma (ccRCC) and ovarian cancers seemed to be the most affected by the homologous loss of the *VDAC1* gene, whereas the heterologous loss was almost ubiquitous. It might be interesting to further examine these data to check if the *VDAC2* and *VDAC3* genes are also deleted or if compensation has taken place to counterbalance for the homologous loss of *VDAC1*. New aspects of the function of VDAC1 were highlighted in our recent transcriptome analysis of mouse embryonic fibroblasts (MEFs) knocked out for *Vdac1* (41). We characterized the cellular and molecular phenotype of both *Vdac1*$^{-/-}$ MEF and MEFs transformed with the pBabe-RAS^{V12} vector, *Vdac1*$^{-/-}$ RAS MEF. Our results pointed to alterations in programs controlling HIF-1, cell death, and survival, as well as cell proliferation and motility. We confirmed the presence of alterations in OXPHOS and glycolysis in knocked out cells, which was accompanied by

a higher level of apoptosis. Of note, *Vdac1*$^{-/-}$ MEF and *Vdac1*$^{-/-}$ RAS MEF grew better in hypoxia, by maintaining respiration and promoting glycolysis. *Vdac1*$^{-/-}$ RAS MEF formed tumors faster than Wt RAS MEF in nod-scid mice. Moreover, after dissection of the various mechanisms involved, our results showed a strong impact of VDAC1 on tumor development, through alterations in the inflammatory response as a result of an abnormal vasculature due to ROS production and HIF-1α stabilization. Changes in metabolism were also observed in both *Vdac1*$^{-/-}$ MEF and *Vdac1*$^{-/-}$ RAS MEF. The first surprise came from the impact of hypoxia on the behavior of *Vdac1*$^{-/-}$ cells, in particular on cell proliferation. These initial results lead us to revisit the Mendelian ratio observed in the *Vdac1*$^{-/-}$ mice. Indeed, a large number of *Vdac1*$^{-/-}$ embryos did not survive (60%), whereas the *Vdac1*$^{-/-}$ embryos that survived gave an almost normal phenotype. Is it possible that the non-viable *Vdac1*$^{-/-}$ embryos were not exposed to low oxygen concentrations during development and, therefore, apoptosis prevailed? Our second hypothesis is that non-viable *Vdac1*$^{-/-}$ embryos may have developed dysfunctional blood vessels during embryogenesis. The second surprise came from our result that suggested the involvement of VDAC1 in modulating the structure of blood vessels and in enhancing the inflammatory response (41). Indeed, accumulation of ROS in *Vdac1*$^{-/-}$ RAS MEF-derived tumors triggered HIF-1α stabilization, abnormal vasculature, and leakage of red blood cells, thus generating an inflammatory response that resulted on a strong impact on VDAC1 tumor development. To our knowledge, this is the first time that VDAC1 has been connected to such events. These pathways should not be neglected in the future when considering VDAC1 as a therapeutic target.

Exogenous over-expression of VDAC1 in different cell lines always seems to be linked to apoptosis (24, 25, 42, 43). However, the impact of VDAC1 directly or indirectly on apoptosis is not yet clear. It may depolarize the inner membrane (44) as it could trigger the mitochondrial permeability transition pore (MPTP) (45). However, we showed that VDAC1 was over-expressed in lung adenocarcinomas tumor tissue from 44 patients (36). More recently, after analysis of the same TCGA data sets, as described above, expression of *Vdac1* was also gained and amplified (41). The *in vitro* results contrast with the *in vivo* ones. It is easy to hypothesize that as VDAC1 regulates metabolism through its association with HK, cancer cells draw a substantial profit from increasing glycolysis. Similarly, because of its association with members of the Bcl-2 family, cancer cells, again, take advantage of such an association by minimizing apoptosis.

THE HYPOXIC MITOCHONDRIAL PHENOTYPE AND VDAC1-ΔC

Mitochondria are dynamic organelles that undergo membrane remodeling through cycles of fusion and fission (**Figure 1**). This balance controls the mitochondrial structure and, thus, mitochondrial activity. The key factors regulating fusion are the dynamin-related GTPases mitofusin 1 (Mfn1) and 2 (Mfn2), and optic atrophy 1 (OPA1) that mediate the OMM, while the dynamin-related protein 1 (DRP1) regulates the opposing process of fission of the inner mitochondrial membrane (IMM) (46). Cells lacking mitochondrial fusion show changes in mitochondrial shape associated with a loss of their membrane potential, a reduced growth rate, and a lower activity of respiratory complexes (47). By contrast, forced expression of Mfns resulted in the formation of clusters with enlarged mitochondria due to the fusion of the OMM (48). In 2010, we reported that a number of human cancer cells, including colon carcinoma cells (LS174) and non-neoplastic CCL39 lung fibroblasts exposed to long-term hypoxia (72 h – 1% O_2) showed a change in mitochondrial phenotype from a tubular network to an enlarged morphology (49). The modification of the shape of mitochondria observed in LS174 cells was HIF-1-dependent (36). The formation of these enlarged mitochondria resulted from hyperfusion as expression of Mfn1 was increased in hypoxia and as silencing of the expression of Mfn1 reverted the mitochondrial morphology. Moreover, we reported that Bcl-2/adenovirus E1B 19-kDa interacting protein (BNIP3) and BNIP3 like (BNIP3L), two pro-autophagy proteins from the Bcl-2 family, also participated in the dynamic process of fusion induced in hypoxia. Finally, cytochrome *c* was retained inside these structurally unusual mitochondria when the cells were treated with staurosporine, a pro-apoptotic drug. We concluded that cells with enlarged mitochondria were more resistant to cell death than normoxic cells and that these cells possessed a selective growth advantage.

We were, therefore, faced with the challenge of exploring the underlying mechanisms that lead to the protective phenotype of hypoxic cancer cell. We found that VDAC1 was detected in hypoxia as a smaller than usual form (26 kDa rather than 30 kDa on SDS-PAGE). The amount of the 30 kDa form was decreased by around 50% with a parallel increase in the smaller 26 kDa form in hypoxia. Using a VDAC1 antibody directed to the C-terminus of VDAC1 that did not detect this fast migrating form of VDAC1 on immunoblots (36) and after analysis by mass spectrometry (50), we concluded that it was a form truncated in the C-terminal region: VDAC1-ΔC. After reconstitution into a planar lipid bilayer system, VDAC1-ΔC presented a similar but not identical channel activity and voltage dependency as VDAC1. At a higher voltage of −40 mV, the full-length channel showed two major conducting states with higher occupancy at the closed substate, whereas VDAC1-ΔC showed a higher open-state occupancy in comparison to the occupancy of low-conducting substates. Moreover, at the high voltages, VDAC1-ΔC showed a slightly higher conductance than VDAC1 (36). Indeed, we observed an increase in ATP levels in cells in hypoxia when VDAC1-ΔC and enlarged mitochondria were present. We also demonstrated that VDAC1-ΔC associated with the same partners as VDAC1, i.e., HKI/II and Bcl-X_L. Anchoring of HKI/II is probably involved in the exacerbated metabolism of tumor cells in hypoxia in the presence of VDAC1-ΔC. Both the association of VDAC1-ΔC with HK and the increase in the expression of HKI/II in hypoxia increased OXPHOS and glycolysis. Thus, VDAC1-ΔC seems to control cell survival in hypoxia by regulating the export of ATP

and probably NADH and brings advantage to cancer cells in promoting survival *via* mitochondria that are probably not as dormant as previously described. We do not know yet whether the conformation and the oligomerization of VDAC1-ΔC are different to that of VDAC1.

The VDAC1–HK complex has been reported to also play an important role in apoptosis (14). Thus, the VDAC1–HK complex already represents a target for cancer therapy, using, for example, specific VDAC1-based peptides that disrupt the connections between these proteins (51). HK provides an apoptosis-suppressive capacity by interfering with the ability of Bax to bind to mitochondria and induce apoptosis (52, 53). In addition, Bcl-X_L, which interacts with VDAC1-ΔC (36), was found to protect cells from apoptosis via a block in the Bax–Bak interaction, subsequently preventing cytochrome *c* release (54, 55). Currently, cytochrome *c* is considered to play an important role in the resistance to cell death observed in cells with enlarged mitochondria and VDAC1-ΔC. We have previously shown that resistance to chemotherapy was linked to the phenotype of enlarged mitochondria. Subsequently, we demonstrated that silencing of VDAC1/VDAC1-ΔC in hypoxia or re-exposure of cells to normoxia, which inhibited the formation of VDAC1-ΔC, restored the sensitivity of the cells to apoptosis. We also showed that the mitochondrial transmembrane potential was unchanged in hypoxia and that cytochrome *c* was not released in the presence of staurosporine-induced apoptosis, whereas the transmembrane potential was decreased in normoxic cells under these conditions. We showed that cytochrome *c* was trapped inside the mitochondrial intermembrane space due to a change in the mitochondrial conformation and a hypothetical modification to VDAC oligomerization. These interactions occur specifically in hypoxia, when cancer cells are known to be highly resistant to anti-cancer treatments, and may, therefore, be exploited for therapy in the future.

Finally, we investigated further the mechanism behind the hypoxic regulation of the truncated form of VDAC1. We found that the cleavage of VDAC1 was dependent on TP53 or p73 as only cells expressing p53 (LS174, A549, or HepG2 cells) or p73 (HeLa cells) contained the hypoxic VDAC1-ΔC. Moreover, silencing of *TP53* and/or *HIF-1α* diminished VDAC1 truncation and, thus, cell survival in the presence of staurosporine. Mieap, a TP53-inducible protein that controls mitochondrial quality was also involved, as silencing of *MIEAP* diminished also the hypoxic VDAC1-ΔC. We found that bafilomycin A1 and chloroquine, two compounds that increase the lysosomal pH, inhibited the cleavage of VDAC1 to VDAC1-ΔC, suggesting implication of lysosomes. This was confirmed by electron microscopy, which showed microfusion between mitochondria and endolysosomes and by cleavage of VDAC1 by an endolysosomal asparagine endopeptidase (AEP). Analysis by mass spectrometry of VDAC1-ΔC showed cleavage at asparagine 214 and glycine 213. Of note, AEP is also regulated by TP53 (56). Moreover, we reported that BNIP3, already known to be involved in regulating mitochondria morphology in hypoxia (36), acted as a docking site for lysosomes together with clathrin, a protein involved in multiple membrane vesicle trafficking pathways (57). This mechanism was identified not only *in vitro* but also *in vivo* in patients with lung cancer. We propose that this novel mechanism is a readout of mitochondrial–endolysosomal microfusion in hypoxia, *in vitro* and most importantly *in vivo*, and represents an additional defense mechanism that cancer cells have developed to resist chemotherapy.

TARGETING VDAC1 FOR THERAPY

Studies into cellular metabolism have lead to the characterization of a number of drugs that have already showed promise in pre-clinical and clinical trials. However, the quest for new therapeutic targets is hampered by the fact that cells show a great degree of plasticity, which already augurs the challenges we face. Studying metabolism *per se* is important and will allow us to identify new targets. However, metabolism should be studied in the context of a changing microenvironment (hypoxia, pH, changes in concentrations of metabolites, etc.) and in the context of malignant transformation. Thus, VDAC1-ΔC appears to be an interesting therapeutic target. Various compounds have already been identified for their capability to directly interact with and modify the activity of VDAC. Avicins (closes VDAC) (58), acrolein (used in Alzheimer's disease to carbonylate VDAC) (59), erastine (binds to VDAC2) (60), endostatin (inducing PTP opening) (61), fluoxetine and cisplatin (inhibition of PTP opening and apoptosis) (62, 63), furanonaphthoquinones (induces VDAC-dependent apoptosis) (64), and oblimersen (blocks channel activity) (65, 66) are chemicals that will be tested under hypoxic conditions in the near future. In addition, VDAC-based peptides, novel pro-apoptotic agents that specifically target domains for interaction with HK, Bcl-2, and Bcl-X_L, could be an interesting alternative to chemicals to restore the sensitivity to apoptosis in hypoxia (67).

We hope that, in the near future, this hypoxia/VDAC1-ΔC duo will meet the expectations that we have discussed in this review.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Hypoxia and Hypoxia-Inducible Factors in Leukemias

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Despite huge improvements in the treatment of leukemia, the percentage of patients suffering relapse still remains significant. Relapse most often results from a small number of leukemic stem cells (LSCs) within the bone marrow, which are able to self-renew, and therefore reestablish the full tumor. The marrow microenvironment contributes considerably in supporting the protection and development of leukemic cells. LSCs share specific niches with normal hematopoietic stem cells with the niche itself being composed of a variety of cell types, including mesenchymal stem/stromal cells, bone cells, immune cells, neuronal cells, and vascular cells. A hallmark of the hematopoietic niche is low oxygen partial pressure, indeed this hypoxia is necessary for the long-term maintenance of hematopoietic stem/progenitor cells. Hypoxia is a strong signal, principally maintained by members of the hypoxia-inducible factor (HIF) family. In solid tumors, it has been well established that hypoxia triggers intrinsic metabolic changes and microenvironmental modifications, such as the stimulation of angiogenesis, through activation of HIFs. As leukemia is not considered a "solid" tumor, the role of oxygen in the disease was presumed to be inconsequential and remained long overlooked. This view has now been revised since hypoxia has been shown to influence leukemic cell proliferation, differentiation, and resistance to chemotherapy. However, the role of HIF proteins remains controversial with HIFs being considered as either oncogenes or tumor suppressor genes, depending on the study and model. The purpose of this review is to highlight our knowledge of hypoxia and HIFs in leukemic development and therapeutic resistance and to discuss the recent hypoxia-based strategies proposed to eradicate leukemias.

Keywords: hypoxia, hypoxia-inducible factors, leukemia, microenvironment, cancer

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LEUKEMIAS AND HYPOXIA

Leukemia is characterized by uncontrolled proliferation of hematopoietic cells within bone marrow (BM). Lymphoid leukemias can be distinguished from myeloid according to the abnormal cell lineage, and acute from chronic leukemias according to the maturity of the blood cells involved and progression rate. Acute leukemias are characterized by rapid proliferation of immature hematopoietic cells, termed blasts, which fail to differentiate into mature cells. Their accumulation in BM also prevents growth and differentiation of normal hematopoietic cells. The clinical evolution is fast (1). In contrast, in chronic leukemia, the growth advantage of neoplastic cells leads to the generation of a more mature cell population that outcompetes normal hematopoiesis, and clinical evolution is longer (several months to years) (2). To date, although the majority of pediatric acute lymphoid

leukemia (ALL) and chronic myeloid leukemia (CML) cases (3) are cured or well controlled under treatment, chronic lymphoid leukemia (CLL) and, even more, acute myeloid leukemia (AML) have a high risk of relapse, despite therapeutic progressions (4). While treatments often target cycling cells, the idea that a small population of quiescent leukemic cells survive and trigger relapse regardless of treatment has emerged (5). In the early 90s, the team of John E. Dick established a hierarchy in leukemic cell populations which, by analogy with that of normal hematopoietic cells, led to the introduction of the concept of cancer stem cells for all cancers [reviewied in Ref. (5, 6)]. Their work identified a subpopulation of leukemic cells able to initiate leukemic growth after transplantation into immune-deficient mice. These stem-like cells, named leukemia-initiating cells (LICs) or leukemic stem cells (LSCs) (7, 8), arise from hematopoietic stem/progenitor cells (HSCs) that reside in the most hypoxic tissue areas within the normal HSC niche (9, 10). The oxygen partial pressure (ppO_2) in tissues is much lower compared to that in the atmosphere (160 mmHg corresponding to around 21% oxygen). In particular, in BM an oxygen gradient exists ranging from <6% oxygen, close to the vessels, to anoxia in the most distant regions from blood vessels (11–15). However, O_2 level differs according to the nature of the hematopoietic niche; the sinusoidal niche is around 10 mmHg (equivalent to 1.3% O_2) (16).

In solid cancers, it is well established that uncontrolled proliferation leads to profound hypoxia, associated with tumor development, metabolic changes, metastatic propagation, immune response modulation, and increased mortality (17, 18). Consequently, it could be assumed that intense blast cell proliferation would eventually decrease the oxygen availability by high consumption. This assumption is particularly difficult to validate by direct measurement in human BM. However, Fiegl et al. (19) demonstrated in total BM aspirates from AML patients that oxygen percentage was highly comparable to the normal counterpart. Using a rat model of an acute AML subtype, the promyelocytic leukemia, Jensen et al. (20) noted an increasing level of hypoxia during disease progression, comparable to that observed with solid tumours. In this model, both normal and leukemic cells stained with 2-nitroimidazole (hypoxic marker) underwent decreased proliferation. In agreement with this observation, the hypoxic culture of normal hematopoietic (21–23) and CML cells (24–27) led to decreased proliferation. Nevertheless, a small fraction of leukemic cells remained insensitive to hypoxia-induced proliferation arrest (26), probably triggering tumor growth (20). Moreover, it has been established that mild hypoxia such as $\lesssim$3% O_2 sustains both primary CML (24) and AML cell (28) maintenance longer than normoxia.

Low oxygen might also affect hematopoietic cells through the modulation of the stromal cells. Indeed, hypoxia has been shown to impact on survival, proliferation capability, and differentiation as well as metabolism of mesenchymal stem/stromal cells (MSCs) (29–31). Hypoxia triggers secretion by MSCs of numerous factors, including SDF-1, VEGF, and IL-6, known to promote HSC maintenance. Interestingly, even in normoxia, HSCs present a hypoxic profile when seeded on MSCs, suggesting appearance of "microhypoxic" regions (32, 33). Moreover, MSCs and hypoxic culture synergize to sustain *in vitro* normal stem cells (23) and primary AML cells (28). Finally, the poorly oxygenized niche enhances resistance to treatments (28, 34, 35), thus protecting from various stresses, such as DNA damage, cell death stimuli, or oxidative stress signals (36–38).

HYPOXIA-INDUCIBLE FACTORS IN LEUKEMIAS

The master regulators mediating cell responses to hypoxia are the hypoxia-inducible factors (HIFs). These heterodimer complexes are composed of one of three oxygen-regulated HIF-alpha subunits (HIF-1alpha, HIF-2alpha, and HIF-3alpha) and the constitutively expressed HIF-beta subunit [HIF-1-beta, also known as aryl hydrocarbon receptor nuclear translocator (ARNT)] (39–41). The *HIF1A* gene is ubiquitously expressed (42). *HIF2A*, also termed endothelial Per-ARNT-Sim (PAS) protein 1 (*EPAS1*), is expressed in a more tissue specific manner, particularly in blood vessels (39, 40, 43). Little is currently known about expression and function of *HIF3A*, but at least 10 splice variants have been described to date (44, 45). HIF-1alpha and HIF-2alpha proteins share similar structural domains such as an N-terminal basic helix-loop-helix (bHLH) domain involved in DNA binding, the two PAS domains allowing dimerization, an oxygen-dependent degradation domain (ODDD) plus N- and C-terminal transactivation domains (NTAD and CTAD). Although HIF-3alpha also exhibits high similarity in bHLH and PAS domains, the lack of the CTAD precludes binding to p300 coactivator (45, 46). Under atmospheric conditions, HIF-alpha subunits are differentially hydroxylated by prolyl hydroxylase domain 1–3 (PHDs) on two proline residues in the ODDD, with oxygen and α-ketoglutarate as substrates. The hydroxylated motif allows binding to von Hippel–Lindau (VHL) tumor suppressor, which leads to HIF-alpha ubiquitination and consequent degradation by 26S proteasome (39–41, 47). In parallel, the hydroxylation of HIF-1alpha by factor inhibiting HIF-1 (FIH1) triggers inhibition of p300/CBP coactivator recruitment (48). PHD activity falls with decreasing oxygen levels, thereby triggering HIF-alpha stabilization and nuclear translocation where it heterodimerizes with HIF-1beta. HIF complexes bind to specific HIF-response elements consisting of specific RCGTG sequences within target gene promoters. Although HIF-1 and HIF-2 share common targets, additional genomic regions and cofactor-binding specifically drive the transcriptional initiation of genes involved in many pathways, such as angiogenesis, differentiation, stem cells maintenance, apoptosis, and invasion (35, 39, 40).

HIF-1alpha mainly participates in the initial response to acute hypoxia, whereas HIF-2alpha responds to chronic exposure (47, 49). Additionally, even in prolonged hypoxia HIF-1alpha undergoes feedback control, whereas HIF-2alpha is stabilized. The multiple HIF-3alpha splice variants appear essentially to regulate HIF-1alpha and HIF-2alpha activity by sequestrating HIF-1beta or by acting as dominant negative regulators (40, 44, 45, 50). HIFs, moreover, can be regulated by oxygen-independent mechanisms. Factors involved in hematopoiesis such as MEIS1 (51), TPO (52), and SCF (53, 54) positively regulate expression of HIFs. Conversely, factors implicated in metabolic changes like

the SIRT1 (55, 56) or SDH (57) inhibit the expression of HIFs. Furthermore, downregulation of HIFs may be achieved by tumor suppressor genes like p53 (58, 59) or GSK3 (60) and the upregulation by oncogenes such as PI3K/AKT (60, 61) or mTORC1 (62). Genetic abnormalities encountered in leukemia such as the *IDH* mutation decrease expression of HIFs by stimulating PHD activity (63) or conversely, *FLT3-ITD* stimulates translation of HIFs *via* the PI3K/AKT pathway (40).

Elevated expression of HIFs is considered to be a marker of poor prognosis in solid cancers (64–77). Overall, increased expression of HIFs is correlated with tumor growth and resistance to therapies, which leads to disease relapse (37). The subject is somewhat more complex and controversial in leukemia. Overexpression of HIF-1alpha in leukemia has been suggested as a marker of poor prognosis and chemotherapy outcomes (78–81). Elevated levels of HIF-1alpha are reported, in the majority of studies, in AML (79, 82–84), acute promyelocytic leukemia (APL) (85), ALL (82), and CML (86, 87). Disease severity and survival have been shown to be influenced by HIF-1alpha levels, in AML and myelodysplastic syndromes (84, 88, 89); the protein expression of HIF-2alpha, usually absent in normal cells, has been observed in both AML and ALL, but has not been correlated with outcome (82, 90, 91). Thus, leukemic subtype, disease stage or the molecular abnormality involved might explain the variability. To explore potential functions of HIFs in leukemogenesis, various mouse models have been proposed (**Table 1**). Several studies have shown that inhibition of HIF-1alpha, either by RNAi targeting or by small molecular inhibition, resulted in a failure of primary cells to form *in vitro* colonies and decreased tumor growth and leukemic progression. *In vivo*, dramatic decrease and potential eradication of primary AML cell xenografts have been shown and a complete absence of leukemic induction in secondary transplantation has been reported in cells in which HIF-1alpha was inhibited (79, 84, 85, 92). This has also been confirmed in ALL (93, 94) and CML (83, 87). Similarly, knockdown of HIF-2alpha with shRNA triggers leukemic inhibition (82, 85, 90), which is demonstrated *in vitro* by limited cellular proliferation as well as *in vivo* by absence or poor engraftment.

Hypoxia *via* HIFs may promote disease maintenance and progression through different mechanisms including energy metabolism (98–100), cycle and quiescence (101, 102), and immune function (103) that are important in normal physiology and deregulated in cancer (47, 104). On one hand, HIF-1alpha and HIF-2alpha influence signaling pathways relevant to leukemia maintenance and propagation. HIF-1alpha activates the Notch1 pathway, which leads to leukemia invasion (94), and promotes the Wnt pathway, consequently preserving LSCs (93). On the other hand, HIF-1alpha acts as an inhibitor of the expression of tumor suppressor genes, such as p15, p16, p19, and p53 (79, 87). Indeed, HIF-1alpha-transactivated DNMT3a methylates DNA, which inhibits tumor suppressors and leads to tumor growth (79). In AML, DNMT3a plays a crucial role since more than 20% of patients exhibit *DNMT3A* mutation (105), conferring a global hypomethylation of DNA and predisposition to developing hematological diseases (106). In contrast, in T-ALL this mutation confers hypermethylation, so the contribution of hypomethylation and hypermethylation to disease development remains to be elucidated (106). Interestingly, taken from non-hematopoietic tissue and cancers, studies have explored the role of hypoxia in epigenetic modifications, through HIF-1alpha stabilization, such as DNA methylation, histone modifications, and non-coding RNA expression (107). Promoter methylation is modified by hypoxia and regulates neural progenitor cell fate (108). The histone demethylase JMJD1A and 1B are targets of HIFs in normal and cancer cells (109–111). Finally in HIF-2alpha-deficient cells, transcriptomic approaches have identified deregulated genes involved in energetic and oxidative metabolisms, plus endoplasmic reticulum (ER) stress, indicating that HIF-2alpha protects AML cells from apoptosis induced by ER stress (90).

One consequences of the expression of HIFs is the promotion of quiescence, which favors chemoresistance. Hypoxia-induced HIF-1alpha promotes entry into G_0/G_1 and decreases S phase in AML cells through, in part, upregulation of p27 (112). Quiescence enhances chemoresistance of leukemic cells to cytosine arabinoside (Ara-C) (112, 113) and adriamycin (ADR) (88), since these agents target cycling cells. Coculture of primary AML cells with stromal cells in hypoxia (3% O_2) confers resistance to Ara-C through stabilization of HIFs and induction of quiescence (28). Antiapoptotic signaling is observed through increased XIAP level, an apoptosis-inhibitory protein, and the activation of the PI3K/AKT pro-survival pathway (112). HIF-1alpha activation by a PHD inhibitor, cobalt chloride ($CoCl_2$), protects HL-60 leukemic cells against arsenic trioxide (ATO) by inhibiting BAX and Caspase 3 and 9 and promoting HSP70 protein and p38/ERK pro-survival factors (114). In T-ALL, through Notch1 activation, HIF-1alpha induces BCL2 and BCL-XL upregulation and the downregulation of Caspase 3 and 9 activities, which decreased dexamethasone-induced apoptosis in leukemic cells (94).

Conversely, low oxygen and hypoxia-mimicking agents such as $CoCl_2$ or desferrioxamine induce AML cell differentiation through HIF-1alpha accumulation (115, 116). In fact, HIF-1alpha mediates differentiation by binding to C/EBPα and promoting its transcriptional activity (115–117) as well as that of RUNX1 and PU.1 (118, 119). Additionally, C/EBPα/HIF-1alpha induces AML differentiation through c-MYC inhibition and further suppression of miR17 and miR20a expression. The knockdown of p21 and STAT3, two inhibitory targets of miR17 and miR20A, reverses HIF-1alpha-induced AML differentiation (120). In renal cell carcinoma, HIF-1alpha inhibits c-MYC/MAX association, which decreases *c-MYC* promoter binding and thus blocks cells in G_1 (121). Conversely, HIF-2alpha triggers cell cycle progression and proliferation by enhancing the formation of c-MYC/MAX and its activity. Since HIF-2alpha and HIF-1alpha have dual effects on cell cycle progression according to cell types, more investigations are needed on their antagonistic effects in leukemias. Nguyen-Khac et al. (122) discovered a translocation involving TEL and ARNT in an AML patient exerting a dominant/negative activity on HIF-1alpha. The fusion protein blocks leukemic differentiation, thus conferring a tumor suppressor function for HIF-1alpha. In line with this, data have previously shown that intermittent hypoxia slows down leukemic development in mice (123). However, *in vivo* hypoxia may have unrelated consequences on leukemic cell physiology. More recently, Velasco-Hernandez et al. (124) using different AML models found reduced survival of mice

TABLE 1 | Models used to characterize *HIF1A* and *HIF2A* as oncogenes in leukemias.

Leukemia	Cell types	Mouse models	HIFs inhibition or overexpression	Phenotypes	Reference
CML	**Human cells**				
	K562	Nude (in subcutaneous)	Human *HIF1A* cDNA + L-ascorbic acid[a]	No decreased tumor growth	(83)
	Mouse cells				
	Vav-Cre-HIF1a$^{flox/flox}$ transduced with BCR–ABL retrovirus	C57BL/6 J	*Vav-Cre-Hif1a*$^{flox/flox}$ system	No leukemia induction in second transplantation Decreased LSCs number	(87)
AML	**Human cells**				
	Primary	NOD-SCID	Echinomycin[b]	Decreased/eliminated leukemia cells No leukemia induction in serial transplantation	(84)
	Primary	NOD-SCID gamma	sh*HIF2A*	Decreased/no engraftment	(90)
	HL-60	NOD-SCID gamma	sh*HIF2A*	Increased mice survival	(82)
	NB4	NOD-SCID gamma	sh*HIF1A*, sh*HIF2A*, or EZN-2968[c]	Increased mice survival Delayed leukemia progression	(85)
	SKNO-1	Athymic nude	Echinomycin[b]	Decreased tumor growth	(79)
	HL-60	Nude (in subcutaneous)	L-ascorbic acid[a]	Decreased tumor growth	(83)
	Mouse cells				
	PML-RARα Lin$^-$	129Sv	*Ex vivo* electroporation with EZN-2968[c]	Increased mice survival	(85)
	PML-RARα Lin$^-$	129Sv	EZN-2208[c] ATRA[d] + EZN-2208[c]	Increased mice survival Synergy of both treatments to leukemia eradication No leukemia induction in serial transplantation	(95)
	FDCP1	DBA/2	Human *HIF2A* cDNA	Accelerated leukemia progression	(82)
	Relapsed *Mll*$^{PTD/WT}$:*Flt3*$^{ITD/WT}$	C57BL/6 Ly5.1	Echinomycin[b]	Decreased AML blasts (≤20%) Increased mice survival	(92)
	A/E9a transgenic mouse cells	C57BL/6 J	Human *HIF1A* cDNA si*HIF1A*	Enhanced leukemia disease Suppressed leukemia disease	(79)
ALL	**Human cells**				
	Jurkat Sup-T1	Transwell matrigel-coated chambers	Hypoxia (2% O_2)	Increased tumor invasion	(94)
	Primary T-ALL	NOD-SCID gamma	sh*HIF1A*	Increased mice survival Decreased LSCs frequency	(93)
	Mouse cells				
	Primary (NOTCH1-ΔE/NGFR retrovirus)	NOD-SCID gamma	*Hif1a*$^{loxP/loxP}$/CreERT2/GFP system	Increased mice survival	(93)

[a]*L-ascorbic acid indirectly inhibits HIF-1alpha expression by inhibiting NF-κB translocation into the nucleus (83).*
[b]*Echinomycin binds the core-binding site of HIF-1alpha and inhibits its DNA-binding activity (96).*
[c]*EZN-2968 and EZN-2208 specifically target HIF-1alpha (85, 95, 97).*
[d]*ATRA target RARα moiety in PML-RARα mutation of APL (95).*

transplanted with HIF-1alpha KO cells (**Table 2**). These observations were confirmed in myeloproliferative neoplasia through a FLT3^{ITD}-induced mouse model (125). Overall, *Hifa* KO enhanced disease progression and severity, making it a tumor suppressor gene. However, authors show that HIF-1alpha deletion may promote compensatory effects *via* overexpression of HIF-2alpha, which may eventually mask the role of HIF-1alpha. This elevation was already seen in HIF-1alpha-deficient cells (90). The *Hif2a* KO in MLL-AF9-driven and Meis1/HoxA9-induced murine AML enhances LSCs development but, once leukemias are established, HIF-2alpha has no impact on their maintenance and propagation. Furthermore, double inhibition of HIF-1alpha and HIF-2alpha demonstrated that HIFs synergize to inhibit AML development, without any role in leukemic propagation (126). Transcriptomic analysis reveals that HIF-1alpha and HIF-2alpha promote a set of genes that fosters survival and proliferation of leukemic cells.

Off-target effects of shRNAs and poor specificity of drugs that inhibit HIFs compared to KO might explain this controversy between studies. However, KO may also induce slow cellular adaptation with compensatory effects. Differences may also arise from the model used, mouse versus human, and the different protumoral gene constructions used to generate the leukemia. It will be pertinent to assess the overexpression of HIFs in AML models, and thus observe whether increased HIF delays disease

TABLE 2 | Models used to characterize *HIF1A* and *HIF2A* as tumor suppressor genes in leukemias.

Leukemia	Cell types	Mouse models	HIFs inhibition or overexpression	Phenotypes	Reference
CML	**Human**				
	K562	–	Mixture with 10% O_2 for 5 or 22 + 1 or 24 h of reoxygenation	Survival signals with 5 h hypoxia Death signals with 22 h hypoxia	(127)
AML	**Human**				
	MLL-AF9 THP-1 transduced with *HIF2A* sgRNAs + Cas9 nuclease	–	Two independent sgRNAs against the exon 12 of *HIF2A*	Same leukemic propagation than control	(126)
	Mouse				
	hMRP8-PML-RARα	FVB-NICO	Intermittent hypoxia equivalent to an altitude of 6000 m (≈10% O_2) for 18 h/day	Increased mice survival Decreased tumor infiltration/invasion	
	Hif1a$^{fl/fl}$; *Mx1-Cre* transduced with *A/E9a* retrovirus	B6SJL	*Hif1a*$^{fl/fl}$/CreERT2 system	HIF-1alpha does not affect AML initiation/progression Decreased mice survival in second transplantation	(123)
	Hif1a$^{fl/fl}$; *Mx1-Cre* transduced with *HoxA9-Meis1* retrovirus *Hif1a*$^{fl/fl}$; *Mx1-Cre* transduced with *MLL-AF9* retrovirus *Hif2a*$^{fl/fl}$; *Vav-iCre* transduced with *Meis1* and *HoxA9* retroviruses	C57BL/6 Ly5.1/ Ly5.2	*Hif2a*$^{fl/fl}$; *Vav-iCre* system	HIF-1alpha does not affect AML initiation/progression HIF-1alpha does not affect AML initiation/progression Decreased mice survival Same AML aggressiveness to control mice	(124)
	MLL-AF9$^{KI/+}$; *Hif2a*$^{fl/fl}$; *Vav-iCre* *Hif1a*$^{fl/fl}$; *Hif2a*$^{fl/fl}$; *Vav-iCre* transduced with *Meis1* and *HoxA9* retrovirus	C57BL/6 Ly5.1/ Ly5.2	*Hif1a*$^{fl/fl}$; *Hif2a*$^{fl/fl}$; *Vav-iCre* system	Accelerated leukemia disease Decreased mice survival	(126)

development and, even, eradicate leukemia. HIFs may also differently impact on LSCs and more mature blasts cells, conferring pro-survival effect on LSCs and differentiation on blasts. In favor of this hypothesis, intermittent hypoxia increases survival of APL mice (123). Similarly, in CML, HIF-1alpha induction following short (5 h) hypoxia exposure delivered a survival signal to cells, whereas it promoted cell death within a longer period (22 h) (127). In the ALL model, 24 h-exposure to hypoxia conferred chemoresistance in contrast to longer exposure (48–72 h) (128). These data suggest that duration of hypoxia incubation may promote or inhibit leukemia progression and maintenance, thus explaining the oncogenic or tumor suppressor activity. The link between HIFs and tumor suppressor activity has been previously demonstrated in other cancers (71, 129–131). Taken together, these data suggest that not only the level but also the duration of activity dictates HIF action and hence cellular response in leukemia.

THERAPEUTIC STRATEGIES: TARGETING HIFs OR NOT?

In light of the results summarized above, it could be difficult to affirm that downregulation of HIFs could be a therapeutic approach. Nevertheless, chemical inhibitors have been tested and the proof-of-concept was first illustrated using echinomycin, which blocks HIF-1alpha-binding activity. This drug preferentially targets AML LSCs through apoptosis, decreasing leukemia burden, prolonging mouse survival, and abrogating disease development in secondary transplantation (84, 92). Echinomycin does not impact on self-renewal and differentiation of normal HSCs, which makes it an ideal molecule to treat leukemia (92). L-ascorbic acid has also been shown to inhibit expression of HIF-1alpha in CML cells and consequently reduces tumor growth. This effect is specific to HIF-1alpha since its overexpression in L-ascorbic acid-treated mice antagonizes leukemic growth inhibition (83). In APL, EZN-2968 and EZN-2208 confer antileukemic activity and prolong mouse survival; in combination with all-trans retinoic acid (ATRA), leukemia eradication was observed, along with survival of mice; fortunately, both compounds are non-toxic to normal HSCs (85, 95). Overall, these data offer new therapeutic options, targeting HIF in leukemia with no impact on normal hematopoiesis. Another approach will be the combination of HIF inhibitors with treatments capable of determining the departure of LSCs from quiescence, and then with treatments that target cycling cells, such as Ara-C.

An alternative strategy will consist of taking advantage of hypoxia to activate drugs and thus to target LSCs in the niche. TH-302 is a hypoxia-activated prodrug, which exhibits a specific cytotoxicity in hypoxia (132, 133). In primary AML, TH-302 hampers tumor growth through multiple mechanisms (cycle arrest, DNA cross-linking, DNA damage). In mouse models, it decreases leukemia burden and prolongs survival (133). PR-104 quickly undergoes alcohol hydrolysis and induces DNA cross-linking in hypoxic cells, impairs ALL progression, decreases tissue infiltration, and prolongs mice survival (134, 135). In a phase I/II study, PR-104 reduced the number of AML and ALL cells in refractory and relapsed

patients (136). Despite some side effects, including myelosuppression, febrile neutropenia, and infections, collectively, these results propose innovative therapies for leukemia based on hypoxia-activated prodrugs.

CONCLUSION

Overall, these data argue that hypoxia and HIF-mediated signaling play a crucial role in leukemia and leukemogenic processes. However, they conflict in determining whether HIFs act as oncogenes or tumor suppressors, certainly because of the different leukemic models, study design, oxygen level, and hypoxia duration. However, therapies targeting hypoxia and HIFs have proven their efficacy in treating mouse models and may benefit leukemic patients.

AUTHOR CONTRIBUTIONS

All authors designed, wrote, and revised the manuscript.

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REVIEW
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Chronic Myeloid Leukemia and Hepatoblastoma: Two Cancer Models to Link Metabolism to Stem Cells

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Low oxygen tension is a critical aspect of the stem cell niche where stem cells are long-term maintained. In "physiologically hypoxic" stem cell niches, low oxygen tension restrains the clonal expansion of stem cells without blocking their cycling, thereby contributing substantially to favor their self-renewal. The capacity of stem cells, hematopoietic stem cells in particular, to reside in low oxygen is likely due to their specific metabolic profile. A strong drive to the characterization of this profile emerges from the notion that cancer stem cells (CSC), like normal stem cells, most likely rely on metabolic cues for the balance between self-renewal/maintenance and clonal expansion/differentiation. Accordingly, CSC homing to low oxygen stem cell niches is the best candidate mechanism to sustain the so-called minimal residual disease. Thus, the metabolic profile of CSC impacts long-term cancer response to therapy. On that basis, strategies to target CSC are intensely sought as a means to eradicate neoplastic diseases. Our "metabolic" approach to this challenge was based on two different experimental models: (A) the Yoshida's ascites hepatoma AH130 cells, a highly homogeneous cancer cell population expressing stem cell features, used to identify, in CSC adapted to oxygen and/or nutrient shortage, metabolic features of potential therapeutic interest; (B) chronic myeloid leukemia, used to evaluate the impact of oxygen and/or nutrient shortage on the expression of an oncogenetic protein, the loss of which determines the refractoriness of CSC to oncogene-targeting therapies.

Keywords: cancer stem cells, microenvironment, metabolism, hypoxia, glucose shortage, chronic myeloid leukemia

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INTRODUCTION

Low oxygen tension is a critical aspect of the environment where stem cells reside. On the basis of *in vitro* data, we were the first to put forward, in 1993, the hypothesis that hematopoietic stem cell (HSC) niches, where HSC are physiologically hosted *in vivo*, are bone marrow areas maintained at relatively low oxygen tension (1). Our results, limited to short-term repopulating HSC, were later confirmed by others and extended to comprise long-term repopulating HSC (2). The capacity of HSC, but not of hematopoietic progenitor cells (HPC), to home in low oxygen tissue areas is crucial to ensure HSC self-renewal and long-term maintenance. This capacity is likely due to the specific metabolic profile of HSC. We also found that low oxygen does not inhibit HSC cycling but limits

cycling to support HSC self-renewal (3). Thus, the environment of "physiologically hypoxic" niches (4) contributes substantially to maintain stem cell potential. A number of excellent reviews addressed the relationship of niche environment to oxygen tension and blood supply in bone marrow. Moreover, the overall architecture of niche, which includes stromal cells, extracellular matrix, and soluble or matrix-bound cytokines has also been extensively described (5, 6). The physiological role of relatively low oxygen tensions in the regulation of stem cell compartments was further supported by later studies on pluripotent embryonal stem cells. Indeed, in the developing embryo, the inner cell mass of blastocyst is a relatively "hypoxic" ($pO_2 < 2\%$) structure (7), where embryonal stem cells largely rely on glycolytic ATP generation independently of oxygen shortage (8), i.e., exploiting the "aerobic" glycolysis or Warburg effect (9).

Cancer cell populations are usually characterized by a marked phenotypical heterogeneity to include differentiating cells as well as cancer stem cells (CSC). CSC seem to rely on environmental cues similar to those characterizing physiological stem cell niches for either their survival or the regulation of balance between self-renewal/maintenance and clonal expansion/differentiation. CSC continuously interact with non-neoplastic components of the niche, which decisively contribute to the maintenance of CSC *via* their protection from insults of different nature coming from outside the niche. These insults range from the physiological pressure of cytokines boosting clonal expansion at the expense of stem cell maintenance to the action of therapeutic agents (10, 11). The location itself of the niche may represent a powerful protection factor. Indeed, being far away from blood vessels implies the exposure to significantly lower concentrations of systemically administered drugs than in the rest of tissue. Furthermore, although CSC are allowed to cycle in the niche, their slow cycling or even quiescence provides an obvious protection from the effects of chemotherapeutic agents designed to suppress proliferating cells (12). On the other hand, CSC hosting in niches at extremely low oxygen tension has the straightforward consequence that CSC are protected from the effects of reactive oxygen radicals (ROS), which are instead typically generated in better oxygenated tissue areas. This represents a serious problem in relation to the effectiveness of radiotherapy on CSC, which largely relies on ROS generation to induce DNA damage in tumor cells (13).

Moreover, it is worth noting here that CSC have intrinsic properties that make them resistant to treatments. These properties, including the expression of aldehyde dehydrogenase and of the two ABC transporters MDR1 and ABCG2, which drive drug efflux from cells, have been excellently reviewed elsewhere (14). The association between multidrug resistance (MDR) and stem cell markers in human chronic myeloid leukemia (CML) cell lines has been clearly shown (15). The combination of CSC-intrinsic properties with the CSC-sheltering effects of stem cell niche makes it impossible to obtain a homogeneous therapeutic efficacy on the whole cancer tissue. In this scenario, CSC homing within niches is the best candidate mechanism to sustain minimal residual disease (MRD). Such a clinical state may determine late relapse of disease even in patients who brilliantly responded to therapy undergoing to complete remission. Low oxygen turns out to represent "the," or at least "one of the," most important factors ensuring MRD persistence (16). This implies that CSC, like normal stem cells, are capable to metabolically adapt to low oxygen. More in general, CSC are capable to adopt a nutrient uptake pattern and energy metabolism profile that favors their homing into niches (i.e., an adequate interaction between the oxygen-sensing and the nutrient-sensing signaling pathways). To conclude, one can affirm that the metabolic profile of CSC heavily impacts cancer response to therapy.

Modern oncology is testing selective CSC targeting as a means to eradicate neoplastic disease (17), aiming at their cure rather than care. The latter outcome is, instead, typical of actions directed to suppress the proliferating bulk of cancer population. Our "metabolic" approach to this challenge was based on two different experimental models. We used AH130 ascites hepatoma cells (18) to try to identify some step of metabolic pathways, which is particularly vulnerable in CSC adapted to low oxygen. On the other hand, we choose CML as a model disease to evaluate the therapeutic targeting of CSC in cell populations where low oxygen determines the suppression of an oncogenetic protein and the consequent loss of oncogene addiction.

AH130 CELLS AS A PROTOTYPE OF CANCER STEM CELLS

The metabolic studies we summarize here were necessarily preceded by a reappraisal of the nature of the AH130 cell population. This hepatoma can be maintained indefinitely *via* serial transplantations in the rat peritoneal cavity, where huge amounts of immature cells are produced each time. AH130 cells express fundamental embryonic transcription factors (ETF), such as Nanog, Klf4, and c-Myc. When peritoneal cavity is cell-saturated and cell growth is arrested, glucose concentration as well as oxygen tension approach 0. In spite of that all hepatoma cells are alive, 75% of which expressing Nanog and more than 90% the stem cell marker CD133 (19). Thus, the AH130 hepatoma is an inexhaustible source of stem cells, which provides a decisive advantage in view of the characterization of their metabolic profile.

Well in keeping with the scenario summarized in the Introduction, AH130 cells are, like other stem cells, adapted to low oxygen (20). The metabolic profile of AH130 cells is indeed centered on an extremely high capacity of converting pyruvate into lactate, so that cells, when exposed *ex vivo* to high glucose concentrations (upto 15 mM) in air, convert up to 80% of the available glucose to lactate, exhibiting an excellent example of Warburg metabolism. This enormous energy waste implies that the elimination of pyruvate produced by aerobic glycolysis is a primary exigency, due to a detrimental effect of pyruvate accumulation on G_1–S transition. A key finding to understand the mechanism of this effect was that the addition of exogenous pyruvate to AH130 cells (incubated in air in the presence of 15-mM glucose) faithfully mimicked that of antimycin A, which blocks electron transport chain (ETC) inhibiting its complex III. On the contrary, 2,4-dinitrophenol, which uncouples (but does not block) ETC function from ATP generation, did not mimic the effects of exogenous pyruvate or antimycin A. This indicates that in AH130 cells glycolytic energy production is

sufficient and that the role of ETC is related instead to the fact that some pyruvate cannot be converted to lactate and needs to be oxidized. In other words, ETC seems necessary to prevent a detrimental accumulation of reducing equivalents coming from oxidizable substrates. In turn, an excess of oxidizable substrates can saturate ETC. In this respect, the differentiation state of cells is critical. ETC saturation is easily prevented in cells endowed with large mitochondrial equipment ("transit" progenitor cells characterized by high proliferation rate), but easily produced in cells with few mitochondria (21). AH130 cells possess, as a consequence of their adaptation to low oxygen, a very scanty mitochondrial apparatus, easily saturable by pyruvate at concentrations which are instead easily catabolized by progenitor cells. Thus, AH130 cells represent the prototype of stem cells that are vulnerable to physiological metabolites totally innocuous to non-stem cells. In conclusion, AH130 cell recruitment into S can be limited in two ways: (a) hindering respiration, either under oxygen shortage or by impairing the electron transport to oxygen using ETC inhibitors (antimycin A) and (b) saturating the ETC with an excess of oxidizable substrates (pyruvate). The metabolic network vulnerable to pyruvate is outlined in **Figure 1**. AH130 cell recruitment into S implies, to control the neo-synthesis of DNA, a tight complementation of glycolysis, cellular RedOx state, and folate metabolism. This recruitment is controlled by a cytosolic NADP-dependent step of folate utilization in the synthesis of purine ring, a step where NADPH produced is re-oxidated through the transport of reducing equivalents (electrons) to ETC (21, 22).

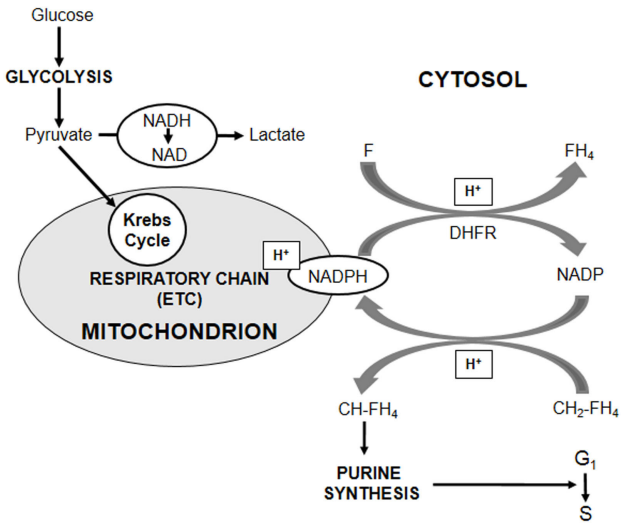

FIGURE 1 | Role of cellular Redox state in the control of cell cycling. The core of the metabolic network controlling AH130 hepatoma cell cycling is the cellular RedOx state expressed by the cytosolic NADP/NADPH ratio. The transfer of reducing equivalents (H^+) from methylene-tetrahydro-folate (CH_2-FH_4) to NADP, generating methenyl-tetrahydro-folate (CH-FH_4) and NADPH, is a limiting step of the assembly of purine ring required for the amplification of purine pools indispensable for the G_1–S transition of mitotic cycle. An accumulation of cytosolic NADPH inhibits cell recruitment into S. A fundamental role in the regulation of NADP/NADPH ratio is played by folate (F), whose reduction to tetrahydro-folate (FH_4) by dehydrofolate-reductase (DHFR) generates NADP. When DHFR activity is impaired by the addition of its inhibitor Methotrexate or of an excess of the reaction product (FH_4), NADPH increases with the consequent reductive shift of NADP/NADPH ratio and the inhibition of purine synthesis. However, the major antagonist of this shift is the transfer of cytosolic reducing equivalents onto the mitochondrial ETC through suitable shuttles, accounting for the crucial role of ETC in purine synthesis. This transfer is antagonized whenever ETC, although not inhibited, is saturated by reducing equivalents produced by oxidizable substrates of the Krebs cycle, such as pyruvate.

CHRONIC MYELOID LEUKEMIA AND THE METABOLIC CONTROL OF ONCOGENE ADDICTION

Chronic myeloid leukemia is determined by a reciprocal translocation between chromosomes 9 and 22, resulting in the formation of the chimeric BCR/Abl protein (hereafter "BCR/Abl") that functions as a constitutively active tyrosine kinase. Tyrosine kinase inhibitors (TKI), such as imatinib-mesylate (IM), are highly effective to suppress BCR/Abl enzymatic activity and to treat chronic phase CML patients. However, in a large majority of patients, IM does not efficiently kill leukemic stem cells (LSC), the crucial event to cure CML (23). It is becoming clear that second-/third-generation TKI remain unable to eradicate LSC. Therefore, strategies directed to hit TKI-resistant LSC aiming at targets different from BCR/Abl are intensely sought after.

Mechanisms traditionally believed to determine resistance to IM and TKI are (24): (a) mutations of *BCR/abl* gene within the tyrosine kinase domain (primary or secondary to treatment), (b) amplification of *BCR/abl*, (c) mutations outside *BCR/abl* determining BCR/Abl-independent survival and proliferation, (d) enhanced activity of drug exporters, and (e) quiescence. A novel mechanism of resistance to IM emerged from our studies *in vitro*. We showed indeed, using CML cell lines, that a very low oxygen tension (0.1% oxygen) in the incubation atmosphere maintains the stem cell potential while cell growth is inhibited and the oncogenic BCR/Abl protein is suppressed (25, 26). Being deprived of BCR/Abl, LSC selected in low oxygen are independent of BCR/Abl signaling, i.e., they lack oncogene addiction (27). In spite of this, LSC selected in low oxygen remain genetically leukemic, so that they are capable to regenerate a BCR/Abl-expressing/dependent progeny (25, 26). Thus, BCR/Abl suppression in low oxygen is not a genetically blocked event, but a fully reversible phenotypical adaptation. This fact is in keeping with the "*chiaroscuro* stem cell" model proposed to describe the relationship between the HSC and HPC phenotypes (28). The refractoriness to TKI of LSC of CML adapted to low oxygen is a straightforward consequence of the transient and reversible suppression of their molecular target. We defined "environment-enforced BCR/Abl suppression" this mechanism of resistance to TKI. This mechanism does not require, to explain the onset of resistance, to postulate the occurrence of permanent changes in a CML subclone due to secondary mutations (29). Our *in vitro* findings are strongly supported by the observation that LSC do not depend on BCR/Abl kinase activity for their survival (30), as well as by the clinical evidences that MRD and the related CML relapse following successful IM treatment is

usually sustained by cells expressing wild-type BCR/Abl (31). On the other hand, our model well explains: (a) the discrepancy observed in CML patients between the expression of *BCR/abl* transcript and that of BCR/Abl protein (32) and (b) the IM resistance of CML progenitors shown to be *BCR/abl*-positive by FISH or PCR (33), which we believe to be in fact transcript-positive/protein-negative cells.

The environment-enforced BCR/Abl-negative/TKI-resistant phenotype implies that LSC are metabolically adapted to home within the "hypoxic" stem cell niches of bone marrow where HSC physiologically reside (see Introduction). Interestingly, we found that LSC adaptation to low oxygen and BCR/Abl suppression are not necessarily linked to quiescence (unpublished data). This is in keeping with the findings we obtained for HSC indicating that low oxygen restrains and redirects their cycling to support self-renewal (3). The capacity of cycling in low oxygen is obviously crucial to allow LSC self-renewal within the stem cell niche and, therefore, to maintain MRD. On the other hand, as cell cycling is necessary for the permanent incorporation of mutations in a cell population, this capacity appears the key factor for neoplastic progression during silent/subclinical phases of the disease. Thus, the metabolic adaptation of LSC seems to warrant all the features necessary to keep CML going in a therapy-resistant fashion.

A first attempt to characterize the adaptation of LSC to a low oxygen environment and its relationship to BCR/Abl suppression showed that this suppression occurs when, in low oxygen, glucose approaches exhaustion (26). Therefore, a low-oxygen/low-glucose environment appeared as the appropriate condition for the maintenance of TKI-refractory CSC sustaining MRD (CSC/MRD). In this respect, it is worth pointing out that glucose exhaustion, under our standard experimental conditions, is reached only after 7 days of incubation in low oxygen. Thus, it is evident that a cell subset exists which can stand low oxygen but still takes advantage of glucose availability and BCR/Abl signaling. It is likely that this cell subset includes CSC that dedicate most of their proliferative potential to oncogene-driven clonal expansion, although they maintain a – probably low – level of self-renewal. We are convinced that this CSC subset and the CSC/MRD subset correspond to the two CSC models originally proposed as alternative: the "CSC in normal stem cell" and the "CSC in normal progenitor cell" (34). Our CML data strongly suggest considering rather these models as complementary. The relationships among CSC models, self-renewal/clonal expansion balance, MRD, role of growth-promoting oncogene signaling, and dependence on oxygen and/or glucose supply are summarized in **Figure 2**.

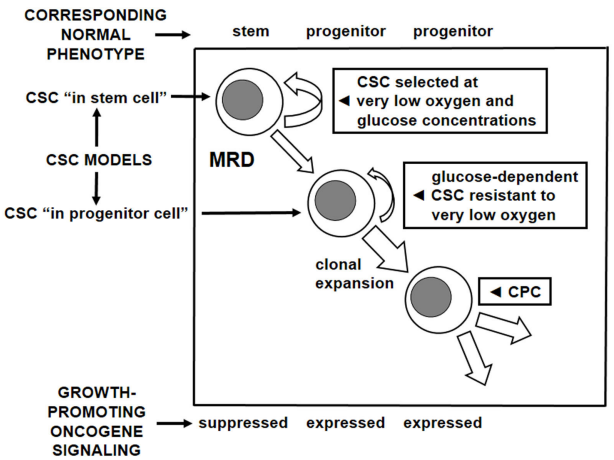

FIGURE 2 | Cancer stem cell models, oncogene dependence, and metabolic profile. Correspondence to the normal stem and progenitor cell phenotypes of two complementary subsets of CSC identified on the basis of two models for their generation. Relationship of these subsets to oxygen and glucose supply in tissue microenvironment as well as to the activity of growth-promoting oncogene signaling. CSC, cancer stem cell; CPC, cancer progenitor cell; MRD, minimal residual disease; the width of arrows corresponds to the hypothesized level of activity.

CONCLUDING REMARKS

A question arising from the information summarized above was whether the Yoshida's hepatoma and the CML models could complement each other contributing to define a unifying scenario for CSC adaptation to low oxygen. We believe that one can answer this question affirmatively. Indeed, AH130 hepatoma cells exhibit a Warburg-type metabolic profile *in vitro* and *in vivo* until oxygen is available, adapt to oxygen shortage relying on glycolysis, and undergo mitotic arrest when glucose shortage complicates oxygen shortage. Thus, the AH130 hepatoma represents a phenotypically homogeneous CSC-like cell population, which is very well suitable for biochemical, molecular, as well as biological studies. CML cell lines, on the other side, are heterogeneous populations, which retain the capacity to differentiate under different environmental conditions, yet, including a cell subset with stem cell traits. However, CML, like hepatoma, cells exhibit the aerobic or the anaerobic glycolytic profile depending on oxygen availability. Moreover, depending on glucose availability or shortage, LSC of CML cells are subjected, rather than to growth promotion/arrest, to a switch between two different CSC phenotypes characterized by different metabolic profiles. This phenotypical difference reflects the expression or suppression of the oncogenetic protein responsible for the disease and underscores the partition of CSC compartment in two subsets, which are dynamically related to each other and reversibly linked to environmental conditions. These subsets drive either the florid state of disease (CSC) or the maintenance of disease during clinical remission (CSC/MRD). Interestingly, the reversibility of the two CSC phenotypes emerged from studies carried out using stabilized CML cell lines, in particular, K562 cells. This strengthens considerably the issue of the genetic identity of the two CSC subsets. A high percentage of K562 cells expresses ETF and exhibits a high level of sensitivity to pyruvate addition. Thus, these cells appear to share with AH130 cells important aspects of gene expression and metabolism profiles. On the other hand, it is also of high interest that the most relevant results obtained using stabilized CML cell lines were confirmed using a number of different primary explants from CML patients (26, 35). These findings allow to export the above conclusions about the CML stem cell compartment to clinical settings.

Stem cells, besides being characterized by a high glycolytic activity, also consume oxygen *via* a functional ETC (36). Referring to CML, the emerging question is whether, when glucose is exhausted, this oxidative activity can be sufficient to sustain energy production from other substrates (29). This scenario may describe appropriately the metabolic profile of CSC/MRD, whereas CSC and cancer progenitor cells (CPC) would follow the AH130-type glucose-dependent metabolic pattern. Metabolic differences between quiescent or slow-cycling CSC/MRD and rapidly proliferating CSC/CPC are emerging from recent literature (37, 38). On the other hand, it has been demonstrated that a dormant subset of pancreatic cancer cells capable to survive oncogene ablation (like CML cells subjected to environment-enforced BCR/Abl suppression) is responsible for tumor relapse, has CSC features, and relies on oxidative phosphorylation for survival (39). CML studies led us to envision a two-tier model of CML stem cell niche where different LSC subsets establish a sort of "metabolic symbiosis" conceptually similar to that shown between cancer cells and stroma within solid tumors (29). According to this model, a drop of oxygen tension in the niche periphery would stimulate glycolysis therein *via* the activation of transcriptional activity mediated by hypoxia-inducible factor 1α (HIF1α). The consequent high rate of glucose consumption would determine a sharp decrease of pH and increase of lactate concentration in the niche core. There, low pH would inhibit HIF1α, and available substrates, such as lactate itself, would be oxidized to produce energy suitable for LSC survival.

In summary, the combination of data from the hepatoma and CML models led us to hypothesize the following coupling between metabolic profiles and functional subsets of cancer cell populations:

(A) Low oxygen-sensitive CPC (proliferation directed to clonal expansion coupled with differentiation); high-level oncogenetic proliferative signaling (e.g., BCR/Abl expressed); high-level oxidative energy production; and many (and elongated/*cristae*-rich) mitochondria.

(B) Low oxygen-resistant self-renewing CSC (proliferation coupled with commitment to clonal expansion); reduced but not suppressed oncogenetic proliferative signaling (e.g., BCR/Abl undergoing suppression); upregulated glycolytic energy production, sustained by glucose availability (maintained even if oxygen supply is restored – Warburg effect); few (and rounded/*cristae*-poor) mitochondria; and low-level oxidative activity directed, rather than to provide energy, to prevent a detrimental accumulation of reducing equivalents produced in high quantities by upregulated glycolysis.

(C) Low oxygen-adapted self-renewing CSC (proliferation uncoupled with commitment to clonal expansion); suppressed oncogenetic proliferative signaling (e.g., BCR/Abl suppressed); downregulated glycolytic energy production due to glucose exhaustion; few mitochondria; and low-level oxidative activity sufficient to sustain energetically the slow-cycling/quiescent cell subset responsible for MRD.

On the basis of all above, the use of metabolic inhibitors to target CSC emerges as a therapeutic strategy well worth being explored if one aims at the cure of disease, i.e., at its eradication. In this respect, a number of attempts have been actually carried out using inhibitors of the best known pathways of energy metabolism, such as glycolysis and mitochondrial function. These inhibitors include the glucose analog 2-deoxyglucose, which accumulates in cells and inhibits hexokinase; dichloroacetate, an inhibitor of pyruvate dehydrogenase kinase, which forces pyruvate to mitochondrial metabolism; metformin, an anti-diabetic drug endowed with insulin-dependent and direct insulin-independent anticancer effects (40–43).

Perhaps even more interesting therapeutic alternatives are represented by the use of physiological substrates related to "energy" metabolism. We have previously shown that peculiar metabolic features of cell adaptation to, and survival in, low oxygen imply growth restriction points that can be targeted by physiological factors, such as pyruvate, tetrahydrofolate, and glutamine (20). For instance, in the presence of pyruvate (upto 20 mM), tumors of various histogenesis (melanoma and neuroblastoma, in addition to AH130 hepatoma and CML) undergo growth inhibition *in vitro*, to levels apparently proportional to their degree of anaplasia, being this inhibition maximal (up to 90%) for AH130 cells. Remarkably, pyruvate is innocuous, even at the highest doses, to normal differentiated cells. Thus, cancer growth can be attacked not only *via* the targeting of metabolic pathways in general but also *via* their targeting, in particular, using physiological substrates. The possibility of transferring the latter strategy to preclinical settings is being addressed in our laboratory.

AUTHOR CONTRIBUTIONS

MGC, IM, and ER carried out personally or supervised the collection of data mentioned in this review. MC and PDS wrote the manuscript. IM and ER made the figures and edited the manuscript.

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REVIEW
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Prognostic Significance of Carbonic Anhydrase IX Expression in Cancer Patients: A Meta-Analysis

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Hypoxia is a characteristic of many solid tumors and an adverse prognostic factor for treatment outcome. Hypoxia increases the expression of carbonic anhydrase IX (CAIX), an enzyme that is predominantly found on tumor cells and is involved in maintaining the cellular pH balance. Many clinical studies investigated the prognostic value of CAIX expression, but most have been inconclusive, partly due to small numbers of patients included. The present meta-analysis was therefore performed utilizing the results of all clinical studies to determine the prognostic value of CAIX expression in solid tumors. Renal cell carcinoma was excluded from this meta-analysis due to an alternative mechanism of upregulation. 958 papers were identified from a literature search performed in PubMed and Embase. These papers were independently evaluated by two reviewers and 147 studies were included in the analysis. The meta-analysis revealed strong significant associations between CAIX expression and all endpoints: overall survival [hazard ratio (HR) = 1.76, 95% confidence interval (95%CI) 1.58–1.98], disease-free survival (HR = 1.87, 95%CI 1.62–2.16), locoregional control (HR = 1.54, 95%CI 1.22–1.93), disease-specific survival (HR = 1.78, 95%CI 1.41–2.25), metastasis-free survival (HR = 1.82, 95%CI 1.33–2.50), and progression-free survival (HR = 1.58, 95%CI 1.27–1.96). Subgroup analyses revealed similar associations in the majority of tumor sites and types. In conclusion, these results show that patients having tumors with high CAIX expression have higher risk of locoregional failure, disease progression, and higher risk to develop metastases, independent of tumor type or site. The results of this meta-analysis further support the development of a clinical test to determine patient prognosis based on CAIX expression and may have important implications for the development of new treatment strategies.

Keywords: cancer, carbonic anhydrase IX, hypoxia, meta-analysis, prognosis

Abbreviations: 95%CI, 95% confidence interval; CAIX, carbonic anhydrase IX; DFS, disease-free survival; DSS, disease-specific survival; HIF, hypoxia-inducible factor; HR, hazard ratio; LC, locoregional control; MFS, metastasis-free survival; NOS, Newcastle–Ottawa scale; OS, overall survival; PFS, progression-free survival; RCC, renal cell carcinoma; TMAs, tissue microarrays; VHL, Von Hippel–Lindau protein.

INTRODUCTION

Hypoxia is a characteristic of many different types of solid tumors and is caused by an inadequate vascular supply. Hypoxic areas are characterized by low oxygen concentrations, limited nutrient supply, and an acidic extracellular environment. Hypoxia is an independent prognostic factor of poor outcome in patients (1) and decreases the efficacy of standard treatment modalities, such as surgery, chemotherapy, and radiotherapy (2–4). Many strategies are therefore being investigated to measure tumor hypoxia to predict treatment outcome and to overcome or target tumor hypoxia with newly designed treatments (5–8).

Tumor cells have adopted several mechanisms to survive the hostile conditions during hypoxia, of which one is the hypoxia-inducible factor (HIF) pathway (9, 10). Upon hypoxic conditions, the expression of the dimeric zinc-containing glycoprotein carbonic anhydrase IX (CAIX) is enhanced as a consequence of HIF stabilization (11, 12). CAIX is important in maintaining the cellular pH regulation and is located on the cell membrane where it hydrolyzes carbon dioxide, produced as a waste product during glycolysis, to bicarbonate and a proton. The bicarbonate is transported intracellularly by different proteins (e.g., anion exchangers), thereby slightly increasing the intracellular pH to promote tumor cell proliferation. The protons in turn add to an acidic extracellular environment causing extracellular matrix degradation favoring invasion, migration, and subsequent metastasis formation (12). Hypoxia-induced CAIX expression, tumor-specific expression of CAIX, and its important role in maintaining the pH balance make CAIX a promising endogenous marker of tumor hypoxia and an attractive target for anti-cancer therapies with newly designed inhibitors (6, 11, 12).

Many clinical studies investigated the prognostic value of CAIX, and a recent meta-analysis of renal cell carcinoma (RCC) concluded that high CAIX expression was associated with a better overall survival (OS) (13). By contrast, a meta-analysis in head and neck cancer patients showed high CAIX expression was associated with a decrease in both OS and disease-free survival (DFS) (14). This discrepancy can be explained by the fact that RCCs are often characterized by an inactive mutant version of the Von Hippel–Lindau (VHL) protein preventing proteasomal degradation of CAIX upon normoxia and making its expression therefore independent of hypoxia (15, 16). To the best of our knowledge, a comprehensive meta-analysis of the association between CAIX expression and treatment outcome in other tumor types has not been performed. The aim of this meta-analysis of published clinical studies is therefore to elucidate the prognostic value of CAIX expression in all solid tumor types besides RCC. In addition, current analysis has included sensitivity and subgroup analysis to be able to determine if the prognostic value of CAIX expression varies in patients with different tumor types.

METHODS

Literature Search

The research question of this meta-analysis was defined as follows: "what is the prognostic value of tumoral CAIX expression in patients with solid tumors?" From this research question, three distinctive keywords were identified, i.e., prognosis, CAIX, and tumor. Different formulations and truncations of the keywords were tested as free text searches to see if appropriate papers could be identified. The search algorithm was applied as a free text search and consisted of the combined mention of all three keywords, in any of the formulations or truncations (Data sheet 1 in Supplementary Material). The search for literature was performed on the 31st of August 2015 in both the PubMed and Embase databases. A total of 958 papers were identified from both databases (**Figure 1**).

Exclusion Criteria

From the total number of papers, 134 reviews, conference abstracts, commentaries, meta-analyses, editorials, or book chapters were

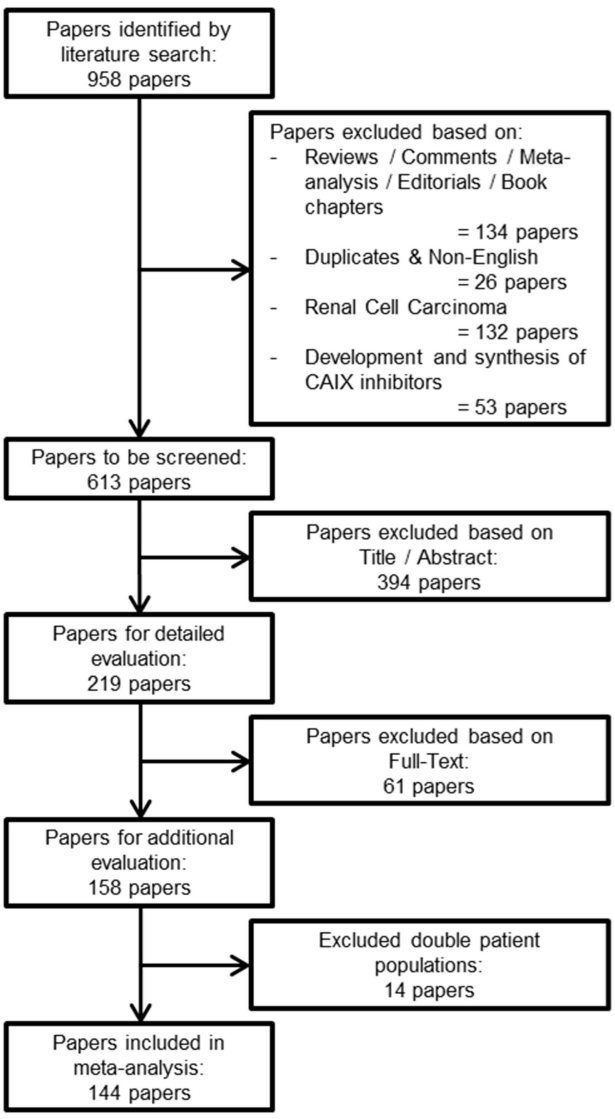

FIGURE 1 | Flowchart of selecting articles describing the association between tumoral CAIX expression and prognosis.

excluded, as were 26 duplicates or non-English papers. From the remaining articles, 132 papers about RCC were excluded, since upregulation of CAIX in RCC is biologically different from other solid tumor types (15, 16). Furthermore, from our experience, we know that papers that describe solely the development and synthesis of CAIX inhibitors do not include patient data, and 53 papers were therefore also excluded. The total number of papers for further screening was thereby reduced to 613 (**Figure 1**).

Screening of Papers

Two researchers (SK and AY) screened the remaining papers independently. The first round of screening was based on the title and abstract, whereas the second round consisted of a detailed evaluation of the full-text. Papers were evaluated based on the predetermined inclusion criteria. First, only solid primary tumors of various types were included, thereby automatically excluding hematological cancer. Second, only immunohistochemical detection of CAIX was included, because mRNA upregulation of CA9 does not fully correlate with an increase in functional protein expression, possibly due to posttranscriptional processing and/or differences in stability (17–19). Third, all endpoints were included (see below) with a minimal median follow-up of 1 year. Fourth, all treatment modalities along with experimental treatments were included. Finally, we included every human patient population without making distinction based on tumor grades or stages. Discrepancies between the included papers by both reviewers were discussed and consensus was reached on all. An additional 14 papers were excluded because their patient populations were similar or overlapping with other papers. Among these repetitive studies, the paper that was included contained the most detailed information about the patient population. A total of 144 papers were included in the meta-analysis (**Figure 1**).

Data Extraction

Several different parameters, if reported, were extracted from each paper, i.e., the number and origin of patients, number of events, treatment modalities, tumor site, tumor stage, tumor type, group dichotomization, antibody supplier, expression pattern, cellular localization, and endpoints. The univariate hazard ratio (HR) was extracted to assess prognostic value of CAIX expression. When the univariate HR with corresponding 95% confidence interval (95%CI) was not reported, the method from Tierney et al. was used to estimate the HR (20). Multivariate HR was only included in the meta-analysis when the univariate HR was not reported or could not be estimated. When insufficient data were reported for estimating HR, the authors were contacted to obtain additional data.

Quality Assessment

The methodological quality of the included papers was evaluated with an adjusted version of the Newcastle–Ottawa scale (NOS) to better suit the study design of the included papers (Data sheet 2 in Supplementary Material). The method of scoring based on awarding stars in different categories remained, however, identical. The NOS was commended in the 2011 version of the Cochrane Collaboration handbook and is an easy method to evaluate the methodological quality of cohort studies (available at http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp) (21).

Statistical Analysis and Sensitivity Analysis

Distribution and frequencies of the extracted data parameters were analyzed using SPSS (version 22). Meta-analysis was performed using R statistical software with the Metafor Library (version 1.9-8) (22). Fixed-effect modeling was performed when no statistical significant heterogeneity between studies was observed. When the heterogeneity between studies was statistically significant, random-effects modeling was applied based on the DerSimonian and Laird method (23). The assigned weight of each study in the analysis was based on its inverse variance. The following endpoints have been addressed: OS, DFS, locoregional control (LC), disease-specific survival (DSS), metastasis-free survival (MFS), and progression-free survival (PFS). Sensitivity analysis was performed by analyzing subgroups of studies separately, e.g., per tumor organ site. Funnel plots were created to visualize possible publication bias or heterogeneity between studies. Asymmetric funnel plots and studies outside the funnel plot suggest heterogeneity between them and/or publication bias (21). p-Values <0.05 were considered as statistically significant.

RESULTS

This meta-analysis encompassed a total number of 24,523 patients across 147 independent studies. Many studies included only a small number of patients (median per study 93, range 15–3630) with a median follow-up time between 12.6 months and 13.9 years and are often inconclusive, which underlines the need for a meta-analysis. All papers were published between 2001 and 2015 of which approximately 50% were published after 2010. The majority of the included studies treated patients with surgery alone (36.7%) or in combination with either chemotherapy (8.8%) or standard radiotherapy (8.8%), or the combination of all three modalities (23.1%). Single radiotherapy treatment or combined with chemotherapy was reported in 5.4 and 6.1% of the papers, respectively. In 4.8% of the studies, a form of experimental treatment was administered, including experimental radiotherapy (24–28), hormonal treatment (29), and VEGF-targeted therapy (30). Most of the studies reported on head and neck cancer patients (21.8%) followed by breast (16.3%) and brain cancer patients (10.2%). By contrast, cancers of the adrenal gland, the cartilage, and the penis were only described once.

Immunohistochemical staining of CAIX was predominantly performed using the M75 antibody (46.3%) targeting the proteoglycan domain of CAIX (31, 32). Other studies used anti-CAIX antibodies obtained from different suppliers. A membranous expression of CAIX was described in 46.3% of the studies, although cytoplasmatic staining or a combination of the two was also reported (4.8 and 17.7%, respectively). Nuclear staining was only reported in one paper, whereas the rest did not state the staining localization. Different quantification methods and thresholds have been applied to stratify patients into groups with

low and high tumoral CAIX expression. Taken together, 33.9% of the total tumors were classified as expressing high levels of CAIX.

Overall, patients suffering from tumors with high CAIX expression had a worse treatment outcome (**Figure 2**). This association was strong and significant for all endpoints. The negative association of CAIX expression with outcome was dominant for DFS and weaker for LC. Systematic heterogeneity in the present meta-analysis as demonstrated by an asymmetric funnel plot (21) for most of the endpoints (Image 1 in Supplementary Material) can at least in part be attributed to the considerable variation in tumor types and sites across the studies. Therefore, in addition, subgroup analysis based on organ site of the tumor was performed. The results of the subgroup analysis demonstrate a significant prognostic value of CAIX in most of the cancer types investigated (see below).

Overall Survival

Effect of pretreatment expression of CAIX on OS could be evaluated in 104 studies. The complete data to estimate the HR could not be retrieved from 11 papers and were therefore not included in the analysis [Table 1 in Supplementary Material (33–43)]. Overall, high CAIX expression was associated with a worse OS (HR = 1.76, 95%CI 1.58–1.98, $p < 0.0001$, **Figure 3**). Subgroup analysis of the different organ sites revealed a similar significant association between tumoral CAIX expression and OS in 11 organ sites: bladder (HR = 1.64, 95%CI = 1.21–2.22), brain (HR = 2.18, 95%CI 1.60–2.96), breast (HR = 1.90, 95%CI = 1.45–2.50), esophagus (HR = 1.97, 95%CI 1.50–2.60), gall bladder (HR = 2.35, 95%CI 1.33–4.15), gastroenteropancreatic tract (HR = 2.57, 95%CI 1.45–4.56), head and neck (HR = 1.66, 95%CI 1.29–2.13), lung (HR = 1.57, 95%CI 1.06–2.33), pancreas (HR = 2.37, 95%CI 1.04–5.43), soft tissue (HR = 2.97, 95%CI 1.65–5.34), and the stomach (HR = 1.92, 95%CI 1.39–2.67). The other six organ sites show a similar trend with worse OS, albeit not statistically significant (**Table 1**). Similar results were often, but not always, observed for different tumor types per organ site (Table 2 in Supplementary Material). A hypoxia-associated perinecrotic staining pattern was reported in 16 of these studies, whereas a diffuse staining pattern was reported in 3 papers. Interestingly, both patterns of CAIX expression significantly associated with OS (perinecrotic: HR = 1.99, 95%CI 1.60–2.48; diffuse: HR = 1.77, 95%CI 1.22–2.56). These results suggest that the expression pattern of CAIX does not affect its prognostic value.

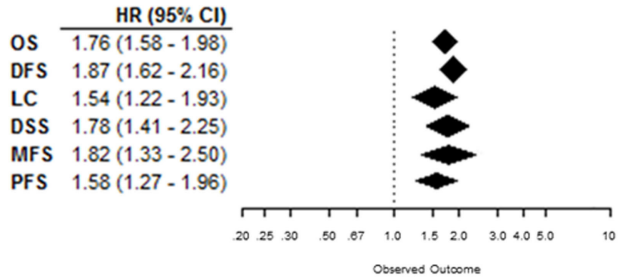

FIGURE 2 | Summary plot of the overall HRs from each endpoint analyzed. Symbols represent the HR with 95%CI, and dashed line indicates no association between CAIX expression and prognosis.

Disease-Free Survival

A total of 40 from the selected 147 studies investigated the association between CAIX expression and DFS. Five studies could not be included in this analysis due to incomplete reporting [Table 1 in Supplementary Material (39, 40, 43, 124, 130)]. Based on 35 studies, high CAIX expression was statistically significantly associated with a decreased DFS (HR = 1.87, 95%CI 1.62–2.16, $p < 0.001$) (**Figure 4**). Subgroup analysis based on organ site of the tumor showed that high CAIX expression was significantly associated with shorter DFS in bladder (HR = 2.63, 95%CI 1.56–4.40), breast (HR = 1.74, 95%CI 1.34–2.27), colorectal (HR = 3.31, 95%CI 1.23–8.89), esophagus (HR = 2.70, 95%CI 2.08–3.50), head and neck (HR = 1.98, 95%CI 1.51–2.61), liver (HR = 1.51, 95%CI 1.26–1.81), lung (HR = 1.87, 95%CI 1.27–2.74), and soft tissue tumors (HR = 3.41, 95%CI 1.58–7.30). By contrast, no significant association with DFS was observed for tumors in the cervix (HR = 1.12, 95%CI 0.75–1.68), pancreas (HR = 2.98, 95%CI 0.56–15.9), penis (HR = 1.35, 95%CI 0.55–3.30), stomach (HR = 1.27, 95%CI 0.77–2.10), and vulva (HR = 1.52, 95%CI 0.79–2.90) (**Table 1**). A similar trend was observed for all different tumor types per organ sites (Table 2 in Supplementary Material).

Locoregional Control

The risk of locoregional relapse associated with CAIX expression was evaluated in 25 studies in 6 different organ sites. **Figure 5** shows the overall LC outcome, which indicates that patients with high tumoral CAIX expression have a higher risk of locoregional recurrences than patients with low expression of CAIX in tumors (HR = 1.54, 95%CI 1.22–1.93, $p = 0.0002$). The negative association between high CAIX expression in tumors and worse LC remained significant in head and neck (HR = 1.54, 95%CI 1.12–2.12) and liver tumors (HR = 1.39, 95%CI 1.09–4.10) (**Table 1**). A similar association was observed in most of the tumor types per organ sites (Table 2 in Supplementary Material).

Disease-Specific Survival

Disease-specific survival was reported in 23 studies, of which 1 study provided incomplete data to estimate the HR [Table 1 in Supplementary Material (43)]. In the remaining 22 studies, patients suffering from tumors with high CAIX expression had a significantly shorter DSS (HR = 1.78, 95%CI 1.41–2.25, $p < 0.0001$) (**Figure 6**). Subgroup analyses by organ site revealed significant associations between high CAIX expression and worse DSS in tumors of the breast (HR = 1.75, 95%CI 1.28–2.38), cervix (HR = 2.19, 95%CI 1.29–3.70), esophagus (HR = 2.78, 95%CI 1.56–5.00), head and neck (HR = 2.21, 95%CI 1.12–4.36), pancreas (HR = 1.49, 95%CI 1.07–2.10), and soft tissue (HR = 1.65, 95%CI 1.11–2.45) (**Table 1**). Subgroup analyses of the tumor types per organ sites revealed a worse DSS to be associated with high CAIX expression in the majority of tumor types (Table 2 in Supplementary Material).

Metastasis-Free Survival

Metastasis-free survival was reported in 12 of the 147 included studies. Based on 11 of these studies, high CAIX

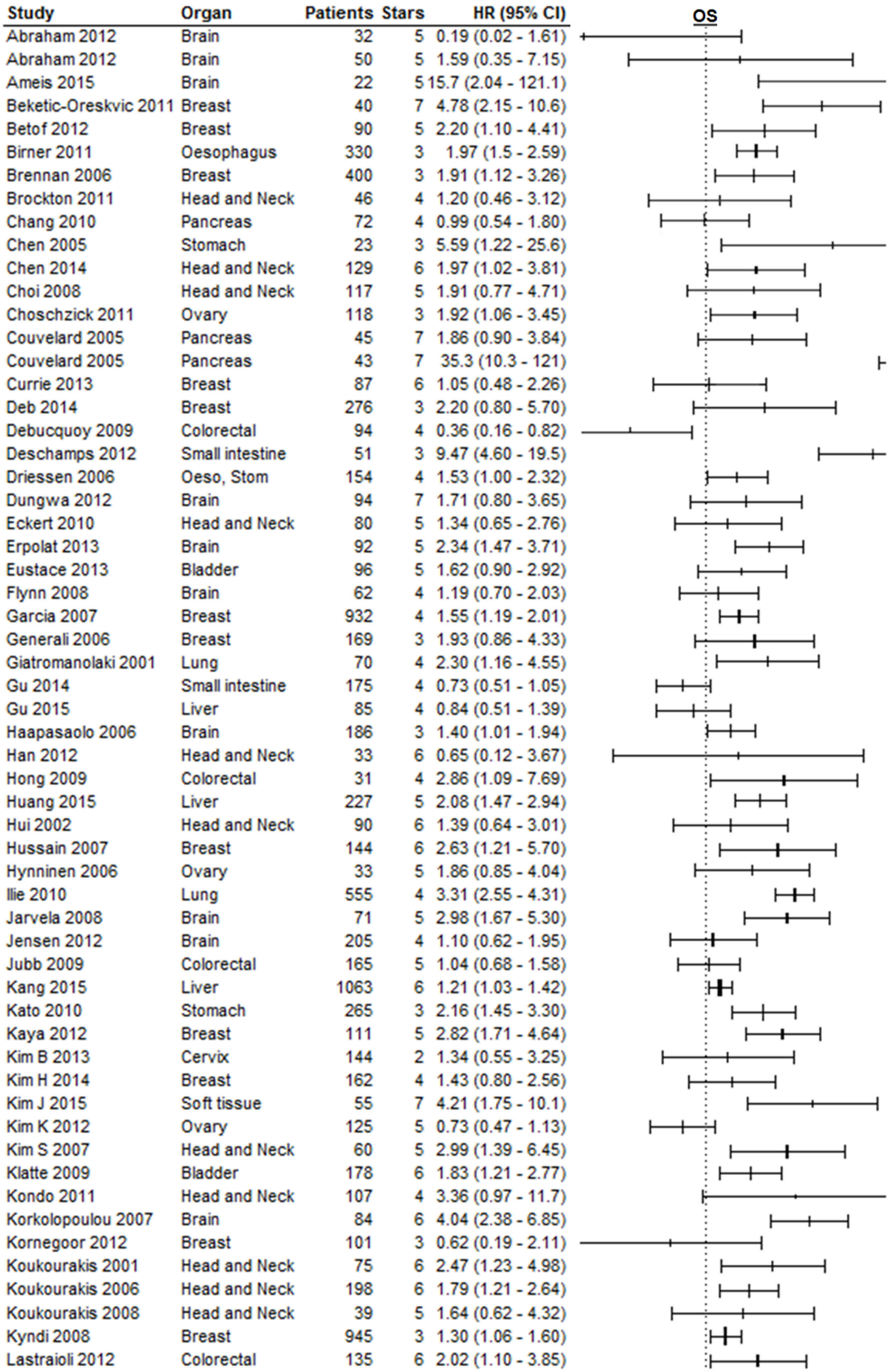

FIGURE 3 | Forest plot of the papers describing the association between CAIX expression and OS. Horizontal bars represent HR with corresponding 95%CI. Symbol size represents the assigned weight of the study. The overall HR with 95%CI is visualized with the diamond shape. Dashed line indicates no association between CAIX and prognosis. Esop, Stom, esophagus and stomach; GEP, gastroenteropancreatic tract (25–27, 29, 30, 44–129).

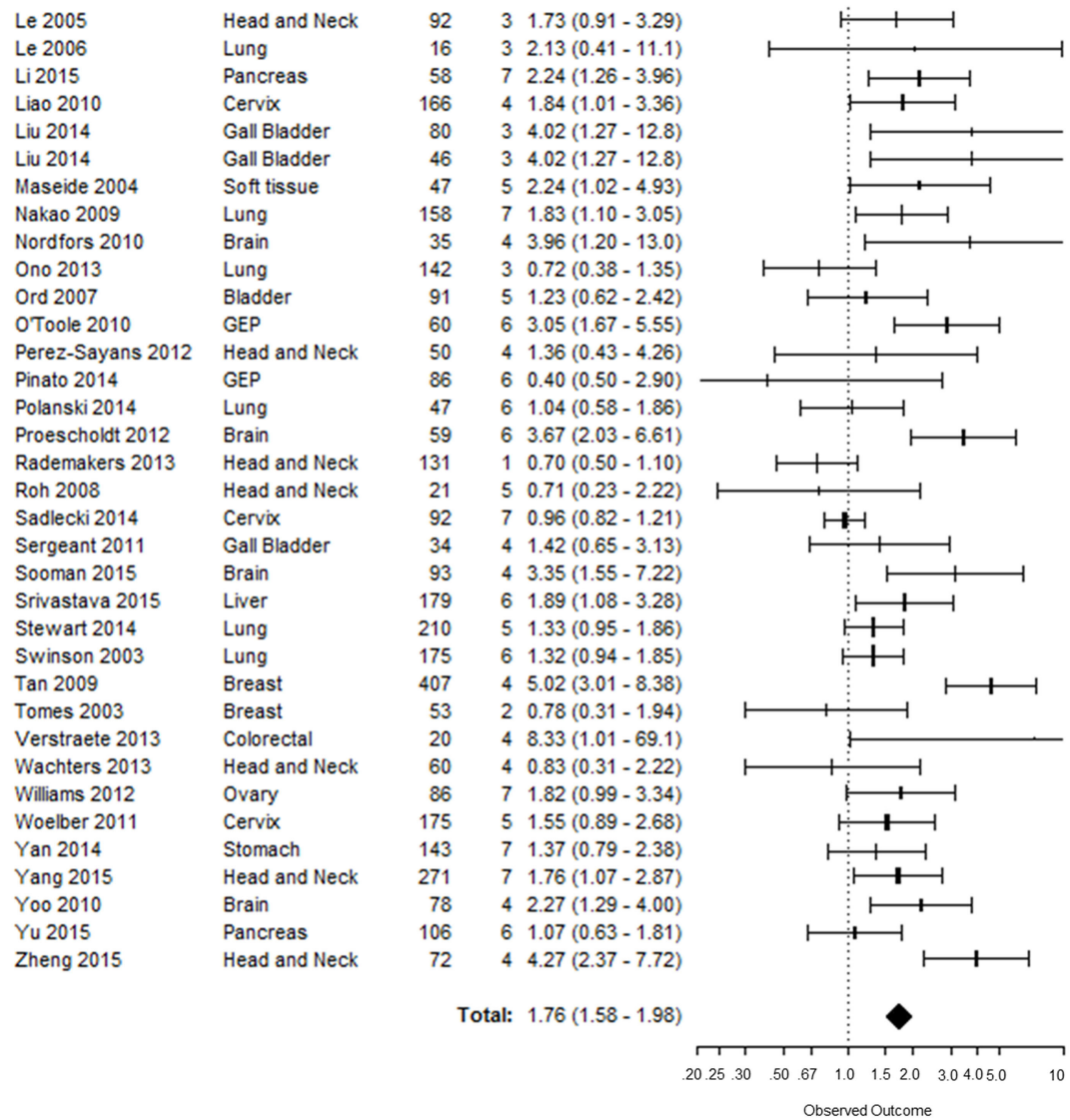

FIGURE 3 | **Continued**

expression was significantly associated with a shorter MFS (HR = 1.82, 95%CI 1.33–2.50, $p = 0.0002$) [**Figure 7**; Table 1 in Supplementary Material (166)]. Subgroup analyses of the different organ sites of the tumors, independent of tumor types, revealed high CAIX expression to be significantly associated with a worse MFS in most of the organ sites reported, i.e., breast (HR = 1.76, 95%CI 1.13–2.74), cartilage (HR = 6.46, 95%CI 2.05–20.0), cervix (HR = 2.37, 95%CI 1.35–4.10), colorectal (HR = 5.17, 95%CI 2.07–13.0), and vulva (HR = 2.25, 95%CI 1.42–3.60), but not in head and neck (HR = 0.77, 95%CI 0.27–2.26) and soft tissue cancers (HR = 1.65, 95%CI 0.72–3.80) (**Table 1**). Interestingly, one study reported a significant positive association between high CAIX expression and better MFS in squamous cell carcinoma of the head and neck (HR = 0.27, 95%CI 0.09–0.80) (Table 2 in Supplementary Material), which may be attributed to the hypoxia-modifying component of the treatment (24).

Progression-Free Survival

Eleven out of 12 studies could be included to estimate the risk of disease progression after treatment based on CAIX expression in tumors [Table 1 in Supplementary Material (169)]. Similar to the other endpoints, PFS was significantly shorter in patients with tumors expressing high levels of CAIX (HR = 1.58, 95%CI 1.27–1.96, $p < 0.0001$) (**Figure 8**). Subgroup analyses per organ site revealed that the association with PFS only remained statistically significant in breast (HR = 1.88, 95%CI 1.13–3.10), colorectal (HR = 2.38, 95%CI 1.06–5.56), and head and neck tumors (HR = 1.62, 95%CI 1.01–2.59) (**Table 1**). The subgroup analyses of tumor types per organ site showed similar associations between

TABLE 1 | Results of subgroup meta-analyses of different organ sites reported.

Organ site	OS	DFS	LC	DSS	MFS	PFS
Bladder	**1.64 (1.21–2.22)**	**2.63 (1.56–4.40)**	0.88 (0.40–1.90)	0.82 (0.47–1.4)		0.68 (0.21–2.20)
Brain	**2.18 (1.60–2.96)**					1.44 (0.91–2.27)
Breast	**1.90 (1.45–2.50)**	**1.74 (1.34–2.27)**	1.37 (0.95–1.96)	**1.75 (1.28–2.38)**	**1.76 (1.13–2.74)**	**1.88 (1.13–3.10)**
Cartillage					**6.46 (2.05–20.0)**	
Cervix	1.11 (0.91–1.35)	1.12 (0.75–1.68)	1.17 (0.74–1.87)	**2.19 (1.29–3.70)**	**2.37 (1.35–4.10)**	1.76 (0.99–3.10)
Colorectal	1.41 (0.67–2.98)	**3.31 (1.23–8.89)**	3.33 (1.76–6.30)	1.31 (0.18–9.41)	**5.17 (2.07–13.0)**	**2.38 (1.06–5.56)**
Esophagus	**1.97 (1.50–2.60)**	**2.70 (2.08–3.50)**		**2.78 (1.56–5.00)**		
Esop, Stom	1.53 (1.00–2.30)					
Gall Bladder	**2.35 (1.33–4.15)**					
GEP	**2.57 (1.45–4.56)**					
H&N	**1.66 (1.29–2.13)**	**1.98 (1.51–2.61)**	**1.54 (1.12–2.12)**	**2.21 (1.12–4.36)**	0.77 (0.27–2.26)	**1.62 (1.01–2.59)**
Liver	1.41 (0.98–2.03)	**1.51 (1.26–1.81)**	**1.39 (1.09–4.10)**			
Lung	**1.57 (1.06–2.33)**	**1.87 (1.27–2.74)**		1.75 (0.59–5.15)		
Ovary	1.42 (0.82–2.45)					1.24 (0.67–2.30)
Pancreas	**2.37 (1.04–5.43)**	2.98 (0.56–15.9)		**1.49 (1.07–2.10)**		
Penis		1.35 (0.55–3.30)				
Small Intestine	2.58 (0.21–31.8)					
Soft tissue	**2.97 (1.65–5.34)**	**3.41 (1.58–7.30)**		**1.65 (1.11–2.45)**	1.65 (0.72–3.80)	
Stomach	**1.92 (1.39–2.67)**	1.27 (0.77–2.10)				
Vulva		1.52 (0.79–2.90)		1.34 (0.67–2.70)	**2.25 (1.42–3.60)**	

The overall HR (with 95%CI) is shown. When HR was available from only one paper, the values were adopted from that single paper. Bold numbers indicate statistical significant associations between CAIX expression and prognosis ($p < 0.01$).
Esop, Stom, esophagus and stomach; GEP, gastroenteropancreatic tract.

high CAIX expression and a worse PFS (Table 2 in Supplementary Material).

High-Quality Papers

This meta-analysis used an adjusted version of the NOS to evaluate the quality of a study. The scores of this quality assessment ranged between 1 and 7 stars, i.e., the maximum, awarded per study. Approximately half of the studies (52.4%) were considered as high-quality studies, i.e., with a number of stars greater or equal to the median (5 stars). Meta-analysis of only the high-quality studies revealed significant prognostic values of CAIX expression for OS (HR = 1.81, 95%CI 1.57–2.09, $n = 50$), DFS (HR = 1.81, 95%CI 1.47–2.23, $n = 18$), DSS (HR = 1.71, 95%CI 1.16–2.51, $n = 10$), and PFS (HR = 1.59, 95%CI 1.21–2.07, $n = 7$). For both LC (HR = 1.90, 95%CI 1.58–2.30, $n = 14$) and MFS (HR = 2.47, 95%CI 1.92–3.19, $n = 7$), the association with CAIX expression became even stronger when only high-quality studies were included.

DISCUSSION

Many clinical studies investigated the prognostic association of CAIX expression with treatment outcome. Most of these studies, however, include only limited numbers of patients and remain inconclusive. This current meta-analysis is the first complete overview of all reported clinical studies investigating the impact of pretreatment CAIX expression in solid tumors on prognosis. Overall, these results clearly show that high CAIX expression is an adverse prognostic marker in solid tumors, irrespectively of the endpoint evaluated, as summarized in **Figure 2**. A strong association between high CAIX expression and poor prognosis was also found in the majority of different tumor sites, supporting an important role of CAIX in disease progression and treatment resistance in many cancer types.

The papers included in the current meta-analysis were all published between 2001 and 2015, which is likely attributed to the identification of the hypoxic responsive element in the promotor region of *ca9* in the end of 2000 (170). This study identified a direct link between CAIX expression and its hypoxic upregulation through HIF stabilization. This crucial finding encouraged research to evaluate CAIX as an endogenous marker of tumor hypoxia, a known biological factor of therapy resistance (2–4). Nevertheless, because alternative mechanisms can also regulate CAIX expression, e.g., *via* PI3K (171) or the unfolded protein response (10, 172), tumoral CAIX expression may not accurately identify hypoxic tumors. Apart from the hypoxia-associated mechanisms underlying resistance of tumor cells to several treatment modalities, CAIX can directly affect cancer prognosis as its main function is to maintain the balance between intracellular and extracellular pH, thereby generating an acidic extracellular microenvironment (11, 12). This is supported by data demonstrating that CAIX is involved in promoting tumorigenesis and leads to a more aggressive phenotype of tumor cells (173). This can partly be explained by the association between CAIX expression and the induction of tumor cell migration and invasion, which could be caused by the reduction in extracellular pH (174–176). In addition, cancer stem cell markers also appear to be enriched in the CAIX expressing population of tumor cells (57, 177). The important role of CAIX, either directly or indirectly, in cancer

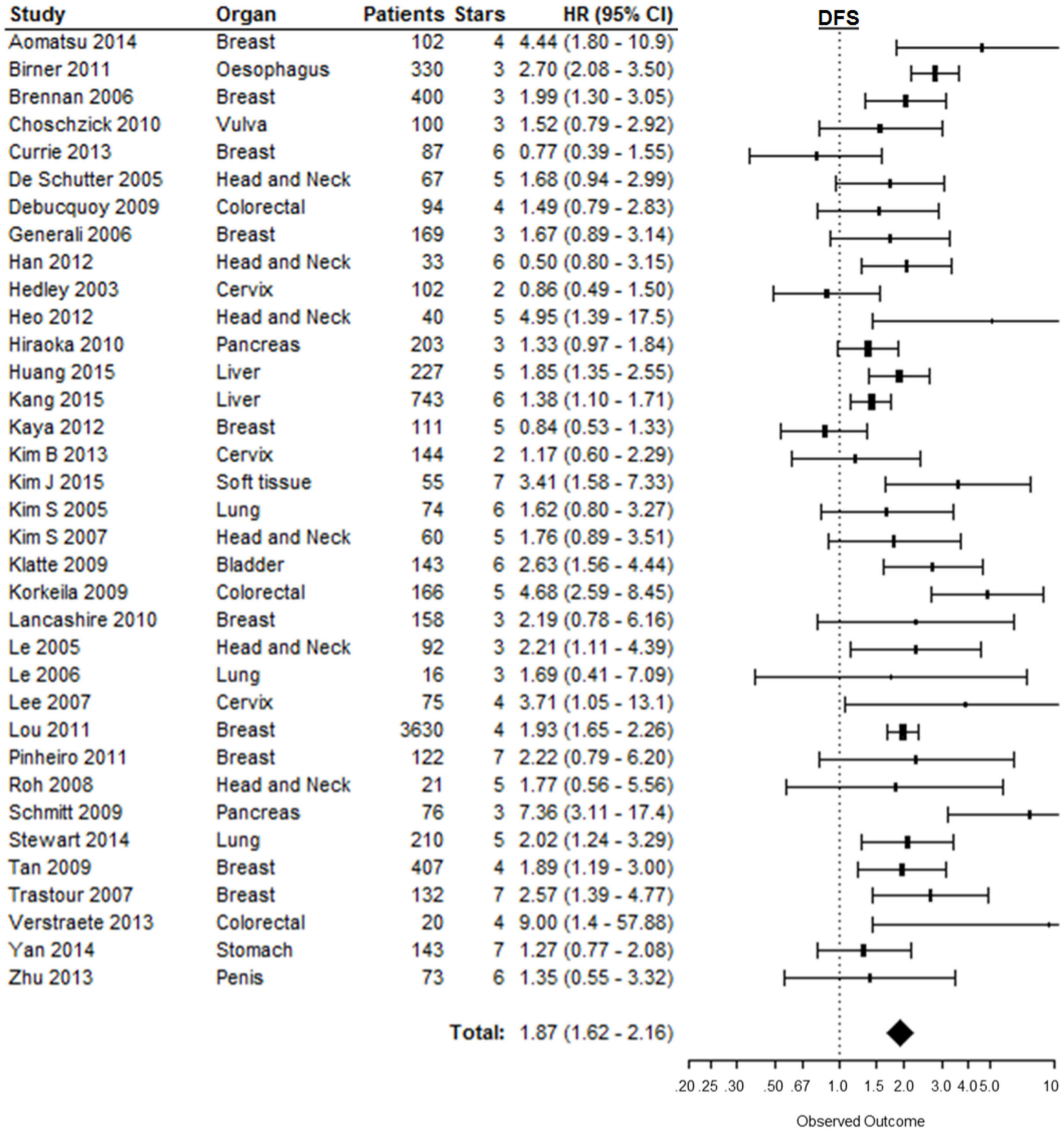

FIGURE 4 | Forest plot of the papers describing the association between CAIX expression and DFS. Horizontal bars represent HR with corresponding 95%CI. Symbol size represents the assigned weight of the study. The overall HR with 95%CI is visualized with the diamond shape. Dashed line indicates no association between CAIX and prognosis (29, 33, 34, 48, 57, 59, 68, 73, 74, 82, 84, 85, 87, 89, 90, 97, 98, 112, 117, 119, 121, 125, 131–143).

prognosis is also supported by the results of the current meta-analysis, which shows that tumors with high CAIX expression have higher risk of locoregional failure, disease progression, and higher risk to develop metastasis. Other proton exchangers and transporters have been shown preclinically and clinically to play an important role in the regulation of cellular pH homeostasis promoting survival and invasion as well as causing treatment resistance (178–180). Therefore, assessment of several major pH regulators in tumors prior and/or during therapy may represent a more powerful prognostic and predictive biomarker as well as important targets for new anti-cancer treatments, which warrants further investigations.

A meta-analysis usually overestimates its results because of selective reporting and publication bias (21). This meta-analysis identified a total of 147 studies reported in 144 papers of which 15 could not be included in final analysis because the HR could not be estimated due to incomplete reporting (33–43, 124, 130, 166, 169). Non-significant association between CAIX and outcome was found in these studies (Table 1 in Supplementary Material). Including these 15 papers in the analysis might therefore decrease the magnitude of the prognostic values of CAIX expression reported here. This overestimation can be further increased by publication bias, i.e., when negative associations are not published at all and can therefore not be identified and included in this meta-analysis. Nevertheless, since the prognostic value of CAIX expression was highly statistically significant, we believe that the possible effect of publication bias on this association is minimal.

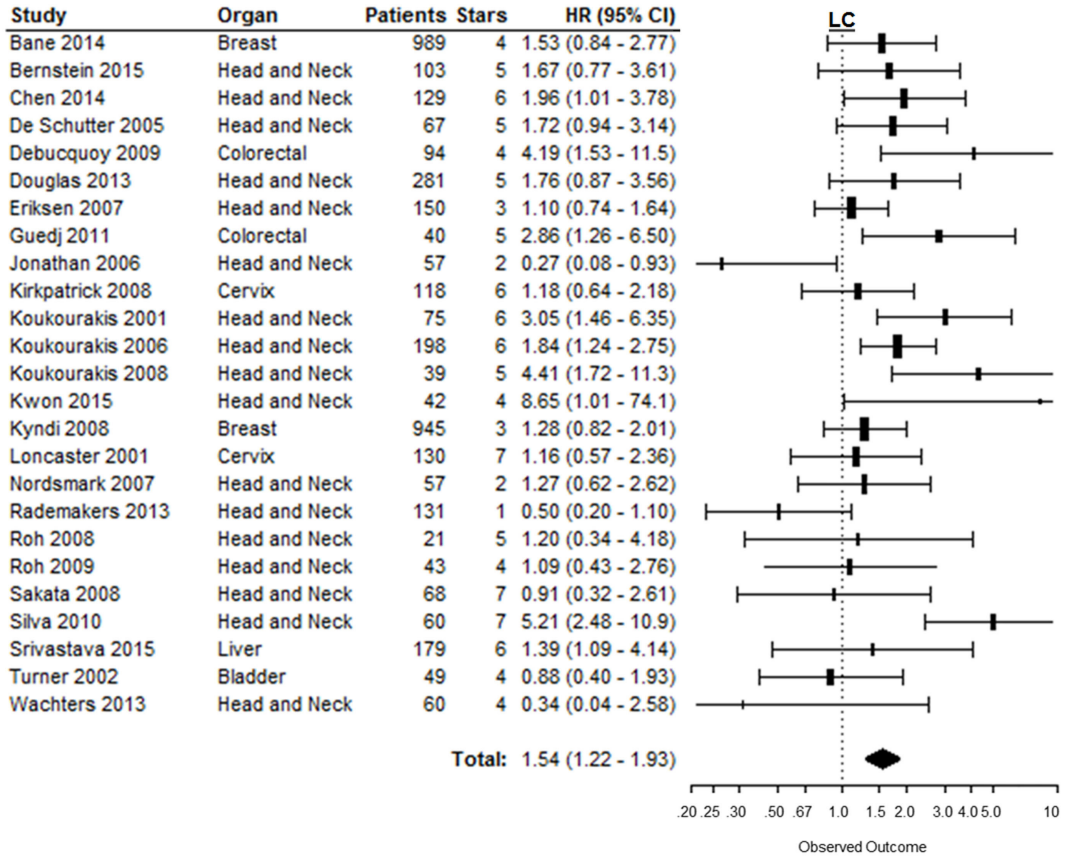

FIGURE 5 | **Forest plot of the papers describing the association between CAIX expression and LC**. Horizontal bars represent HR with corresponding 95%CI. Symbol size represents the assigned weight of the study. The overall HR with 95%CI is visualized with the diamond shape. Dashed line indicates no association between CAIX and prognosis (24–28, 52, 59, 94, 95, 112, 116, 122, 133, 144–155).

The different staining and scoring methods used in the included papers to quantify CAIX expression might be an additional source of bias. Visual quantification was used in the majority of the reports and could either be based on staining intensity, the number of stained cells, or a combination of both. In addition, different thresholds have been used to dichotomize patients based on their CAIX expression. This discrepancy in methods is one of the reasons of significant heterogeneity between studies, which therefore requires the use of a random-effect model in the meta-analysis (181, 182). Additionally, tissue microarrays (TMAs) are used in the majority of included papers to visualize and quantify CAIX expression, even though TMAs may underestimate the actual expression levels of the protein (183). The use of TMAs might therefore bias the prognostic value of CAIX when CAIX expression levels are dichotomized erroneous. Furthermore, this meta-analysis is limited by difficulties in obtaining homogenous endpoints and by non-uniform observation times, although most of the data are based on reports with a median follow-up of more than 1 year.

To identify possible bias in a selected study, an adjusted version of the NOS was used, which is a quick and easy method to assess the quality of studies that has been commended in the Cochrane handbook (21). However, the validity and reproducibility of the NOS have been questioned because of the subjective interpretation of certain criteria, which require detailed guidelines to obtain a better inter-rater agreement (184–186). The test–retest reliability of the NOS is, however, better, which allows for a single reviewer to continuously use uniform criteria while rating papers (185). When only high-quality papers, i.e., those with minimal bias, were included in our analyses, there was no significant difference in the results as compared with all studies included.

It remains impossible to eliminate every source of bias in a meta-analysis. Nevertheless, the high statistical significance of the results presented here clearly show that CAIX expression is associated with worse prognosis in a global patient population and in the majority of tumor sites. These findings are similar to the results of the meta-analysis in head and neck cancer (14), but different from RCC (13) due to the alternative mechanism of CAIX upregulation in RCC (15, 16). New treatment options are currently being developed to specifically inhibit CAIX function (6, 187), of which one is currently in a Phase I clinical trial (NCT02215850). These types of compounds might prove to be beneficial for the specific

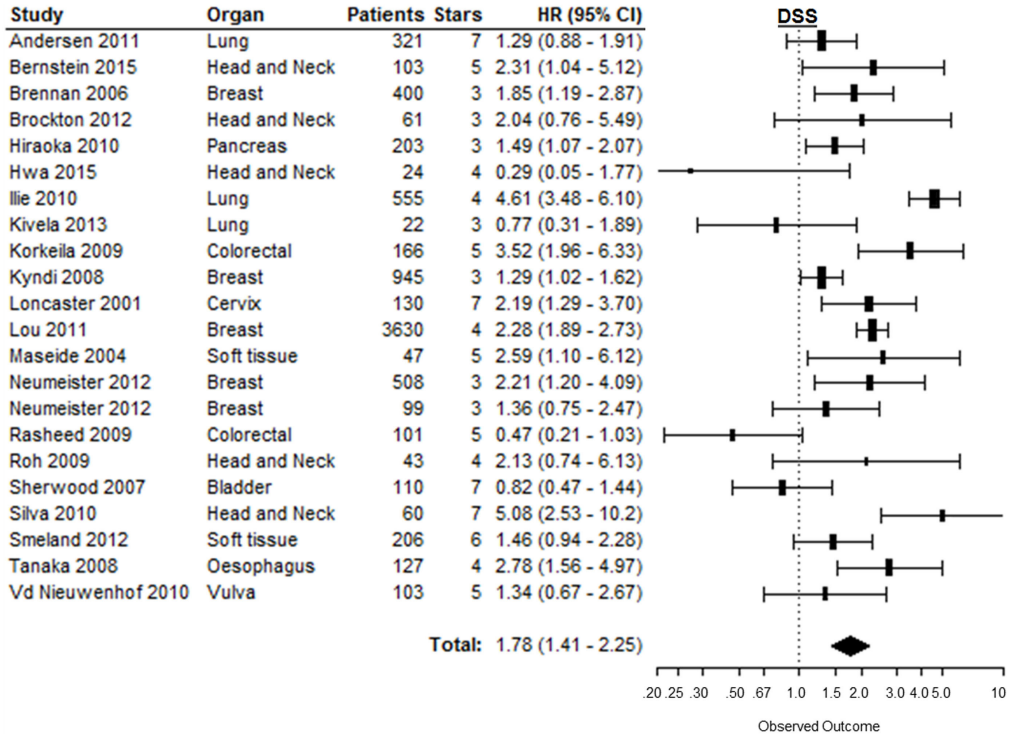

FIGURE 6 | **Forest plot of the papers describing the association between CAIX expression and DSS**. Horizontal bars represent HR with corresponding 95%CI. Symbol size represents the assigned weight of the study. The overall HR with 95%CI is visualized with the diamond shape. Dashed line indicates no association between CAIX and prognosis (29, 78, 95, 102, 136, 138, 140, 144, 150, 152, 154, 156–165).

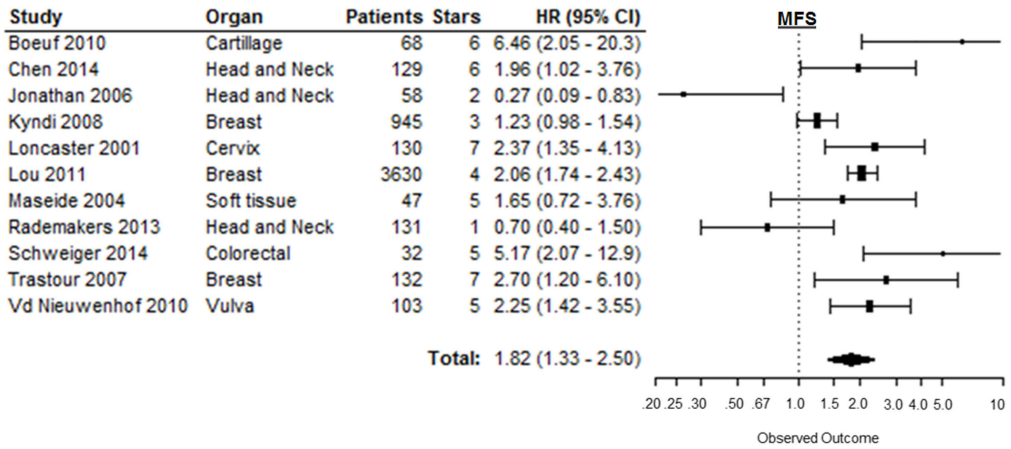

FIGURE 7 | **Forest plot of the papers describing the association between CAIX expression and MFS**. Horizontal bars represent HR with corresponding 95%CI. Symbol size represents the assigned weight of the study. The overall HR with 95%CI is visualized with the diamond shape. Dashed line indicates no association between CAIX and prognosis (24, 27, 34, 52, 95, 102, 140, 34, 150, 165, 167, 168).

treatment of tumors with high CAIX expression. The results of this meta-analysis further support the development of a clinical test to determine patient prognosis based on CAIX expression, although a standardized protocol remains to be developed and validated.

AUTHOR CONTRIBUTIONS

Study was conceived and designed by SK, AY, RN, PL, and LD. Screening of papers and data extraction was performed by SK and AY. Statistical analyses were performed by RH. Writing of the first

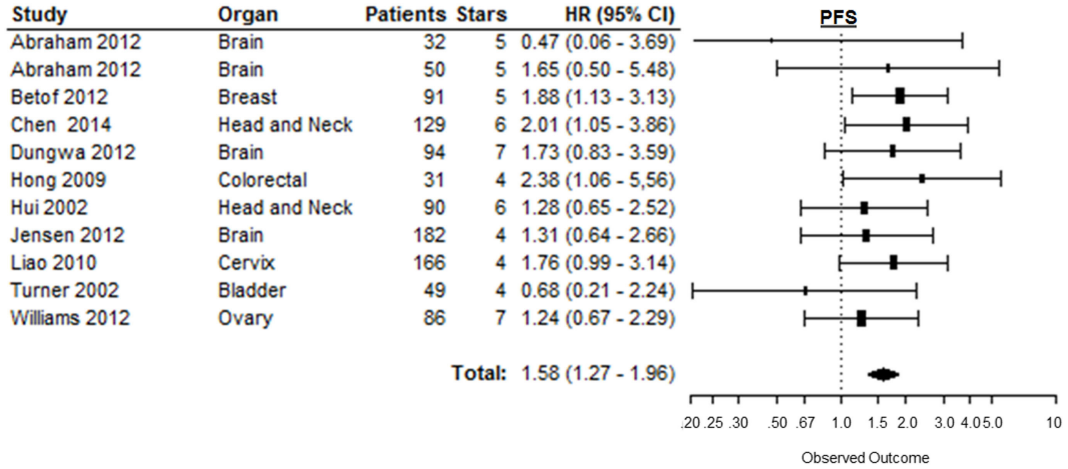

FIGURE 8 | Forest plot of the papers describing the association between CAIX expression and PFS. Horizontal bars represent the HR with corresponding 95%CI. Symbol size represents the assigned weight of the study. The overall HR with 95%CI is visualized with the diamond shape. Dashed line indicates no association between CAIX and prognosis (30, 44, 47, 52, 62, 75, 80, 100, 123, 155).

draft of the manuscript was performed by SK. AY, RH, RN, PL, and LD contributed to the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fonc.2016.00069

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Hypoxia on the Expression of Hepatoma Upregulated Protein in Prostate Cancer Cells

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Hepatoma upregulated protein (HURP) is a multifunctional protein with clinical promise. This protein has been demonstrated to be a predictive marker for the outcome in high-risk prostate cancer (PCa) patients, besides being a resistance factor in PCa. Although changes in oxygen tension (pO_2) are associated with PCa aggressiveness, the role of hypoxia in the regulation of tumor progression genes such as HURP has not yet been described. We hypothesized that pO_2 alteration is involved in the regulation of HURP expression in PCa cells. In the present study, PCa cells were incubated at 2% O_2 (hypoxia) and 20% O_2 (normoxia) conditions. Hypoxia reduced cell growth rate of PCa cells, when compared to the growth rate of cells cultured under normoxia ($p < 0.05$). The decrease in cell viability was accompanied by fivefold ($p < 0.05$) elevated rate of vascular endothelial growth factor (VEGF) release. The expression of VEGF and the hypoxia-inducible metabolic enzyme carbonic anhydrase 9 were elevated maximally nearly 61-fold and 200-fold, respectively ($p < 0.05$). Noted in two cell lines (LNCaP and C4-2B) and independent of the oxygen levels, HURP expression assessed at both mRNA and protein levels was reduced. However, the decrease was more pronounced in cells cultured under hypoxia ($p < 0.05$). Interestingly, the analysis of patients' specimens by Western blot revealed a marked increase of HURP protein (fivefold), when compared to control (cystoprostatectomy) tissue ($p < 0.05$). Immunohistochemistry analysis showed an increase in the immunostaining intensity of HURP and the hypoxia-sensitive molecules, hypoxia-inducible factor 1-alpha (HIF-1α), VEGF, and heat-shock protein 60 (HSP60) in association with tumor grade. The data also suggested a redistribution of subcellular localization for HURP and HIF-1α from the nucleus to the cytoplasmic compartment in relation to increasing tumor grade. Analysis of HURP Promoter for HIF-1-binding sites

Abbreviations: CA9, carbonic anhydrase 9; CO_2, carbon dioxide; DLGAP5, discs, large (*Drosophila*) homolog-associated protein 5; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; HIF-1α, hypoxia-inducible factor 1-alpha; HIFs, hypoxia-inducible factors; HSP, heat-shock protein; HURP, hepatoma upregulated protein; IHC, immunohistochemistry; mRNA, messenger ribonucleic acid; O_2, dioxygen; PCa, prostate cancer; pO_2, oxygen tension; qRT-PCR, reverse transcription polymerase chain reaction; RNA, ribonucleic acid; VEGF, vascular endothelial growth factor.

revealed presence of four putative HIF binding sites on the promoter of *DLGAP5/HURP* gene in the non-translated region upstream from the start codon, suggesting association between HIF-1α and the regulation of HURP protein. Taken together, our findings suggest a modulatory role of hypoxia on the expression of HURP. Additionally our results provide basis for utilization of tumor-associated molecules as predictors of aggressive PCa.

Keywords: HURP, hypoxia, prostate cancer, LNCaP, C4-2B, tumor

INTRODUCTION

Prostate cancer (PCa) remains the most common form of cancer affecting men in the Western Hemisphere. In 2015, an estimated 220,800 new cases of PCa are expected to occur in the US and an estimated 27,540 deaths are expected nationwide (1). On the assessment of the aggressiveness of PCa, factors of tumor microenvironment have received increasing attention. In particular, the role of oxygen tension [potentia oxygenii (pO_2)] in tumor biology is of special relevance. Unappreciated for a long time, tumor hypoxia has been recently linked to malignant progression, metastasis, resistance to therapy, and poor clinical outcomes, particularly in the case of PCa (2–4). Likewise, addition of hypoxia as a variable improved prognostic accuracy of aggressive PCa, when added to currently used clinicopathological variables (5). This demonstrates the relevance of hypoxia, a variable of the microenvironment, as a factor of aggressiveness in PCa.

We previously reported that a subset of transcripts of hypoxia-associated genes are relevant as markers of PCa progression (6). One of these genes, namely hepatoma upregulated protein (HURP), was found to be associated with Gleason score and systemic progression of PCa, in addition to being a potential independent outcome predictor in high-risk PCa (6). Moreover, the induction of HURP expression in PCa cells was shown by us to inhibit γ-irradiation-induced apoptosis *via* destabilization of p53 and ATM, key proteins in the modulation of γ-irradiation-induced apoptosis (7). Thus, in addition to its reliability as a prognostic biomarker in patients at high-risk of developing aggressive PCa, HURP seems to trigger PCa resistance to standard antitumor therapies.

It has been generally accepted that conditions of tumor microenvironment including hypoxia promote disease progression and metastasis *via* mechanisms mediated by chromosomal instability, gene amplification, and decreasing tumor sensitivity to DNA damaging agents (8, 9). The above mentioned tumor-associated aberrations contribute to disease development and resistance to therapies (10–12). HURP expression is tightly regulated during cell cycle progression (13–15) and is a component of the Ran-importin β-regulated spindle assembly pathway (16). HURP possesses significant regions of positive charge that are postulated to interact with microtubules (17), suggesting an essential role for this protein on the regulation of cell cycle control. Accordingly, the overexpression of HURP in 293T cells and NIH3T3 embryonic fibroblasts at low serum levels was associated with the promotion of cell growth and colony formation, respectively (14, 18, 19). In contrast, the knockdown of HURP in SK-Hep-1-derived hepatoma model delayed tumor formation (19).

When analyzed in total, the available information utilizing *in vitro* and *in vivo* models suggest that HURP's biological properties are compatible with its role in carcinogenesis. Changes in pO_2 contribute to the aggressiveness of tumors as well, but it is less clear whether hypoxia affects HURP expression. The present study provides the first insight into the biological properties of HURP as a hypoxia-associated gene in tumor development and progression.

MATERIALS AND METHODS

Cell Culture

The human PCa cell lines LNCaP and C4-2B were obtained from the Characterized Cell Line Core Facility, University of Texas MD Anderson Cancer Center. C4-2B cells represent a human bone metastatic PCa and were derived from LNCaP cells. They have more aggressive characteristics when compared to their parental cells (20). For methylation experiments, we utilized LNCaP, DU-145, and PC3 PCa cell lines purchased from the ATCC. Cells were cultured as recommended by the company. All utilized cell lines were genotyped by STR DNA fingerprinting. They were mycoplasma-free following the detection with the MycoAlert™ Mycoplasma Detection Kit (cat # LT07-218; Lonza, Allendale, NJ, USA). Cells were routinely cultured in phenol red RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (Cellgro, Manassas, VA, USA) at 37°C in humidified air enriched with 5% CO_2 and with O_2 content either 20% (normoxic) or 2% (hypoxic) in a CB-150 (Binder, Germany) CO_2 incubator. The cells were trypsinized at 80–90% confluence and plated at the density of 12,000 cells/cm^2. The medium was not refreshed during the course of the experiments. To evaluate cell viability, cells excluding trypan blue were counted by the aid of a hemocytometer.

Measurement of VEGF Concentration in Conditioned Media

Concentrations of vascular endothelial growth factor (VEGF) in supernatants were measured by a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. This ELISA kit has been shown to recognize recombinant human $VEGF_{165}$, recombinant human $VEGF_{121}$, and recombinant human $VEGF_{165b}$. The lower detection limit of the kit was 31.3 pg/mL. Rate of VEGF secretion was expressed as pg/(mL/cell/day).

Quantitative RT-PCR

Total mRNA was isolated using RNeasy Mini kit (Qiagen, Germantown, MD, USA) according to the manufacturer's

instructions: 1-μg RNA was reversely transcribed using SuperScript III First Strand Synthesis (Invitrogen, Grand Island, NY, USA). Subsequently, quantitative PCR was performed with a LightCycler 480 SYBR Green I Master (Roche, Madison, WI, USA). Levels of mRNA were normalized relative to the levels of control ribosomal protein S28 (RPS28) mRNA (21). Data were analyzed by the Delta Delta Ct (2-ΔΔCT) method using Excel program. Primer sequences used were (forward/reverse): VEGF-α: 5′-AGT CCA ACA TCA CCA TGC AG-3′/5′-TTC CCT TTC CTC GAA CTG ATT T-3′, carbonic anhydrase 9 (CA9): 5′-TTT GCC AGA GTT GAC GAG G-3′/5′-AGC CTT CCT CAG CGA TTT C-3′, HURP: 5′-CAT TTT CCT TCA TAT TAT CAA TG-3′/5′-CAT TAT ATG CTA TAG AAG TGA ACA C-3′, and ribosomal protein S28 (RPS28): 5′-TTT TGG AGT CAG AGC GAG AAG-3′/5′-AGC ATC TCA GTT ACG TGT GG-3′.

Preparation of Protein Extracts

Cells were washed with cold phosphate-buffered saline (PBS) and lysed in RIPA buffer. Cells lysates were sonicated on ice for 2 min with 30 s intervals followed by vortexing every 5 min during 45 min at 4°C. Lysates were centrifuged at 24,000 × *g* for 10 min. Supernatants were collected and saved at −80°C. For flash frozen tissue, prostate obtained from PCa patients ($n = 8$) and cystoprostatectomy patients ($n = 4$; used as control) was utilized. Cystoprostatectomy is a surgical procedure, which combines a cystectomy and a prostatectomy for the removal of bladder cancer tumors. Tissues were homogenized in an IKA Work tissue homogenizer (Wilmington, NC, USA). Proteins were extracted from the homogenate with the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's guidelines. Protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA, USA) using bovine γ-globulin (Pierce, Rockford, IL, USA) as standard.

SDS-PAGE and Western Blot Analysis

Fifty micrograms of protein were separated in a 10.5–14% SDS-PAGE gradient gel, transferred to a nitrocellulose membrane, and incubated with blocking buffer containing primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) specific for HURP (diluted 1/400; sc-68540), β-actin (1/5000; Sc-8432), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1/5000; Sc-25778). The anti hypoxia-inducible factor 1-alpha [HIF-1α antibody (1/500)] was from R&D Systems (AF1935). Bound primary antibodies were visualized with HRP-conjugated antibodies specific for human IgG (diluted 1/1000 in blocking buffer; Abcam, Cambridge, MA, USA). After addition of a chemiluminescent substrate (Rockford, IL, USA), the membrane was immediately exposed on a CL-XPosure film (Thermo Fisher Scientific) and scanned with an Epson Perfection 4490 Photo scanner to detect bands. Relative intensities of the bands were quantified using ImageJ software (NIH online, Bethesda, MD, USA); the recorded values were normalized to the intensity of the respective β-actin signal.

Immunohistochemistry

According to the manufacturer's instruction provided in ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA), immunohistochemistry (IHC) was performed on formalin fixed and paraffin embedded samples of benign prostatic tissues, low grade PCa, and high grade PCa. Briefly, 5-μm sections in thickness were deparaffinized and rehydrated followed by antigen retrieval with citrate buffer (pH 6.0) for 20 min. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 min and unspecific bindings were blocked with 10% normal serum in room temperature for 1 h. Next, the slides were incubated with primary antibodies against HURP [rabbit polyclonal diluted 1/100 (cat # ab84509; Santa Cruz Biotechnology, Inc., Dallas, TX, USA)]; hypoxia-inducible factor 1-alpha (HIF-1α) [H-206, rabbit polyclonal diluted 1/50 (cat # sc-10790; Santa Cruz Biotechnology)]; heat-shock protein 60 (HSP60) [mouse monoclonal (LK1) diluted 1/50 (Abcam, Cambridge, MA, USA)]; and VEGF [VEGF (147), rabbit polyclonal diluted 1/50 (cat # sc-507; Santa Cruz Biotechnology)]. Sections were incubated overnight at 4°C. Negative controls were prepared by using a normal anti-rabbit IgG. After PBS wash, the slides were incubated with the components in ABC kits, and with 3,3′-diaminobenzidine (DAB) for color development. Finally, slides were counterstained in hematoxylin and mounted. Under microscope, subcellular localizations of IHC signals for each antibody were defined as membranous, cytoplasmic, or nuclear stain. The stain intensity was graded as no stain, weak, moderate, and strong.

Methylation Assay

DNA (500 ng) from log growing LNCaP, DU-145, and PC3 cell lines was treated with sodium bisulfite and later purified using the EZ DNA Methylation Kit from Zymo Research (San Diego, CA, USA), as recommended. The bisulfite-treated DNA (BST-DNA) was denatured with 0.1 N NaOH for 10 min at room temperature and then amplified at 37°C for 24 h following Illumina's recommended protocols, as we have previously published (22). The amplified DNA sample was then fragmented at 37°C for 1 h and precipitated with 2-propanol by centrifugation at 3000 × *g* for 20 min at 4°C. The DNA pellet was air dried, resuspended in buffer, and hybridized for 24 h at 48°C to the Illumina chips HumanMethylation27 to interrogate 27,000 CpG in more than 14,000 genes. A single-base extension protocol is followed by staining of the beadchip and several washing steps, as recommended by the manufacturer (Illumina). The chips were dried under vacuum for 55 min and scanned using the BeadArray reader (Illumina). The beta fraction (β value) of the HURP gene was obtained utilizing the GenomeStudio software v2011.1. For methylation analysis of HURP, we focused on cg 25465634-4010161.

Bioinformatics Analysis for HURP Promoter

The promoter region of *DLGAP5/HURP* was studied for the binding sites of the transcription factor HIF using reported evidence (23, 24) on the minimal cis-regulatory elements required for HIF-dependent transactivation. Identified potential binding sites were analyzed in context of their location in methylated DNA regions.

The possible binding sites were defined by those having at least 80% of nucleotide content identical to the reported canonical binding site for HIF (23, 24).

Statistics

All reported values represent three independent cell culture experiments expressed as means ± SEM. Data for cell numbers, VEGF release, and mRNA expression were analyzed by two-way ANOVA (pO_2 vs. day), followed by a *post hoc* Student–Newman–Keuls multiple comparisons test. Difference in HURP protein between tumor and normal tissue lysates were analyzed by the Mann–Whitney *U* test. A difference was considered significant at $p < 0.05$.

RESULTS

Sensitivity to Hypoxia

We first evaluated the effect of hypoxia on cell viability in C4-2B cells exposed to 20% O_2 (normoxia) or 2% O_2 (hypoxia). Accordingly, the number of living cells was counted in a time course experiment. Under normoxia (**Figure 1**), a significant increase of cell numbers was noted on day 2 ($p < 0.05$), and increased thereafter to reach a maximum on day 8, while under hypoxia, the increased cell numbers were markedly higher (three-fold), when compared to the cell numbers on day 0 ($p < 0.05$). Numbers remained unchanged until day 6, and slightly increased by 20% at day 8. Cell numbers under normoxia, in contrast, did not experience further variation until completion of the experiment at day 8. Taken together, these findings show that C4-2B cells cultured under hypoxia have a significantly reduced growth kinetics relative to those cultured under normoxia.

Rate of release of VEGF and the hypoxia-inducible pro-angiogenic factor associated with growth and aggressiveness (25) was next evaluated in cell culture supernatants by ELISA. As shown in **Figure 2**, under normoxia, the rate of released VEGF to the conditioned media was constant (2.5 pg/cell/day) up to day 8 (≈2.5 pg/cell/day). Cells cultured under hypoxia had constant induction until day 6. At that time point, a maximum release rate at day 6 (12.3 pg/cell/day) was noted. The release rate of VEGF decreased thereafter ($p < 0.05$) on day 8. At that last time point a release rate of 8.3 pg/cell/day was observed. The results indicate that exposure of C4-2B cells to low levels of oxygen enhances the production of the angiogenic factor, VEGF.

We next assessed whether transcriptional regulation of VEGF was associated with its production in cells cultured under hypoxia. Cells were incubated under normoxia or hypoxia over a period of 8 days, mRNA was extracted, and qRT-PCR was performed. The induction of VEGF expression (61-fold) was noted first on day 4 ($p < 0.05$) in cells growing under hypoxia, when compared with control cells (**Figure 3A**). In cells growing under normoxia, induction of VEGF transcripts was noted as well ($p < 0.05$). Particularly, on day 8, a 10-fold induction was evidenced. Observed levels in cells under normoxia at that time point were comparable to those noted in cells growing at 2% O_2 (15.9-fold over base line).

Similar to VEGF, hypoxia-inducible metabolic enzyme CA9 is overexpressed in cancer cells (26) and has been proposed as a useful marker for hypoxic exposure (27). When measured, the transcripts of CA9 had a sustained induction over the 8 days of exposure to low oxygen (**Figure 3B**). On day 8, nearly 200-fold induction over baseline on day 0 was observed in hypoxic C4-2B cells ($p < 0.05$). In cells cultured under normoxia, no expression of CA9 was noted for most of the analyzed time course, but for

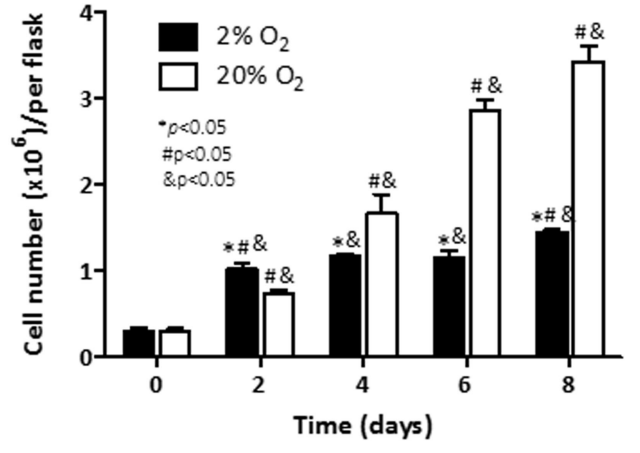

FIGURE 1 | Effect of hypoxia on cell viability: C4-2B cells were cultured at under hypoxia (■) or normoxia (□). Alive cells were counted in triplicate flasks using trypan blue exclusion to differentiate dead cells. *$p < 0.05$ when data at 2% O_2 are compared to values at 20% O_2 at the same time point; #$p < 0.05$ when data at 2% O_2 or 20% O_2 are compared to a previous time point within the same O_2%; and &$p < 0.05$ when a specific data point is compared to the point at day 0 within the same O_2%.

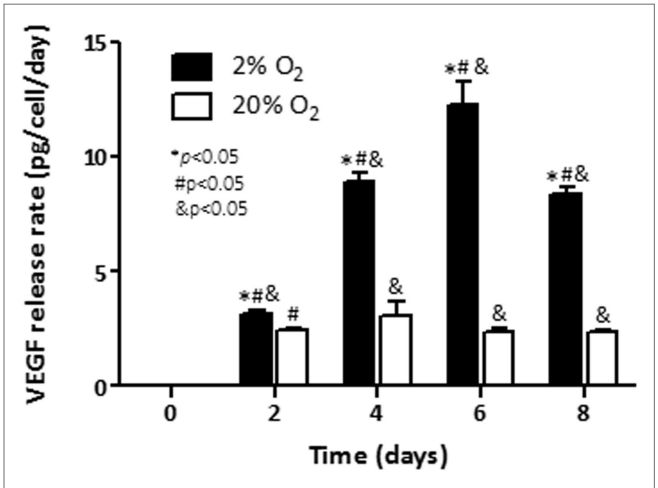

FIGURE 2 | Effect of hypoxia on VEGF production: rates of vascular endothelial growth factor (VEGF) release in conditioned culture media were measured by ELISA for C4-2B cells growing under hypoxia (■) or normoxia (□). *$p < 0.05$ when data at 2% O_2 are compared to values at 20% O_2 at the same time point; #$p < 0.05$ when data at 2% O_2 or 20% O_2 are compared to a previous time point within the same O_2%; and &$p < 0.05$ when a specific data point is compared to the point at day 0 within the same O_2%.

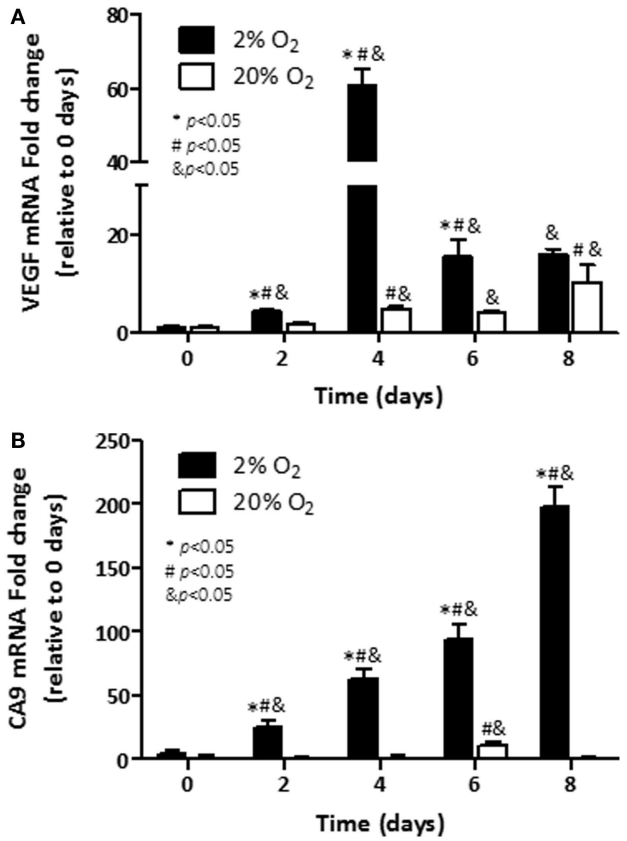

FIGURE 3 | Assessment transcripts of hypoxia-responsive genes: mRNA levels of; (A) Vascular endothelial growth factor (VEGF) and; (B) carbonic anhydrase 9 (CA9) were analyzed in C4-2B cells growing under hypoxia (■) or normoxia (□). $^{*}p < 0.05$ when data at 2% O_2 are compared to values at 20% O_2 at the same time point; $^{\#}p < 0.05$ when data at 2% O_2 or 20% O_2 are compared to a previous time point within the same O_2%; and $^{\&}p < 0.05$ when a specific data point is compared to the point at day 0 within the same O_2%.

11.4-fold induction over basal levels, observed at day 6 ($p < 0.05$). In consistency with our recent publication (28) and reports from others (29, 30), our findings show that PCa cells exhibit features of increased aggressiveness when they are cultured under hypoxia.

Effects of Oxygen Tension on the Expression of HURP

We previously reported on the value of hypoxia-associated genes as prognostic markers of aggressive PCa (6). Among identified genes, transcripts of HURP independently predicted outcome in high-risk PCa (6). Because the role of hypoxia in the regulation of HURP expression is poorly understood, we set out to investigate the effect of varying oxygen levels on its expression. We assessed protein levels by Western blot analysis (**Figures 4A,B**) in C4-2B cells and their precursor cells, the LNCaP cell line (20). HURP protein levels were relatively low in LNCaP cells, compared to those detected in C4-2B at day 0. When quantified (**Figures 4C,D**), HURP protein increased to a similar extent at day 2 in LNCaP cells under normoxia (7.7-fold) and hypoxia (6.8-fold). In C4-2B cells, the increase was more pronounced in cells cultured under normoxia (1.6-fold) relative to those cultured at 2% O_2 (1.2-fold). Irrespective of cell line and oxygen levels, HURP protein was drastically reduced over time. Under normoxia, the reduction of HURP expression was first noted at culture day 4 (21 and 61% decrease for LNCaP and C4-2B cells, respectively) and was reduced thereafter close to 90% over maximum induction, regardless of the cell line, on day 8. Under hypoxic conditions the reduction of HURP protein was more pronounced. This observation was particularly evident in the case of C4-2B cells; HURP protein was reduced by 93% on day 4 and was undetectable at days 6 and 8 in this cell line.

We next studied the expression level of HIF-1α protein (**Figures 4A,B**), a key regulator of cellular responses to variation in pO_2. Expression level of HIF-1α quantified on day 2 (**Figure 4E**) was increased in LNCaP cultured under normoxia (3-fold) and hypoxia (1.8-fold). At that time point, HIF-1α protein was reduced (**Figure 4F**) by 10% in normoxia and 20% in hypoxia in C4-2B cells. From day 4, HIF-1α protein was considerably reduced under normoxic condition. In C4-2B cells, however, a more pronounced decrease (68% reduction at day 8), relative to that observed in LNCaP cells (55% reduction at day 8) was noted when the maximum level of HIF-1α for each corresponding condition was used as reference. The expression of HIF-1α was differentially affected by hypoxia in LNCaP and C4-2B cells. Following maximal expression at day 2 (1.8-fold), levels of expression of HIF-1α remained unchanged until day 8. C4-2B cells growing in hypoxia had 50% reduction in HIF-1α protein from base level after 4 days of culture; however, the decrease was not as pronounced as that observed in cells growing in normoxia. Under low oxygen level, HIF-1α protein levels remained unchanged (42% of base level) until culture day 8.

Next, we studied the transcriptional regulation of HURP under hypoxia using qRT-PCR analysis. In LNCaP cells, induction of HURP transcripts on day 2 (1.3-fold, $p < 0.05$) was evident for cells cultured under normoxic condition (**Figure 5A**). At that time point, cells growing under hypoxia had a 65% decrease in HURP transcripts ($p < 0.05$). From day 4 and thereafter, a pronounced reduction in HURP transcripts was noted irrespective of pO_2 in LNCaP cells. Accordingly, at day 8, a decline of 98 and 94% over maximum levels was noted for cells growing under normoxia and hypoxia, respectively ($p < 0.05$). In accordance with protein expression data in C4-2B cells, early induction of mRNAs for HURP (**Figure 5B**) was noted on day 2. The increase in HURP transcripts observed at 20% O_2 (3-fold, $p < 0.05$), was more pronounced than that noted in cells growing at 2% O_2 (1.2-fold, n.s.). In C4-2B cells growing under normoxia, a reduction to baseline level was noted on day 4 ($p < 0.05$). At this oxygen level, additional decline in HURP transcripts was noted as function of time ($p < 0.05$), 68% on day 6 and 95% decrease on day 8. Under hypoxia, HURP transcripts increased by 27% on day 2 ($p < 0.05$), relative to the levels on day 0. Moreover, a time-dependent reduction of HURP expression was noted on day 4 (~70%), on day 6 (~70%), and on day 8 (85%). These findings suggest that HURP expression is associated to adaptive mechanisms of cellular response to varying oxygen tension.

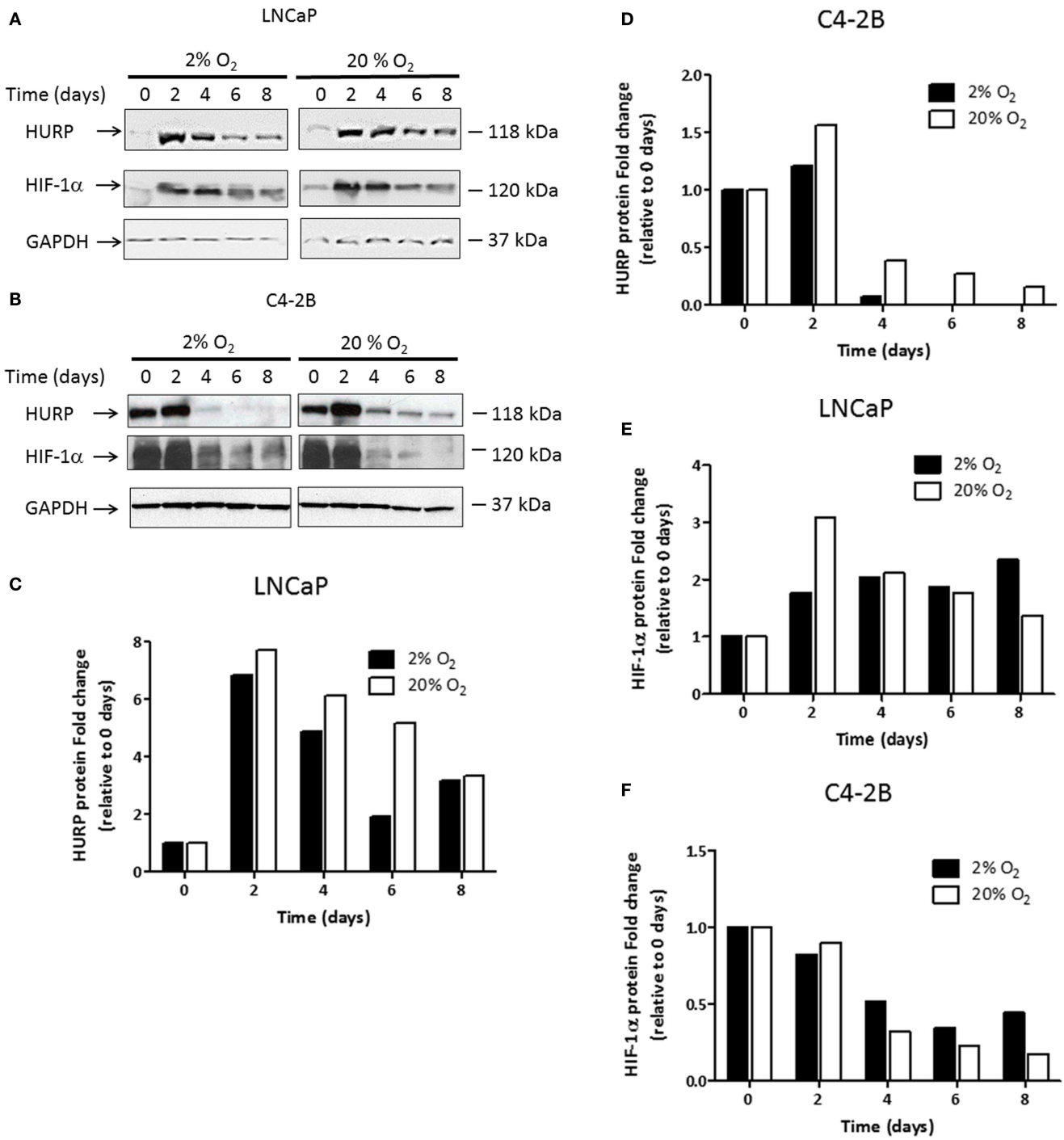

FIGURE 4 | **Assessment transcripts of hypoxia-responsive genes: prostate cancer cells were cultured under hypoxia or normoxia during the indicated time intervals**. Protein lysates were prepared and 50 μg of the total protein was fractionated on the 12% SDS-PAGE, blotted onto the nitrocellulose membrane, and tested against antibodies. Western blot analysis using anti-hepatoma upregulated protein (HURP), hypoxia-inducible factor 1-alpha (HIF-1α), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies in **(A)** LNCaP cells and **(B)** C4-2B cells. The relative intensities of the bands under hypoxia (■) or normoxia (□) were quantified using the ImageJ software, and all the values were normalized to the intensities of the respective GAPDH signal. Data are expressed as the fold change obtained after dividing the optical density of HURP **(C,D)**; and HIF-1α **(E,F)** proteins for a given cell line and time point, relative to the OD observed of the respective protein under the corresponding value at 0 days.

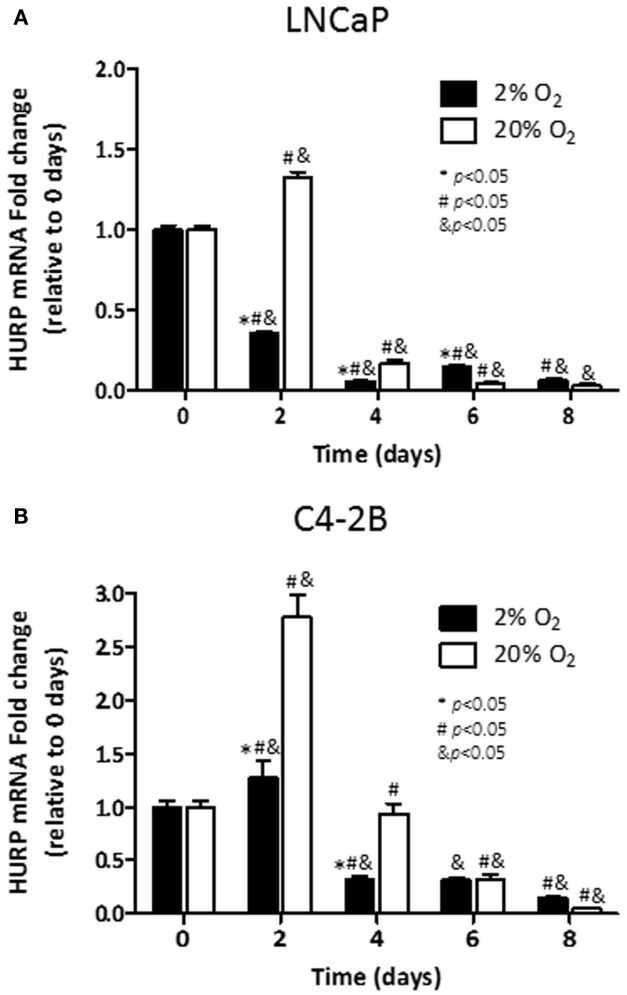

FIGURE 5 | Effect of hypoxia on HURP mRNA: mRNA levels of hepatoma upregulated protein (HURP) were analyzed in LNCaP (A) and C4-2B (B) cells growing under hypoxia (■) or normoxia (□) during 8 days. $^{*}p < 0.05$ when data at 2% O_2 are compared to values at 20% O_2 at the same time point; $^{\#}p < 0.05$ when data at 2% O_2 or 20% O_2 are compared to a previous time point within the same O_2%; and $^{\&}p < 0.05$ when a specific data point is compared to the point at day 0 within the same O_2%.

Levels of HURP Protein in PCa Tissues

Based on our publication regarding the prognostic value of HURP in aggressive PCa (6), we next explored the expression of HURP protein in PCa tumors. For this purpose, we prepared protein lysates from PCa tumors obtained from patients selected over the basis of their HURP mRNA expression and analyzed them by Western blot. Prostatic tissue was obtained from patients suffering with PCa or cystoprostatectomy (controls). Separated proteins were transferred onto a nitrocellulose membrane and blotted against anti-human HURP antibodies. The level of HURP protein (**Figure 6A**) was analyzed by Western blot. Densitometry analysis revealed almost fivefold higher relative in PCa tissue relative to control (cystoprostatectomy) tissue ($p < 0.05$) (**Figure 6B**). These data suggest that protein expression for HURP can be elevated in PCa tumors at the protein level.

Histological Localization of HURP in the Context of Hypoxia-Responsive Molecules

We next performed IHC in FFPE tissue blocks obtained from benign, low grade, and high grade PCa, as shown in **Figure 7A** (low magnification, 10×) and 7B (high magnification, 40×). IHC signals for HURP were moderate in nuclei staining and weak in cytoplasm localization of both benign prostatic epithelia and low grade PCa. High staining was found in the nuclei and cytoplasm of high grade PCa. Since expression of hypoxia-regulated molecules has been found associated with pathology and aggressive phenotype in PCa (3, 29, 31), we next evaluated their histological localization in relation with tumor grade. Similarly to HURP, IHC signals for HIF-1α were moderate in nuclei, and weak in cytoplasm of benign prostatic epithelia, moderate in both cytoplasm and nuclei of low grade PCa, and strong in both nuclei and plasma of high grade PCa. The immune intensity of VEGF, a HIF-1α induced protein, was also analyzed. IHC signals for VEGF were strong in nuclei and moderate in cytoplasm of benign prostatic epithelia, moderated in both nuclei and cytoplasm of low grade PCa, and strong in both nuclei and cytoplasm of high grade PCa. Finally, histological expression of HSP60, the mitochondrial chaperonin, actively involved in the accumulation of HIF-1α (32) was also analyzed. The pattern of IHC signals for HSP60 was different to

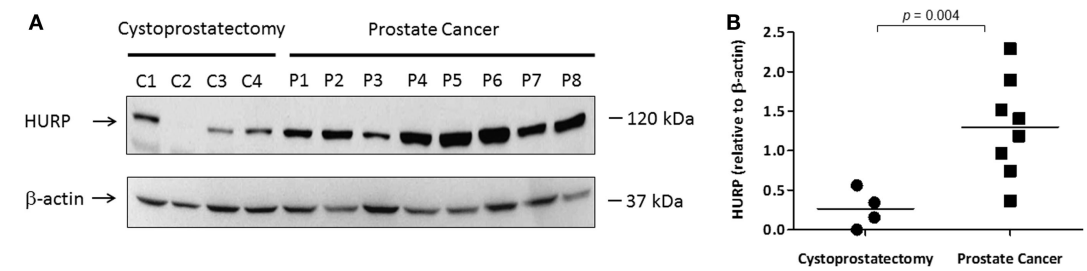

FIGURE 6 | Expression of HURP protein in PCa tissues. Protein lysates (50 μg) extracted from frozen prostate tissues (C1–C4 samples from cytoprostatectomy patients and P1–P8 samples from PCa patients) were separated by SDS-PAGE. **(A)** Hepatoma upregulated protein (HURP) and beta (β)-actin levels in the samples were identified by Western blot as described in Section "Materials and Methods." **(B)** The results were normalized to β-actin. Normalized ratios were compared between controls and PCa tumors. The level of significance was set at $p < 0.05$ between PCa and cytoprostatectomy samples.

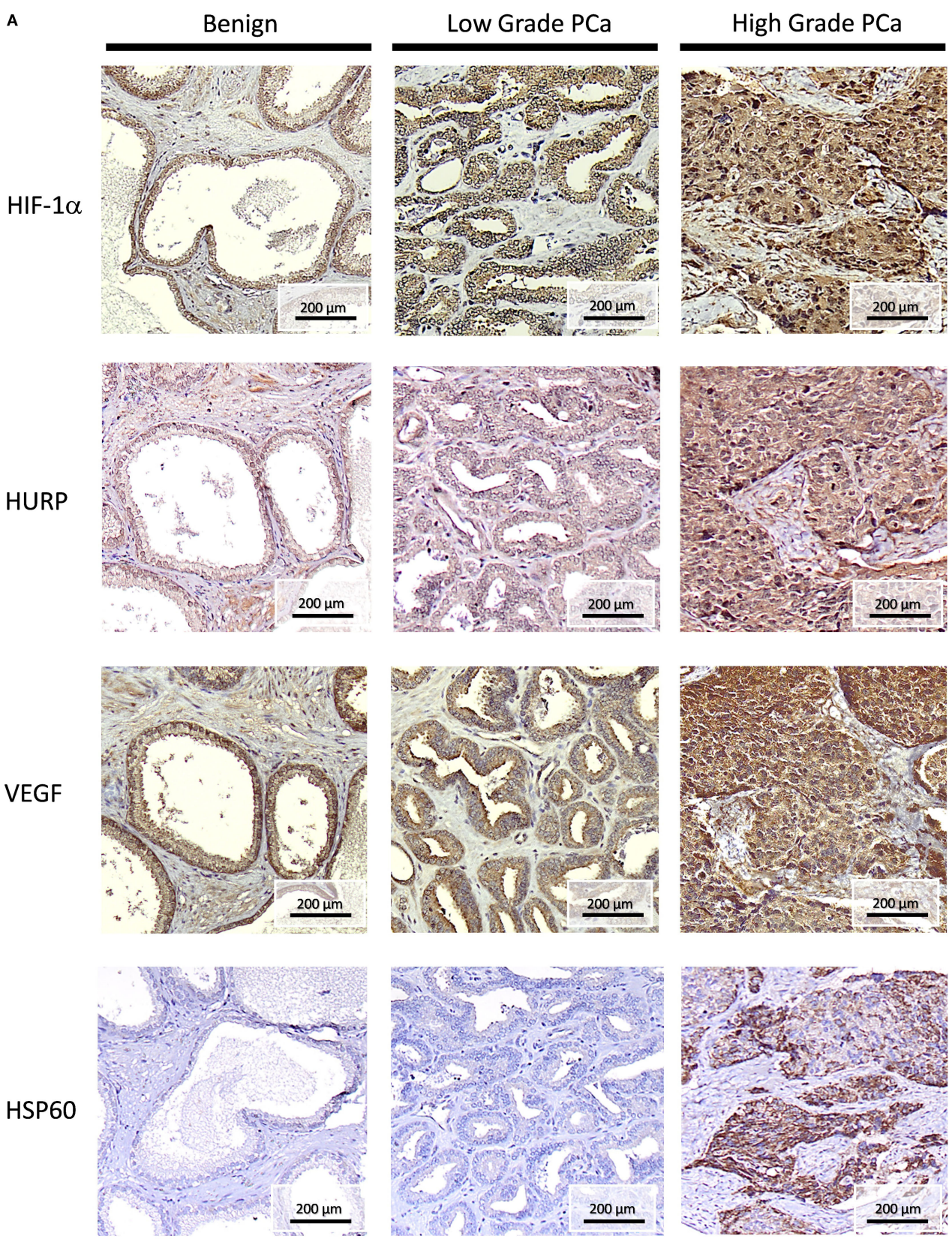

FIGURE 7 | **Continued**

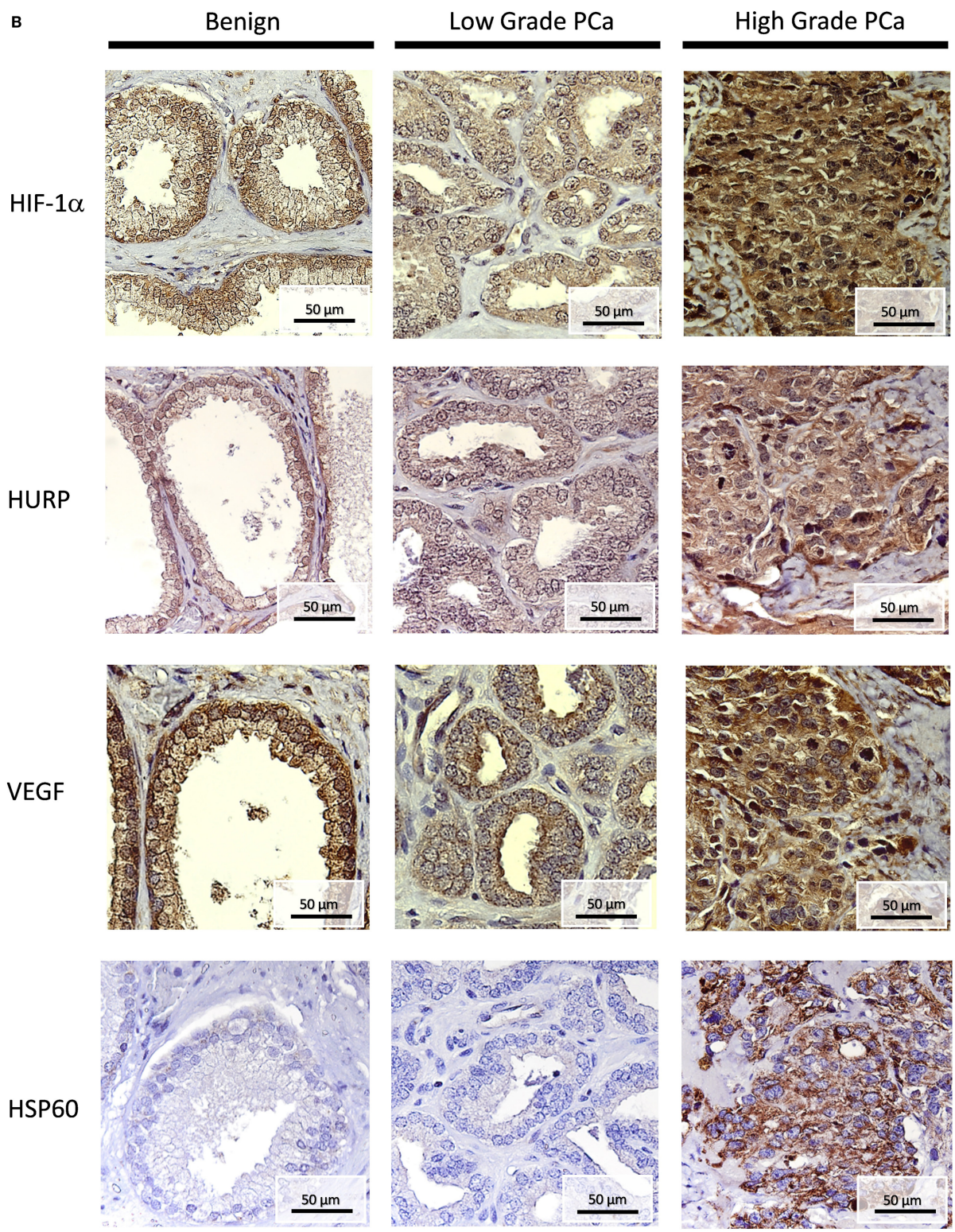

FIGURE 7 | **Histopathology of HURP and hypoxia-sensitive molecules in association with prostate cancer progression**. Hepatoma upregulated protein (HURP), hypoxia-inducible factor 1-alpha (HIF-1α), Vascular endothelial growth factor (VEGF), and Heat-shock protein 60 (HSP60) immunostaining was assessed in benign tissues, Gleason <7 low grade PCa, and Gleason >7 high grade PCa tumors. Slides were counterstained in hematoxylin and mounted. Representative images of HURP, HIF-1α, VEGF, and HSP60 expression in bening tissue, low grade and high grade PCa tissues are shown. **(A)** Analysis of immunostaining intensity. Under a 100× magnification, the stain intensity was graded as no stain, weak, moderate, and strong stain. **(B)** Subcellular localization is shown under a 400× magnification.

that of HIF-1α, HURP, and VEGF: IHC signals were not present in benign prostatic epithelia and in low grade of PCa, and not present in nuclei of high grade PCa cells. However, staining was strong in cellular membrane and cytoplasm of high grade PCa.

Methylation of HURP Promoter Is Associated with the Decrease of HURP Expression in PCa Cell Lines

Aberrant DNA methylation is a common epigenetic aberration in PCa and has led to the identification of markers for disease diagnosis and prognosis (33). To determine whether an epigenetic mechanism contributes to the downregulation of HURP expression in PCa cells, DNA methylation status at the promoter region of the HURP gene was examined in the PCa cell lines LNCaP, DU-145, and PC3. Analysis of the methylation status of the promoter for two independent experiments in the cell lines revealed a significant increase of the methylation level of the promoter of HURP (cg 25465634-4010161) in LNCaP cells, when compared to the methylation level noted in DU-145 or PC3 cell as shown in **Figure 8A**. Interestingly, the noted increase of the promoter methylation was associated with decrease of protein level of HURP (4.5, 1.1, and 0.5 relative density units for PC3, DU-145, and LNCaP, respectively) as evidenced by densitometry analysis of the Western blot signals (**Figures 8B,C**).

Analysis of HURP Promoter for HIF-1-Binding Sites

To show whether the regulation of *DLGAP5/HURP* gene expression is HIF-1-dependent, we analyzed the putative HIF binding sites on the promoter of *DLGAP5/HURP* gene. The analysis of the non-translated region upstream from the start codon (9057 nt), using the 5′-rcgtg-3′ (R = g/a) motif identified by Wenger et al. (24), revealed four putative HIF binding sites, 5′-GCGTG-3′ at position: −8310; 5′-ACGTG-3′ at position: −3815; 5′-ACGTG-3′ at position: −2616; and 5′-GCGTG-3′ at position: −1134 upstream from the start codon (+1). The positions of the identified binding sites are outlined in **Table 1**. This information suggests an essential role for HIF-1 transcription factor in the transcriptional activation *DLGAP5/HURP* promoter in response to hypoxia.

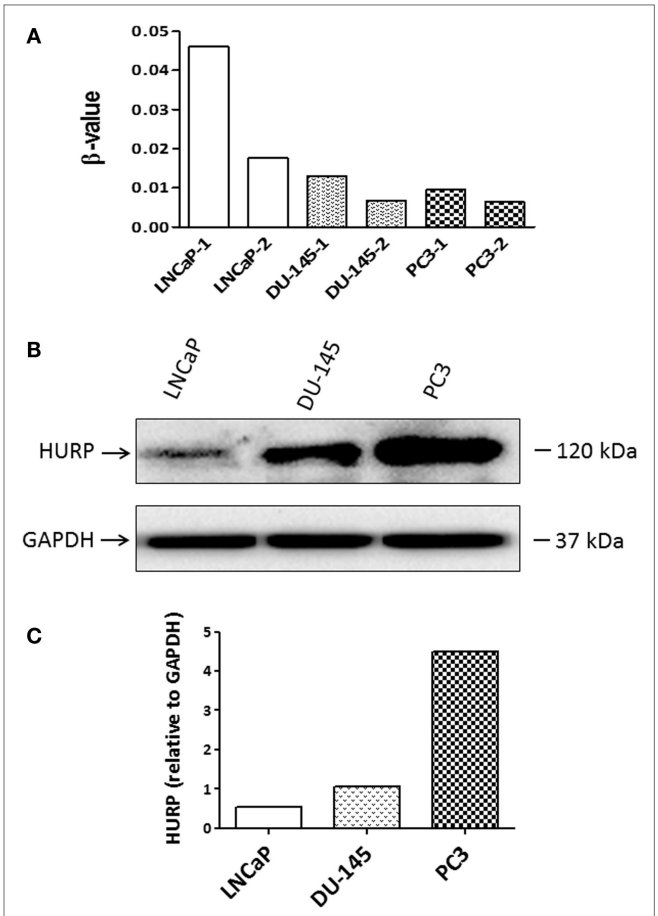

FIGURE 8 | Methylation of HURP promoter and HURP expression in PCa cell lines. **(A)** Epigenetic analysis of the promoter of HURP demonstrated the methylation status in LNCaP, DU-145, and PC3 cells in two independent experiments per cell line. **(B)** Protein lysates (50 μg) extracted from whole cell lysates were separated by SDS-PAGE. Hepatoma upregulated protein (HURP) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels in the samples were identified by Western blot as described in Section "Materials and Methods." GAPDH was used as internal control for loading and transfer. **(C)** The relative intensities of the bands were quantified using the ImageJ software, and all the values were normalized to the intensities of the respective GAPDH signal.

TABLE 1 | Identified putative HIF-1-binding sites in the promoter sequence of the *DLGAP5* gene.

Upstream position	Sequence (5′–3′)
8310–8306	GCGTG
3815–3811	ACGTG
2616–2612	ACGTG
1134–1130	GCGTG

DISCUSSION

We investigated the effect of hypoxia on the expression of HURP in human PCa cells. In the C4-2B cell line hypoxia, as expected, modified cell viability and increased the rate of release and mRNA expression of VEGF, the pro-angiogenic factor. Likewise, mRNA expression of CA9, a marker for hypoxic exposure and tumor aggressiveness, was highly induced in response to low pO_2. Both Western blot and mRNA expression analyses in LNCaP and C4-2B cells showed that HURP expression was reduced by time of culture in normoxia. Hypoxia, however, accelerated the rate of decrease of HURP expression. In contrast to what was observed *in vitro*, HURP expression was increased in PCa tumors, and its elevated expression seems to be associated with tumor grade. Overall, our studies suggest that tumor-associated hypoxia is a relevant determinant of the expression of HURP in PCa cells.

We and others have demonstrated the effects of low pO_2 on viability responses in cultured PCa cells (28, 34–40). In agreement with our previous findings (28) the C4-2B subline, a derivative of the LNCaP cell line, grew slower in hypoxia than cells cultured under normoxia (**Figure 1**). The methodology for assessment of

cell viability was validated by us in the initial phase of our study. We used different cell densities and various timelines including the one referred herein (8 days) or even longer time points. As expected, effects on cell numbers and VEGF production were proportional to cell densities. Although our studies have the limitation of the lack of information regarding the effect of oxygen on cells of those with prostatic carcinoma origin, they add to the information indicating the effect of low pO_2 on the viability responses in cultured PCa cells.

The adaptive response of PCa cells to a changing metabolism and survival environment has been considered a relevant factor of tumor aggressiveness. A component of the response of cancer cells to hypoxia is VEGF. Since hypoxia promotes secretion of this pro-angiogenic factor (30, 41, 42), assessment of its release to the culture medium can be used as an indicator of low pO_2. C4-2B cells cultured under low pO_2 showed increased VEGF release rate. The increase in VEGF secretion rate was transcriptionally driven, as it could be expected (30, 41, 42). The hypoxia-inducible enzyme CA9, overexpressed in cancer cells (26), has been proposed as a marker for hypoxic exposure and tumor aggressiveness (27). Accordingly, we demonstrated that under hypoxic culture CA9 transcripts were elevated nearly by 200-fold in hypoxic C4-2B cells, when compared to cells growing under normoxic conditions. All together these results show that, in agreement to reported findings obtained in other cell lines (28, 43), the C4-2B subline shows characteristics of increased aggressiveness when cultured under hypoxia.

In a previous report, using a validated data mining approach (21, 44–46), we identified hypoxia-associated genes that can be utilized as markers of aggressive PCa (6). Among these genes, transcripts of HURP were associated with Gleason score and systemic progression. We further validated HURP as an independent outcome predictor in high-risk PCa (6). Our published data supported the association of the hypoxic transcriptome and PCa, and provided evidence to sustain the participation of hypoxia-associated genes into the mechanisms of PCa progression. To further support this hypothesis, we scrutinized the promoter region of HURP for binding sites of HIF-1α (**Table 1**). We found four putative HIF binding sites upstream of the HURP transcription start.

Most of the identified binding sites for HIF-1α on the promoter of HURP reside in highly methylated regions, known to be commonly inversely correlated with gene expression and gene methylation in cancer cells (47), including PCa cell lines (48). In support of a notion for the regulatory effects of promoter methylation on HURP expression, we found that the increased methylation of HURP promoter (**Figure 8A**) is associated with a reduction in protein levels in the cell lines LNCaP, DU-145, and PC3 (**Figure 8B**). Epigenetic modification of the HURP promoter, therefore, correlates with reduction of HURP expression. Although the data does not include hypoxia as an experimental variable, these findings suggest that the methylation of HURP promoter is responsible for the reduction of the basal expression of HURP in PCa cell lines. It may be anticipated that low oxygen, a condition known to increase tumor aggressiveness will have regulatory effects on HURP expression. Increased methylation of promoters can repress gene expression by directly preventing binding of transcription factors (49). It may be plausible to suggest that methylation of HURP promoter may prevent the binding of HIF to its promoter region. As a consequence, inhibition of HURP expression may occur. This highly speculative hypothesis is interesting since it suggests a regulatory effect of methylation in silencing HURP gene expression in PCa cells exposed to hypoxia. Future studies are necessary to directly prove the importance of HIF-1α binding sites on HURP promoter, or that of other signaling factors associated with cellular responses to hypoxia, on the regulation of HURP expression under hypoxia condition. Utilization of epigenetic drug treatment of PCa cell lines followed by gene expression analysis for HURP may allow us to assess the effects of pO_2 on the expression of HURP. Those experiments deserve special attention due to the reported effects that promoter methylation exerts on expression of hypoxia-controlled genes (e.g., CA9, studied herein) in context of tumor microenvironment (23).

Analysis of protein levels of HIF-1α showed that expression of this transcription factor, essentially involved in the cellular response to hypoxia, was observed in PCa cells cultured under normoxia. Those findings are consistent with literature showing that HIF-1α is expressed in normoxic conditions as a means to carry out a regulatory role in response to regulatory factors such as cytokines, hormones, and genetic alterations (50, 51). Independent of the cell line, HIF-1α expression was maintained at a higher level under prolonged (8 days) hypoxia. Elevated and sustained HIF-1α expression *via* a non-transcriptional mechanism has been demonstrated to block DNA replication (52). By binding to components of the pre-replicative complexes that assemble at origins of replication, HIF-1α inhibited the activation of minichromosome maintenance helicase, consequently hindering unwinding of the DNA during replication (52). It is likely, that the transcription-independent mechanism of cell cycle arrest in response to hypoxia may be operating in the studied PCa cell lines. Additional experiments are needed to clarify this point.

We analyzed protein expression of HURP in tumors and made associations with the expression of hypoxia-associated molecules. As a follow-up of our report showing increased transcripts of HURP in association with increase in the Gleason score and systemic progression (6), the level of HURP protein analyzed by Western blot (**Figure 6**) and IHC (**Figures 7A,B**) was higher in tumors relative to control tissue. These results agree with a previous study showing elevated expression of HURP protein in fine needle cell aspirates obtained from hepatocellular carcinoma patients (53). In the cited study, further analysis revealed association between positive HURP staining and a shorter disease-free survival (53). In our case, analysis of larger number of samples will allow us to establish an association between HURP expression and aggressive PCa. This study is certainly needed given the inter-individual heterogeneity in HURP expression noted by us in non-tumor tissue as well as in prostate carcinomas. We cannot provide explanation for those findings at this point. However, we suggest that histological observation is certainly a good complement to Western blot analysis. Histological techniques helped us to reveal with higher sensitivity distinct features for tissue expression of HURP not clearly revealed by Western blot analysis.

Our results in PCa tumors, however, somehow, contradict evidence in PCa cell line cultures showing that HURP expression was reduced as a function of incubation time and hypoxia. We attribute the apparent discordant results in part to the presence of complex interactions of the tumor microenvironment observed in tumors (54). Equally relevant is the variability provided by the well-known heterogeneity in PCa (55). A reduction in HURP expression in relation with incubation time is expected based on the cell cycle-associated nature of this protein (13–15). We still do not have direct evidence experimental of the direct role of hypoxia in HURP's expression and the status of other signaling pathways involved in its regulation. Despite of the lack of information, the literature has illustrated pathway-specific differences between tumor cell lines and tumor cells (56). Use of pathway-specific enrichment analysis of publicly accessible microarray data and quantified the gene expression differences between cell lines, tumor, and normal tissue cells for different tissue types including PCa, revealed substantive numbers of genes and associated pathways common between cell lines and tumor cells (56). In that study, however, a fraction of pathways showed expression profiles that differed significantly between cell lines and tumors included cell cycle and a number of metabolic and transcription-related pathways (56). Among them, metabolic pathways closely sensitive to variation of the tumor microenvironment (e.g., ATP synthesis, oxidative phosphorylation, pyrimidine and purine metabolism, and proteasome) were significantly altered in cell lines compared to tumors (56). Over the basis of those results, we can postulate that characteristics of the tumor microenvironment, including presence of metabolites, cell-to-cell interaction in the multilayered structure of solid tumors, among many others may be particularly relevant for the expression of HURP in tumors. Differences in environmental selection pressure between *in vitro* culture and tumor tissue may help to explain, in part, our dissimilar results.

Hypoxia in tumor regions exists due to multiple factors, such as low blood irrigation, aberrant angiogenesis, and excessive oxygen consumption by cancer cells. The hypoxic areas are characterized by variable blood flow and pO_2. In common with other solid tumors, pO_2 in PCa fluctuates, resulting in acute and chronic hypoxia (3). Mostly because of the conflicting information showing lack of correlation between pO_2 values measured in the PCa nidus and clinical outcome (3), assessing pO_2 in PCa tissue is at preliminary stages. A number of publications have indicated a correlation of hypoxia-associated molecules, such as VEGF, HIF-1α, osteopontin, lysil oxidase, and glucose transporter-1 with pathology and patient features in PCa [reviewed in Stewart et al. (3)]. Therefore, hypoxia-associated molecules are reliable as subrogates of hypoxia and indicators of aggressive tumors. In agreement with this notion, tumor expression of HURP, along with expression of HIF-1α, VEGF (a HIF-1α target), and HSP60 (involved in the regulation of HIF-1α protein stability) were associated with PCa progression. It is therefore likely that the relationship between expression of HURP and hypoxia-responsive molecules that we observed *in vitro* has a histological counterpart in PCa tumors. We postulate that localization of HURP protein in PCa tumors should be further explored as subrogate of tumor hypoxia and as marker of aggressive disease.

CONCLUSION

The role of pO_2 in PCa pathobiology has been underappreciated. Tumor-associated hypoxia has been associated with malignant progression, metastasis, resistance to therapy, and poor clinical outcome. Among hypoxia-associated proteins, HURP has multifunctional biological properties, which are compatible with its role in carcinogenesis. A direct cause and effect relationship between hypoxia and HURP expression is yet to be established. Nevertheless, our findings bring insight into the effect of low oxygen on expression of HURP in PCa. Additionally our results provide basis for utilization of tumor-associated molecules as relevant tumor markers. The expression of HURP, together with that of hypoxia-responsive molecules, such as HIF-1α, VEGF, and HSP60, may serve as predictor of aggressive tumors.

AUTHOR CONTRIBUTIONS

IE performed experiments, drafted the manuscript, edited, and approved the final version; MS performed experiments, drafted the manuscript, edited, and approved the final version; TM performed experiments and approved the final version; LF assisted with pathological data analysis and approved the final version; MH contributed to conception and design, edited and approved the final version; XZ performed experiments, wrote the manuscript, edited, and approved final version; JZ performed experiments, edited, and approved the final version; CG contributed to conception and design, wrote the manuscript, edited, and approved final version.

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REVIEW
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Overcoming Hypoxia-Mediated Tumor Progression: Combinatorial Approaches Targeting pH Regulation, Angiogenesis and Immune Dysfunction

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Hypoxia is an important contributor to the heterogeneity of the microenvironment of solid tumors and is a significant environmental stressor that drives adaptations which are essential for the survival and metastatic capabilities of tumor cells. Critical adaptive mechanisms include altered metabolism, pH regulation, epithelial-mesenchymal transition, angiogenesis, migration/invasion, diminished response to immune cells and resistance to chemotherapy and radiation therapy. In particular, pH regulation by hypoxic tumor cells, through the modulation of cell surface molecules such as extracellular carbonic anhydrases (CAIX and CAXII) and monocarboxylate transporters (MCT-1 and MCT-4) functions to increase cancer cell survival and enhance cell invasion while also contributing to immune evasion. Indeed, CAIX is a vital regulator of hypoxia mediated tumor progression, and targeted inhibition of its function results in reduced tumor growth, metastasis, and cancer stem cell function. However, the integrated contributions of the repertoire of hypoxia-induced effectors of pH regulation for tumor survival and invasion remain to be fully explored and exploited as therapeutic avenues. For example, the clinical use of anti-angiogenic agents has identified a conundrum whereby this treatment increases hypoxia and cancer stem cell components of tumors, and accelerates metastasis. Furthermore, hypoxia results in the infiltration of myeloid-derived suppressor cells (MDSCs), regulatory T cells (Treg) and Tumor Associated Macrophages (TAMs), and also stimulates the expression of PD-L1 on tumor cells, which collectively suppress T-cell mediated tumor cell killing. Therefore, combinatorial targeting of angiogenesis, the immune system and pH regulation in the context of hypoxia may lead to more effective strategies for curbing tumor progression and therapeutic resistance, thereby increasing therapeutic efficacy and leading to more effective strategies for the treatment of patients with aggressive cancer.

Keywords: hypoxia, tumor microenvironment, carbonic anhydrase IX, monocarboxylate transporter, angiogenesis, immune checkpoint inhibitors

INTRODUCTION

The tumor microenvironment (TME) is heterogeneous in its composition and dynamic in its evolution. Hypoxia is a prominent component of the TME of solid cancers and is a result of an imbalance between the increasing demand for oxygen and nutrients by rapidly proliferating tumor cells and an inadequate, dysfunctional blood supply resulting from tumor angiogenesis (Lendahl et al., 2009; Bailey et al., 2012). The presence of hypoxia is an independent marker of poor prognosis in many types of human cancer, including breast, non-small cell lung, head and neck, ovarian and cervical cancer (Brahimi-Horn et al., 2007; Semenza, 2014). Furthermore, hypoxia is a functional contributor to several biological processes critical for cancer progression, including angiogenesis, epithelial-mesenchymal transition (EMT), migration/invasion, maintenance of cancer stem cells (CSCs) and the associated CSC niche, metastasis, immune surveillance and resistance to chemotherapy and radiotherapy (Gillies et al., 2012; Parks et al., 2013). Thus, it is not surprising that hypoxia is associated with reduced patient survival in many cancers (Semenza, 2014). Importantly, the development of hypoxia in the TME produces substantial cellular stress that drives adaptive responses by cancer cells aimed at providing advantages for survival, growth and metastasis (Gatenby and Gillies, 2008; Kroemer and Pouyssegur, 2008; Damaghi et al., 2013; Marchiq and Pouysségur, 2015). Targeting the molecular machinery necessary for driving these adaptations is a critical strategy required for the development of effective cancer therapy.

Role of Metabolic Reprogramming and Angiogenesis in pH Dysregulation

Early in tumor development, cancer cells proliferate beyond the limit of diffusion and confront hypoxia, resulting in the inhibition of energy production by oxidative phosphorylation. The increasing demand for energy in the form of ATP and for anabolic precursors by rapidly proliferating cancer cells in this low oxygen environment triggers rapid metabolic reprogramming (Marchiq and Pouysségur, 2015). Tumor cells undergo a "metabolic switch" and, through activation of HIF-1α, shift toward increased use of glycolysis to efficiently maintain cellular bioenergetics and macromolecular biosynthesis in increasingly restrictive growth conditions (Pouyssegur et al., 2006; Marchiq and Pouysségur, 2015). As a result of metabolic reprogramming, cancer cells display increased heterogeneity in glucose metabolism, investing most heavily, but not exclusively, in glycolysis, while continuing to utilize glucose oxidation, albeit at a reduced level in hypoxic regions (Sonveaux et al., 2008; Hensley et al., 2016; Pavlova and Thompson, 2016). Furthermore, it is now recognized that cancer cells use alternative carbon sources, especially glutamine, to promote the tricarboxylic acid (TCA) cycle (Sonveaux et al., 2008; Hensley et al., 2016; Pavlova and Thompson, 2016). The perpetuation of a level of oxidative phosphorylation in the hypoxic microenvironment further reduces the amount of oxygen available, effectively exacerbating hypoxia (Sonveaux et al., 2008; Hensley et al., 2016). Importantly, glycolytic metabolism is associated with increased metabolic plasticity, allows cancer cells to utilize glucose for both energy and biosynthesis (Payen et al., 2015a,b), and fuels the "Warburg effect" in which cancer cells maintain highly glycolytic metabolism even in the presence of oxygen (Marchiq and Pouysségur, 2015). However, a major consequence of the switch to glycolytic metabolism by hypoxic cancer cells is the production of acidic metabolites, including lactate and protons (H^+), which result in increased tumor acidosis and further challenge cell survival (Brahimi-Horn et al., 2011; Webb et al., 2011; Damaghi et al., 2013; Gillies and Gatenby, 2015).

In addition to regulating metabolic reprogramming, the HIF-1-mediated transcriptional program induced by cancer cells in hypoxia drives the process of tumor angiogenesis, a hallmark of cancer progression (Hanahan and Weinberg, 2011). Angiogenesis may be viewed as an initial attempt by the growing tumor to alleviate hypoxia and provide vital nutrients and oxygen to cancer cells (Kerbel, 2008; Rapisarda and Melillo, 2012; Welti et al., 2013; McIntyre and Harris, 2015). However, in contrast to the balanced, tightly regulated multistage process of angiogenesis in normal tissues, tumor angiogenesis is aberrantly regulated and leads to vessels that are tortuous, leaky and dysfunctional (Kerbel, 2008; De Bock et al., 2011; Rapisarda and Melillo, 2012; Welti et al., 2013). As a result, tumor tissues generally exhibit poor perfusion and increased interstitial pressure, a situation that leads to significant biological consequences, including the further development of hypoxic regions, a reduction in nutrient delivery and a reduced ability to remove rapidly accumulating acidic metabolites from the TME (Gillies et al., 2012; Rapisarda and Melillo, 2012). Therefore, the switch to glycolytic metabolism by cancer cells and the angiogenic response to tumor hypoxia collude to create an increasingly acidic, hypoxic TME that fuels adaptations by tumor cells that are geared toward enhanced survival and growth in an otherwise hostile environment.

TARGETING pH REGULATION AND HYPOXIA-DRIVEN ACIDOSIS

A major ramification of the generation of large amounts of acidic metabolites by glycolytic cancer cells, coupled with impaired perfusion and diffusion capacity in hypoxia, is increasing intracellular and extracellular acidosis (Webb et al., 2011; Damaghi et al., 2013; McIntyre and Harris, 2015). The accumulation of lactate and H^+ in glycolytic cells, if left unbuffered, leads to intracellular acidification and apoptosis (Webb et al., 2011; Damaghi et al., 2013), while a slightly alkaline intracellular pH (pHi) is permissive for proliferation (Webb et al., 2011). In contrast, acidification of the TME stimulates breakdown of the extracellular matrix, and promotes migration, invasion and metastasis (Webb et al., 2011; Damaghi et al., 2013). Therefore, to both mitigate the potentially lethal consequences of increasing intracellular acidosis and exploit the advantages of a hypoxic, acidic TME, cancer cells upregulate the molecular machinery necessary to maintain a reverse pH gradient (i.e., alkaline pHi and acidic pHe) that acts to promote survival, proliferation, invasion and metastasis (Webb et al., 2011; Damaghi et al., 2013). Key pH regulatory components that

cancer cells upregulate in hypoxia include membrane-bound, extracellular carbonic anhydrases (CAs), particularly CAIX and CAXII, which maintain an intracellular and extracellular acid-base balance (McDonald et al., 2012; Damaghi et al., 2013; McDonald and Dedhar, 2014), and monocarboxylate transporters (MCTs), especially MCT4, which facilitate lactate extrusion (Marchiq and Pouysségur, 2015). The importance of these factors in regulating pH in hypoxia has resulted in their exploitation as therapeutic targets across a broad spectrum of solid tumors, as discussed below.

Carbonic Anhydrase IX

Carbonic Anhydrase IX (CAIX) is a major effector of the HIF-1-mediated transcriptional response to tumor hypoxia and its critical role in tumor progression is well-recognized (McDonald et al., 2012; McDonald and Dedhar, 2014; Pastorek and Pastorekova, 2015). It is highly expressed in the hypoxic regions of many types of solid tumors, has a very restricted expression profile in normal tissues and is a well-established marker of poor prognosis across a wide spectrum of solid cancers (McDonald et al., 2012; Pastorek and Pastorekova, 2015). Of importance for its utility as a cancer therapeutic target, CAIX is a critical, hypoxia-induced functional effector of several biological processes necessary for cancer growth and metastasis, including pH regulation and cell survival, migration and invasion, maintenance of cancer stem cell (CSC) function, development of the pre-metastatic niche and acquisition of chemo and radioresistant properties (McDonald et al., 2012; McDonald and Dedhar, 2014; Chafe and Dedhar, 2015; Pastorek and Pastorekova, 2015). By catalyzing the reversible hydration of CO_2 to bicarbonate (HCO_3^-) and protons (H^+) at the extracellular surface (Gillies et al., 2008; McDonald et al., 2012; Parks et al., 2013; Sedlakova et al., 2014), CAIX controls an intracellular and extracellular acid-base balance that regulates both survival and invasive properties (**Figure 1**). The HCO_3^- produced by CAIX re-enters the cell through bicarbonate transporters and anion exchangers, thereby buffering intracellular acidosis and facilitating tumor cell survival and growth. The H^+ participate in the generation of an increasingly acidic extracellular environment, a phenomenon recently demonstrated in models of colorectal cancer *in vivo* using hyperpolarized ^{13}C-magnetic resonance spectroscopy (Gallagher et al., 2015), fueling the breakdown of the extracellular matrix and facilitating tumor cell invasion and metastasis (Swietach et al., 2010; McDonald et al., 2012; Parks et al., 2013; Sedlakova et al., 2014). Congruent with its role in regulating pH, several studies have demonstrated that perturbing CAIX function in hypoxia elicits biological consequences that impede cancer progression and demonstrate its utility as a therapeutic target.

Stable depletion of CAIX expression or inhibition of its activity with small molecule inhibitors (discussed in detail below) in the context of hypoxia results in the inhibition of tumor growth across multiple models, including breast cancer (Lou et al., 2011), colorectal cancer (Chiche et al., 2009; McIntyre et al., 2012) and glioblastoma (McIntyre et al., 2012), and demonstrates a critical role for CAIX in cancer cell survival *in vivo*. Perturbation of CAIX function in hypoxia also blunts migration and invasion of cancer cells *in vitro* (Proescholdt et al., 2012; Lock et al., 2013), and inhibits the formation of metastases *in vivo* (Lou et al., 2011; Gieling et al., 2012). Importantly, the role of CAIX in migration, invasion and metastasis is linked to its catalytic activity and the production of H^+, which helps to drive development of acidosis within the extracellular environment and facilitates local invasion through disruption of the extracellular matrix, activation of metalloproteases and increased cell invasiveness (Estrella et al., 2013; Svastova and Pastorekova, 2013; Sedlakova et al., 2014; Pastorek and Pastorekova, 2015). Furthermore, evidence now strongly suggests that CAIX is an integral functional component of CSCs. Several studies have shown that CAIX is required for stemness properties of CSCs in hypoxia (Lock et al., 2013; Papi et al., 2013; Ledaki et al., 2015; Pore et al., 2015), and treatment of orthotopic human breast cancer xenografts with specific small molecule inhibitors of CAIX significantly reduced the CSC population. Increased CAIX expression was also observed in the tumor initiating cell fraction of pancreatic ductal adenocarcinoma in a patient-derived xenograft cell line and targeting CAIX expression in this population of cells with shRNA greatly reduced their tumor initiating capacity (Pore et al., 2015). Together, these studies demonstrate a functional role of CAIX in maintenance of the CSC population *in vivo* and suggest that pharmacologic targeting of CAIX may be effective at reducing or eliminating CSCs in hypoxia, a cell population that is resistant to conventional chemotherapy and radiotherapy. These attributes, together with its ease of accessibility to pharmacologic agents due to its membrane-bound, extracellular catalytic domain, have made CAIX a very attractive target for cancer therapy (Neri and Supuran, 2011; Wilson and Hay, 2011; McDonald et al., 2012; Supuran, 2012; Pastorek and Pastorekova, 2015).

It is clear from the discussion above that therapeutic targeting of CAIX holds potential for enhanced treatment efficacy through the elimination of aggressive cancer cells that have adapted to hypoxia, a realization that has spawned extensive efforts to develop therapeutics targeting CAIX. Two overarching and complementary approaches have been utilized to target CAIX for cancer treatment. One approach has involved the development of therapeutic modalities, especially CAIX-specific small molecule inhibitors that directly target the catalytic activity of CAIX, thereby exploiting the biological roles of CAIX, including pH regulation, migration and invasion, and CSC maintenance (**Figure 1**). A second, complementary approach exploits the tumor-specific upregulation of CAIX as a highly selective "address" to which to deliver CAIX-specific monoclonal antibodies, either alone or in combination with cytotoxic or radioactive warheads to elicit killing of hypoxic tumor cells. Both of these approaches are discussed below.

Small Molecule Inhibitors

The development of small molecule inhibitors of CA activity that are selective for cancer associated extracellular CAs such as CAIX (and CAXII) over other, closely related "off-target" CA isoforms has been an area of intense investigation during the past few years (Neri and Supuran, 2011; Supuran, 2012). These studies have resulted in a large number of novel, potent

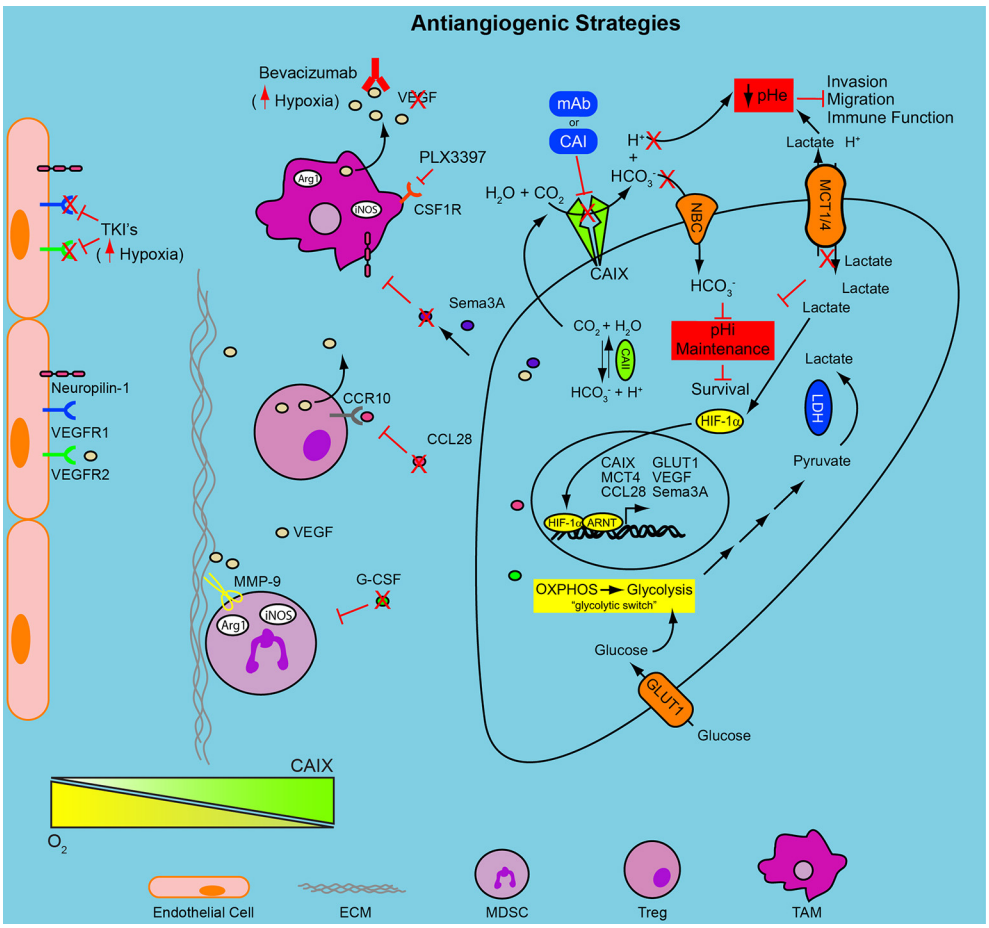

FIGURE 1 | Combinatorial approaches to target the hypoxic TME and anti-angiogenic resistance. Hypoxia induces a HIF-1-mediated signaling cascade that results in nuclear translocation of HIF-1α and activation of hypoxia-regulated genes, including GLUT1, MCT4, and CAIX. The decreased oxygen availability forces tumor cells to become reliant upon glycolysis for energy production, the "glycolytic switch"; the concomitant accumulation of intracellular glycolytic byproducts forces the upregulation of transporters, MCT1/4, to cope with the declining pHi, thus acidifying the extracellular environment. The upregulation of CAIX contributes to the decreasing pHe, through the production of H^+, and to the regulation of pHi through the production of HCO_3^-, which re-enters the cell and buffers intracellular acidosis. The declining pHe activates proteases, increasing migration and invasion and reduces immune function. The use of anti-angiogenic drugs, tyrosine kinase inhibitors or VEGF antibodies (Bevacizumab and TKI's), increases the hypoxic fraction of the tumor and engages the HIF program. This adaptation may then render those cancer cells vulnerable to the metabolic adaptations initiated above. Blocking the pH regulatory machinery, CAIX and MCTs, may be an effective combinatorial strategy for reducing tumor cell survival and overcoming resistance to anti-angiogenic therapy. Stromal cell populations (MDSC, TAM, and Treg) recruited to the hypoxic niches of solid tumors contribute to resistance to anti-angiogenic therapy. Limiting their recruitment to the TME by reducing circulating levels of soluble factors (e.g., G-CSF, CCL28) or antagonizing cellular receptors (PLX3397) needed for their chemotaxis in combination with the modalities mentioned above may reduce resistance to anti-angiogenic therapy and improve therapeutic outcome. pHe, extracellular pH; pHi, intracellular pH; mAb, monoclonal antibody; CAI, carbonic anhydrase inhibitor; HCO_3^-, bicarbonate; H^+, proton; CO_2, carbon dioxide; H_2O, water; NBC, sodium/bicarbonate cotransporter; MCT1/4, monocarboxylate transporter 1 and 4; HIF-1α, hypoxia-inducible factor 1 alpha; ARNT, aryl hydrocarbon receptor nuclear translocator; LDH, lactate dehydrogenase; OXPHOS, oxidative phosphorylation; CAII, carbonic anhydrase II; CAIX, carbonic anhydrase IX; GLUT-1, glucose transporter 1; Sema3A, semaphorin 3A; VEGF, vascular endothelial growth factor; CCL28, chemokine (C-C motif) ligand 28; CCR10, chemokine (C-C motif) receptor 10; Arg1, arginase 1; iNOS, inducible nitric oxide synthase; CSF1R, colony stimulating factor 1 receptor; TCR, T cell receptor; G-CSF, granulocyte colony stimulating factor; TAM, tumor associated macrophage; MDSC, myeloid-derived suppressor cell; Treg, regulatory T cell; ECM, extracellular matrix; MMP9, matrix metalloprotease 9; VEGFR1, vascular endothelial growth factor receptor 1; VEGFR2, vascular endothelial growth factor receptor 2; TKI, tyrosine kinase inhibitor.

CAIX-selective inhibitory compounds, some of which are now being evaluated *in vivo* and in the clinic (McDonald et al., 2012; McDonald and Dedhar, 2014). While initial strategies to enhance CAIX selectivity of the pan CA inhibitor, acetazolamide, were met with limited success (Ahlskog et al., 2009; Chiche et al., 2009), a series of novel, potent, CAIX-selective "next generation" small molecule inhibitors are showing great promise. Several classes of novel CAIX inhibitors, including ureidosulfonamides (Pacchiano et al., 2011), glycosyl coumarins (Touisni et al., 2011) and indanesulfonamides (Dubois et al., 2011) have been used successfully to inhibit tumor growth in preclinical models of hypoxic, CAIX-positive breast cancer (Lou et al., 2011; Pacchiano et al., 2011; Touisni et al., 2011) and colorectal cancer (Dubois et al., 2011), demonstrating that the selective pharmacologic inhibition of CAIX activity elicits an anti-tumor effect *in vivo*. Furthermore, the ureidosulfonamide and glycosyl coumarin

TABLE 1 | Clinical trials targeting pH regulators in cancer.

Target	Drug	Intervention	Phase	Identifier
CAIX	SLC-0111	Advanced solid tumors	1	NCT02215850
CAIX	Girentuximab (cG250)	Kidney Cancer	3	NCT00087022
CAIX	DTP-348	Solid tumors	1	NCT02216669
CAIX	^{177}Lu-cG250	Metastatic RCC	2	NCT00142415
CAIX	AdGMCAIX transduced DC	Metastatic RCC	1	NCT01826877
MCT1	AZD3965	Solid tumors, Gastric and prostate cancer, DLBCL	1	NCT01791595

DC, dendritic cell; RCC, renal cell carcinoma; DLBCL, diffuse large B cell lymphoma.

inhibitors were effective in reducing lung metastases (Lou et al., 2011; Pacchiano et al., 2011), as was a sulfamate inhibitor of CAIX (Gieling et al., 2012), and in depleting CSCs in models of breast cancer metastasis (Lock et al., 2013), showing the value of CAIX inhibitors as therapeutic agents for use in targeting metastasis and chemoresistant tumor initiating cells.

These exciting data and further assessment of lead compounds resulted in the selection of a novel, first in class, highly selective ureidosulfonamide inhibitor of CAIX and CAXII, SLC-0111 (aka U-104; Lou et al., 2011; Pacchiano et al., 2011), for clinical development. Pharmacokinetic and toxicology studies demonstrated negligible toxicity and >10-fold therapeutic index, and SLC-0111 is orally bioavailable. SLC-0111 is now the subject of a first in man, multi-center phase 1 clinical trial (NCT02215850) in patients with solid tumors and is nearing completion of recruitment (**Table 1**). In addition to this pivotal clinical trial, data demonstrating preclinical efficacy of another CAIX inhibitor/radiosensitizer, DTP-348, in breast and colorectal models alone and in combination with either ionizing radiation (Dubois et al., 2013; Ward et al., 2015) or doxorubicin (Rami et al., 2013) has resulted in reports of a phase 1 clinical trial (NCT02216669) in patients with solid tumors (McIntyre and Harris, 2015; **Table 1**). However, this study is not currently open for recruitment of patients. Importantly, if clinical trials utilizing inhibitors of CAIX activity are to be successful, selection of those patients likely to respond to therapy is critical. Only patients whose tumors require CAIX function for survival, growth and metastasis are likely to benefit. Thus, predictive biomarkers, including CAIX expression and additional measures of hypoxia response, will be necessary to guide patient selection for these therapies.

Therapeutic Antibodies against CAIX

Immunotherapy using CAIX-specific monoclonal antibodies (mAbs) may derive its therapeutic efficacy through several mechanisms (McDonald et al., 2012; Pastorek and Pastorekova, 2015) (**Figure 1**). For example, direct binding of the mAb to CAIX can elicit an anti-tumor response due to antibody-mediated cell cytotoxicity (ADCC). Alternatively, high affinity CAIX mAbs capable of receptor-mediated internalization have the potential to provide effective vehicles for targeted delivery of various therapeutic compounds, including cytotoxins and radionuclides, as has been demonstrated clinically for antibody-drug conjugates such as ado-trastuzumab emtansine (T-DM1) in breast cancer (Lambert and Chari, 2014).Thus, the development of CAIX-specific therapeutic mAbs is an active area of research and one with potential to yield important advances in cancer therapy.

Girentuximab (cG250) is arguably the most clinically advanced mAb against CAIX, particularly for renal cell carcinoma (Oosterwijk, 2008; Oosterwijk-Wakka et al., 2013). Initial studies demonstrated that cG250 could elicit antibody-dependent cellular cytotoxicity (ADCC) (Surfus et al., 1996), an established mechanism by which therapeutic mAbs function to destroy tumor cells. This property of therapeutic mAbs is well-established clinically, and a recent study has demonstrated that modification of the Fc region of a CAIX mAb originally identified in a high throughput screen (Xu et al., 2010) increased ADCC *in vitro* and was effective at targeting orthotopic RCC tumors in an immunocompromised mouse model following allogeneic transplantation of human peripheral blood mononuclear cells (Chang et al., 2015). Girentuximab is marketed by WILEX AG under the trade name RENCAREX® and Phase I and II trials demonstrated that this Ab was safe, well-tolerated and able to positively impact disease burden, alone and together with IL-2 treatment (Davis et al., 2007; Zatovicova et al., 2010; Neri and Supuran, 2011; Siebels et al., 2011). However, a phase 3 trial (ARISER; NCT00087022) targeting patients with non-metastatic renal cell carcinoma failed to show an improvement in disease-free survival with treatment (Pastorek and Pastorekova, 2015). It should be noted that this study lacked stratification of patients based on CAIX expression which, if accounted for, showed significant improvement in the subset of patients with high CAIX expression, demonstrating the need for guided patient selection for CAIX-based therapies.

In addition to direct stimulation of the host immune response, mAbs may be used as a target-specific vehicle for the delivery of therapeutic payloads (Scott et al., 2012). Internalization of mAbs is required for delivery of radioisotopes and cytotoxic drugs to cancer cells, and CAIX mAbs have been developed that exploit this functionality. For example, the cG250 mAb can be internalized by cancer cells (Zatovicova et al., 2014), and treatment of xenograft tumors with radioimmunoconjugates employing the cG250 mAb has demonstrated a delay in growth (Brouwers et al., 2004). Recently, a phase II trial in patients with metastatic clear cell renal carcinoma (mccRCC) treated with ^{177}Lu-Girentuximab achieved stable disease in 9 of 14 patients (Muselaers et al., 2015; **Table 1**), demonstrating the potential utility of such conjugates in CAIX-positive disease. Furthermore, a study in which a novel CAIX mAb was conjugated to the microtubule inhibitor monomethyl auristatin E (MMAE) (BAY79-4620) demonstrated efficacy in several preclinical human xenograft tumor models in which the level of efficacy correlated with the level of CAIX expression (Petrul et al., 2011). A phase 1 clinical trial (NCT01028755) involving the treatment of patients with solid tumors with BAY79-4620 was initiated, but was terminated early due to issues related to patient safety. While the basis for the adverse safety profile of this ADC is not known, recent improvements in ADC technology,

including the use of non-cleavable linkers, will certainly provide avenues for continued development of ADCs using CAIX as a target.

In addition to small molecule inhibitors and antibodies, other modalities for targeting CAIX *in vivo* are showing promise. For example, small molecule drug conjugates comprised of an acetazolamide derivative linked to the maytansinoid DM1 were found to accumulate in CAIX-positive lesions and have antitumor effects using the SKRC52 renal cell carcinoma model (Krall et al., 2014). Such conjugates have the advantage of increased access to tumor tissue while enabling delivery of potent cytotoxic compounds (Wichert and Krall, 2015) and engineering of conjugates using current, highly CAIX-specific small molecules may prove effective. Interestingly, a dendritic cell (DC) based vaccine designed to use CAIX as a tumor associated antigen for immune targeting where DCs are engineered to express the GM-CSF-CAIX (AdGMCAIX) fusion protein has been developed. This vaccine has demonstrated preclinical success in a model of RCC when treated in an immunization or intervention setting (Birkhäuser et al., 2013). Based on the recent FDA approval of the DC based vaccine Sipuleucel-T and with other vaccines in phase III evaluation (Palucka and Banchereau, 2013), it will be interesting to see how the AdGMCAIX vaccine progresses clinically as a trial has been initiated (**Table 1**) and is recruiting patients to test this vaccine in metastatic RCC (NCT01826877). Together, these therapeutic strategies provide a robust platform for targeted treatment of hypoxic tumors in patients.

MCT1 and MCT4

Cancer cells relying on glycolysis to support survival and rapid proliferation in hypoxia produce large amounts of acidic byproducts, particularly lactate (Gatenby and Gillies, 2008; Gillies et al., 2012; Marchiq and Pouysségur, 2015; Parks et al., 2015). The production of lactate contributes to intracellular acidosis, a situation requiring an adaptive response to increase lactate efflux and potentiate survival (Parks et al., 2015). Cancer cells enhance the efflux of lactate through the upregulation of members of the MCT family of lactate/H^+ symporters (Doherty and Cleveland, 2013). Two members of this family, MCT1 and MCT4, are upregulated in several cancers, including breast, colorectal, lung, kidney, and glioblastoma (Doherty and Cleveland, 2013; Doyen et al., 2014), and function to regulate lactate transport across the plasma membrane (Halestrap, 2013; Marchiq and Pouysségur, 2015). The differential distribution of MCT1 and MCT4 in cancer cells highlights their distinct roles in lactate transport and demonstrates cooperativity between the two transporters. MCT4 is upregulated in hypoxia as a direct target of HIF-1α and functions to export lactate from hypoxic tumor cells (Doherty and Cleveland, 2013; Parks et al., 2015). Importantly, MCT-mediated extrusion of lactate contributes to acidosis of the TME and plays a role in tumor cell migration and invasion, angiogenesis and immunosuppression (Marchiq and Pouysségur, 2015; **Figure 1**). MCT1, in contrast, is expressed on oxidative tumor cells and functions to import lactate to feed the TCA cycle through conversion to pyruvate, forming a lactate shuttle and engaging a process termed "metabolic symbiosis" (Payen et al., 2015b). The reliance of hypoxic tumor cells on MCT function to adapt to the potentially detrimental consequences of acidosis and the cooperativity that exists between MCT1 and MCT4 in regulating lactate levels in hypoxia has opened the door to targeting both of these transporters for cancer therapy.

Significant efforts are currently underway to target the MCTs with small molecule inhibitors. While the first generation MCT inhibitors were not clinically viable, owing to a lack of MCT specificity and associated toxicity (Marchiq and Pouysségur, 2015), a potent, second generation MCT1 inhibitor from AstraZeneca, AZD3965, has shown anticancer effects in a variety of cancer cell lines (Bola et al., 2014; Polanski et al., 2014) and treatment of tumors *in vivo* reduced tumor growth and increased sensitivity to radiation (Bola et al., 2014). AZD3965 is undergoing phase 1 clinical trials (NCT01791595) for solid tumors and diffuse large B cell lymphoma (Marchiq and Pouysségur, 2015; **Table 1**). There have also been efforts to target CD147/Basigin, a transmembrane glycoprotein that functions as a chaperone for folding and trafficking of MCT1 and MCT4 to the plasma membrane (Doherty and Cleveland, 2013; Marchiq and Pouysségur, 2015). Genetic depletion of CD147, when coupled to MCT1 and MCT4, reduced the growth of colon carcinoma tumors (Le Floch et al., 2011). Directed targeting of CD147 has focused on antibody-based therapeutics and mAbs against CD147 have shown efficacy in preclinical cancer models. However, studies have shown that functional redundancy exists between MCT1 and MCT4, and genetic silencing or pharmacological inhibition of MCT1 in human colon adenocarcinoma cells was effective only when combined with MCT4 depletion (Le Floch et al., 2011; Marchiq and Pouysségur, 2015). These results suggest that combinatorial targeting of MCT1 and MCT4 may be required to elicit a robust therapeutic response. It has also been suggested that a limitation of targeting MCTs may be the potential for on-target toxicity in normal tissues and dose limiting side effects in humans due to their ubiquitous expression and involvement in multiple functions, including metabolism, pH regulation, angiogenesis and the immune response (Marchiq and Pouysségur, 2015). However, despite these potential challenges, emerging data demonstrates the potential power of targeting MCT4, particularly for hard-to-treat cancers.

Upregulated MCT4 expression has been reported in human breast cancer, with especially high levels in aggressive, triple negative breast cancer cells that correlated with decreased overall survival (Doyen et al., 2014). MCT4 was also found to be a key regulator of breast cancer cell metabolism and survival in an unbiased, functional RNAi screen and silencing its expression reduced glycolytic flux, increased dependence on oxidative phosphorylation and glutamine metabolism, and reduced spheroid growth (Baenke et al., 2015). It has also been shown recently that MCT4 is over-expressed in a glycolytic subtype of pancreatic cancer and its depletion in xenograft models significantly impacted tumor metabolism and rapid tumor growth (Baek et al., 2014). To date, inhibitors specific for MCT4 have not become available, although one report has suggested that AstraZeneca is currently testing a potent, specific

MCT4 inhibitor (Marchiq and Pouysségur, 2015). It remains to be seen whether co-targeting of MCT1 and MCT4 in the context of hypoxia will provide therapeutic benefit. However, together with CAIX, MCT4 plays an important role in the maintenance of glycolytic flux and pH regulation in hypoxic tumor cells and suggests that cotargeting of MCTs and CAIX may serve to further limit the growth of hypoxic solid tumors.

TARGETING ANGIOGENESIS

Inhibition of vascularization by treatment with inhibitors of angiogenesis, generally termed anti-angiogenic agents, thereby effectively starving tumor cells of nutrients and oxygen, is a clinically validated strategy for cancer therapy (De Bock et al., 2011; Jain, 2014). However, what was seen initially as a panacea for cancer patients has fallen short of expectations. In particular, many patients fail to respond to anti-angiogenic therapy and those who do often show only a modest survival benefit (Rapisarda and Melillo, 2012; McIntyre and Harris, 2015). While vascular "normalization" can occur and leads to reduced hypoxia and interstitial pressure in the TME, thereby increasing perfusion and improving delivery of chemotherapeutic agents (Jain, 2014; McIntyre and Harris, 2015), the most frequent response to anti-angiogenic therapy is vascular regression and vessel pruning, resulting in increased intratumoral hypoxia (Rapisarda and Melillo, 2012; McIntyre and Harris, 2015), as assessed by hypoxic gene signatures and increased expression of hypoxia-induced effectors, including CAIX.

Many patients are innately resistant to anti-angiogenic agents or rapidly develop acquired resistance in response to treatment. Amongst several resistance mechanisms that have been identified to enable cancer cells to circumvent angiogenesis blockade (van Beijnum et al., 2015), the development of increased intratumoral hypoxia as a result of extensive vessel pruning is of critical importance (Rapisarda et al., 2009; Hu et al., 2012; McIntyre et al., 2012; Rapisarda and Melillo, 2012; Kim et al., 2013; McIntyre and Harris, 2015). Treatment with anti-angiogenic agents has resulted in increased invasiveness and consequently metastases preclinically (Ebos et al., 2009; Pàez-Ribes et al., 2009). However, at least one retrospective study suggests that this is not the case in the clinical setting, although the patients investigated often had metastatic disease at the time of treatment initiation (Miles et al., 2011). In addition, it was recently demonstrated that this difference between the preclinical and clinical realms was likely the result of patients being treated with chemotherapy in combination with anti-angiogenic agents, as this combination tested preclinically eliminated the increased metastasis observed from treatment with anti-angiogenic agents alone (Paez-Ribes et al., 2015).

Since the hypoxia induced by anti-angiogenic therapy stimulates adaptations by the tumor cells that promote therapeutic resistance, it has been suggested that these adaptations may now be critical for survival, producing a type of synthetic lethality termed "induced essentiality" (McIntyre and Harris, 2015). Targeting critical downstream effectors of hypoxia, or HIF-1α itself, has provided a therapeutic advantage in preclinical models (Rapisarda et al., 2009; Hu et al., 2012; McIntyre et al., 2012; Kim et al., 2013). Furthermore, as a consequence of treatment induced hypoxia, CAIX expression is significantly upregulated, suggesting that targeting CAIX in combination with anti-angiogenic agents may provide an effective therapeutic strategy (Hu et al., 2012; Kim et al., 2013). McIntyre and colleagues provided proof of principle data for this co-targeting strategy by showing that genetic depletion of CAIX in combination with bevacizumab in models of colorectal cancer and glioblastoma resulted in a significant delay in tumor growth (McIntyre et al., 2012). Similar results were observed upon treatment with the broad spectrum carbonic anhydrase inhibitor, acetazolamide. Acetazolamide was also utilized recently in combination with bevacizumab in a model of cholangiocarcinoma. While bevacizumab alone showed considerable efficacy in this model, the combination of bevacizumab with acetazolamide further delayed tumor growth (Vaeteewoottacharn et al., 2016). However, the lack of specificity of acetazolamide is a significant caveat of these studies, and investigation of therapeutic combinations using inhibitors specific for CAIX, such as SLC-0111, should be undertaken. In addition to VEGF antibodies, tyrosine kinase inhibitors have been utilized to block angiogenic signaling at the level of the cellular receptors. Treatment of patients with metastatic clear cell renal cell carcinoma with sunitinib results in objective response rates of 40% (Molina et al., 2014). Combinatorial treatment with the CAIX antibody, cG250/Girentuximab, in combination with sunitinib was tested clinically in patients with metastatic RCC (NCT00520533). Unfortunately, the trial ended early due to toxicity issues. Based on preclinical studies, it is clear that this is an area that warrants careful evaluation clinically with rational therapeutic combinations targeting treatment induced hypoxia.

The hypoxic niche of solid tumors is an environment that drives aggressive tumor cell behavior and is a known source of cancer stem cells (CSCs; Currie et al., 2013). As conventional chemotherapy and radiation treatment are known to be less effective in this microenvironment, rational drug combinations need to be developed in order to effectively overcome treatment failure due to therapy induced adaptations. Interestingly, sunitinib and bevacizumab treatment of breast cancer xenografts was demonstrated to increase intratumoral hypoxia and the population of stem cells found within the tumor (Conley et al., 2012). Therefore, it is conceivable that combining drugs that increase intratumoral hypoxia with a drug that can effectively eradicate the CSC pool in the hypoxic niche, such as CAIX inhibitors, may overcome treatment resistance. Alternatively, therapeutic initiatives aimed at promoting re-oxygenation of tumor tissues, including vessel normalization strategies (Jain, 2014) and the use mild hyperthermia (Moon et al., 2010; Datta et al., 2015) in combination with radiotherapy or chemotherapy are proving successful in the clinic. Recent technological advances in the delivery of hyperthermia as a radio- and chemosensitizer, together with the relative absence of additional significant toxicity, have reinvigorated efforts using thermoradiotherapy and thermochemotherapy (Moon et al., 2010; Datta et al., 2015) as combinatorial treatment strategies for hypoxic, solid tumors.

TARGETING IMMUNE DYSFUNCTION

The cellular composition of solid tumors consists of many stromal cell types in addition to the epithelial cancer cells (Gabrilovich et al., 2012). There exists an extensive communication network between the stroma and the tumor that ultimately results in the hijacking of the stroma by the tumor supporting disease progression, increased invasiveness and enhanced metastatic propensity, angiogenesis, therapeutic resistance and resistance to immune cell eradication mechanisms (Gabrilovich et al., 2012; Quail and Joyce, 2013). The contribution of the various stromal cell types to the tumor have been reviewed extensively elsewhere (Hanahan and Coussens, 2012; Junttila and de Sauvage, 2013). Here, we will focus on three very prominent stromal cell types that mediate immunosuppression in the hypoxic TME and allow the tumor to escape immune detection: myeloid-derived suppressor cells (MDSC), regulatory T cells (Treg) and tumor associated macrophages (TAMs). We will discuss the various mechanisms by which these cell types contribute to tumor progression and therapeutic resistance in the context of hypoxia, as well as the efforts to target the immunosuppressive functions of these cell populations, especially using immune checkpoint inhibitors.

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSC) are an immature myeloid cell population named for their potent immunosuppressive ability toward T and NK cells (Bronte, 2009; Gabrilovich and Nagaraj, 2009) which play a prominent role in tumor progression (Talmadge and Gabrilovich, 2013). In healthy individuals this population is present in very small numbers in the bone marrow, lacks immunosuppressive activity and readily differentiates into mature macrophages and neutrophils. However, in mice and patients with cancer this differentiation is diverted by the abnormally high levels of tumor-derived myeloid growth factors in the blood (Messmer et al., 2015) and circulating MDSC levels have been shown to correlate with increased disease burden in patients with cancer (Diaz-Montero et al., 2009; Messmer et al., 2015). Recruitment of MDSC to tumors has been shown to occur in response to multiple soluble factors, although the precise chemotactic factors involved vary depending on the tumor model interrogated (Acharyya et al., 2012; Gabrilovich et al., 2012; Wesolowski et al., 2013; Highfill et al., 2014; Palazon et al., 2014). Together with these soluble factors, tumor hypoxia, via HIF-1α stabilization and the production of downstream effectors, plays a role in facilitating MDSC mobilization into the circulation and recruitment to tumors (Du et al., 2008; Erler et al., 2009; Wong et al., 2011; Sceneay et al., 2012; Chafe et al., 2015; **Figure 2**), and regulates the immunosuppressive functions of MDSC (Corzo et al., 2010). Recently, hypoxia was further implicated in expanding the immunosuppressive arsenal of MDSC through the HIF-1 mediated upregulation of programmed cell death 1 ligand 1 (PD-L1; Noman et al., 2014). Moreover, the response to hypoxia has been shown to impact MDSC by triggering rapid differentiation into TAM (Corzo et al., 2010). The same study also identified increased F4/80 (macrophages) positivity relative to Gr1 (MDSC) in hypoxic, pimonidazole staining regions of tumors, suggesting that TAM arising from MDSC may be vitally important for tumor progression in these niches.

MDSC suppress the anti-tumor immune response (Gabrilovich et al., 2012), therapeutic efforts to restore this response (Highfill et al., 2014; Kim et al., 2014), as well as limit the efficacy of anti-angiogenic (Shojaei et al., 2007, 2009) and chemotherapies (Acharyya et al., 2012). Consequently, a number of therapeutic approaches to target MDSC are being explored which can be grouped into 4 distinct categories: (1) forced differentiation (2) targeting soluble factors mediating expansion and/or recruitment (3) direct targeting with the use of chemotherapy (4) reducing their immunosuppressive capacity. Each of these therapeutic strategies has been reviewed extensively in Wesolowski et al. (2013) and targeting MDSCs using immune checkpoint inhibitors is discussed below.

Regulatory T Cells

Regulatory T cells (Treg) are another important stromal cell population that supports tumor progression by contributing to immune evasion (Facciabene et al., 2012). Similar to MDSC, Treg are normally required to keep the immune system tightly regulated and prevent autoimmunity. Treg are found in one of two forms: natural Treg (nTreg) which are thymically derived, or induced Treg (iTreg) which can be induced from naïve CD4 T cells. The fate and function of these populations are controlled by the expression of the FoxP3 transcription factor (Facciabene et al., 2012). Treg have been identified in many cancers where they have been associated with worse prognosis (Zou, 2006; Facciabene et al., 2012).

Like MDSC, Treg have a number of weapons to reduce anti-tumor immunity such as the secretion of immunosuppressive cytokines, direct killing of effector cells, increasing local pools of the toxic metabolite adenosine and disruption of dendritic cell function (Facciabene et al., 2012). Hypoxia-induced CCL28 secretion by the tumor has been shown to induce the CCR-10-dependent recruitment of Treg to tumors (**Figure 2**), exacerbating the immunosuppressive pressure that allows the tumor to evade host destruction (Schlecker et al., 2012). It has also been shown that Treg are important for the formation of new blood vessels in the tumor (Facciabene et al., 2011). Importantly, these cells can be targeted by immune checkpoint inhibitors such as ipilimumab (anti-CTLA-4; Selby et al., 2013).

Tumor Associated Macrophages (TAM)

Tumor-associated macrophages make up the largest population of stromal cells within growing solid tumors and while they are generally described as belonging to the classically activated M1 or the alternatively activated M2 phenotype, these cells actually display a great degree of phenotypic plasticity (Noy and Pollard, 2014). The M1 phenotype is tumoricidal and pro-inflammatory, whereas the M2 phenotype is pro-tumoral and suppresses the inflammatory response (Noy and Pollard, 2014). The recruitment of macrophages to tumors is largely dependent upon soluble factors released into the circulation

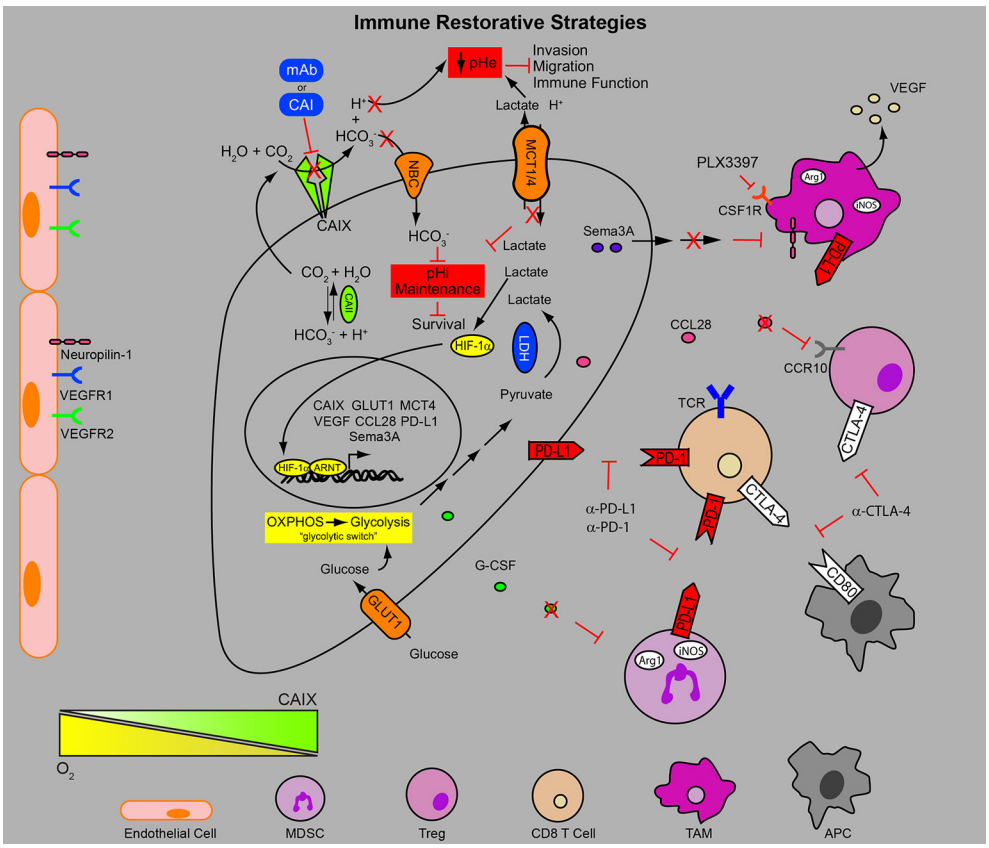

FIGURE 2 | Combinatorial approaches to target the hypoxic TME and immune dysfunction. In addition to the metabolic adaptations initiated by engagement of the HIF program in the hypoxic niches of the tumor contributing to the migratory and invasive phenotype of the cancer cells, metabolic byproducts (e.g., low pH) reduce anti-tumor immune function. Furthermore, many HIF target genes (e.g., VEGF, Sema3A, CCL28) secreted by the tumor result in chemotaxis of immune suppressive cell populations (MDSC, TAM, and Treg), limiting the function of anti-tumor immune response. In addition HIF-1-mediated upregulation of PD-L1 by the tumor, MDSC and TAM populations provides an additional layer of resistance to immune surveillance mechanisms. Consequently the use of checkpoint inhibitors to restore anti-tumor immunity to eradicate the therapy resistant cells in the hypoxic TME in combination with inhibitors of pH regulation and recruitment of immune suppressive stromal cell populations may circumvent many of the hurdles facing anti-tumor immunity. pHe, extracellular pH; pHi, intracellular pH; mAb, monoclonal antibody; CAI, carbonic anhydrase inhibitor; HCO_3^-, bicarbonate; H^+, proton; CO_2, carbon dioxide; H_2O, water; NBC, sodium/bicarbonate cotransporter; MCT1/4, monocarboxylate transporter 1 and 4; HIF-1α, hypoxia-inducible factor 1 alpha; ARNT, aryl hydrocarbon receptor nuclear translocator; LDH, lactate dehydrogenase; OXPHOS, oxidative phosphorylation; CAII, carbonic anhydrase II; CAIX, carbonic anhydrase IX; GLUT-1, glucose transporter 1; Sema3A, semaphorin 3A; VEGF, vascular endothelial growth factor; CCL28, chemokine (C-C motif) ligand 28; CCR10, chemokine (C-C motif) receptor 10; Arg1, arginase 1; iNOS, inducible nitric oxide synthase; CSF1R, colony stimulating factor 1 receptor; TCR, T cell receptor; G-CSF, granulocyte colony stimulating factor; TAM, tumor associated macrophage; MDSC, myeloid-derived suppressor cell; Treg, regulatory T cell; APC, antigen presenting cell; VEGFR1, vascular endothelial growth factor receptor 1; VEGFR2, vascular endothelial growth factor receptor 2; CTLA-4, cytotoxic T lymphocyte antigen-4; PD-1, programmed cell death receptor 1; PD-L1, programmed cell death 1 ligand.

by the tumor, including CCL2, CCL5, VEGF, Endothelins, endothelial monocyte activating polypeptide (EMAP) II and colony stimulating factor 1 (CSF1), resulting in the mobilization of monocytes from the bone marrow (Murdoch et al., 2004; Franklin et al., 2014). Once macrophages reach the tumor, migration to the hypoxic regions is driven by VEGF, EMAPII, Endothelin-2, CXCR4 and Semaphorin3A (Murdoch et al., 2004; Casazza et al., 2013; **Figure 2**). While tumor hypoxia was thought to play a prominent role in polarizing TAMs, recent findings suggest that tumor hypoxia merely enhances the expression of pro-angiogenic and metastasis promoting genes (Laoui et al., 2014). TAMs expressing M2 markers were found in the same proportion in well oxygenated and poorly oxygenated tumors in a model of PHD2 haploinsufficiency (Laoui et al., 2014). However, immunosuppressive M2 macrophages are found in greater number in hypoxic regions of solid tumors compared to M1 macrophages (Murdoch et al., 2004; Movahedi et al., 2010; Laoui et al., 2014), supporting a role for hypoxia in M2 TAM chemotaxis and retention in these niches. Hypoxic TAMs provide a rich source of VEGF, augmenting tumor angiogenesis, blood vessel dysfunction and exacerbation of the hypoxia (Stockmann et al., 2008; **Figure 2**). VEGF secreted by macrophages is also responsible for a role of macrophages in assisting the transit of migratory tumor cells into the vasculature to facilitate their entrance into the circulation (Harney et al., 2015). These macrophages, together with the migratory cancer cells and the endothelial cells, form the "tumor microenvironment of metastasis" (TMEM). The TMEM has been observed in the

tumors of breast cancer patients where it has been shown to be predictive of metastatic potential in $ER^{+}HER2^{-}$ cases (Rohan et al., 2014).

The highly immunosuppressive nature of macrophages poses a significant hurdle to anti-tumor immunity especially in hypoxic regions of tumors and TAM presence has been shown to impact breast cancer patient survival (Doedens et al., 2010; DeNardo et al., 2011; Casazza et al., 2013). The stabilization of HIF-1α is required for the immunosuppressive activity of macrophages by increasing the expression of Arg1 and iNos activity (Doedens et al., 2010). Therefore, eliminating this immunosuppressive population is critical for restoring anti-tumor immunity. Recently it was shown that blocking the recruitment of macrophages using antibody blockade of CSF1 or CSF1R antagonism with the small molecule inhibitor PLX3397, which is being evaluated clinically, resulted in a CD8 T cell dependent reduction in tumor burden in the MMTV-PyMT model (DeNardo et al., 2011). As hypoxia has also been shown to upregulate PD-L1 expression on TAMs, treatment of tumors with high TAM presence with immune checkpoint inhibitors may also restore anti-tumor immunity (Noman et al., 2014).

Targeting the Immune Response with Checkpoint Inhibitors

Recent clinical breakthroughs in targeting tumor immunology through the use of immune checkpoint inhibitors have revitalized interest in the field (Hodi et al., 2010; Brahmer et al., 2012; Topalian et al., 2012; Wolchok et al., 2013). However, despite the significant measurable responses observed clinically, treatment with inhibitors to PD-1 and CTLA-4 have only produced objective responses in 10–28% of patients treated when utilized as a monotherapy (Hodi et al., 2010; Brahmer et al., 2012; Topalian et al., 2012). Achieving an understanding of the mechanisms limiting the response to these antibodies as well as the identification of biomarkers that can be utilized to stratify patients that will respond to this therapy alone or may require a combinatorial approach will improve patient outcome.

In order to achieve activation, a T cell must recognize a peptide antigen presented by a major histocompatibility complex (MHC) molecule on the surface of an antigen presenting cell (APC) or cancer cell via its T cell receptor (TCR). When this is done in the context of appropriate stimulating signals through the engagement of costimulatory molecules (e.g., CD28) with their cognate receptors (e.g., CD80), activation of a T cell occurs (Pardoll, 2012; Mahoney et al., 2015). Fail safe mechanisms exist to regulate the activity of the T cell and the duration of the response through the activity of inhibitory receptors or checkpoint molecules. As an example, molecules such as PD-L1 expressed on the surface of APCs or cancer cells binds to its cognate receptor PD-1. This engagement reduces T cell activation and cytolytic activity (Pardoll, 2012; Mahoney et al., 2015; Topalian et al., 2015). Similarly, the expression of cytotoxic T lymphocyte antigen (CTLA)-4 binds, with higher affinity than CD28, to CD80 and CD86 on APCs achieving the same reduction in T cell activation (Pardoll, 2012; Mahoney et al., 2015; Topalian et al., 2015). This is the basis for immune checkpoint blockade where antibodies such as ipilimumab (α-CTLA-4), pembrolizumab (α-PD-1) and nivolumab (α-PD-1) interfere with the engagement of the inhibitory ligand with the receptor to maximize anti-tumor immunity (**Figure 2**). However, there are significant hurdles to initiating and maintaining this immune response in various cancers, which include the presence of a hypoxic, acidic tumor microenvironment (Kareva and Hahnfeldt, 2013; Motz and Coukos, 2013; Palazon et al., 2014; Joyce and Fearon, 2015; Wherry and Kurachi, 2015).

Recently it has been shown that hypoxia contributes to the limited success of an anti-tumor immune response (Barsoum et al., 2014a). Hypoxia triggers the shedding of major histocompatibility complex (MHC) class I chain-related molecule A (MICA), a ligand required for natural killer (NK) cell and effector cell activation, in an ADAM10 dependent manner, contributing to the evasion of immune surveillance mechanisms (Siemens et al., 2008; Barsoum et al., 2011). Furthermore, hypoxia-induced autophagy was demonstrated to limit NK-mediated cell death in models of breast cancer and melanoma due to autophagosome degradation of granzyme B (Baginska et al., 2013). However, it appears that NK cell mediated tumor killing is one of several immune surveillance mechanisms in place in the syngeneic models interrogated as complete tumor regression was not observed upon autophagy inhibition (Baginska et al., 2013). Nevertheless, it is clear that cancer cells in low oxygen become increasingly more difficult for the immune system to eliminate.

It was identified recently that hypoxia, through the action of HIF-1α, increases the expression of PD-L1 by tumor cells and by tumor-infiltrating MDSC (Barsoum et al., 2014b; Noman et al., 2014). In both cases, HIF-1α was found to bind directly to the hypoxia response element (HRE) in the PD-L1 promoter. It has been known for a number of years now that cancer cells within the tumor are capable of expressing PD-L1 in response to interferon-γ, a significant counter measure to the activation of T cells (Dong et al., 2002; Curiel et al., 2003; Pardoll, 2012). Barsoum and colleagues demonstrate *in vitro* that tumor-educated T cells are much less capable of eliciting a cytolytic effect on hypoxic cancer cells in a PD-L1 dependent manner (Barsoum et al., 2014b). However, preclinical studies in multiple tumor models where antibodies to PD-L1, PD-1 and CTLA-4 are used as a monotherapy have not achieved much success (Grosso and Jure-Kunkel, 2013; Highfill et al., 2014; Kim et al., 2014; Guo et al., 2015; Hu-Lieskovan et al., 2015; Ngiow et al., 2015). Recent success has been achieved by depleting MDSC in combination with checkpoint inhibitors (Highfill et al., 2014; Kim et al., 2014). Depletion through the use of epigenetic modifiers 5-azacytidine and entinostat, a combination currently being explored clinically (Kim et al., 2014) (NCT01928576), in models of colorectal and breast cancer achieved significant anti-tumor effects. A similar enhancement was observed upon blocking MDSC recruitment through CXCR2 antagonism in a model of sarcoma (Highfill et al., 2014). Both studies achieved significant enhancement of immune checkpoint inhibitor efficacy as a result of removing multiple layers of immune suppression within the TME. Since hypoxia is known to recruit MDSC to tumors and induce their immunosuppressive behavior it is intriguing to explore hypoxia

driven therapies that may limit the recruitment of MDSC to the tumor. Indeed, CAIX was shown to be required for mobilization of MDSC in an implantable model of breast cancer in a G-CSF-dependent manner facilitating establishment of a breast cancer pre-metastatic niche (Chafe et al., 2015). Thus, targeting CAIX in combination with checkpoint inhibitors may prove effective in this regard.

HYPOXIA-INDEPENDENT MECHANISMS FOR CAIX INDUCTION

Although hypoxia is arguably the major pathophysiological stimulus for HIF-1-mediated upregulation of CAIX in solid tumors, several alternative "provocateurs" present in the TME may contribute to its induction. For example, it has been demonstrated that lactate promotes HIF-1α accumulation in a hypoxia-independent manner in tumor cells through the inhibition of proline hydroxylation, driving expression of downstream effectors such as VEGF (Lu et al., 2002; De Saedeleer et al., 2012), and, potentially, CAIX. The common chemotherapy drugs paclitaxel, gemcitabine and carboplatin have been recently shown to induce HIF-1α expression in various models of breast cancer in a reactive oxygen species dependent manner (Samanta et al., 2014; Lu et al., 2015). Combination treatments of tumor xenografts with paclitaxel or gemcitabine in combination with the HIF-1α inhibitor digoxin proved more effective than either of the drugs tested as a single agent (Samanta et al., 2014). As the chemotherapy induced expression of HIF-1α, and downstream targets, increased the tumorsphere forming capacity of the basal breast cancer lines tested, it is conceivable that chemotherapy induced HIF-1α stabilization and a concomitant increase in the CSC population is a contributing factor to treatment resistance *in vivo*. However, randomized clinical trials are needed to confirm these preclinical findings. In line with these findings, we have demonstrated in both human and murine breast cancer models that the combination of paclitaxel with the small molecule CAIX inhibitor SLC-0111 significantly delayed tumor growth over either single agent (Lock et al., 2013). Based on the data presented by Samanta et al. (2014) and Lu et al. (2015), our observations may be related to increased CAIX expression in the tumor models tested in response to the chemotherapy induced initiation of the HIF program in a hypoxia independent manner, especially in the CSCs. Furthermore, while paclitaxel and doxorubicin treatment of the MMTV-PyMT model of breast cancer was previously shown to result in increased TAM presence in the tumor, hypoxia was not investigated in this setting (DeNardo et al., 2011). Based on recent findings and the role of tumor hypoxia in TAM recruitment and promotion of the alternatively activated, immunosuppressive phenotype, it would be worthwhile to test inhibitors of HIF targets in combination with the above chemotherapy drugs in this model to prevent TAM enrichment and immune evasion following treatment. It has also recently been demonstrated that estrogen signaling induces HIF-1α expression by direct binding of the estrogen receptor (ER)-α to the estrogen response element (ERE) in the HIF-1α promoter (Yang et al., 2015). HIF-1α expression was associated with resistance to tamoxifen in *in vitro* studies and was correlated with reduced overall survival in ER^+ patients treated with tamoxifen and chemotherapy, correlating HIF-1α with tamoxifen resistance in patients (Yang et al., 2015). Interestingly, the CAIX promoter was also found to have an ERE and was shown to be induced in rat uteri following estrogen treatment (Yang et al., 2015; Karim et al., 2016). Given the correlation with HIF-1α and tamoxifen resistance, it would be interesting to investigate whether CAIX expression is increased in this patient population. If so, CAIX inhibition might offer a second-line treatment for ER^+ breast cancer patients that have relapsed on hormone ablation therapy.

CONCLUSION

Therapeutic resistance is a critical determinant of cancer patient outcome. Identifying biomarkers of response and determinants of treatment failure is paramount to improving treatment modalities. The acidic pH and low oxygen tension within the hypoxic tumor microenvironment of solid tumors poses a significant hurdle to the efficacy of chemo-, radio- and immunotherapies. The body of work described here points to combinatorial treatment approaches designed to eliminate hypoxic niche cells and improve the response to immunotherapy and chemotherapy. For example, combining immune checkpoint inhibitors such as α-PD-1 or α-CTLA-4 with inhibitors of pH regulatory enzymes such as CAIX and MCT-4 may result in a reduced response to hypoxia while simultaneously stimulating the anti-tumor immune response. Furthermore, the application of CAIX inhibitors in combination with anti-angiogenic agents has the potential to reduce the cancer stem cell compartment in the hypoxic niche that is generated as a byproduct of use of therapies such as bevacizumab and sunitinib. Rational combinatorial approaches such as these may lead to improved clinical trial design and ultimately identify effective regimens to overcome treatment resistance.

AUTHOR CONTRIBUTIONS

PM, SC, and SD conceived and designed the manuscript. PM and SC drafted the manuscript. PM, SC, and SD critically revised and approved the final manuscript.

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