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Transcriptional control of the tumor- and hypoxia-marker *carbonic anhydrase 9*: a one transcription factor (HIF-1) show?

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SUMMARY

Transcriptional activation by hypoxia is mediated by the hypoxia-inducible factor (HIF) via binding to the hypoxia-responsive element (HRE). Hypoxia in solid tumors associates with poorer outcome of the disease and reliable cellular markers of tumor hypoxia would represent a valuable diagnostic marker and a potential therapeutic target. In this category, carbonic anhydrase IX (CAIX) is one of the most promising candidates. Here, we summarize the knowledge about transcriptional regulation of *CA9*. The HRE is the central regulatory element in the *CA9* promoter, whereas other elements are limited to lesser roles of amplification of signals received at the HRE. The analysis of known mechanisms of activation of *CA9* reveals the prominent role of the HIF-1 pathway. Experimental paradigms with uncoupled HIF-1α stability and transcriptional activity (pericellular hypoxia, proteasomal inhibitor) provide evidence that *CA9* expression monitors transcriptional activity of HIF-1, rather than the abundance of HIF-1α. Furthermore, these paradigms could provide a corollary to some of the apparently discordant cases (CAIX+, HIF-1α−) or (CAIX−, HIF-1α+) observed *in vivo*. In conclusion, the existing data support the notion that *CA9*, due to the unique structure of its promoter, is one of the most sensitive endogenous sensors of HIF-1 activity.

Keywords

Carbonic anhydrase IX; transcriptional regulation; hypoxia; hypoxia-inducible factor

INTRODUCTION

Celluar hypoxia in locally advanced solid tumors correlates with poor prognosis and resistance to conventional therapy [1]. Because of the medical significance of hypoxia in tumors, there is an ongoing search for reliable biomarkers of this condition as they would represent both a valuable diagnostic marker of hypoxia and a potential therapeutic target. In this category,

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carbonic anhydrase IX (CAIX) has recently emerged as one of the most promising endogenous markers of cellular hypoxia.

CAIX, one of the 15 CA isoforms in humans, is a transmembrane protein with an extracellular CA domain. In human malignancy, high levels of CAIX expression are consistently seen in a strikingly high proportion of carcinomas of the cervix [2] and clear cell carcinoma of the kidney [3], and, to a lesser degree, in other types of human tumors, such as carcinomas of the breast [4], head and neck [5], lung [6], and tumors of the brain [7]. High CAIX expression is associated with poor survival and is considered an independent predictor of poor disease-specific survival for astrocytoma [8], sarcoma [9], and carcinomas of cervix [10], breast [4], non-small cell lung [6], and head and neck [11]. In contrast, high CAIX expression in renal clear cell carcinoma (RCCC) cells is a factor of good prognosis [12]. However, this is a relatively unique situation since RCCC is a VHL "disease" where HIF-1α, when expressed, is stable in both normoxic and hypoxic conditions. Thus, in those cases where there is a lack of CA-IX expression, the tissues may be hypoxic and expressing only HIF-2α. This intriguing possibility remains to be investigated. With few exceptions, in the majority of carcinomas CAIX expression is restricted to peri-necrotic areas and/or to the hypoxic regions [13,14]. Conversely, CAIX is seldom expressed in postnatal normal tissues and, when it is expressed, the expression is restricted to highly specialized cells, as discussed in Section III E. Overall, CAIX expression in normal tissues appears to closely relate to the cell origin, cell differentiation, ion transport, and cellular hypoxic condition, respectively, and may also serve as a biomarker of stem or transit amplifying cells [15]. In most instances the pattern of CAIX expression and hypoxia, as measured by HIF-1α protein expression, are coincident (CAIX+, HIF-1α+, Fig. 1 A and B). However, substantive exceptions, exemplified by CAIX−, HIF-1α+ (Fig. 1 C and D) and CAIX+, HIF-1α− (Fig. 1 E and F) cases, have also been observed [14,16].

The expression pattern of CAIX in solid tumors, unique among the CA isoforms, has led to suggestions that CAIX could be used as a biomarker of cellular hypoxia [13,17]. Furthermore, due to its transmembrane cellular location, it has been suggested that its ectopic expression in tumors could be exploited for immunotherapeutic targeting [12,18]. In this respect, the monoclonal antibody G250 that recognizes a non-denatured form of CAIX has been extensively characterized [19]. Given the known distribution of CAIX protein expression in normal and tumor tissues, it is imperative that a full understanding of the regulation of *CA9* gene expression be attained in order to evaluate its utility as a tumor and hypoxia biomarker.

CAs are zinc metalloenzymes that catalyze the reversible conversion of CO_2 to bicarbonate and proton and can thus be involved in regulation of pH. Tumor cells, utilizing predominantly glycolytic metabolism, maintain intracellular pH (pH_i) within the physiological limits by extruding lactate and protons. More recent evidence indicates that CAs also participate in maintaining a microenvironmental pH gradient by forming cooperative complexes (metabolons) with bicarbonate transporters [20]. Intracellular CAs facilitate neutralization of actively imported bicarbonate with protons in the cytoplasm in a process generating CO_2. CO_2. freely diffuses through the cell membrane into the extracellular space where it is captured by extracellular CAs and converted back into bicarbonate and proton. The whole process is equivalent to exporting protons from cells and further contributes to acidosis, a hallmark of solid tumors [17].

The utility of CAIX as a tumor/hypoxia biomarker, as a prognostic factor predictive of relative survival, and as a potential tumor therapeutic target have been extensively reviewed [21–23]. In this review, we will focus on mechanisms of transcriptional regulation of *CA9*. Regulation of transcription in general can be viewed as an integrative process in which transcription factors, activated by specific signaling cues, bind DNA sequences (*cis*-acting elements) within regulatory regions, and increase or decrease activity of the transcriptional machinery.

Transcriptional responses will be determined by the sum of respective signals (positive and negative) that is received at the regulatory region of a gene. By connecting the regulatory elements in the *CA9* promoter with various stimuli and signaling pathways, we hope to provide a functional summary of the accumulated knowledge of regulation of this important protein. We will focus especially on experimental evidence that may help explain the complexity of the patterns of CAIX expression observed *in vivo*, in normal and tumor tissues. In the closing part, we will also discuss the potential value of designing expression constructs, consisting of cytotoxic gene(s) whose expression is driven by the *CA9* promoter or its regulatory regions, for use in tumor therapy.

I. The *CA9* gene and its transcription

The protein coding sequence of *CA9* is divided into 11 exons, covering ~11 kb [24] in the p12–p13 region of the chromosome 9 [25]. Northern blot analysis revealed that *CA9* is highly inducible, being transcribed into a single 1.5 kb mRNA, upon appropriate activation, but no mRNA is detected in the basal, uninduced state [26]. This mRNA is processed into two transmembrane proteins with apparent molecular weights of 54 and 58 kDa. Although both variants are glycosylated, glycosylation alone does not account for the difference in molecular weights [26], and the underlying cause, along with any functional consequence, of this variation remains to be established. Sequencing of *CA9* cDNAs from cancer cell lines and normal tissues detected no mutations that would indicate different function in tumor versus normal cells [27].

In addition to the predominant, full-length *CA9* mRNA, an alternatively spliced variant, lacking exons 8/9, has been detected by RT-PCR. This mRNA variant is constitutively expressed at a very low level in various cell lines and codes for a truncated form of CAIX with decreased enzymatic activity [28]. Although it was suggested that it could behave in a dominant negative fashion, the physiological role of this variant is unknown.

II. *Cis*-acting elements of *CA9*

A *CA9* promoter construct, corresponding to the (−173; +31) region, relative to the transcription start site (most likely more distal regions do not contribute significantly to promoter activity [29], Kaluz, unpublished), reconstitutes the tightly controlled pattern of endogenous *CA9* transcription: it has a very low basal activity but it is readily induced by the same stimuli as the endogenous *CA9* promoter [13,30]. The GC-rich *CA9* promoter does not have a TATA-box and neither of the two initiator-like sequences overlaps with the transcription start site [24]. Six *cis*-acting elements were functionally characterized in the *CA9* promoter, five with a positive and one with a negative influence on promoter activity [13,30] (Fig. 2A).

Hypoxia-responsive element (HRE)—HREs are composite *cis*-acting elements, activated by the hypoxia-inducible factor (HIF). All endogenous HREs characterized so far are structurally complex, having in common a conserved core HIF-binding site (HBS) with a G/ACGTG consensus sequence. However, a single HBS is necessary but not sufficient for activation by hypoxia and a non-conserved but functionally critical flanking sequence is an integral part of the HRE. This suggests that HIF has an intrinsically cooperative nature and, in order to be transcriptionally active, the HBS-bound HIF needs to engage the juxtaposed transcription factor(s) [31].

The TACGTG HBS, located immediately upstream of the transcription start (-3;-10), is the most critical regulatory element in the *CA9* promoter [13,32]. The evidence for this conclusion is that mutations within the core HBS inactivate the *CA9* promoter and no promoter activity was observed in HIF-1α-deficient cells. This suggests that binding of the HIF-1 complex to the *CA9* HRE is a prerequisite for recruiting the transcriptional machinery to the *CA9* promoter.

In the absence of HIF-1, no activation of the promoter is seen. This assumption regarding the tight control of the *CA9* promoter by HIF-1 appears to be amply supported by experimental evidence: the *CA9* promoter is transactivated in conditions of limiting O_2 (hypoxia) and other HIF-activating stimuli (as discussed in Section III A), in a HIF-1α-dependent manner whereas it is practically inactive in normoxia, except in cells with a mutant or epigenetically silenced *von Hippel-Lindau* (*VHL*) gene [13,33]. The complete structure of the *CA9* HRE may still not be fully characterized: it is possible that in addition to the HBS and cooperating SP1/SP3-binding protected region 1 (PR1) (see below), other sequences are functionally important and may play a critical role in the transcriptional response of *CA9*.

Other regulatory elements—Two of the positive *CA9* regulatory elements, PR1 (-45;-24) and PR5 (-163;-145), bind SP1/SP3 factors [29,32,34]. The large family of SP/Kr ppel-like factors, binding to various forms of “SP1 sites”, comprises transcription factors that can positively or negatively affect transcription [35]. The simplistic view that clusters of SP1/SP3-binding sites are involved in constitutive gene transcription has been abandoned in favor of highly complex regulation [35], a notion strongly supported by the tightly controlled *CA9* promoter. Under all conditions examined (normoxia, hypoxia, and high cell density, which corresponds to mild hypoxia), binding of SP1/SP3 to PR1/PR5 was constitutive [32,34]. Constitutive binding of SP1/SP3 to PR1/PR5, in conditions where HIF-1 is active, is critical for *CA9* transcription and mithramycin, an inhibitor of SP1/SP3 binding, effectively prevents CAIX expression [32,36].

The minimal form of the *CA9* promoter that reflects patterns of endogenous *CA9* expression encompasses PR1 and is dependent on SP1/SP3 activity; therefore we have proposed that PR1 and HRE in the *CA9* promoter form a novel type of hypoxia-responsive enhancer element [32]. SP1/SP3-binding sites were later found also to be part of the HRE in other genes, e.g. *endoglin* [37], *retinoic acid receptor-related orphan receptor α 4*[38], and *visfatin* [39]. None of these genes, however, behave like CAIX with respect to its tightly controlled expression in hypoxic versus normoxic conditions. The HRE and SP1/SP3-binding PR1 sites are conserved between human, mouse, and cow *CA9* (Fig. 2B), further attesting to their importance in physiological regulation of CAIX expression.

The PR2 (-71;-56) was identified as an AP1-binding site [30,34]. Activity of AP-1 complexes, usually comprised of heterodimers of the Jun, Fos, ATF, MAF, and Nrf families of transcription factors, can be induced by hypoxia [40]. Cell type-specific activation of AP-1 by hypoxia is mediated by the extracellular signal-regulated (ERK) and Jun N-terminal kinase/stress-activated protein kinases of the MAP kinase family as well as the phosphatidylinositol 3-kinase (PI-3K) pathway [40,41]. AP-1 sites have the potential to integrate signals from these oncoprotein-mediated signaling pathways and PR2 thus provides a means to further amplify the level of transcription from the *CA9* PR1-HRE enhancer.

The PR3 (-101;-85) regulatory element binds proteins from nuclear extracts and has a positive effect on *CA9* promoter activity [30]. However, no further information is available as to the functional importance of this element.

Deletions and mutations within the remaining regulatory element, PR4 (-134;-110), increased *CA9* promoter activity, confirming its negative effect [30]. The presumed repressor binding PR4 has not been identified, but electromobility shift assays revealed that its binding inversely correlated with CAIX expression in the model of HeLa x normal fibroblast hybrids [42], being significantly stronger in the CAIX-negative CGL1 (non-tumorigenic) than in the CAIX-positive CGL3 (tumorigenic) hybrids [30]. However, the PR4-binding protein was not observed in RCCC cells, leading to the suggestion that in these cells the presumed repressor is either absent or is prevented from binding to the *CA9* promoter [29]. These data suggest that

PR4 may, in a cell-type specific manner, contribute to the tight control of the *CA9* promoter by repressing its transcriptional activity in the basal state.

Epigenetic factors controlling *CA9* expression—Epigenetic modifications, such as methylation of CpG sites in the proximity of promoters and/or post-translational modifications of histones, play a significant role in the control of gene expression, presumably by limiting access of transcription factors to *cis*-acting elements. The methylation status of the −74 and −6 CpG sites in the *CA9* promoter (located near PR2 and within the TACGTG *CA9* HBS, respectively) has been reported to negatively correlate with *CA9* expression in renal cells: the *CA9* promoter is hypomethylated in CAIX-positive RCCC cell lines, while showing complete methylation in CAIX-negative cells, including normal kidney tissues [43–46]. Intriguingly, compared with normal kidney tissue, no hypomethylation was observed in primary RCCC: investigated CpG dinucleotides in RCCC as well as in normal kidney tissue were generally completely methylated [46].

Methylation of the *CA9* promoter in non-renal cell lines has not been studied as systematically, although it could play a similar restrictive role. For example, the endogenous *CA9* promoter is hypermethylated in CAIX-negative colon carcinoma HCT 116 cells (I. Kuzmin, personal communication) and yet the exogenous *CA9* promoter construct was strongly induced by hypoxia, suggesting that the transcriptional machinery required for activation of *CA9* in these cells is intact [47]. Promoter methylation was also proposed to represent an accessory mechanism that controls CAIX expression in dense cultures of non-RCCC cells [48]. However, it should be noted that there are no reports of methylation “switching” at *CA9* CpG sites upon induction of *CA9* transcription.

Epigenetic silencing of the *CA9* promoter may have important implications for CAIX-targeted therapeutic regimens as it can represent an “escape” route for tumor cells subjected to a strong selection process. In order to prevent them from evading the therapeutic pressure, it will be important to understand in detail how CAIX-negative tumor cells compensate for the lack of CAIX activity, especially whether resorting to other transmembrane CAs (e.g. CAXII, CAXIV) is the only escape route or whether there are other “back-up options”.

III. Pathways controlling *CA9* transcription

Due to the possibility of inactivation of endogenous *CA9* by epigenetic factors, activity of the ectopically introduced *CA9* promoter may be a better readout of *CA9*-regulating pathways than endogenous mRNA or CAIX protein. The consensus from available experimental data is that, with the notable exception of cells with disrupted HIF-1 signaling, proper experimental conditions activate the *CA9* promoter in all cells, transformed as well as normal, regardless of the expression status of endogenous *CA9*. This suggests that the primary mechanism(s) responsible for activation of *CA9* is widespread and readily activated by proper stimuli. Fig. 3 provides an overview linking pathways, factors, and experimental conditions that are known to control *CA9* transcription with the *cis*-acting elements in the *CA9* promoter and their cognate transcription factors. In the following sections we will discuss the underlying mechanisms.

A. Regulation of *CA9* by the HIF pathway

1. Hypoxia and other inhibitors of the HIF-1α degradative pathway: The transcription factor HIF that transactivates hypoxia-inducible genes by binding to HREs is a heterodimer of the O_2-regulated α and constitutive β subunits. Of the three different α subunits, HIF-1α, HIF-2α, and HIF-3α, 1α and 2α are the most important and best studied [49]. Interestingly, *CA9*, in contrast to most other hypoxia-inducible-genes, is exclusively transactivated by HIF-1α. The α subunits are regulated by two O_2-dependent molecular switches that control their abundance and transcriptional activity, respectively. In the presence of O_2, prolyl

hydroxylase domain proteins (PHDs) hydroxylate HIF-α on two prolines in the O_2-dependent degradation domain (ODDD), initiating VHL-dependent polyubiquitylation and degradation in the proteasome. The asparaginyl hydroxylase factor inhibiting HIF-1 (FIH-1) prevents interactions between HIF-α and the transcriptional co-activators, p300/CBP, by hydroxylating the C-terminal activation domain (CAD) of HIF-α. Hypoxia, the main physiological activator of HIF, leads to stabilization (via inactivation of the PHDs) and transcriptional activation (via inactivation of the FIH-1) of α subunits ([50], and references therein). HIF-1α and HIF-2α also have an N-terminal activation domain (NAD) that is contiguous with the ODDD (Fig. 4).

Since identification of the HRE in the *CA9* promoter [13], the critical role of the HIF-1 pathway in regulation of *CA9* has been supported by overwhelming evidence from numerous studies. It is noteworthy that the vast majority of the pathways, factors, and experimental conditions that are known to control *CA9* transcription converge on the HIF-1 pathway (Fig. 3) and regulate HIF-1α stability, HIF-1 transcriptional activity or both (Table 1). This provides further support for the notion that the HIF-pathway is the integrator and central mediator of signaling cues for *CA9.* The direct role of hypoxia in regulation of *CA9 in vivo* has been deduced from CAIX immunostaining in tumor specimens, where it is mostly restricted to hypoxic regions (Fig. 1 A and B) [13,17]. The striking effect of hypoxia/HIF-1 on CAIX expression can be also documented *in vitro*, as the large majority of cell lines express CAIX only when exposed to some level of hypoxia [13,51].

In addition to hypoxia, other agents and genetic factors can inhibit the HIF-degradative pathway and induce CAIX expression (with the exception of proteasomal inhibition, discussed separately below). Inactivating mutations or epigenetic silencing of VHL, frequently associated with RCCC, are directly linked to overexpression of CAIX and other hypoxia-inducible genes in these tumors, even under normoxic conditions [13,29,33]. These VHL mutations disrupt the HIF-degradative pathway at the level of polyubiquitylation and lead to accumulation of transcriptionally active HIF [52]. PHDs and the FIH-1 hydroxylases are dependent on the same factors (O_2, Fe(II)+, 2-oxoglutarate, (2-OG)), suggesting that their co-inhibition (in most cases) will also result in accumulation of active HIF [50]. Inhibitors of these hydroxylases, e.g. iron chelators (deferoxamine mesylate), iron analogues (Co^{2+}, Ni^{2+}), 2-OG analogues (dimethyloxalylglycine), are all strong activators of CAIX expression [13,36,53]. In agreement with the critical role of p300/CBP transcriptional co-activators in HIF function, overexpression of adenoviral E1A, a competitive inhibitor of p300/CBP, significantly down-regulated CAIX expression in RCCC cells [29].

Collectively, the available evidence strongly implicates hypoxia in regulation of CAIX but the correlation between CAIX and hypoxia and its markers in solid tumors *in vivo* is not absolute and other molecular mechanisms of control may operate.

2. Discrepancies between CAIX expression and hypoxia/hypoxic-markers: CAIX expression does not always correlate with pO_2 measurements and other hypoxic markers. For example, there seems to be a lack of consistency in relating CAIX expression with low O_2 levels. Whereas an earlier report described a significant positive correlation between the level of tumor hypoxia and CAIX expression in cervical carcinoma [10], later studies in the same tumor type did not find significant associations between CAIX expression and tumor pO_2 [16,54]. In addition, as documented in cervical carcinomas, CAIX staining typically extends beyond the region binding the chemical marker of hypoxia, pimonidazole [14]. Immunohistochemical studies of intratumoral distribution of CAIX often identify certain proportions of tumors that do not show signs of hypoxia, e.g. absence of necrotic areas, expression of HIF-1α, pimonidazole staining, expression of other hypoxia markers (VEGF and/or Glut-1), and yet they express CAIX. Conversely, some tumors with apparent hypoxic regions are CAIX negative [21].

Some of the discrepancies may be explained in terms of the causal relationship between HIF-1α and CAIX. An important consideration is, of course, that the cells/tissues examined are a "snapshot" in time. HIF-1α is rapidly degraded in normoxic conditions (on the order of a few minutes) and just as rapidly stabilized in hypoxic conditions. This very dynamic kinetic will have significant effects on transcription of HIF-1 target genes and subsequent protein expression. In this regard, upon initiation of *CA9* transcription, it takes several hours before measurable CAIX protein is seen (Kaluz, unpublished). Thus, several scenarios may be envisaged: in a situation where the cells/tissue have only recently become hypoxic, HIF-1α+, CAIX− cells may have been analyzed before they had time to activate CAIX expression. Alternatively, different stabilities of HIF-1α (labile) and CAIX (extremely stable) could be responsible for the HIF-1α−, CAIX+ phenotype in cells that had been hypoxic and then were reoxygenated (loss of HIF-1α) yet they retained expression of the long-lived CAIX. Also, in respect to the duration and severity of hypoxia there is a significant heterogeneity within the tumor that could be identified as acute, cycling (acute hypoxia followed by rapid reoxygenation), and chronic hypoxia [55,56]. The complex inter-relationships between these different forms apparently lead to differential effects on transcription and translation [57]. Even when exposed to similar levels of O_2, a short-term, acute hypoxic exposure versus a longer-term, chronic hypoxic exposure might lead to a different biology within the tumor [58].

It should be also noted that although HIF-1 was initially identified because of its response to low O_2 concentrations, it is now apparent that HIF-1 can be regulated by other, O_2-independent, factors. For example, upon activation of oncogenes, such as Ras, SRC, and PI-3K, or inactivation of tumor suppressor PTEN, HIF-1α accumulates in tumor cells even in normoxia [59]. The role of free radicals, in particular reactive oxygen species and nitric oxide, in activating HIF-1 was suggested to be at least as important as hypoxia *per se* and their roles are more complex than hypoxia itself [56]. In summary, the complexity of regulation of HIF-1 (by hypoxia and other factors) may affect the extent of co-localization of HIF-1α and CAIX expression.

3. Paradigms with uncoupled HIF-1 transcriptional activity and stabilization of HIF-1α: implications for CAIX/HIF relationship: In Section III A 1, we focused on situations where CAIX expression is strictly correlated with both increased abundance and transcriptional activity of HIF-1α. However, alternative phenomena, that reflect an uncoupling of HIF-1α stability and transcriptional activity, have been observed and we will discuss the effect of this uncoupling on CAIX expression.

In vitro, cells grown at ambient O_2 levels at high cell densities become mildly hypoxic (pericellular hypoxia) when the rate of O_2 consumption exceeds the rate of unassisted O_2 diffusion. In some cells, pericellular hypoxia activates HIF and HRE-dependent transcription (including *CA9*) but does not appear to be severe enough to stabilize detectable levels of HIF-1α [53]. Despite the apparent absence of HIF-1α stabilization, the phenomenon of cell density-induced CAIX expression is strictly dependent on HIF-1α and does not occur in HIF-1α-deficient cells. On the other hand, improved oxygenation of dense cultures (lower volume of medium, stirring) prevented CAIX expression, confirming that this process is indeed driven by limited O_2 availability [53]. Regulation of constructs with the Gal-4 DNA-binding domain fused to the HIF-1α ODDD (comprising NAD, aa 401–601) and the regulated CAD (aa 744–826) further support the proposition that there is a preferential modulation of transcriptional activity of HIF-1α in the absence of increased stability. Increasing cell density activated the CAD construct (reflecting transcriptional activity) but had no effect on the ODDD construct (reflecting stability) (Fig. 5).

Original measurements, using oligopeptides that spanned the ODDD and CAD regions, respectively, indicated that the K_m of FIH-1 for O_2 is less than half that of the PHDs, suggesting

that, as the O_2 levels drop, the PHDs would be inactivated first, while FIH-1 would require more severe hypoxia to lose activity [60]. This led to the proposition that stabilization of HIF-1α precedes its transcriptional activation and that there might be a hypoxic window in which HIF-1α would be stable due to the absence of prolyl hydroxylation and yet would be transcriptionally inactive due to continued asparaginyl hydroxylation [60]. However, more recent studies with longer peptides have established that the K_m of FIH-1 for O_2 *in vitro* is much closer to that of the PHDs (~ 250 μM)[61], casting doubt on the linear, progressive model. More importantly, cell-based assays indicated that FIH-1 was less sensitive to decreasing O_2 levels than the PHDs in some cell types, whereas in other cell types it was more sensitive, demonstrating a cell type-specific sensitivity of FIH-1 to lowered O_2 [62]. Inactivation of FIH-1, resulting in activation of the HIF-1α CAD prior to inactivation of PHDs, would thus be compatible with the phenomenon observed in pericellular hypoxia.

Pericellular hypoxia in densely plated cells is accompanied by activation of other signaling pathways, notably the PI-3K pathway. Activation of the PI-3K pathway by increased cell density was observed in most cell lines and PI-3K inhibitors potently suppressed cell density-induced CAIX expression [53]. Interestingly, activation of the PI-3K pathway in dense cultures did not result in increased HIF-1α levels, despite the previous findings that PI-3K activators increase the abundance of HIF-1α by promoting translation of its mRNA [63]. In contrast, in dense plated cells it is the selective upregulation of HIF-1 activity by PI-3K that is responsible for transactivation of *CA9*. Pericellular hypoxia thus provides an experimental paradigm of uncoupling the stability and transcriptional activity of HIF-1α, in which CAIX expression is driven solely by increased transcriptional activity of HIF-1 (presumably due to the combination of mild hypoxia and activation of PI-3K), without apparent stabilization of HIF-1α. This provides a corollary for some of the *in vivo* cases where CAIX expression is detected in the absence of HIF-1α signal.

The effect of inhibitors of proteasome activity on HIF-1 is another example of uncoupling of HIF-1α stability and HIF-1 transcriptional activity that is essentially the converse of the situation in pericellular hypoxia. Proteasomal inhibitors (PIs), by inactivating the 26S proteasome, increase the stability of numerous proteins, including HIF-1α, that are normally degraded by the ubiquitin-proteasome pathway. However, PIs not only did not activate CAIX expression in normoxic cells, they also prevented induction of CAIX and other hypoxia-inducible expression in hypoxic cells, indicating a strong inhibitory effect on HIF-1 despite the presence of significant levels of HIF-1α [51,64]. The prominence of the HIF pathway in regulation of *CA9* is further underscored by the fact that the inhibitory effect on *CA9* occurred against the background of potent activation of AP-1 (binding PR2) and p300/CBP co-activators by PIs, presumably due to their increased stability and, as a consequence, a higher level of the respective protein. Inactivation of HIF-1 by a putative labile corepressor that is stabilized in the presence of PIs, and possibly by other factors, thus provides a corollary for those *in vivo* cases where HIF-1α signal is detected in the absence of CAIX expression.

Recently, a total disconnection of hypoxia from the HIF pathway has been described in leiomyomas, benign tumors of the uterus. Leiomyomas were found to be severely hypoxic (1 to 5 mm Hg), but neither HIF-1α/HIF-2α stabilization nor CAIX (or any other hypoxia-inducible gene) expression was detected [65]. Why hypoxia apparently failed to activate the HIF pathway by either inhibiting degradation of HIF-α and/or increasing its transcriptional activity in this model is not known at present but this example further illustrates the complexity of conveying the hypoxic signal to the HIF pathway.

The effects of pericellular hypoxia and PIs on HIF-1 and *CA9* document that the stability and transcriptional activity of HIF-1α are not necessarily co-regulated. In situations where these two attributes of HIF-1α are uncoupled, CAIX expression always reflects transcriptional

activity rather than the relative abundance of HIF-1α. Thus, although the underlying mechanisms of pericellular hypoxia-induced CAIX expression and suppression of hypoxia-induced CAIX expression by PIs have not been completely elucidated, these two examples indicate that the induction of transcription of *CA9* provides a very sensitive endogenous sensor of the activation of HIF-1.

4. Regulation of *CA9* by HIF-1α and CAD but not by HIF-2α: Among the HIF-α isoforms, HIF-1α and HIF-2α are physiologically the most important. These isoforms, despite sharing a highly conserved domain architecture, are functionally distinct and show selectivity with respect to their transcriptional targets. *CA9* is exclusively responsive to HIF-1 in all cell types tested [29,66]. Interestingly, contrary to most carcinomas, decreased CAIX expression in RCCCs is an independent prognostic indicator of poor survival [12], and downregulation of CAIX in more advanced tumor stages of RCCCs could be related to gradual replacement of HIF-1α with HIF-2α protein expression [66]. Chromatin immunoprecipitation revealed that both HIF-1α and HIF-2α bind to the *CA9* HRE *in vivo* and it was proposed that the selective activation of *CA9* by HIF-1α is determined at a post-DNA binding stage by the non-equivalent C-terminal portions, comprising transactivation domains [67]. The sequence(s) in the *CA9* promoter responsible for its exclusive responsiveness to HIF-1 has not been identified, although SP1/SP3 occupancy at PR1, in what was originally defined as a novel type of hypoxic enhancer [32], may be involved.

Of the two transactivation domains, namely CAD and NAD (Fig. 4), the NAD has been reported to regulate the target gene specificity of HIF-1α [68], but it is the CAD that dominantly regulates transcription of *CA9*. This was inferred from the observation that modulation of FIH-1 levels (controlling CAD activity) exerted a more significant effect on *CA9* transcription than modulation of PHD2 levels (controlling NAD activity) [69]. The same authors proposed a model in which induction of HIF-1α CAD/FIH-1-dependent genes like *CA9* would occur at the lower end of the hypoxic window [69]. However, for reasons discussed in the preceding Section, this linear, progressive model may not apply universally and may thus be an oversimplification.

B. Regulation of *CA9* by other microenvironmental conditions (acidosis, glucose deprivation)—Apart from hypoxia or, more specifically, activation of HIF-1, the role of other microenvironmental conditions, most notably acidosis and glucose deprivation, in regulating *CA9* have also been considered. Low extracellular pH could be a factor responsible for physiological expression of CAIX in the normal digestive tract, although it has not been formally proven due to the lack of proper experimental models.

Acidification of growth media increased the level of CAIX protein, mRNA, and activity of the minimal *CA9* promoter in normoxic cells derived from glioblastoma, a tumor type particularly linked with acidosis. In these cells, normoxic acidosis stabilized HIF-1α, transiently activated the ERK pathway, and a combination of ERK and PI-3K inhibitors abolished induction of *CA9* by acidosis [70]. However, in an earlier report by the same group, neither acidosis nor low glucose concentrations induced CAIX in normoxic HeLa cells, but each factor alone further moderately enhanced hypoxia-induced CAIX expression [71]. Contrary to this, in yet another report the low glucose levels were found to reduce or abolish hypoxia-induced CAIX expression and it was argued that the 5.5 mM concentration of glucose used by Rafajova and colleagues [71] was within the physiological range and not genuinely “low” [72]. Thus far, the available data on effects of microenvironmental factors, other than hypoxia, on CAIX appear to indicate a cell type-specific effect and the strong activation of *CA9* occurred only with the concurrent stabilization/activation of HIF-1α. The significance of the putative hypoxia-independent mechanism in acidosis-increased CAIX expression, previously suggested to operate through modulation of the basic *CA9* transcriptional machinery [70], will have to be

evaluated. It is also apparent that a set of standard experimental conditions (concentrations of glucose, bicarbonate, cell lines) will be required before the putative role of acidosis and/or low glucose on CAIX expression is elucidated.

C. Tumor suppressors—The most significant connection between *CA9* and a tumor suppressor has been established for the *VHL* gene. The loss of VHL function is the underlying genetic cause of RCCC, hemangiomas of the retina, and hemangioblastomas of the retina and central nervous system, and results in constitutive, hypoxia-independent expression of hypoxia-inducible genes, including *CA9*. Inactivation of VHL, a substrate recognition subunit of the HIF-α E3 ubiquitin ligase, disrupts polyubiquitylation of HIF-α and leads to constitutive activation of HIF whereas introduction of a functional VHL restores the O_2-dependent regulation of HIF activity [52] and CAIX expression [13]. Additionally, VHL negatively affects expression of a subset of hypoxia- inducible genes (e.g. *vascular endothelial growth factor* (*VEGF*), *platelet-derived growth factor B-chain*) via suppressing the SP1 function [73,74]. Although this mechanism has not been directly shown for *CA9*, SP1 plays a prominent role in regulation of *CA9* and inhibition of SP1 may contribute to the strong inhibitory effect of VHL on *CA9*.

The p53 tumor suppressor has also been reported to down-regulate CAIX expression. Upon activation by genotoxic stress, the wild type p53 mediated increased proteasome-dependent degradation of HIF-1α thereby suppressing hypoxia-inducible CAIX expression, whereas p53 with inactivating mutations had no effect [47]. Another mechanism by which activated p53 can inhibit CAIX expression is competition for p300/CBP cofactors [75]. Both VHL and p53 tumor suppressors inhibit *CA9* expression and it is evident that the primary target in each case is HIF-1α. By virtue of being one of the key components of the HIF-α degradative pathway, VHL exerts a significantly more pronounced effect on *CA9* transcription than p53.

D. Oncogenic pathways—Two oncogenic pathways have been directly implicated in regulation of *CA9*. Increased PI-3K activity correlates with cell density-induced CAIX expression and there is a strong inhibitory effect of PI-3K inhibitors on CAIX expression [36,53]. The PI-3K pathway can exert its positive effect on *CA9* via either upregulation of HIF-1α levels or HIF-1 activity in a cell type-specific manner. To a lesser extent, the PI-3K pathway may also stimulate activity of AP-1, the cognate transcription factor of PR2 [40].

Although inhibitors of the ERK pathway also down-regulate CAIX expression, there was not a compelling correlation between the level of ERK activation and CAIX expression [36,76]. This was most conspicuously manifested in HeLa cells where CAIX and ERK activity correlated inversely: a maximal CAIX expression was observed in dense cultures with a minimal ERK activation whereas in sparse cultures the situation was reversed [76]. An intriguing disconnect between the ERK pathway and *CA9* expression was also observed in RCCC cells where interferons, potent inhibitors of the ERK pathway [77], upregulated CAIX [78]. These data suggest cell type-specific effects and the requirement for rather limited ERK activation in regulation of CAIX expression. The ERK pathway could modulate *CA9* at three different levels: by stimulating activities of HIF-1 (indirectly through p300/CBP [79]), SP1/SP3 [76], and also AP1 [40]. In summary, the PI-3K and ERK (less prominently) pathways can stimulate CAIX expression by targeting cognate transcription factors of multiple *cis*-acting elements, including HRE, in the *CA9* promoter. Both pathways appear to play an auxiliary role, amplifying the underlying signal, and are significantly less important in regulation of *CA9* than the HIF pathway.

E. Expression of CAIX in normal tissues and HIF-independent CAIX expression —Apparently, hypoxia-independent CAIX expression is observed in a few fetal and adult normal tissues. They include the following: (1) lining epithelium of the body cavity and visceral

organs (mesothelium), and remnants of the coelomic epithelium (rete ovarii, rete testis, etc.); (2) the epithelium of the stomach and biliary trees, including the gallbladder, Brunner's glands, and crypt cells of the duodenum and small intestine, and rare columnar cells in the crypts of the large intestine [17,27]. Developmental regulation of CAIX expression in human embryonic, fetal and postnatal tissues was the subject of a recent study [15]. During human development, there are certain cell types that either show persistent CAIX expression from the very early fetal stage to adult life, such as the derivative of coelomic epithelium, the infundibulum and the outer sheath of hair follicles and the sebaceous units of the skin, or exhibit transient expression limited to immature tissues. These immature tissues are mainly that of mesenchyma, cartilage and muscle of mesodermal origin, adrenal cortical cells and Müllerian epithelium of the female genital organ, primitive ependymal cells and those cells involved in chondrogenesis and skin development. Interestingly, targeted disruption of the *Car9* gene in the mouse resulted only in mild phenotypic changes, most prominently gastric hyperplasia, but no further changes, e.g. in gastric acid secretion, were observed, suggesting that other CA isoenzymes or related enzymes are relatively efficient in substituting for the CAIX function in normal tissues [80]. The regulatory mechanisms responsible for expression of CAIX in normal tissues have not been studied and at present it is not known whether HIF is somehow implicated or a completely different mechanism, e.g. action of tissue-specific transcription factors, is employed. The mechanism(s) governing *CA9* expression in normal tissues remains one of the most important outstanding questions and definite answers to this question, in particular the role of HIF-1, would allow one to draw a more complete picture of transcriptional regulation of *CA9*.

Because of the discrepancies between CAIX expression and other hypoxia biomarkers observed primarily *in vivo*, it has been repeatedly proposed that hypoxia is not the only factor driving CAIX expression and *CA9* may be under control of other, hypoxia-independent pathway(s). To this end, however, little evidence has been offered so far that would unambiguously rule out involvement of HIF-1. For example, short inhibitory RNA directed against SLUG, a member of the Snail family, potently down-regulated *CA9* but the effect on HIF-1α was not investigated, thus leaving open the possibility of an indirect effect via HIF-1α down-regulation [81].

IV. Therapeutic application of *CA9* regulatory elements

The tight control of the *CA9* promoter by hypoxia/HIF-1 led to expectations that it could be used for the specific targeting of cytotoxic gene expression in hypoxic tumor cells or cells containing a mutated or silenced *VHL* gene. As a proof of principle, a conditionally replicating adenovirus was designed, with E1A, essential for the lytic life cycle of adenovirus [82], under the control of the *CA9* promoter (Ad-MN/*CA9*-E1A). The virus replicated only in CAIX-positive cells and, compared to CAIX-negative cells, infection of CAIX-expressing cervical carcinoma cells indeed resulted in much stronger E1A protein expression [83]. *In vitro* and *in vivo* experiments revealed that the growth of CAIX-positive cells was significantly inhibited at a multiplicity of infection (MOI) of 0.01–1, whereas the CAIX-negative cells were only inhibited at a MOI of 100 [83]. Apparently, expression of E1A from the *CA9* promoter provides an intriguing feed-back mechanism as it was previously reported that overexpression of E1A down-regulates the *CA9* promoter by 75% via competition for p300/CBP [29]. Presumably, during the viral life cycle in the CAIX-positive cells, accumulation of E1A occurs that may eventually sequester p300/CBP, thereby significantly inhibiting *CA9* promoter activity.

However, there are some important limitations for practical applications of the *CA9* promoter. First, the *CA9* promoter is HIF-1-specific and therefore will not be induced in HIF-1 negative cells. Second, the rather limited activity of the *CA9* promoter, even in hypoxic conditions, or in CAIX-positive cells, may not allow for truly high level expression to be achieved with other engineered expression systems. For example, the *CA9* promoter-based constructs, even those

containing multiple HREs, were invariably inferior to constructs with HREs upstream of a minimal TATA-box (Fig. 6). On the other hand, the PR1HRE *CA9* module performed significantly more efficiently in an enhancer mode, upstream of the TATA-box (Fig. 6). One explanation for this could be that, compared to other promoters, the TATA-less *CA9* promoter is deficient in recruiting basal transcription factors and/or in initiating transcription; inclusion of the TATA-box greatly stimulates both recruitment of the basal transcription machinery and initiation. For this reason, in the future, constructs based on the TATA-box and artificial hypoxic enhancers generated by assembling optimized HBSs with other regulatory elements [31] might hold more promise than *CA9* promoter-based constructs for not only therapeutic but imaging/monitoring purposes [84] as well. In conclusion, despite enthusiasm and positive early reports, the *CA9* promoter-based constructs have not met expectations and may require significant engineering before they do.

Concluding remarks and future perspectives

In recent years, strong, but not absolute, evidence has correlated CAIX expression with HIF-1 activity. New paradigms of uncoupled HIF-1α stability and HIF-1 transcriptional activity make this connection even stronger and could help explain those instances where an apparent discordance between CAIX expression and stable HIF-1α expression has been observed *in vivo*. The present experimental data evidently link *CA9* expression with HIF-1 activity, rather than HIF-1α abundance. Although, in the majority of cases, CAIX expression correlates with increased levels of HIF-1α, there are notable exceptions, identification of which will require additional methodology on top of the currently used immunohistochemical staining for CAIX and HIF-1α.

The basis of the unique properties of *CA9* gene expression is the organization of its promoter. In contrast to other hypoxia-inducible genes, the HRE, encompassing the transcription start site, is central to *CA9* promoter activity whereas the remaining *cis*-acting elements are subordinated to HRE and play significantly lesser roles. The cognate factors of these elements are clearly regulated by multiple pathways but in the context of the *CA9* promoter they appear to amplify signal(s) received at the HRE, but are unable to transactivate *CA9* on their own, in the absence of HRE-targeting signal(s). The available evidence is compatible with the notion that the presence of the HIF-1 complex on the HRE is necessary for recruitment of the transcriptional machinery and initiation of *CA9* transcription. In the basal state, in the absence of HIF-1 activation, the *CA9* promoter is shut off, but even small increases in HIF-1 activity will be integrated by the *CA9* HRE and further amplified by the juxtaposed regulatory elements. This arrangement would make *CA9* a universal sensor of HIF-1 activation. This does not necessarily mean that *CA9* is a universal marker of cellular hypoxia as one must bear in mind that *CA9* expression, cellular hypoxia, and activation of HIF-1 are distinct entities that overlap only partially (Fig. 7). On the one hand, *CA9* expression is more restricted than cellular hypoxia, as it fails to be activated in cells in which hypoxic signaling is mediated by HIF-2 or when the *CA9* promoter is methylated. On the other hand, activation of *CA9* expression will occur even when HIF-1 is activated by non-hypoxic stimuli, thus separating = *CA9* expression from cellular hypoxia (Fig. 7). *CA9* expression will, therefore, be only as good as a surrogate marker of cellular hypoxia as HIF-1 activity is.

In future cytologic studies, in addition to conventional immunostaining to detect HIF-1α, a new methodology will be required for monitoring/detecting HIF-1 transcriptional activity that may occur in the absence of HIF-1α stabilization. Unraveling the mechanism of regulation of *CA9* expression in normal tissue will be critical for the concept of universal dependence of *CA9* transcription on HIF-1 activity, posited in the previous paragraph. Finally, an ideal endogenous diagnostic marker of hypoxia/therapeutic target should equally respond to HIF-1 as well as HIF-2 activation, and exclusive dependence on HIF-1 is one of the most obvious

shortcomings of CAIX. It remains to be seen, however, whether future studies will identify hypoxia-inducible protein(s) that are comparably transactivated by HIF-1 and HIF-2 (or HIF-2 alone that could complement CAIX) and would, at the same time, display the whole set of unique features of CAIX, such as the extremely tight control by hypoxia, high protein stability, and accessibility of the extracellular part of the transmembrane protein for therapeutic targeting.

Nevertheless, the data discussed in this review support the conclusion that CAIX remains, at least for the time being, the best endogenous marker of cellular hypoxia, encompassing the range of mild to severe hypoxia. As such it might be considered a “gold standard” to which other prospective biomarkers of hypoxia will be compared.

Abbreviations

2-OG
: 2-oxoglutarate

CAD
: C-terminal activation domain

CAIX
: carbonic anhydrase IX

ERK
: extracellular signal-regulated protein kinase

HBS
: HIF-binding site

HIF
: hypoxia-inducible factor

HRE
: hypoxia-responsive element

FIH-1
: factor inhibiting HIF-1

MOI
: multiplicity of infection

NAD
: N-terminal activation domain

ODDD
: O_2-dependent degradation domain

PHDs
: prolyl hydroxylase domain proteins

PI-3K
: phosphatidylinositol 3-kinase

PIs
: proteasomal inhibitors

PR
: protected region

RCCC

renal clear cell carcinoma

VEGF

vascular endothelial growth factor

VHL

von Hippel-Lindau protein

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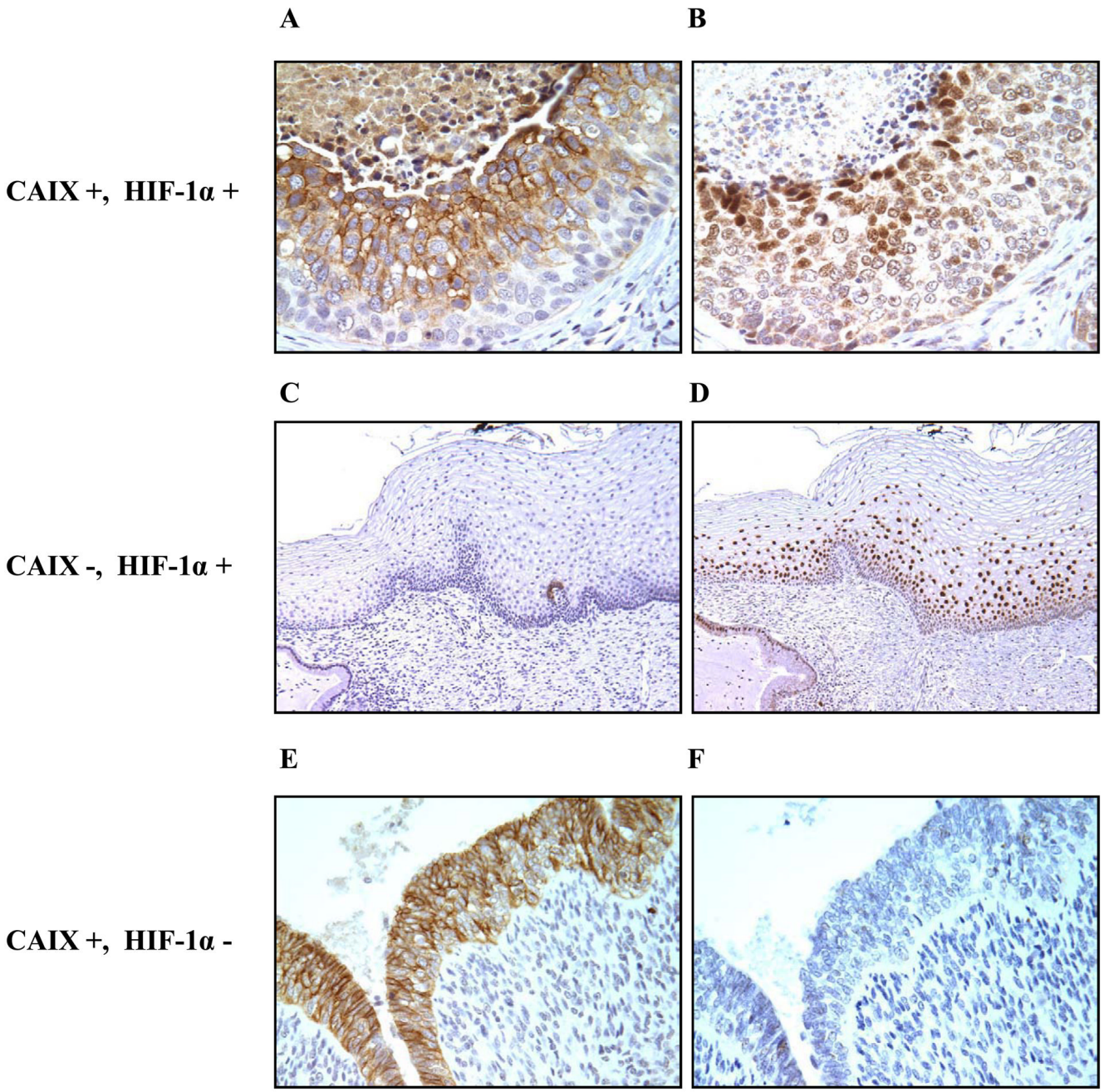
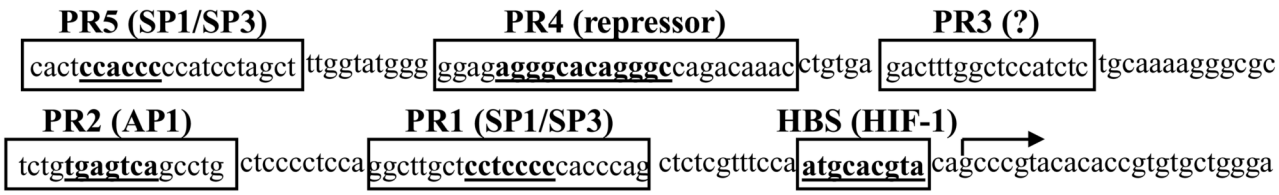


FIGURE 1. ***In vivo*** **relationships between CAIX and HIF-1α expression**

For detection of the CAIX protein, immunohistochemical staining of tissue sections with the mouse monoclonal antibody M-75 (1:10,000) was done using a peroxidase technique as described previously [17]. HIF-1α was detected on tissue sections with the anti-HIF-1α mouse monoclonal antibody (BD Biosciences) at a 1: 25 dilution. Sections A, C, and E were immunostained for CAIX, sections B, D, and F were immunostainedstained for HIF-1α. A and B, cervical carcinoma; C and D, adult cervix; E and F, fetal endometrium. The corresponding CAIX and HIF-1α immunostains were serial sections.

A



B

FIGURE 2. (A) Sequence of the *CA9* promoter and its *cis*-acting elements

PR, protected region; HBS, HIF-binding site; the arrow indicates the transcription start site. Each PR is boxed, the cognate transcription factors are in brackets, and their binding sites are in underlined bold. **(B) Comparison of the human, bovine, and murine minimal *CA9* promoter sequence.** ATG initiation codons are in underlined bold.

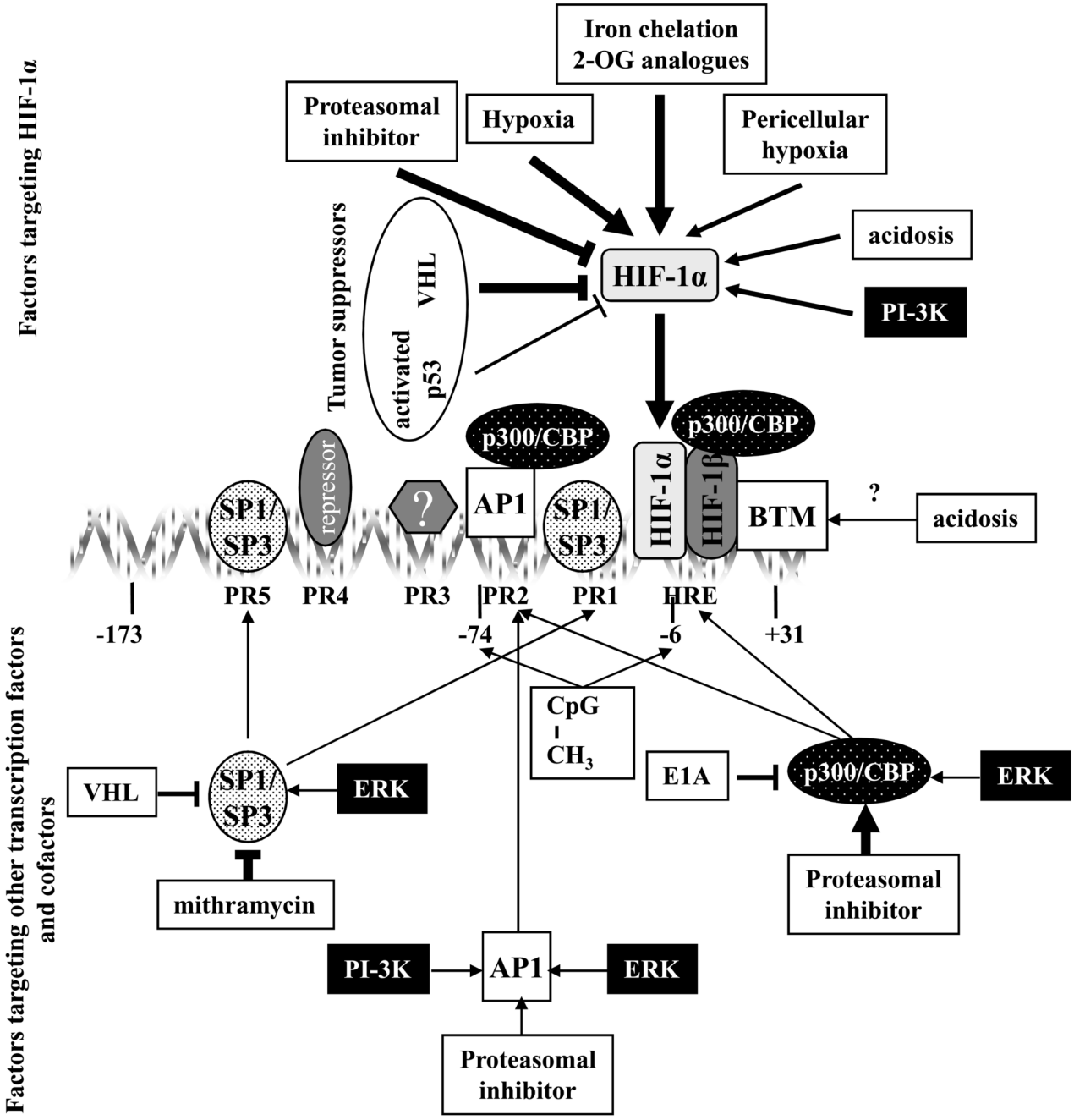


FIGURE 3. Pathways/factors regulating activity of the *CA9* promoter

→, activation; ⊣, inhibition; increasing line thickness denotes increased intensity of the effect. BTM, basal transcriptional machinery.

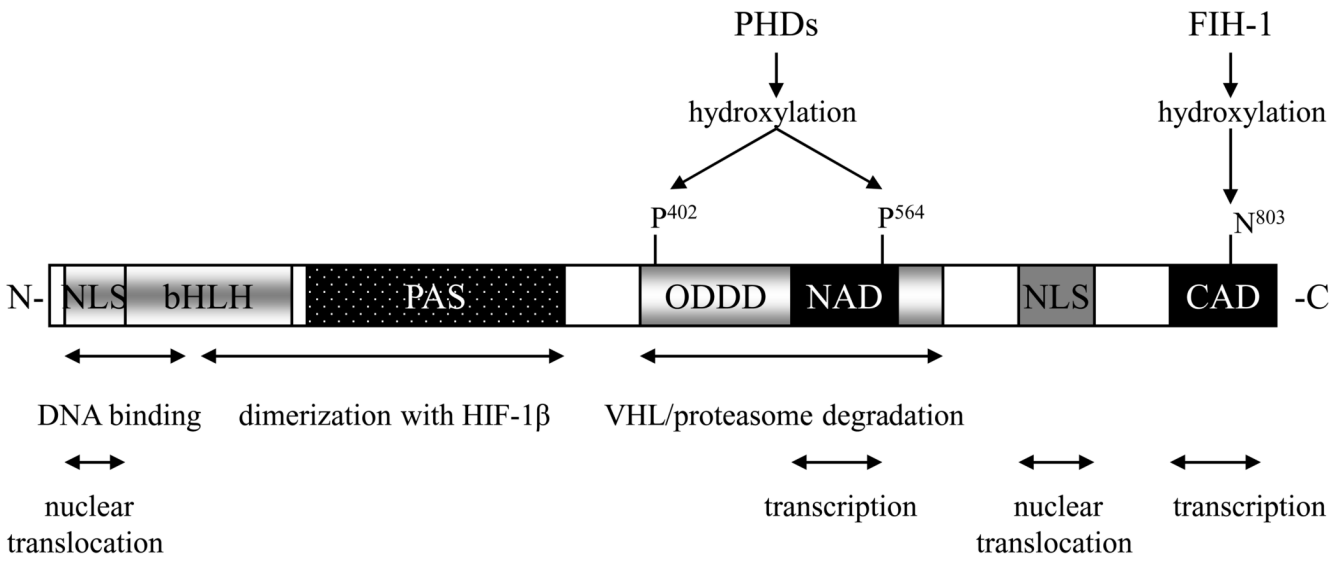


FIGURE 4. HIF-1*α*: schematic outline of the domain structure, function of individual domains, and sites hydroxylated by PHDs and FIH-1

bHLH, basic loop-helix-loop; CAD, C-terminal activation domain; FIH-1, factor inhibiting HIF 1; NAD, N-terminal activation domain; NLS, nuclear localization signal; ODDD, O_2–dependent degradation domain; PAS, PER-ARNT-SIM; PHDs, prolyl hydroxylases.

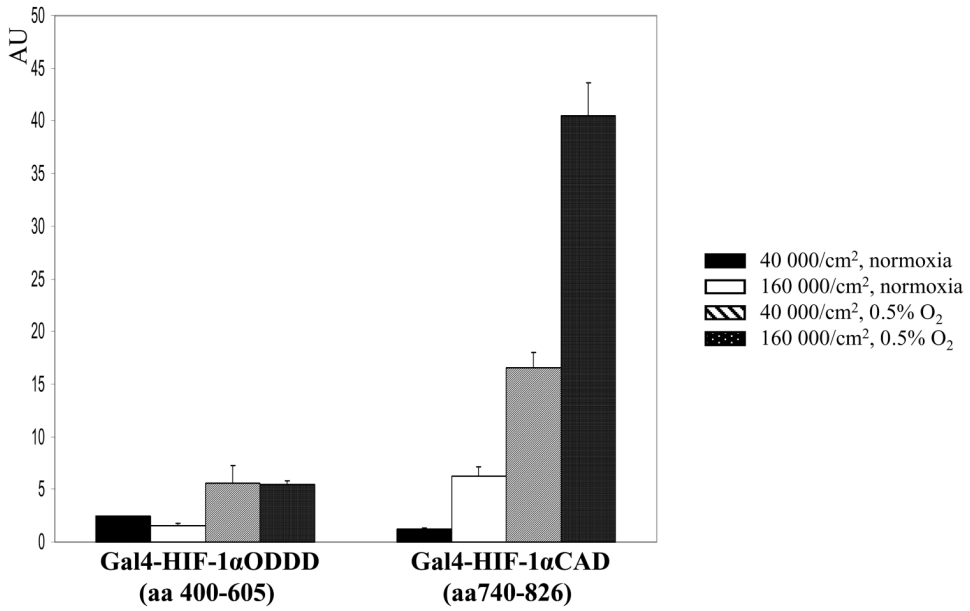


FIGURE 5. The effect of cell density and 0.5% O_2 on activity of Gal4 DNA-binding fusion constructs

Saos-2 cells were transiently co-transfected with the indicated Gal4 expression construct, pFR-Luc (a reporter vector in which the firefly luciferase gene is under the control of a minimal E1B promoter and upstream five copies of a Gal4 binding site, Stratagene), and pRL-CMV vector (internal control expressing *Renilla* luciferase, Promega) using Effectene reagent (QIAGEN). After exposure to the transfection mixture for 16 h, the cells were trypsinized, plated at the indicated cell density, allowed to adhere for 5 h, and exposed to normoxia or 0.5% O_2 for 24 h. Reporter assays were performed with the Dual-Luciferase Reporter Assay System (Promega). Activities of the Gal4-fusionconstructs are expressed as the average ratios of firefly to *Renilla* luciferase activities (± SD) from at least three independent experiments.

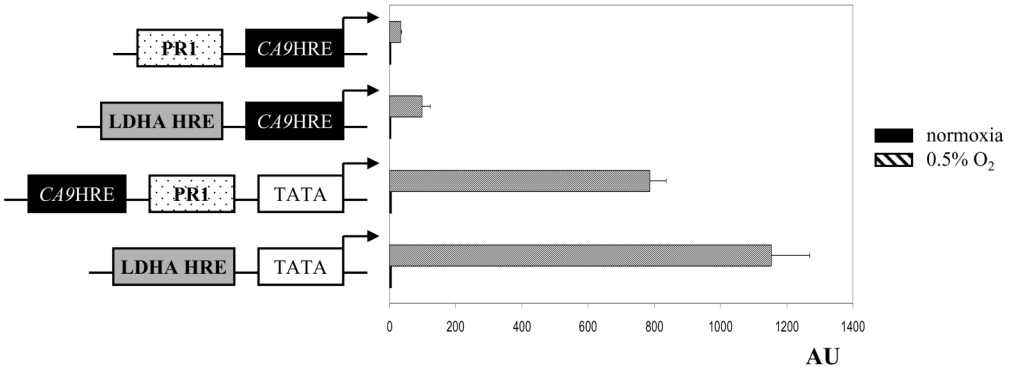


FIGURE 6. Hypoxic inducibility of the *CA9* promoter- and TATA-box based reporter constructs

Transient co-transfection of Saos-2 cells with a reporter construct in which the firefly luciferase gene is under the control of the indicated promoters along with pRL-CMV, and expression of promoter activities is as described in Fig. 5 (except trypsinization).

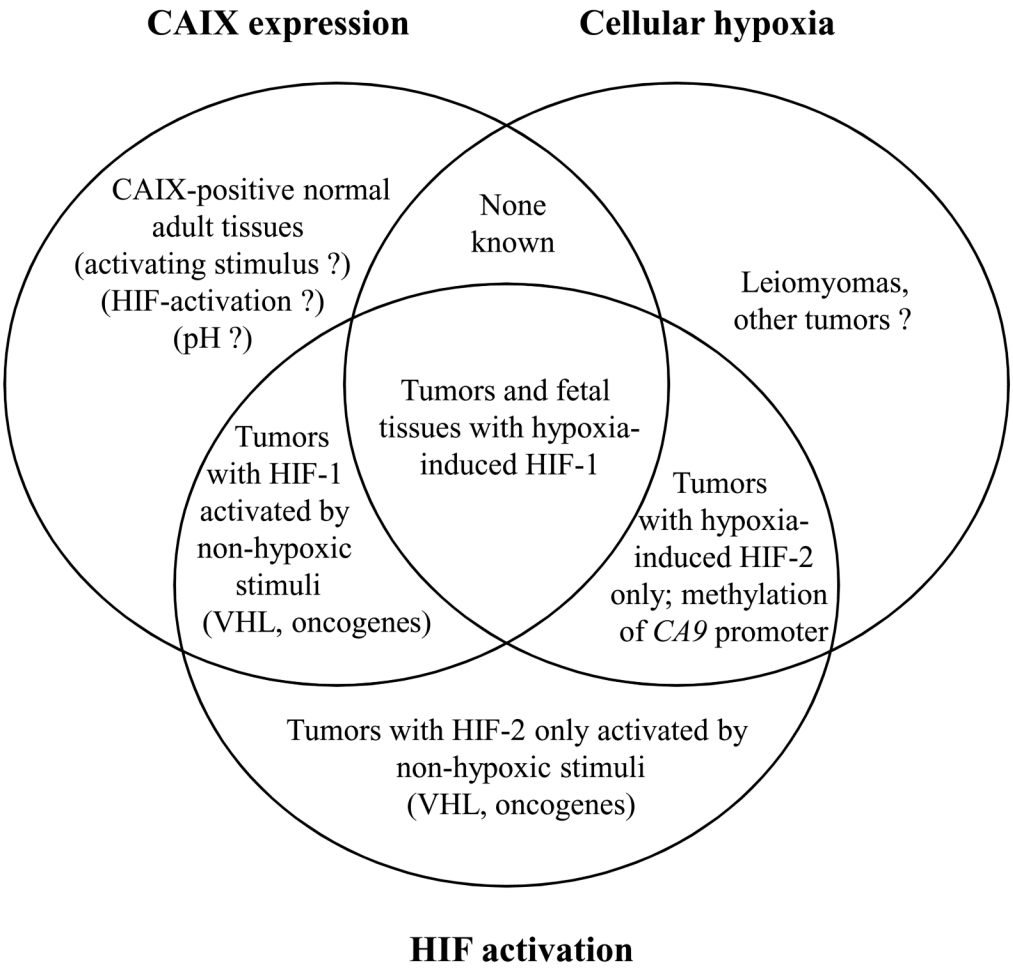


FIGURE 7.
A Venn diagram depicting the relationship between CAIX expression, cellular hypoxia, and HIF activation *in vivo*.

Table 1

Factors regulating *CA9*, their effects on CAIX expression, HIF-1α stability, HIF-1α transcriptional activity, and proposed mechanism of action.

Factor	CAIX expression	HIF-1α stability	HIF-1α transcriptional activity	Mechanism of action	Ref.
Hypoxia	+++	+++	+++	inhibition of PHDs and FIH-1	[8,12]
Iron chelation	+++	+++	+++	⊣	[8,41]
2-OG analogues	+++	+++	+++	⊣	[31]
VHL inactivation	+++	+++	+++	inhibition of proteasome-mediated degradation of HIF-α at the level of polyubiquitylation	[27,8]
Pericellular hypoxia	++	+/−	++	inhibition of FIH-1 (by mild hypoxia and PI3-K?)	[47]
Proteasomal inhibitors	− − −	+++	− − −	inhibition of proteasome-mediated degradation of HIF-α at the level of the proteasome	[45,59]
acidosis	+++*	+++*	+++*	sequestration of VHL in the nucleolus, activation of HIF	[65,66]
low glucose	+/−	ND	ND	?	[66]
PI-3K pathway	+	+	+	increased HIF-α translation, activation of AP1	[47,31]
ERK pathway	+	+/−	+	activation of p300/CBP, AP1, SP1/SP3	[31,71]
p53	− −	−	−	increased degradation of HIF-α	[41]
mithramycin	− − −			inhibition of SP1/SP3 activity	[26]

+++, ++, + strong, intermediate, and weak activation; − − −, − −, − strong, intermediate, and weak inhibition; +/−, no effect;

* cell type-specific; ND not done; ? not known.