Research Paper

Cancer-Associated Carbonic Anhydrases IX and XII: Effect of Growth Factors on Gene Expression in Human Cancer Cell Lines

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AIM: **Carbonic anhydrase IX (CA9) and carbonic anhydrase XII (CA12) are cancer-associated enzymes that are strongly up-regulated by hypoxia via hypoxia-inducible factor HIF-1, but whether these isozymes are regulated by other mechanisms is not well understood. In the present study, we investigated the effects of certain hormones and growth factors on the levels of CA9 and CA12 mRNA expression in human cancer cell lines.**

METHODS: **Seven human cell lines were selected for the study. The cells were treated with several hormones and growth factors for 24 h. Changes in the levels of human CA9 and CA12 transcripts were detected using quantitative real-time PCR.**

RESULTS: **Different growth factors or hormones had different effects on CA9 and CA12 mRNA expression in different cancer cells. The strongest up-regulation of CA9 and CA12 expression was observed after deferoxamine mesylate treatment, which was used to induce a hypoxia-like response. Additionally, CA12 expression could be stimulated by growth factors like IGF-1, TGF-β1 and EGF in U373, MCF-7, Caki-1, and A-498 cells. Induction of CA9 expression was obvious only in U373 cells. Conversely, CA12 expression was reduced in human endothelial cells after growth factor treatments.**

CONCLUSION: **The results suggest that the increase in CA9 and CA12 stimulated by IGF-1, TGF-α, TGF-β1 and EGF is mediated through HIF-1α protein expression, which has been shown to be up-regulated by several growth factors under normal oxygenation conditions in human cell lines. This could represent a novel regulatory mechanism for CA9 and CA12 expression.**

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Keywords:

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Introduction

Carbonic anhydrases IX (CA9[2]) and XII (CA12) are cancer-associated zinc metalloenzymes belonging to a large enzyme family of mammalian α-carbonic anhydrases that contains 13 active isoforms (i.e., I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII, XIV, and XV). These enzymatically active CAs catalyze the reversible hydration of carbon dioxide in the reaction $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$. This reaction affects various biological processes, such as regulation of pH homeostasis, CO_2 and ion transport, respiration, bone resorption, ureagenesis, gluconeogenesis, lipogenesis, production of body fluids, and fertilization [1,2]. Each isozyme has a characteristic subcellular localization, distribution within the body, enzyme activity, and affinity for inhibitors.

Both CA9 and CA12 are transmembrane proteins. CA9 is an unusual isoform because it is present in few normal human tissues, but is ectopically expressed in some tumor cells whose normal counterparts contain none or low levels of this protein. CA9-positive tumors include colon, cervical, breast and renal carcinomas and brain tumors [3]. In addition, the few normal tissues with high natural CA9 expression, such as the stomach and gallbladder, have been demonstrated to lose some, or all, of their CA9 upon conversion to carcinomas [4,5]. The presence of CA9 in various tumor types has been found to correlate with poor prognosis [6-9]. CA9 has also been implicated in the regulation of pH balance during carcinogenesis and tumor progression, but this hypothesis still requires further proof.

In contrast to CA9, CA12 is expressed in a variety of normal human tissues including kidney, colon, prostate, pancreas, ovary, testis, lung, and brain [10,11]. Expression of CA12 usually becomes stronger or more widespread in tumors compared to the corresponding normal tissues [12-15].

Tissue oxygen content has been considered to be the main regulatory mechanism for CA9 and CA12 expression, as both CA9 and CA12 transcript levels are strongly up-regulated via hypoxia-inducible transcription factor 1 (HIF-1). HIF-1 is a heterodimer that consists of an oxygen-sensitive α subunit and a constitutively expressed β subunit. Under normal oxygenation conditions, prolyl-4-hydroxylases (PHDs) hydroxylate two conserved proline residues of HIF-1α. The von Hippel-Lindau protein (pVHL) binds hydroxylated HIF-1α and targets it for degradation by the ubiquitin-proteasome system, and thus abrogates its function in the

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[2]**Abbreviations:** CA, carbonic anhydrase; HIF-1, hypoxia-inducible transcription factor 1; pVHL, von Hippel-Lindau protein; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase.

transcriptional activation of downstream genes [16,17]. In hypoxia, which occurs frequently in tumors as a result of aberrant vasculature, HIF-1α is not hydroxylated, because PHDs are inactive in the absence of oxygen. Non-hydroxylated HIF-1α is not recognized by pVHL; instead, it is stabilized and accumulates in the cell. HIF-1α then translocates to the nucleus and dimerizes with the HIF-1β subunit to form the active transcription factor. The HIF-1 then binds to hypoxia-responsive elements (HREs) within the promoter regions of hypoxia-inducible genes, leading to gene expression. In addition to *CA9* and *CA12*, target genes include glucose transporters (*GLUT-1* and *GLUT-3*), vascular endothelial growth factor (*VEGF*), erythropoietin (*EPO-1*), and a number of other genes with functions in cell survival, proliferation, metabolism, and other cellular processes [18,19]. Thus, activation of the HIF-1 pathway substantially alters the expression profile of pre-malignant lesions by generating tumor cells which either die via apoptosis or adapt to hypoxia. As a result, hypoxia selects more aggressive tumor cells with an increased capability to metastasize, and is therefore often associated with poor prognosis and resistance to anti-cancer therapy [20].

In addition to hypoxia, increased activity of the HIF-1 pathway can result from genetic events such as inactivating mutations in the *VHL* gene or activation of oncogenic pathways, e.g. mitogen-activated protein kinase (MAPK) and/or phosphatidylinositol 3-kinase (PI3K)[20-22]. Notably, suppression of *CA12* expression requires both a central VHL domain involved in the HIF-1α binding and a C-terminal elongin-binding domain, whereas only the latter is needed for the negative regulation of *CA9*. Therefore, CA12 is regulated by hypoxia, in a similar manner to CA9, but using different biochemical pathways [23,24]. Furthermore, CA9 is directly induced by binding of HIF-1 to the HRE within the basal promoter, whereas the *CA12* gene does not contain any HRE that corresponds to the HRE of *CA9*. In fact, the upstream region of the *CA12* gene possesses several putative HREs with a core HIF-binding sequence, but their functionality has not been examined to date. According to tissue distribution analyses and *in vitro* experiments, CA12 does not appear to be as tightly regulated by the hypoxia/pVHL pathway and as strongly linked to cancer as CA9 [11,23].

Apart from hypoxia, very little is known about the mechanisms that regulate CA9 and CA12 expression. It is possible that these cancer-associated enzymes are regulated at higher levels of the biosynthetic pathway in a similar manner to some other hypoxia-induced genes. However, it is also possible that CA12 has an unknown role in cancer progression, which is not directly linked to the hypoxia-associated pathway. In the present study, we examined whether different hormones and growth factors affected the levels of CA9 and CA12 mRNA in human cancer-derived and normal cell lines.

Materials and Methods

Cell lines and treatments

Caki-1 and A-498 human renal carcinoma cell lines and HepG2 human hepatocellular carcinoma cell line were obtained from the American Type Culture Collection (Manassas, VA, USA). HeLa human cervical carcinoma cell line and MCF-7 human breast adenocarcinoma cell line were kindly provided by Professor Jorma Isola (Institute of Medical Technology, University of Tampere, Tampere, Finland). U373 human glioblastoma cell line and HUVEC normal human umbilical vein endothelial cell line were purchased from the European Collection of Cell Cultures (Porton Down, Salisbury, UK).

Caki-1, A-498, and HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Lonza, Venviers, Belgium) supplemented with 10% (v/v) FBS (Biochrom, Cambridge, UK), 2 mM L-glutamine, 100 unit/ml penicillin, and 100 μg/ml streptomycin (Lonza). MCF-7 cells were cultured in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate (Lonza), 1% (v/v) non-essential amino acids (Lonza), and 10 μg/ml insulin (Sigma-Aldrich Finland Oy, Helsinki, Finland). HeLa cells were maintained in RPMI-1640 medium (Lonza) supplemented with 10% FBS and 2 mM L-glutamine. U373 cells were cultured in Alpha MEM (Lonza) supplemented with 10% FBS, 2 mM L-glutamine, 100 unit/ml penicillin, and 100 μg/ml streptomycin. HUVEC cells were maintained in Endothelial Cell Basal Medium-2 (EBM®-2) supplemented with EGM™-2 SingleQuots® kit (Lonza, Walkersville, MD, USA).

All cell cultures were grown in 75-cm^2 flasks in a 37°C incubator with humidified 5% CO_2/95% air. When the cultured cells reached 80-90% of confluence, they were trypsinized and plated in 6-well plates (except for the HUVEC cells which were plated in 59-cm^2 dishes) at appropriate densities to obtain a sufficient quantity of cells for RNA extraction. After 24 h, the cells were changed to fresh serum-free medium with the tested hormones and growth factors. Only serum-free medium was added to two control wells/dishes. The cells were incubated for 24 h in the presence of different hormones or growth factors. The treatments were performed using recombinant human growth hormone (400 ng/ml), hydrocortisone (10 μM), insulin (30 nM), tri-iodothyronine (T3; 10 nM), estradiol (E2; 2.5 μM), recombinant human insulin-like growth factor-1 (IGF-1; 50 ng/ml), recombinant human transforming growth factor-alpha (TGF-α; 10 ng/ml), recombinant human transforming growth factor-beta 1 (TGF-β1; 10 ng/ml), recombinant human epidermal growth factor (EGF; 10 ng/ml), and deferoxamine mesylate (200 μM), an iron chelator commonly used to induce the hypoxia regulatory pathway. Every cell line was treated with the same hormones or growth factors except for MCF-7, which was not treated with insulin because these cells require a high insulin concentration in their culture medium. The growth hormone, IGF-1, TGF-β1 and EGF were purchased from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel). TGF-α was purchased from PromoCell GmbH (Heidelberg, Germany). The rest of the chemicals were obtained from Sigma-Aldrich Finland Oy, and diluted when necessary according to the manufacturers' instructions.

RNA extraction and first-strand cDNA synthesis

Total RNA from cultured cells was extracted using the RNeasy RNA isolation kit (Qiagen, Valencia, USA). Residual DNA was removed from samples using RNase-free DNase (Qiagen). The RNA concentration and purity was determined by measurement of the optical density at 260 and 280 nm. Different quantities of RNA (U373, 410 ng; HeLa, 600 ng; A-498, 850 ng; HepG2, 1000 ng; HUVEC and MCF-7, 1100 ng; Caki-1, 1400 ng) were converted into first-strand cDNA using the First Strand cDNA synthesis kit (Fermentas, Burlington, Canada) using random hexamer primers according to the protocol recommended by the manufacturer.

Quantitative real-time PCR

The levels of human *CA9* and *CA12* transcripts in the different cell lines after indicated treatments were assessed by quantitative real-time PCR using the Lightcycler detection system (Roche, Rotkreuz, Switzerland). Real-time PCR primers were designed based on the complete cDNA sequences deposited in GenBank (accession numbers: NM_001216 for *CA9*, NM_001218 for *CA12*, and NM_001530 for *HIF-1α*). The house-keeping genes *ubiquitin C* (*UBC*) and *β-2-microglobulin* (*B2M*) were used as internal controls to normalize the cDNA samples for possible differences in quality and quantity. The *UBC* primers were obtained from RTprimerDB database (under the identification number 8), and the *B2M* primers were designed on the basis of the complete *B2M* cDNA sequences (accession number: NM_004048). In order to avoid amplification of genomic DNA, the primers from each primer pair were located in

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Table 1: Primer sequences for quantitative real-time PCR used in the study

Gene	Forward primer	Reverse primer	PCR product size	T_a (°C)**
CA9	5'- GGAAGGCTCAGAGACTCA-3'	5'- CTTAGCACTCAGCATCAC-3'	160	53
CA12	5'- CTGCCAGCAACAAGTCAG-3'	5'- ATATTCAGCGGTCCTCTC-3'	179	53
HIF-1α	5'- TCACCTGAGCCTAATAGTCC-3'	5'- GCTAACATCTCCAAGTCTAA-3'	161	52
*UBC**	5'- ATTTGGGTCGCGGTTCTTG-3'	5'- TGCCTTGACATTCTCGATGGT-3'	133	57
B2M	5'- GTATGCCTGCCGTGTGAA-3'	5'- CTCCATGATGCTGCTTAC-3'	84	52

*obtained from RTprimerDB database (http://medgen.ugent.be/rtprimerdb/index.php)
**T_a: annealing temperature

Table 2: *CA9* expression in different cell lines after the treatments (fold change values are shown)**

	U373	HeLa	MCF-7	Caki-1	A-498	HepG2	HUVEC
EGF	2.2	0	0	0	0	0	0
GH	-1.3	0	0	0	0	0	0
HC	1.0	0	0	0	0	0	0
INS	1.4	0	0	0	0	0	0
T3	1.4	0	0	0	0	0	0
DFM	4.2	137.5	0	0	0	0	0
E2	-1.2	0	0	0	0	0	0
IGF-1	2.4	0	0	0	0	0	0
TGF-α	2.6	0	0	0	0	0	0
TGF-β1	1.7	0	0	0	0	0	0

*EGF, epidermal growth factor; GH, growth hormone; HC, hydrocortisone; INS, insulin; T3, tri-iodothyronine; DFM, deferoxamine mesylate; E2, estradiol; IGF-1, insulin-like growth factor-1; TGF-α, transforming growth factor-alpha; TGF-β1, transforming growth factor-beta 1.
**0 = copy number < 100

different exons. The primer sequences used in this study are shown in Table 1.

Each PCR reaction was performed in a total volume of 20 µl containing 1.0 µl of first-strand cDNA, 1× QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany), and 0.5 µM of each primer. The amplification and detection were carried out as follows: after an initial 15 min activation step at 95°C, amplification was performed in a three-step cycling procedure for 45 cycles: denaturation at 95°C for 15 sec, annealing at a temperature determined according to the T_m for each primer pair for 20 sec, and elongation at 72°C for 15 sec (the ramp rate was 20°C per sec for all the steps), and a final cooling step. Melting curve analysis was always performed after the amplification to check the specificity of the PCR reaction. To quantify the levels of transcripts in the cell lines studied, a standard curve was established for each gene using five-fold serial dilutions of known concentrations of purified PCR products generated with the same primer pairs. Every cDNA sample was tested in triplicate, and the obtained crossing point (Cp) value permitted the levels of the starting mRNA to be determined using a specific standard curve. The geometric mean of the two internal control genes was used as an accurate normalization factor for gene expression levels [25]. The final relative mRNA expression was indicated as the copy number of the target gene divided by the corresponding normalization factor and multiplied by 10^3.

Statistical analyses

After a statistical consultation, it was decided that the obtained results should be presented using descriptive methods rather than using extensive statistical comparisons. The median values were calculated from the technical triplicates for the quantitative real-time PCR experiments. Subsequently, the median values for treatments were compared to the median values for negative controls.

Results and Discussion

CA9 had a narrow expression profile and was detected in only two out of seven cell lines (Table 2). In a human glioblastoma cell line, U373, the basal level of *CA9* expression was relatively high (Figure 1). This is in accordance with the fact that the strongest CA9 expression among different gliomas has been observed in the most malignant forms, i.e. glioblastomas [26]. Compared to the control sample, *CA9* expression increased 4.2-fold after deferoxamine mesylate treatment, which was used as a positive control to induce a hypoxia-like response (Table 2 and Figure 1). In addition, the IGF-1, TGF-α and EGF treatments notably increased *CA9* expression compared to the control sample (fold changes of 2.4, 2.6 and 2.2, respectively). After TGF-β1 treatment, *CA9* expression was moderately elevated (1.7-fold).

Interestingly, the inhibition of MAPK and PI3K pathways has been shown to result in reduced CA9 expression under both standard and acidic conditions in human glioblastoma 8-MG-BA cells [27]. It is also known that HIF-1α can be up-regulated under normoxic conditions via the MAPK and PI3K/Akt pathways [22]. Furthermore, it has been demonstrated that EGF up-regulates HIF-1α protein expression in several cell lines, including human embryonic kidney 293 cells [28] and human prostate cancer TSU cells [29]. IGF-1, among other growth factors, also increases the levels of HIF-1α protein [28]. The increase in the level of CA9 mRNA induced by EGF and IGF-1 is therefore likely to be mediated through the stabilization of HIF-1α protein rather than via increased mRNA levels. This was supported by our finding, confirming that HIF-1α mRNA expression was not up-regulated after growth factor treatments in U373 cells (data not shown). This result was expected, as HIF-1α regulation mainly involves post-transcriptional changes [30]. One hypothesis is that up-regulation of *CA9* by EGF and IGF-1 treatments is mediated through activation of the MAPK and/or PI3K pathway. In fact, Dorai and co-workers have demonstrated that the tyrosine moiety of CA9 present in its intracellular domain can be phosphorylated in an EGF-dependent manner in the renal cell carcinoma cell line SKRC-01 [31]. Their results also suggested that tyrosine-phosphorylated CA9 interacted with the regulatory subunit of PI3K, leading to Akt activation. Similarly, TGF-α could increase the level of *CA9* expression via these pathways, since TGF-α is one of the ligands of the EGF receptor [32]. It has also been shown that TGF-β1 can contribute to the accumulation of HIF-1α in normoxic conditions. Stimulation with TGF-β1 increased HIF-1α expression levels and DNA

Table 3: ***CA12*** **expression in different cell lines after the treatments (fold change values are shown)****

	U373	HeLa	MCF-7	Caki-1	A-498	HepG2	HUVEC
EGF	2.9	0	2.4	1.8	2.6	0	-1.3
GH	-1.6	0	1.3	-1.5	-1.3	0	-1.2
HC	1.0	0	-1.1	1.4	-1.6	0	-2.0
INS	1.2	0	ND	1.3	-1.3	0	-1.1
T3	-1.3	0	1.3	1.2	-1.4	0	-1.4
DFM	3.8	0	-1.3	10.2	-1.3	0	1.3
E2	1.0	0	1.9	1.5	1.2	0	1.1
IGF-1	2.0	0	1.3	1.5	1.7	0	-2.2
TGF-α	3.1	0	-1.1	1.1	2.2	0	-1.8
TGF-β1	1.6	0	2.8	1.7	2.5	0	-1.6

***EGF, epidermal growth factor; GH, growth hormone; HC, hydrocortisone; INS, insulin; T3, tri-iodothyronine; DFM, deferoxamine mesylate; E2, estradiol; IGF-1, insulin-like growth factor-1; TGF-α, transforming growth factor-alpha; TGF-β1, transforming growth factor-beta 1.**

****0 = copy number < 100, ND = not done**

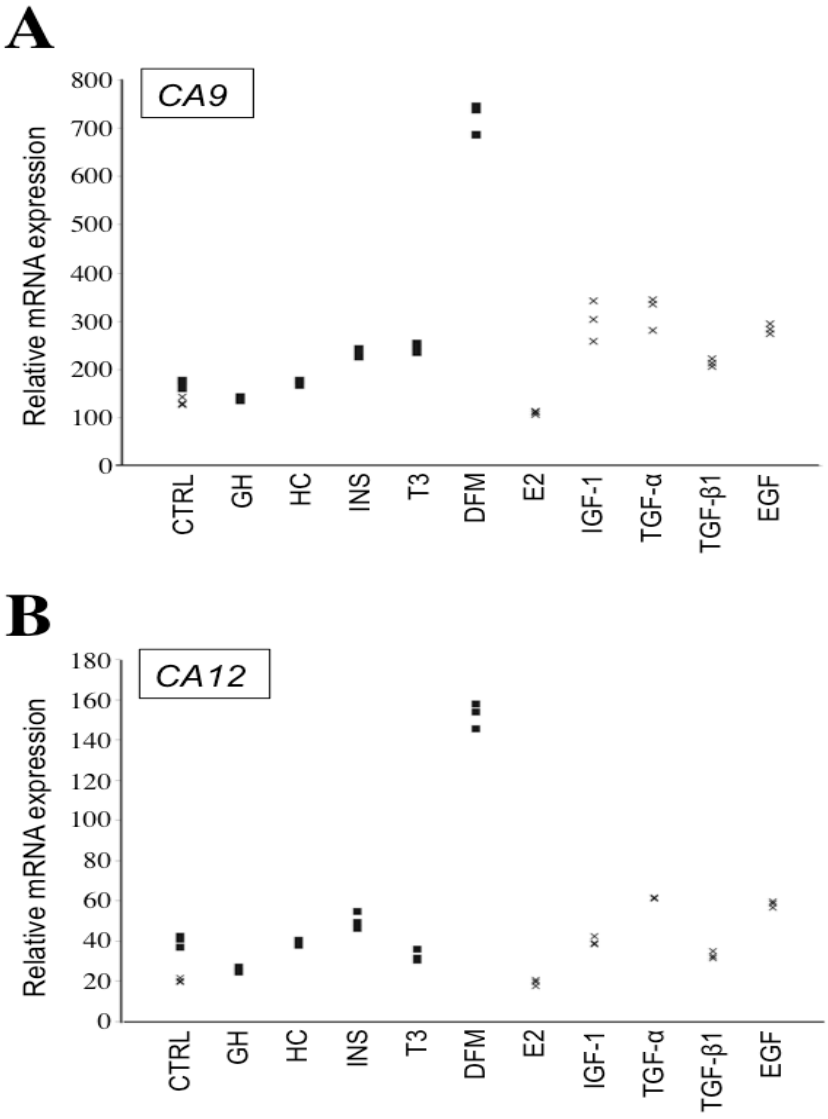

Figure 1: **Effects of different treatments on *CA9* and *CA12* expression in U373 cells, detected by quantitative real-time PCR. The treatments included growth hormone (GH), hydrocortisone (HC), insulin (INS), tri-iodothyronine (T3), deferoxamine mesylate (DFM), estradiol (E2), insulin-like growth factor-1 (IGF-1), transforming growth factor-alpha (TGF-α), transforming growth factor-beta 1 (TGF-β1), and epidermal growth factor (EGF). Both *CA9* (A) and *CA12* (B) expression were maximally induced by DFM treatment. Likewise, growth factor treatments elevated the transcription levels of both isozymes. The normalized values of triplicate experiments are shown. Two control (CTRL) groups were included in the study. The values of the first five treatments are compared to control 1 (squares), and the values of the last five treatments are compared to control 2 (crosses).**

binding activity in human HT-1080 fibrosarcoma cells and in human vascular smooth muscle cells [33,34]. The observed induction of *CA9* by TGF-β1 may also act through accumulation of HIF-1α, but this remains to be tested.

In HeLa cells, *CA9* was expressed only when stimulated with deferoxamine mesylate (Table 2). This change in expression was notable and indicates that hypoxia is an important regulator of *CA9* expression in this cell line, which was already reported [23]. Basal *CA9* expression below the detection limit (copy number < 100) was observed with MCF-7, Caki-1, A-498, HUVEC, and HepG2 cell lines (Table 2). Furthermore, *CA9* expression was not induced by any treatment in these cell lines, suggesting that critical element(s) necessary to drive its transcription could be missing or underrepresented. The fact that *CA9* expression was not induced in Caki-1 cells expressing wild-type pVHL after deferoxamine mesylate treatment is in line with a previous publication showing no *CA9* expression in Caki-1 cells with or without a hypoxic stimulation [23]. It is unclear why the levels of CA9 mRNA were below the detection limit in A-498 cells. It was presumed that there were high basal *CA9* expression levels in these cells due to pVHL inactivation.

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Furthermore, these cells produced CA9 protein under standard cell culture conditions [35]. The most plausible explanation for this discrepancy is that *CA9* transcription was decreased due to the absence of serum in the cell cultures.

CA12 mRNA expression was detected in U373, MCF-7, Caki-1, A-498 and HUVEC cell lines (Table 3). In U373 cells, the CA12 mRNA levels were not as high as the CA9 levels, but the expression patterns were strikingly similar (Table 3 and Figure 1). Deferoxamine mesylate treatment markedly increased *CA12* expression compared to the control sample (3.8-fold). In addition, stimulation with TGF-α and EGF both caused a notable increase in *CA12* expression (fold changes of 3.1 and 2.9, respectively). Likewise, IGF-1 treatment elevated *CA12* levels two-fold and TGF-β1 treatment increased *CA12* levels 1.6-fold. Addition of growth hormone slightly decreased the levels of *CA12* expression (1.6-fold).

In MCF-7 cells, *CA12* was present at relatively high levels with an unusual expression profile (Table 3). *CA12* transcription was clearly induced after TGF-β1 and EGF treatments (fold changes of 2.8 and 2.4, respectively). Estradiol treatment also increased the CA12 mRNA levels (1.9-fold). A similar effect was demonstrated in MCF-7 cells [36]. The study showed that *CA12* was strongly up-regulated by estradiol via the estrogen receptor α (ERα), and that this regulation involved a distal estrogen-responsive enhancer region. We anticipated that the hypoxia pathway would have been induced in this cell line by deferoxamine mesylate treatment, but this was not the case. In fact, deferoxamine mesylate treatment somewhat decreased the levels of CA12 mRNA, and CA9 mRNA remained completely absent. It is notable that in invasive breast cancer, CA12-positive tumors are associated with positive ERα status, lower grade disease, lower relapse rate, and better overall patient survival [37]. Since hypoxia is usually linked to poor prognosis, it is possible that CA12 is not mainly regulated through the hypoxia-induced pathway in breast cancer, but regulation may involve an alternative pathway triggered by estradiol and certain growth factors.

Caki-1 and A-498 human renal carcinoma cell lines were selected for the study, because the first cell line produces wild-type pVHL and the latter represents a VHL-null cell line. In Caki-1 cells, *CA12* showed the highest signal after deferoxamine mesylate treatment (10.2-fold), as expected (Table 3). Again, IGF-1, TGF-β1 and EGF also induced a moderate increase in CA12 mRNA levels (fold changes of 1.5, 1.7 and 1.8, respectively). Moreover, estradiol induced a 1.5-fold increase in *CA12* expression. Similar to U373 cells, growth hormone treatment caused a decrease in *CA12* expression (1.5-fold). In A-498 cells, *CA12* transcript was present in high levels and, as predicted, deferoxamine mesylate treatment had no effect on gene expression (Table 3). The CA12 mRNA levels were also elevated after growth factor treatments. The highest levels of induction were obtained with TGF-β1 and EGF (fold changes of 2.5 and 2.6, respectively), while TGF-α had a slightly weaker effect (2.2-fold). Moreover, IGF-1 stimulation elicited a 1.7-fold increase in the CA12 mRNA levels. Addition of hydrocortisone moderately decreased the CA12 mRNA levels (1.6-fold). It is worth noticing that the mRNA levels of another hypoxia-induced gene, *VEGF*, have also been demonstrated to be down-regulated in A-498 cells after hydrocortisone treatment [38]. Although this effect was suggested to associate with the glucocorticoid receptor pathway, the exact mechanism for the reduced expression was not reported.

In HUVEC cells, *CA12* was expressed at a low level. None of the treatments markedly increased *CA12* expression (Table 3). Interestingly, several treatments reduced *CA12* expression. Hydrocortisone and IGF-1 caused an approximately two-fold decrease in CA12 mRNA levels whereas TGF-α and TGF-β1 treatments resulted in minor decreases (1.8- and 1.6-fold, respectively). In HeLa and HepG2 cells, the levels of *CA12* expression were below the detection limit of the quantitative PCR.

Like *CA9*, *CA12* expression was also elevated in the cancer-derived cell lines after IGF-1, TGF-α, TGF-β1, and EGF treatments. Since *CA12* is not strongly regulated by hypoxia, it is not surprising that it has additional regulators. It is plausible that the main stimulatory effects observed in this study are mediated through up-regulation of HIF-1α protein, which has been shown to be activated by several growth factors under normoxia. Our data suggests that this effect could be specific to cancerous environments, since it was not observed in normal HUVEC cells. However, in contrast, some inhibition of *CA12* was detectable after growth factor treatment. It is also worth noticing that similar to *CA9*, *CA12* expression could not be induced in cell lines lacking *CA12* expression under control conditions.

In summary, an understanding of *CA9* and *CA12* regulation has been mainly confined to hypoxia. Therefore, the object of the present study was to use several hormones and growth factors in order to find novel potential regulators of *CA9* and *CA12* transcription. In conclusion, we have shown that *CA9* and *CA12* are up-regulated by certain growth factors in human cancer-derived cell lines. For *CA9*, this effect was only observed in one cell line. In contrast, *CA12* expression was elevated in several cancer-derived cell lines after growth factor treatment. This induction may represent a novel regulatory mechanism for *CA12* expression in a cancerous environment and merits further investigation. The regulation of CA isozymes has been generally an understudied research area, and future studies should therefore include investigation of the regulation of other CAs. In the present study, insulin and T3 remained the only representatives of hormones or growth factors which elicited no substantial effects in any of the analyzed cell lines.

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