

## The role of hypoxia response element in TGF $\beta$ -induced carbonic anhydrase IX expression in Hep3B human hepatoma cells

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**Abstract:** Carbonic anhydrase IX (CAIX) is a hypoxia-regulated gene. It is overexpressed in a variety of cancers, including hepatocellular cancer. Transforming growth factor  $\beta$  (TGF $\beta$ ) is considered to have an impact on cancer biology due to its important roles in cell proliferation and differentiation. The effect of the TGF $\beta$  on CAIX expression under hypoxia and the mechanism underlying the role of the hypoxia response element (HRE) on this expression are unknown. In this study, we demonstrate that TGF $\beta$  upregulates CAIX expression under hypoxic conditions in the Hep3B hepatoma cell line, indicating that the mitogen-activated protein kinase (MAPK)- and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-signaling pathways might be responsible for this response. Site-directed mutagenesis of the HRE region in CAIX promoter reduced the TGF $\beta$ -induced CAIX promoter activity, pointing to the significance of HRE for this response. Upregulation of TGF $\beta$ -stimulated CAIX expression was consistent with the upregulation of promoter activity of five different truncated constructs of the CAIX promoter under hypoxia. Our findings show that the HRE region is critical for TGF $\beta$ -induced CAIX expression, which is mainly controlled by MAPK and PI3K pathways.

**Key words:** carbonic anhydrase IX (CAIX); hypoxia response element (HRE); transforming growth factor  $\beta$  (TGF $\beta$ ); mitogen-activated protein kinase (MAPK) signaling; phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling

### INTRODUCTION

Hypoxia is the most important feature of tumor microenvironments and is directly associated with metastasis and malignant progression [1]. Hypoxia-inducible factor 1 (HIF1) has a significant role in the adaptation of tumor cells to hypoxic conditions through the activation of the transcription of hypoxia-regulated genes that are involved in several biological processes, such as angiogenesis, cell proliferation and survival, glucose metabolism, pH regulation and migration [2]. Hypoxia response elements (HREs) contain consensus binding sites for HIF1 and HREs are found in the regulatory regions of several genes [3,4]. Carbonic anhydrase IX (CAIX), which is associated with the hypoxic phenotype, is overexpressed in a wide variety of cancers, and its overexpression is clearly related to tumor aggressiveness and poor outcome [5]. CAIX expression is principally correlated with the activation of CAIX gene transcription via HIF1, which binds to the HRE region of the CAIX

promoter, localized at position -10/-3 bp with respect to the transcription start site [6]. Transforming growth factor (TGF)  $\beta$  is involved in a variety of physiological processes, including chronic liver disease related to fibrogenesis in inflammation [7]. TGF $\beta$  has been shown to play both tumor-suppressive and tumor-promoting roles. TGF $\beta$  inhibits hepatocyte proliferation, but it also promotes hepatocellular carcinoma (HCC) [8-10]. Hypoxia leads to the death of cells at the center of the tumor via as a result of decreased oxygen concentration. Hung et al. [11] reported that hypoxia increased the expression of TGF $\beta$ 1. Zhang et al. [12] demonstrated that TGF $\beta$ 2 gene expression was upregulated by hypoxic stimulation. Similarly, Nishi et al. [13] reported that hypoxia transcriptionally upregulates TGF $\beta$ 3 promoter activity, resulting in increased TGF $\beta$ 3 expression. While our understanding of the effect of hypoxia on the expression of the TGF $\beta$  gene family has increased, there is very little information on the effect of TGF $\beta$ s on the expression of hypoxia-regulated genes. Specifically, there is virtu-

ally no study on the hypoxia-regulated and potential hypoxia marker gene, CAIX.

The HRE region, specifically nucleotides between -10/-3 bp, has a pivotal role in the transcriptional regulation of CAIX. As HIF1 $\alpha$ -deficient cells have no CAIX promoter activity, HIF bound to the HRE region appears to be important for the binding of other transcription factors [6,14]. In our previous work, we reported that TGF $\beta$  transcriptionally upregulates CAIX expression at normoxia [14]. Considering several reports on CAIX upregulation under hypoxia and the induced release of TGF $\beta$  in hypoxic conditions, we examined the relationship between the HRE region and TGF $\beta$ -mediated upregulation of CAIX promoter activity under hypoxia. Thus, the aim of this study was to analyze the effect of TGF $\beta$  on CAIX expression in the human hepatoma cell line under hypoxic conditions. In the present work we utilized site-directed mutagenesis of the HRE region in the CAIX promoter to obtain a better understanding of the TGF $\beta$ -induced regulation of CAIX under hypoxia. The findings of this study shed light on the mechanism regulating CAIX, one of the most important hypoxia-regulated genes that serves as an essential target for anticancer therapy.

## MATERIALS AND METHODS

### Materials

The human hepatoma cell line, Hep3B, was obtained from Dr. Dipak Ramji (Cardiff University School of Medicine, England). All tissue culture reagents were purchased from Invitrogen; recombinant Human TGF $\beta$  was obtained from Cell Signaling Technology; the antibodies were from Abcam, Santa Cruz Biotechnology and Sigma-Aldrich; wortmannin and PD98059 were purchased from Cell Signaling; phorbol 12-myristate 13-acetate (PMA) was obtained from Santa Cruz Biotechnology. The luciferase reporter assay kits were from Clontech and Promega. The primers for site-directed mutagenesis were obtained from Macrogen.

### Plasmids and site-directed mutagenesis

The following 5' truncated CAIX promoter constructs, 1289 bp (-1251/+38), 973 bp(-935/+38), 504 bp (-466/+38), 304 bp (-266/+38) and 154 bp (-116/+38),

were cloned and verified previously by Yildirim and Kockar [14]. A 154-bp promoter construct served as a template DNA for subcloning in to the pMetLuc reporter vector. The sequence of the PCR-amplified 154-bp wild-type (WT) promoter fragment was verified by automated sequencing. PCR-based site-directed mutagenesis was carried out using the mutant oligonucleotides as follows: forward 5'-AGCTCTCGTTTC-CAAAAAAAAAAAGCCCGTACACA-3', reverse 5'-TGTGTACGGGCTTTTTTTTTTTTGGAAAC-GAGAGCT-3'. Mutant plasmids were transformed into *Escherichia coli* XL1-Blue cells and the sequence of the PCR-amplified mutated (MUT) promoter fragment was verified by automated sequence analysis.

### Cell culture, cytokine treatment and the hypoxia model

Hep3B cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) in humidified air containing 5% CO<sub>2</sub>, at 37°C. Cell viability was controlled by trypan blue exclusion. Cells were treated with 150 $\mu$ M of a final concentration of CoCl<sub>2</sub> to obtain hypoxic conditions [15]. Before the cytokine treatment, cells were grown overnight in a serum-free media supplemented with 0.1% bovine serum albumin (BSA); serum-starved cells were treated with 200U/mL of TGF $\beta$  for different time intervals (24, 48 and 72h).

### MTT assay

Cell viability was determined by the MTT assay, as described previously [16]. Briefly, the cells were plated onto a 96-well plate and incubated with 200 U/mL of TGF $\beta$  for 24 and 48h. At the end of the incubation times, 20  $\mu$ L of MTT (5 mg/ml) per well were added and the cells were incubated for 4 h at 37°C. The medium was removed and formazan crystals were dissolved in 100  $\mu$ L of acidified isopropanol (0.04 N HCl). Absorbance was measured at 550 nm using a spectrophotometer (Thermo Scientific). Two independent experiments were performed in triplicate.

### Transient transfection

Transient transfection was carried out by the calcium phosphate precipitation method [14,16]. Different

lengths of the 5' truncated constructs of the CAIX promoter were transiently transfected into Hep3B cells. The cells were co-transfected with cytomegalovirus (CMV)- $\beta$ -galactosidase plasmid, which served as an internal control of transfection efficiency. Luciferase/ $\beta$ -galactosidase activities in cell extracts were determined using Promega luciferase kits. The luciferase activity was normalized to the  $\beta$ -galactosidase value, and each transfection was repeated at least three times.

The luciferase activities of WT and MUT -116/+38 bp promoter constructs of CAIX, luciferase and secreted alkaline phosphatase (SEAP) activities were measured using the Ready-To-Glow™ Secreted Luciferase Reporter Systems (Clontech) and Fluoroskan Ascent FL Luminometer (Thermo Electron Co.). Luciferase activity was normalized to the SEAP values and each transfection was repeated at least three times.

### RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using the GeneJET™ RNA Purification Kit according to the manufacturer's recommendations (Thermo Scientific). cDNAs were generated using RevertAid Reverse Transcriptase (Fermentas). qRT-PCR (SYBR Green I Master, Roche) was used to measure the mRNA expression levels of CAIX, HIF1 $\alpha$  and human  $\beta$ 2 microglobulin (h $\beta$ 2). The PCR reaction was performed using 10  $\mu$ L total volume; verification of product specificity was performed by melting curve analysis. The  $\Delta$ -Ct method was used for analysis of the results [17].

### Western blotting

Protein samples were prepared using radio immunoprecipitation assay (RIPA) buffer and size-fractionated under reducing conditions by electrophoresis in 10% (w/v) polyacrylamide gels, followed by transfer to Immobilon-P PVDF membranes (Millipore) by blotting. After incubation of the blotted membranes with antibodies, bands were detected using an enhanced chemiluminescence detection kit (Pierce) and X-ray sensitive film (Kodak). Image J software<sup>19</sup> was used for quantitative analysis of relative protein expression of CAIX and  $\beta$ -actin.

### Electrophoretic mobility shift assays (EMSA)

EMSA, using whole cell extracts from Hep3B cells, were performed using the Pierce kit according to the manufacturer's instructions (Thermo Scientific). WT sequences of the oligonucleotides used as probes were 5'-CTC TCG TTT CCA ATG CAC GTA CAG CCC GTA CAC A-3'; 5'-TGT GTA CGG GCT GTA CGT GCA TTG GAA ACG AGA G-3'. MUT oligos were 5'-AGC TCT CGT TTC CAA AAA AAAAAA GCC CGT ACA CA-3'; 5'-TGT GTA CGG GCT TTTT'TTTT TGG AAA CGA GAG CT-3', respectively. Briefly, the DNA binding reaction was performed using 4  $\mu$ g nuclear extracts and DNA-binding buffer according to the manufacturer's instructions. Samples were loaded on 5% native polyacrylamide gels; the gels were transferred to nylon membranes. Complexes were detected by autoradiography (Thermo Scientific). Anti-HIF1 $\alpha$  antibody was used for the super-shift assay.

### Cell pathway inhibitors

The experimental setup for the inhibition studies was as follows: the cells were seeded in 6-well plates and incubated overnight, followed by CoCl<sub>2</sub> treatment for hypoxic conditions. Next, the cells were treated with a 10- $\mu$ M final concentration of PD98059 (MEK-1 inhibitor, Cell Signaling 9900S); 1- $\mu$ M final concentration of wortmannin (PI3K inhibitor, Cell Signaling 99515); 3.125-nM final concentration of PMA, protein kinase C (PKC) activator, Santa Cruz 16561-29-8) for 60 min and 200-U/mL TGF $\beta$  stimulation for 6 h. Finally, cells were harvested for qRT-PCR and western blotting experiments.

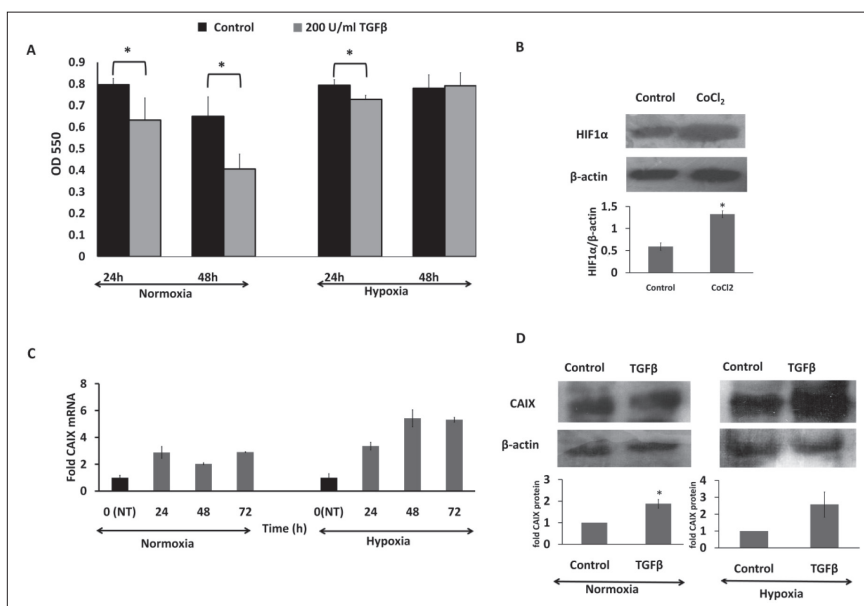
### Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA). A probability (P) of 0.05 or less was deemed statistically significant.

## RESULTS

### TGF $\beta$ increases CAIX expression under hypoxic conditions in Hep3B Cells

The effects of TGF $\beta$  on the proliferation of Hep3B cells were determined under normoxic and hypoxic



**Fig. 1.** TGF $\beta$  stimulates CAIX gene expression. Hep3B cells were serum-starved, treated with CoCl<sub>2</sub> to produce hypoxia, cultured in the presence of 200 U/mL of TGF $\beta$  and harvested as indicated. **A** – MTT assay was performed as described above. Treatment with 200 U/mL of TGF $\beta$  inhibited the proliferation of Hep3B cells. Non-treated cells served as the control. **B** – HIF1 $\alpha$  protein levels in Hep3B cells under normoxic (control) and hypoxic (CoCl<sub>2</sub>) conditions. A 72 h CoCl<sub>2</sub> treatment increased the level of HIF1 $\alpha$  protein. **C** – TGF $\beta$ -induced CAIX mRNA expression in Hep3B cells under normoxia and hypoxia. Total RNA was isolated, reverse transcribed and 1  $\mu$ g cDNA was used as a template for qRT-PCR. The results are presented as the fold induction compared with each non-treated control group. Non-treated cells served as the control. **D** – TGF $\beta$ -induced CAIX protein expression in Hep3B cells under normoxia and hypoxia. 200 U/mL TGF $\beta$  treated and control cell lysates were used for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Anti CAIX antibody was used to detect CAIX protein in Hep3B lysates.  $\beta$ -actin served to normalize data using the IMAGE J program.

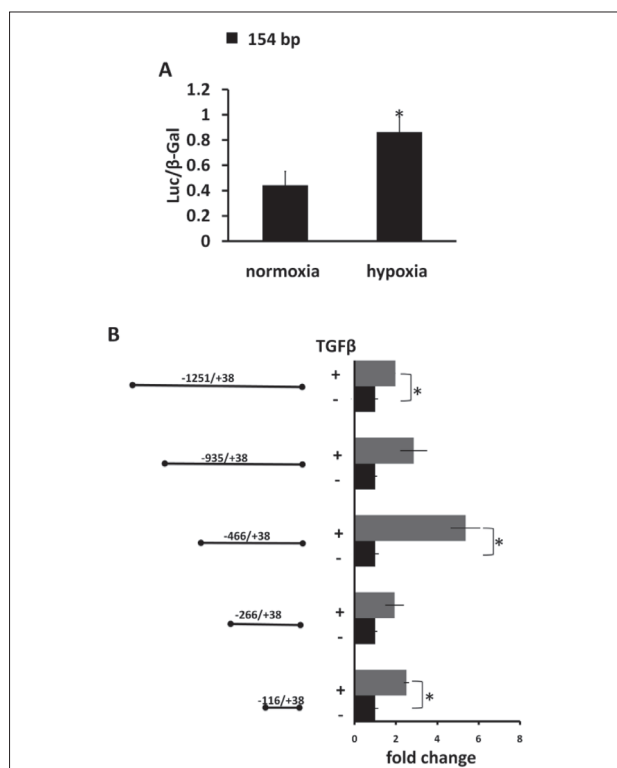
conditions by the MTT assay. Hep3B cells were serum-starved and treated with 200 U/mL of TGF $\beta$  for 24 and 48 h under normoxic and hypoxic conditions. For the hypoxic condition, the cells were treated with CoCl<sub>2</sub> before the cytokine treatment. The CoCl<sub>2</sub>-induced hypoxic condition is used in mammalian systems, and several studies described the induction of a response similar to that observed under low-oxygen conditions [15,18-25]. TGF $\beta$  caused a statistically important reduction in the proliferation of Hep3B cells under normoxic conditions for 24 and 48h. TGF $\beta$  treatment under hypoxia also reduced the proliferation of Hep3B cells; precisely, the reduction was statistically significant at 24 h (Fig. 1A).

The effect of TGF $\beta$  on CAIX expression under hypoxic conditions was determined by qRT-PCR and western blot analysis. The CoCl<sub>2</sub>-induced hypoxic

conditions were verified by determining HIF1 $\alpha$  protein expression. As can be seen in Fig. 1B, the CoCl<sub>2</sub>-treatment increased the HIF1 $\alpha$  protein level 2.2-fold when compared to the normoxic condition, which served as the control for Hep3B cells for 72h. CAIX mRNA levels were increased up to 3-fold at 72h under normoxia, whereas the hypoxic condition led to increased CAIX expression, resulting in a 5.4-fold increase as compared to the control group. TGF $\beta$  stimulation caused a statistically significant increase in CAIX mRNA levels in Hep3B cells (Fig. 1C). Western blot analysis (Fig. 1D) revealed a 1.8-fold increase in CAIX protein level as compared to the control group under normoxia. The effect of TGF $\beta$  on CAIX protein expression under hypoxia was greater than in normoxia, causing a 2.5-fold increase in CAIX protein expression as compared to the non-treated group.

## Transcriptional activity of mutated HRE CAIX promoter was not affected by TGF $\beta$

The effect of TGF $\beta$  under hypoxia on the transcriptional activities of the CAIX promoter constructs was examined in Hep3B cells. The transcriptional activity of the 154-bp promoter construct was about 2-fold higher under hypoxia (Fig. 2A) in Hep3B cells transfected with 5' truncated promoter regions and

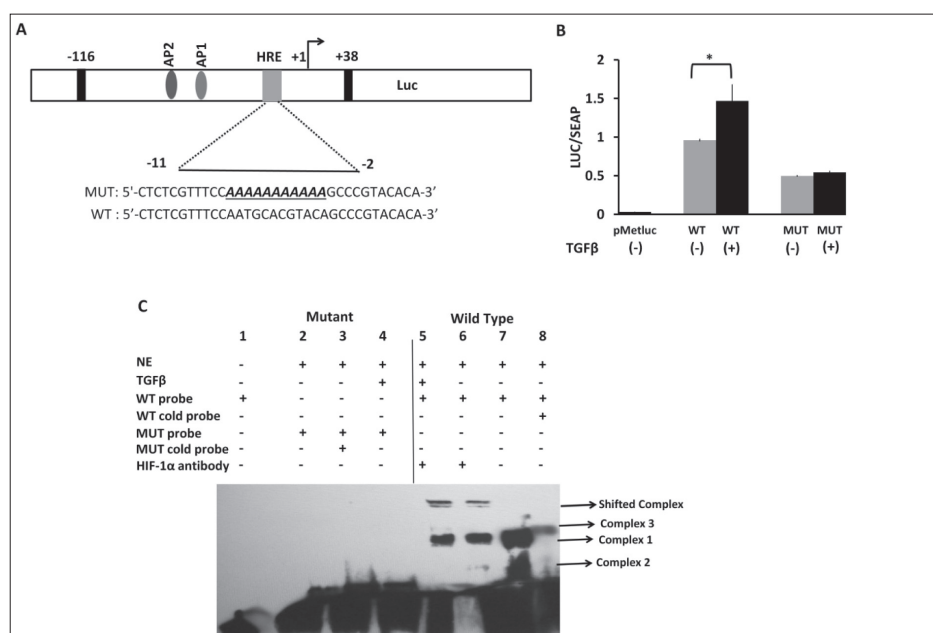


**Fig. 2.** Transcriptional regulation of CAIX gene under hypoxia. **A** – Transcriptional activity of a 154-bp promoter construct in normoxic and hypoxic Hep3B cells. The 154-bp CAIX promoter construct was transiently transfected into Hep3B cells under normoxia and hypoxia. Cells were cotransfected with cytomegalovirus (CMV)- $\beta$ -galactosidase plasmid to provide an internal control for transfection efficiency. After 72 h of transfection, luciferase activity was determined using a luminometer and normalized to the  $\beta$ -galactosidase value. Results are expressed as means $\pm$ standard error of normalized promoter activity (luciferase/ $\beta$ -gal). **B** – The effect of TGF $\beta$  on the transcriptional activity of 5' truncated CAIX promoter constructs. The effect of TGF $\beta$  on basal promoter activity was detected by calcium phosphate transfection. Different lengths of the CAIX promoter 5' truncated constructs were transiently transfected into Hep3B cells. Cells were cotransfected with CMV- $\beta$ -galactosidase plasmid to provide an internal control for transfection efficiency. After 24 h of transfection, cells were treated with TGF $\beta$  for 72h. Luciferase activity was normalized to the  $\beta$ -galactosidase value. Results are expressed as the fold activity of normalized data compared to each non-treated control (\* $P$ <0.05).

stimulated by 200 U/mL of TGF $\beta$  for 72 h (Fig. 2B). While all promoter-luciferase DNA constructs were upregulated by TGF $\beta$ , the level of stimulation for the 504-bp promoter construct was 5.3-fold higher than in the non-treated group, which is consistent with our previous results under normoxic conditions [14].

To determine the role of the HRE region in response to TGF $\beta$  stimulation in Hep3B cells, the HRE region of the CAIX promoter was modified by site-directed mutagenesis (Fig. 3A). Briefly, all the nucleotides of the HRE (-11/-2 bp) in the CAIX promoter were replaced with adenine nucleotide to prevent the binding of HIF protein complex to this region. WT CAIX promoter-luciferase DNA and CAIX-HRE MUT promoter-luciferase DNA constructs were transfected into Hep3B cells under hypoxia, along with the SEAP expression plasmid. Mutation in the HRE region of the CAIX promoter caused a significant decrease in basal promoter activity when compared to the wild-type promoter. TGF $\beta$  upregulated promoter activity was also reduced in the HRE-mutated CAIX promoter construct (Fig. 3B).

To characterize the DNA-binding activity on the HRE sequence of the CAIX promoter, we performed EMSA with hypoxic Hep3B nuclear extracts with mutated and wild-type oligos of the HRE region contained in the CAIX promoter (Fig. 3A). In EMSA with wild-type oligos, we could clearly identify two binding complexes, 1 and 2, in WT HRE (Fig. 3C, lane 7). TGF $\beta$ -treated nuclear extract leads to a new complex formation – complex 3, as indicated in lane 5 (Fig. 3C). The intensities of complexes 1 and 2 were reduced when a 500-fold excess of the non-radiolabeled WT HRE oligonucleotide was used, indicating that these DNA-binding complexes are specific (Fig. 3C, lane 8). To confirm that the complex was formed with HIF1 $\alpha$  transcription factors, an antibody super-shift assay was performed in which nuclear extracts were incubated with HIF1 $\alpha$  antibody. Super-shifted bands were detected, as can be seen in Fig. 3C, lanes 5 and 6. However, these DNA-protein complexes were completely absent in the presence of HRE mutated oligos (lane 2), which indicated that this region is important for hypoxia-mediated transactivation.



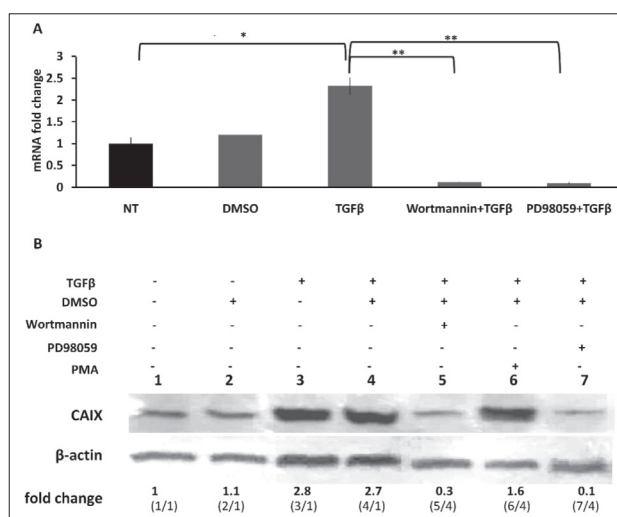
**Fig. 3.** Site-directed mutagenesis of CAIX. **A** – Schematic representation of the mutated CAIX promoter-luciferase DNA construct. The sequence of the mutated HRE region of the CAIX gene is underlined. The HRE region of the promoter was replaced with adenine nucleotides by site directed mutagenesis. The transcription start site is indicated as +1. The positions of binding sites on HRE for transcription factors, p53, AP1 and AP2 are shown in different symbols. **B** – TGFβ increases the transcriptional activity of the WT human CAIX gene promoter, but not the mutated MUT promoter under hypoxia. Hep3B cells were transiently transfected with 1 μg/mL wild/mutated promoter constructs. A SEAP control (0.5 μg/mL) vector was used to assess transfection efficiency in cells cotransfected with all promoter constructs. After 24 h of transfection, the cells were treated with TGFβ for 48h. Hypoxic conditions were created in cells by treatment with CoCl<sub>2</sub> before the cytokine treatment. Luciferase activity was normalized to SEAP activity. Results are expressed as means ± standard error of normalized promoter activity (luciferase/SEAP) (\*P<0.05). **C** – Characterization of the DNA-binding activity on the HRE sequence of CAIX promoter. EMSA was performed using hypoxic Hep3B nuclear extracts with mutated and wild type oligos of the HRE region. Arrows indicate the positions of the complexes. (NE: nuclear extracts; WT: wild type; MUT: mutated).

### The effect of PI3K and MAPK pathway inhibitors on TGFβ-induced CAIX expression

CAIX protein expression was induced in Hep3B cells by treatment with TGFβ under hypoxic conditions. The roles of MAPK and PI3K pathways in the stimulation were investigated in experiments in which specific pathway inhibitors were used. The effect of pathway inhibitors on TGFβ-induced CAIX expression was studied by qRT-PCR and Western blotting (Fig. 4A and B). Both the PI3K-specific inhibitor wortmannin, and the MAPK pathway-specific inhibitor PD98059 abolished the TGFβ-induced CAIX expression. In addition, treatment with PMA, the endogenous activator of PKC, resulted in a 1.6-fold increase in CAIX protein (Fig 4B).

### DISCUSSION

CAIX is a cell-surface protein frequently expressed in different tumor types but not in corresponding normal tissues. CAIX expression is primarily determined by the strong transcriptional activation of the gene encoding CAIX by hypoxia via the hypoxia-inducible transcription factor HIF1α [26]. In the present study, we showed for the first time that TGFβ-mediated CAIX expression in the hepatoma cell line under hypoxia was abolished by site-directed mutagenesis of the HRE region contained in the CAIX promoter. Pathway inhibition studies demonstrated that PI3K and MAPK/ERK pathways are involved in the TGFβ-mediated CAIX upregulation.



**Fig. 4.** PI3K and MAPK pathway inhibitors suppressed CAIX expression in Hep3B cells. **A** – CAIX mRNA expression in Hep3B cells in the presence of 200 U/mL TGFβ, 10 μM PD98059 (PI3K inhibitor), and 1 μM wortmannin (MAPK inhibitor) determined by qRT-PCR. **B** – Western analysis of CAIX in Hep3B cells after additional treatments (DMSO, TGFβ, PMA, PD98059 and wortmannin). Densitometric analysis was performed using Image J (NIH); CAIX bands were normalized to β-actin bands. Values represent the fold change relative to control cells (given in brackets). \*P<0.05, \*\*P<0.01.

TGFβ is a pleiotropic cytokine that transcriptionally regulates the expression of genes that are important in cell growth, proliferation, differentiation and migration [27,28]. Hypoxia has a pivotal role in hepatocellular cancers. Although one study showed that treatment of rat hepatocytes with 100-1000 μM CoCl<sub>2</sub> leads to a decrease in cell viability in a dose-dependent manner [29], the CoCl<sub>2</sub>-induced hypoxic model has been used in several studies [15,18-25]. Our results also showed that treatment of Hep3B cells with 150 μM of CoCl<sub>2</sub> did not significantly reduce cell viability in comparison to control groups that were exposed to normoxia and hypoxia. Some researchers have suggested that there is some variation in the responses of different cells to TGFβ [30,31]. Consistent with reports showing the inhibitory effect of TGFβ in different hepatoma cell lines, TGFβ stimulation caused a statistically significant reduction in proliferation of Hep3B cells.

TGF-β plays a critical role from the initial liver injury up to the end-stage of HCC; this is supported by the finding that inhibition of the TGF-β-signaling pathway attenuates HCC progression [32,33]. Sev-

eral epidemiologic studies have shown that patients with hepatocellular carcinoma have higher circulating TGFβ concentrations in comparison to healthy individuals. A positive relationship between increased concentrations of circulating TGFβ and increased risk of hepatocellular carcinoma was also demonstrated in meta-analyses [34,35]. Our previous studies demonstrated that TGFβ upregulates CAIX expression at mRNA and protein levels in hepatocellular cell lines under normoxia and hypoxia [36]. However, the mechanism underlying this upregulation remains unclear. In agreement with previous reports, TGFβ was observed to stimulate the time-dependent increase in CAIX mRNA and protein under hypoxia. As none of the previous studies have addressed the mechanism of the TGFβ-dependent CAIX gene expression under hypoxic conditions, we analyzed the effect of TGFβ on the activities of CAIX promoter constructs under hypoxia. Transient-transfection analysis in Hep3B showed that all promoter-luciferase DNA constructs were upregulated by TGFβ; specifically, a higher stimulation of activity was observed with the 504-bp promoter construct, as in the normoxic condition [14]. The importance of response of the HRE region to TGFβ stimulation was tested by site-directed mutagenesis. Mutation of the HRE region completely ablated TGFβ-induced and basal promoter activities. The role of the HRE region in hypoxia-dependent activity of the CAIX promoter was demonstrated by small fragments of the HRE region [6]; our mutation studies concentrated on the more extensive area around the HRE region. EMSA analysis of the -11/-2 bp promoter region revealed the formation of three complexes, whereas the antibody super-shift assay demonstrated that complexes 1 and 2 were super-shifted by the HIF1α antibody. EMSA analysis suggested that the TGFβ treatment induced the formation of a new complex (complex 3); this was absent when the mutated -11/-2 bp CAIX promoter region was used, and no shift was observed in complex 3 in the antibody super-shift assay. These results suggest that TGFβ stimulation induced other transcription factors to bind to the CAIX promoter region located between -11/-2 bp.

Members of the TGFβ superfamily mediate downstream intracellular signaling via the Smad-dependent (canonical) or Smad-independent (non-canonical)

pathways. In non-canonical pathways, TGF- $\beta$  moieties transduce their signals via MAPK, PI3K-Akt, AMP-activated kinase (AMPK) and others [37-39]. Our pathway inhibition studies for TGF $\beta$  focused on the non-canonical pathways, which involve PI3K and MAPK/ERK, since there are no consensus Smad-binding sites in the CAIX promoter [14]. The effect of inhibitors on TGF $\beta$ -induced CAIX expression was investigated by qRT-PCR and Western blot analysis. Inhibitor studies showed that the TGF $\beta$ -mediated upregulation in CAIX expression was removed by inhibition of the MEK1 and PI3K pathways. These results indicate that PI3K and MAPK/ERK pathways are involved in the TGF $\beta$ -mediated upregulation of CAIX mRNA and CAIX protein expressions.

To conclude, TGF $\beta$ -induced upregulation of CAIX under hypoxia is tightly controlled by the HRE element contained in the promoter. PI3K and MAPK/ERK pathways are also involved in this regulation. As hypoxia-induced proteins are important targets for anticancer therapy, understanding the mechanisms underlying TGF $\beta$ -mediated upregulation of CAIX in hepatoma cells could assist in the development of novel anti-hepatocellular carcinoma strategies.

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**Authors' contribution:** HY and FK conceived and designed the study. HY and MK performed the experiments. HY wrote the paper.

**Conflict of interest disclosure:** The authors state no conflict of interest.

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