Dexamethasone downregulates expression of carbonic anhydrase IX via HIF-1α and NF-κB-dependent mechanisms

VERONIKA SIMKO, MARTINA TAKACOVA, MICHAELA DEBREOVA, KATARINA LAPOSOVA, ELENA ONDRISKOVA-PANISOVA, SILVIA PASTOREKOVA, LUCIA CSADEROVA and JAROMIR PASTOREK

Institute of Virology, Biomedical Research Center, Slovak Academy of Sciences, Bratislava, Slovak Republic

Received April 12, 2016; Accepted June 21, 2016

DOI: 10.3892/ijo.2016.3621

Abstract. Dexamethasone is a synthetic glucocorticoid frequently used to suppress side-effects of anticancer chemotherapy. In the present study, we showed that dexamethasone treatment leads to concentration-dependent downregulation of cancer-associated marker, carbonic anhydrase IX (CA IX), at the level of promoter activity, mRNA and protein expression in 2D and 3D cancer cell models. The effect of dexamethasone on CA IX expression under hypoxic conditions is predominantly mediated by impaired transcriptional activity and decreased protein level of the main hypoxic transcription factor HIF-1α. In addition, *CA9* downregulation can be caused by protein-protein interactions between activated glucocorticoid receptors, major effectors of glucocorticoid action, and transcription factors that trigger *CA9* transcription (e.g. AP-1). Moreover, we identified a potential NF-κB binding site in the *CA9* promoter and propose the involvement of NF-κB in the dexamethasone-mediated inhibition of *CA9* transcription. As high level of CA IX is often linked to aggressive tumor behavior, poor prognosis and chemo- and radiotherapy resistance, uncovering its reduction after dexamethasone treatment and implication of additional regulatory mechanisms can be relevant for the CA IX-related clinical applications.

Correspondence to: Dr Lucia Csaderova, Department of Molecular Medicine, Institute of Virology, Biomedical Research Center, Slovak Academy of Sciences, Dubravska cesta 9, 84505 Bratislava, Slovak Republic
E-mail: lucia.csaderova@savba.sk

Abbreviations: CA IX, carbonic anhydrase IX; DEX, dexamethasone; GC, glucocorticoid; GR; glucocorticoid receptor; GRE, glucocorticoid response element; HD, Huntington's disease; HIF-1, hypoxia-inducible factor-1; HRE, hypoxia response element; Hy, hypoxia; LDHa, lactate dehydrogenase A; NF-κB, nuclear factor-kappaB; TIS, transcription initiation site; VEGF, vascular endothelial growth factor

Key words: dexamethasone, hypoxia-inducible factor-1α, carbonic anhydrase IX, transcriptional regulation, nuclear factor-kappaB

Introduction

Hypoxia as a consequence of low oxygenation caused by impaired and aberrant vascularization is a common feature of many malignant tumors. Hypoxia leads to reduced apoptosis, increased proliferation and angiogenesis predominantly via altered gene expression. The main factor mediating this oxygen sensitive response is the hypoxia-inducible factor-1 (HIF-1) that consists of a constitutive β subunit and an oxygen-sensitive α subunit that is regulated through O_2-dependent degradation by prolyl hydroxylation. Oxygenation of cells results in the binding of the von Hippel-Lindau tumor-suppressor protein specifically to hydroxylated HIF-1α which ensures its ubiquitylation and rapid proteasomal degradation (1). During hypoxia these processes are inhibited, HIF-1α accumulates, dimerizes with HIF-1β to generate the functional HIF-1 that regulates many genes responsible for adaptive responses, which are important for cell survival at low oxygen levels (2).

One of the proteins, which support adaptation of tumor cells to hypoxia is carbonic anhydrase IX (CA IX). CA IX is a tumor-associated, membrane located metalloenzyme catalyzing the reversible conversion of carbon dioxide to bicarbonate ion and proton (reviewed in ref. 3). Its activity, dependent on the phosphorylation of Thr443 residue by protein kinase A contributes to intracellular pH maintenance (4), supporting cancer cell survival in the conditions of hypoxia and related acidosis and promoting their migration and invasion (5). Moreover, through its unique extracellular proteoglycan domain it is involved in cell adhesion and spreading (6). Transcription of *CA9* gene is primarily regulated by the hypoxia-inducible HIF-1 transcription factor that binds to the hypoxia response element (HRE) located next to the transcription initiation site (7) and CA IX is considered as a marker of tumor hypoxia. CA IX is expressed in a broad range of tumors where its strong expression often associates with worse prognosis. Due to its tumor-related expression and its role in pro-survival and pro-metastatic processes of tumor cells CA IX represents a promising target for antitumor therapy (8).

Recently, it was described that hypoxia also occurs as a result of the inflammation when HIF-1α can be regulated independently of the vascularization level induced by growth factors, pro-inflammatory cytokines, reactive oxygen and nitrogen species or mitochondrial stress (9). Moreover, hypoxia can actively participate in the development of the

inflammatory microenvironment through the promotion of many pro-inflammatory genes (10) governed by nuclear factor-kappaB transcription factor (NF-κB) (11,12). Mammalian NF-κB family consists of: NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB and c-Rel. The active NF-κB is a heterodimer typically consisting of NF-κB1 and RelA subunits. In unstimulated cells, a latent protein complex is sequestered in the cytoplasm by the associated inhibitor IκB. The activation of the NF-κB is initiated by the phosphorylation of IκB proteins mediated via the signal-induced activation of IκB kinase (IKK). This leads to the ubiquitylation and degradation of IκB in the proteasome which results in a release of the NF-κB complex and its subsequent relocation to the nucleus (13,14). Transcriptional targets of NF-κB transcription factor include mostly pro-inflammatory genes encoding cytokines, chemokines, adhesion molecules as well as angiogenic factors and key enzymes involved in prostaglandin synthase or nitric oxide synthase pathways. In this way, NF-κB directly contributes to the development of inflammation. A number of studies provided evidence on aberrant regulation of NF-κB in many cancers where it actively participates in tumor initiation and progression (15).

Immunosuppressive properties and their potent ability to reduce inflammation make synthetic glucocorticoids (GCs) the most prescribed drugs used in the treatment of different disorders, such as asthma, arthritis or dermatitis. GCs are also used to treat patients suffering from a wide range of hematological and non-hematological cancers either because of their inhibiting effects on cell cycle progression and apoptosis promotion or for their beneficial properties, e.g. decreasing oedema, pain, nausea and reducing toxicity of the standard chemotherapy regimens in healthy tissues (16,17). The effect of GCs is executed through glucocorticoid receptors (GRs) which belong to the nuclear receptor family of ligand-dependent transcription factors (18).

Among synthetic GCs a potent and widely used one is dexamethasone (DEX). In multiple cases dexamethasone was shown to affect angiogenesis. This action is achieved by dexamethasone regulation of mRNA and protein levels of VEGF, which belongs to HIF-1 targets. Decrease of VEGF levels by DEX was reported in brain astrocytes and pericytes (19), hyalocytes (20) and also in several cell lines derived from tumors, such as renal carcinoma (21), rat glioma (22), prostate tumor (23) and meningiomas (24), where it was linked with HIF-1 involvement. A substantial reduction of HIF-1 and VEGF expression also occurred in hypoxic mice treated with dexamethasone (25). Microarray analysis of renal proximal tubular epithelial cells showed that hypoxia transcriptionally upregulated glucocorticoid receptors, and this effect was confirmed at the protein level (26). These results indicate a possible cross-talk between glucocorticoid and hypoxia-dependent signaling pathways.

In the present study, we have investigated the interplay between GCs and hypoxia-mediated signaling and a possible involvement of dexamethasone, a potent synthetic GC commonly used in various clinical treatments, in the regulation of tumor-associated carbonic anhydrase IX level. We have confirmed that DEX reduced HIF-1α expression and activity, showed that it decreased CA IX expression in 2D and 3D cellular models and proposed a mechanism of *CA9* transcriptional regulation by glucocorticoids. The obtained results linking *CA9* expression with dexamethasone bring new insight into a clinically relevant situation which can occur during dexamethasone treatment of patients with solid tumors.

Materials and methods

Cell culture and spheroids preparation. RKO colorectal carcinoma and MCF-7 breast cancer cell lines were cultured under standard conditions in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; Biowhittaker, Lancaster, MA, USA) and gentamicin (Sandoz) in humidified air containing 5% CO_2 at 37˚C (normoxia). Cells were obtained from the ATCC and regularly tested for mycoplasma contamination. Before experiments, cells were counted, seeded in culture dishes and incubated in normoxic conditions for 24 h. The following day, culture medium was replaced with the fresh one containing 10 or 100 μM dexamethasone (Sigma-Aldrich, dissolved in ethanol) or ethanol only (in appropriate concentrations, as control samples) and moved to the anaerobic workstation (Ruskinn Technology Ltd., Bridgend, UK) with hypoxic conditions (2% O_2, 2% H_2, 5% CO_2, 91% N_2) for additional 24 h.

MCF-7 spheroids were pre-formed from 600 cells/20 μl of culture medium in drops hanging on the lid of tissue culture dish for 7 days at 37˚C. The resulting cell aggregates were transferred to Petri dish with a non-adherent surface and cultivated in suspension for additional 14 days. During this period dexamethasone (treated groups) or ethanol (control groups) were added every fourth day in fresh medium. At the end of the experiment, images were taken and spheroids size of at least 20 aggregates from each group was analyzed using AxioVision software. Finally, spheroids were collected by centrifugation and used for RNA or protein isolation.

Real-time cell proliferation assays. To assess the cell growth of cells treated with dexamethasone, a real-time characterization was performed using the xCELLigence system (ACEA Biosciences Inc., San Diego, CA, USA). The xCELLigence® system was placed in a hypoxic cabinet with the O_2 controller at 2% O_2 (Coy Laboratory Products Inc., Grass Lake, MI, USA), 5% CO_2 and 37˚C. For the proliferation assay, $10x10^3$ RKO colorectal cancer cells/well were seeded into an E-plate 16 (ACEA Biosciences) in DMEM medium with dexamethasone or ethanol as a control. The plates were placed into the xCELLigence system performing a real-time, impedance-based, label-free monitoring of cell proliferation. Data were collected every 15 min and are presented as a dimensionless parameter called the cell index. Cell index reflects an increase in the area covered by cells, corresponding to increasing cell number during proliferation.

Transient transfections and promoter analysis. The cells were plated onto 35-mm Petri dishes to reach ~70% monolayer density on the following day. For luciferase reporter assay, transfection was performed with 2 μg of the pGL3 luciferase vector containing *CA9* promoter (-1500/+37, -174/+37 or -174/+37_HRE-mut) or luciferase vector containing hypoxia-response elements (HRE-luc) and 100 ng pRL-TK *Renilla* vector using Attractene transfection reagent (Qiagen).

Human promoter constructs were generated by an insertion of PCR-amplified -1500/+37 and -174/+37 *CA9* genomic fragments upstream of the firefly luciferase gene in pGL3-Basic vector (Promega) (27). The pGL3-174/+37_HRE-mut construct with mutated HRE upstream of the firefly luciferase gene was created from the original pBMN5HREmut plasmid (28). shRNA targeting human HIF-1α cloned in pSUPER plasmid was kindly provided by Dr Nicholas Denko, (Ohio State University Wexner Medical Center, Columbus, OH, USA). Luciferase vector containing a trimer of hypoxia-response elements (HRE-luc) of the lactate dehydrogenase A gene was kindly provided by Dr Kaye Williams (University of Manchester, Manchester, UK). Transient silencing of HIF-1α and NF-κB was performed using specific si/shRNA: 2 μg of plasmid vector pSuper_shHIF-1α vs. pSuper_shCtrl containing non-targeting sequence, 50 nM siNF-κB (Thermo Fisher Scientific) vs. non-targeting control (Thermo Fisher Scientific). The following day, the transfected cells were plated according to the type of further experiment and cultured in hypoxia for additional 24 h. Reporter gene expression was assessed using the Dual-luciferase reporter assay system (Promega), and the luciferase activity was normalized against the *Renilla* activity.

Quantitative PCR. Total RNA was isolated using TRIzol solution (Sigma-Aldrich) and reverse transcription of 3 μg RNA for each sample was performed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative PCR was carried out using Maxima SYBER-Green PCR Master Mix (Thermo Fisher Scientific). Sample Ct values were normalized to actin. Relative expression was calculated using the ΔΔCt method. All amplifications were performed in triplicate. Oligonucleotides used for real-time qPCR were as follows: *Actin* sense 5'-CCAACCGCGAGAAGATGACC-3' and *actin* antisense 5'-GATCTTCATGAGGTAGTCAGT-3'; *VEGF* sense 5'-CTTGCTGCTCTACCTCCACCAT-3' and *VEGF* antisense 5'-CACACAGGATGGCTTGAAGATG-3', *Glut1* sense 5'-CTCCTTTCTCCAGCCAGCAATG-3' and *Glut1* antisense 5'-CCAGCAGAACGGGTGGCCATAG-3'; *LDHa* sense 5'-TGGCAGCCTTTTCCTTAGAA-3' and *LDHa* antisense 5'-ACTTGCAGTTCGGGCTGTAT-3'; *HIF-1α* sense 5'-GCTTGGTGCTGATTTGTGAACC-3' and *HIF-1α* antisense 5'-GCATCCTGTACTGTCCTGTGGTG-3'; *CA9* sense 5'-CCGAGCGACGCAGCCTTTGA-3' and *CA9* antisense 5'-GGCTCCAGTCTCGGCTACCT-3'.

Western blotting. Protein extracts were prepared using lysis buffer (1% Triton X-100; 50 mM Tris pH 7.5; 150 mM NaCl; 0.5% Nonidet P-40) containing protease (Roche) and phosphatase inhibitor cocktail (Sigma-Aldrich), disrupted by sonication and cleared by centrifugation. Concentrations were quantified using the BCA protein assay kit (Pierce). A total of 100 μg of proteins/lane were resolved in 8% SDS-PAGE, transferred to a PVDF membrane (Macherey-Nagel, Düren, Germany) and visualized using an enhanced chemiluminescence kit (GE Healthcare Life Sciences). Protein bands were quantified in ImageJ software, all results were normalized to actin. Antibodies used for specific proteins were as follows: HIF-1α (dilution 1:250, 610959; BD Transduction Laboratories, San Jose, CA, USA), CA IX (in-house generated M75, dilution 1:3), actin (dilution 1:1,000, sc1615; Santa Cruz Biotechnology, Santa Cruz, CA, USA), NF-κB1-p105/p50 (dilution 1:1,000, 13586; Cell Signaling Technology, Danvers, MA, USA), appropriate secondary antibodies conjugated with horseradish peroxidase were purchased from Dako.

Proteome profiler array. For analyzing the expression profile of cell stress-related proteins in MCF-7 spheroids treated with dexamethasone, we performed Human Cell Stress Proteome Profiler Array kit (R&D Systems, Minneapolis, MN, USA). All steps were carried out according to the manufacturer's instructions.

Cignal finder reporter array. For analyzing the activity of cancer-related transduction pathways, we performed Cignal finder reporter array (SABiosciences) according to the instructions of the manufacturer. Dual-luciferase results were calculated for each transfectant and analyzed by the data analysis software (SABiosciences). Changes in the activity of each signaling pathway were determined by comparing the normalized luciferase activities of the reporter in treated vs. untreated transfected cells or hypoxic vs. normoxic cells.

Bioinformatics. *In silico* analysis of the *CA9* promoter was performed using MatInspector program (https://www.genomatix.de) (29,30). Promoter sequence (663 bp) was extracted directly from ElDorado genome database after gene submission. Accurate positions of predicted binding elements were calculated according to the transcription start site.

Statistical analysis. Results were analyzed by two-tailed unpaired t-test (Student's t-test), and $P<0.05$ was considered significant. $P<0.05$ is denoted as *, $P<0.01$ as ** and $P<0.001$ as ***.

Results

Dexamethasone reduces protein level of HIF-1α as well as its activity and affects transcription of HIF-1 target genes. We investigated the effect of dexamethasone on the regulation of hypoxia-induced genes in colorectal carcinoma RKO cells cultured for 24 h under 2% hypoxia. As shown in Fig. 1A, both doses of dexamethasone (10 and 100 μM) reduced the expression of several HIF-1 targets at mRNA level, such as VEGF, Glut1 and LDHa in our colorectal carcinoma model.

We then performed a real-time monitoring of the proliferation of hypoxic RKO cells under dexamethasone treatment (appropriate ethanol concentrations were used as negative controls) using an impedance-based xCELLigence platform. As our results show (Fig. 1B) DEX (10 μM) did not affect the growth of hypoxic RKO cells. The higher dose of dexamethasone (100 μM) slightly decreased the growth rate of RKO cells which is in agreement with a known dose-dependent bimodal effect of glucocorticoids on cell proliferation (31).

As HIF-1 is a major regulator of hypoxic responses at the transcriptional level, we investigated if dexamethasone influenced its transcriptional activity. Using luciferase reporter containing HRE, we detected a significant dose-dependent decrease in the activity of HIF-1 transcription factor with an increasing concentration of dexamethasone (Fig. 2A). At the

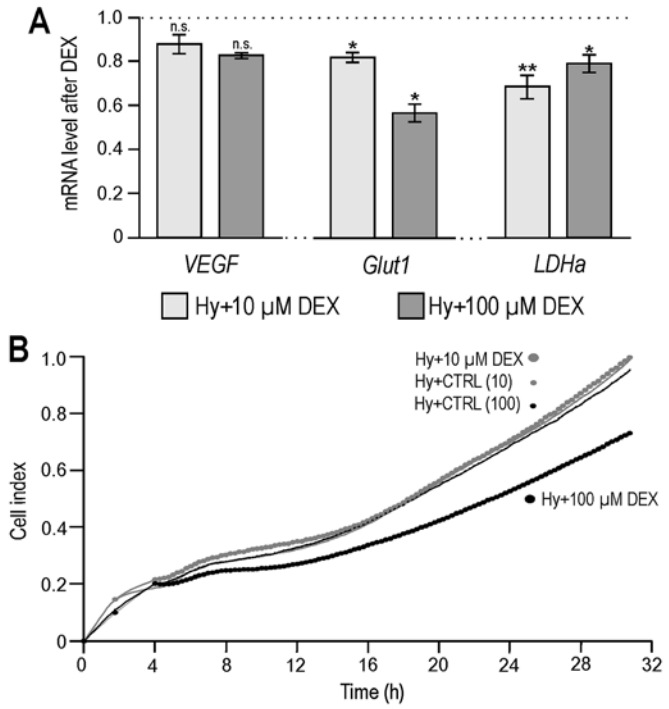

Figure 1. Effect of dexamethasone (DEX) on the transcription of HIF-1 targets and on a proliferation rate of colorectal RKO cells exposed to hypoxia. (A) RKO cells were cultured in hypoxia and treated with two concentrations of DEX (10 and 100 μM) for 24 h. Quantitative PCR analysis shows that selected concentrations of DEX decreased mRNA level of VEGF, Glut1 and LDHa, all normalized to actin. The results represent the mean from three independent biological experiments, all performed in triplicate. Control samples, containing appropriate concentrations of ethanol for separate DEX doses were set to 1. T-tests were performed among DEX -treated samples and their appropriate untreated controls. P<0.05 was considered significant. *P<0.05, **P<0.01. (B) The graph shows a proliferation rate of RKO cells cultured in hypoxia with and without DEX (10 and 100 μM) expressed as the cell index. Cell index reflects an elevation in the number of cells during proliferation measured as an increased impedance due to raising cell coverage of the electrode area. The results show that only the higher dose of DEX slightly slowed the proliferation rate. No cytotoxic effect of administered compounds (ethanol vs. DEX dissolved in ethanol) was observed. Data represent the mean from three independent biological experiments, all performed in quadruplicate.

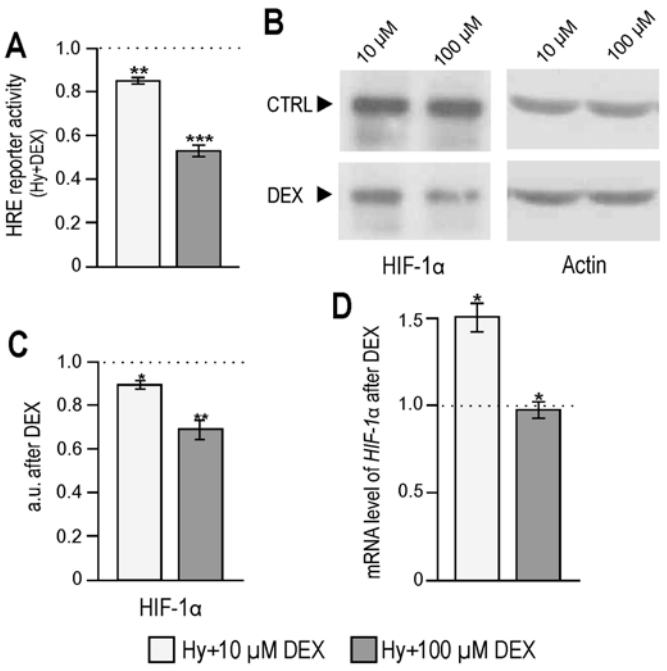

Figure 2. Effect of dexamethasone (DEX) on HIF-1α transcriptional activity, mRNA and protein levels. (A) Analysis of the HIF-1α transactivation potential in hypoxic RKO cells treated with DEX (10 and 100 μM). RKO cells were co-transfected with pGL3-HRE-luc and pRL-TK plasmids, incubated in hypoxia, treated with DEX and analyzed by Dual-luciferase assay. The graph shows that DEX administered during hypoxia reduced activity of HIF-1α in a dose-dependent manner. (B) Representative western blot analysis of hypoxic RKO cells demonstrated DEX-mediated reduction of HIF-1α protein level. (C) The HIF-1α bands were quantified using ImageJ software. (D) The graph represents quantitative PCR analysis from hypoxic RKO cells treated with DEX. The results show that 10 μM DEX caused an 1.5-fold increase of *HIF-1α* mRNA, while 100 μM DEX did not cause any marked changes at the transcriptional level. (A, C and D) The results represent the mean from three independent biological experiments, all data are related to control samples (containing ethanol) which were set to 1. T-tests were performed among DEX-treated samples and their appropriate untreated controls. P<0.05 was considered significant. *P<0.05, **P<0.01 and ***P<0.001.

same time, the levels of the HIF-1α protein in the hypoxic RKO cells subjected to dexamethasone treatment were reduced when compared to their controls (Fig. 2B and C). Quantitative PCR analysis revealed 1.5-fold elevation of the HIF-1α mRNA level when hypoxic RKO cells were treated with 10 μM dexamethasone, whereas no change was detected after 100 μM concentration (Fig. 2D) suggesting that DEX interferes with the translation and/or degradation pathways of HIF-1α.

Dexamethasone decreases expression of CA IX in hypoxic cancer cell monolayer and in spheroids. Tumor-associated carbonic anhydrase IX is one of the best hypoxia-responsive targets which is often used as a marker of tumor hypoxia in clinical samples. Here, we show that dexamethasone reduced the activity of the *CA9* promoter in RKO cells cultured in hypoxia in a dose-dependent manner (Fig. 3A and B). Reduced activity was accompanied by a significantly decreased expression of CA IX at both protein and mRNA levels (Fig. 3C-E). Measurement of extracellular pH of hypoxic RKO monolayers showed a significantly reduced acidification after 24 h of 100 μM DEX treatment (ΔpH=0.047 compared to control) which is in agreement with the reduced CA IX expression described above. As HIF-1 is the main regulator of *CA9* transcription this result is in agreement with the reduced HIF-1α protein and its lower transcriptional activity after dexamethasone treatment. Testing of dexamethasone treatment on another tumor cell line (MCF-7 monolayer) yielded similar results as on RKO cells, decreased transcriptional activity of HIF-1 and reduced activity of the *CA9* promoter (Fig. 4A and B).

Several studies showed reduced primary tumor growth and proliferation after knocking down the CA IX expression (32-34). Well described was also an inhibitory effect of dexamethasone on tumor volume e.g. in *in vivo* models of the brain or prostate cancer (23,35). As possible effects of dexamethasone on CA IX which promotes tumor cell survival could be important, especially during DEX treatment of cancer patients, we tested the effect of dexamethasone in the physiologically more relevant 3D environment. We used the established MCF-7 breast carcinoma 3D model of the spheroid formation in hanging drops. Spheroids were treated every 4th day during a two-week period. At the end of the experi-

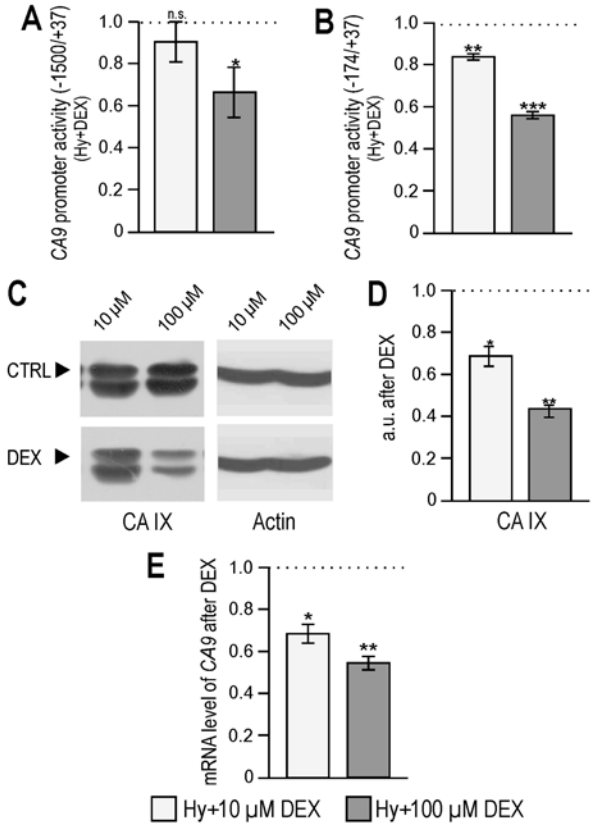

Figure 3. Dexamethasone (DEX) affects the expression of the HIF-1α target CA IX. (A and B) Analysis of the *CA9* promoter activity in hypoxic RKO cells treated with DEX (10 and 100 μM). RKO cells were co-transfected with pGL3-*CA9* (-1500/+37, -174/+37) and pRL-TK plasmids, incubated in hypoxia, treated with DEX and analyzed by Dual-luciferase assay. The data show that in hypoxic RKO cells, DEX reduced *CA9* promoter activity in a dose-dependent manner. (C) Representative western blot analysis of hypoxic RKO cells demonstrates DEX-mediated reduction of CA IX protein. (D) The CA IX bands were quantified using ImageJ software. (E) The graph gives quantitative PCR analysis of mRNA from hypoxic RKO cells treated with DEX. The results show that DEX decreased CA IX also at mRNA level. (A, B, D and E) The results represent the mean from three independent biological experiments, all data are related to control samples (containing ethanol) which were set to 1. T-tests were performed among DEX-treated samples and their appropriate untreated controls. P<0.05 was considered significant. *P<0.05, **P<0.01 and ***P<0.001.

ment, spheroids exposed to dexamethasone had a significantly smaller diameter than controls, by almost 20% in 10 μM group and 35% in 100 μM group (Fig. 4C) and their morphology was also altered (Fig. 4D). Analysis of spheroids by quantitative PCR and proteome profiler array showed that an increasing amount of dexamethasone considerably lowered transcription (Fig. 4E) and protein level of CA IX (Fig. 4F) also in this breast cancer 3D model. Our findings indicate that dexamethasone-mediated effect of spheroid growth inhibition is at least partially associated with its effect on CA IX levels. In addition, reduction of HIF-1α protein was confirmed in MCF-7 spheroids. Notably, the protein level of hypoxia-inducible factor-2α, which is also capable of activating transcription via binding of HRE and is regulated in the same way as HIF-1α (36,37), was unchanged (Fig. 4F).

In silico analysis predicts potential NF-κB binding sites in the promoter of CA9 gene. As mentioned above HIF-1 tran-

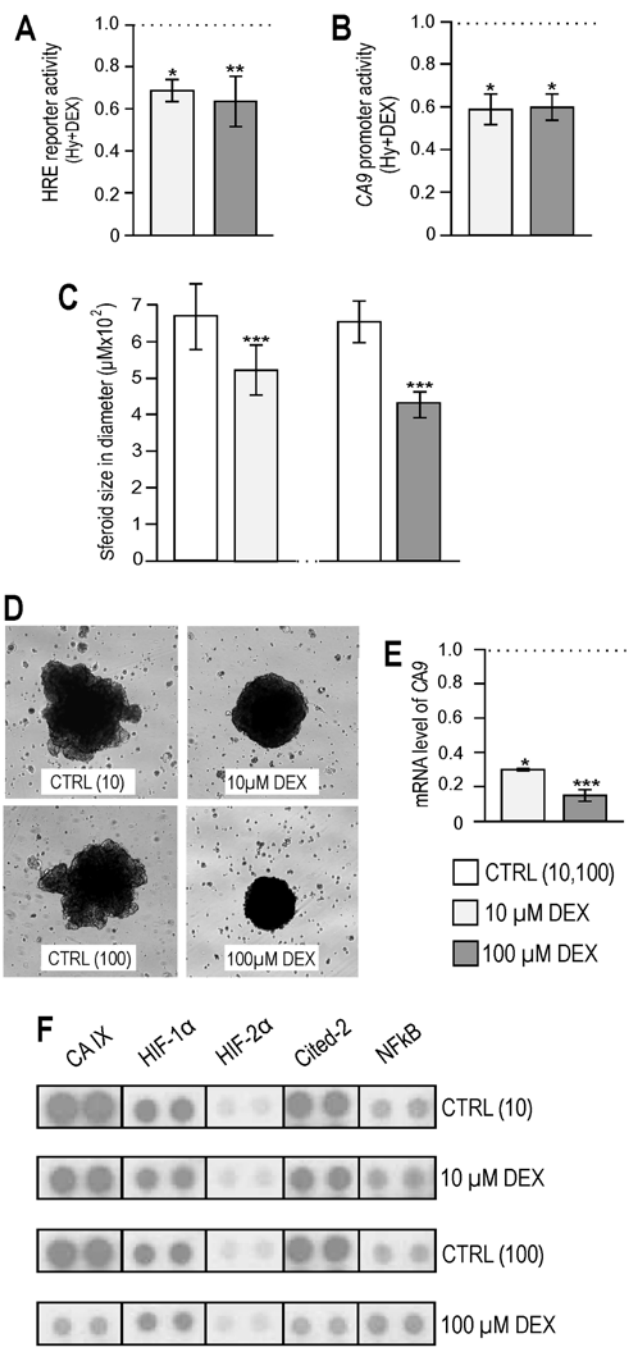

Figure 4. Effect of dexamethasone (DEX) treatment on MCF-7 spheroid size and expression of CA IX and HIF-1α. (A) Analysis of the HIF-1α transactivation potential in hypoxic MCF-7 cells treated with DEX (10 and 100 μM). MCF-7 cells were co-transfected with pGL3-HRE-luc and pRL-TK plasmids, incubated in hypoxia, treated with DEX and analyzed by Dual-luciferase assay. The graph shows that DEX administered during hypoxia reduced activity of HIF-1α in a dose-dependent manner. (B) Analysis of the *CA9* promoter activity in hypoxic MCF-7 cells treated with DEX (10 and 100 μM). MCF-7 cells were co-transfected with pGL3-*CA9* (-174/+37) and pRL-TK plasmids, incubated in hypoxia, treated with DEX and analyzed by Dual-luciferase assay. The data show that in hypoxic MCF-7 cells DEX reduced *CA9* promoter activity. (C) Analysis of MCF-7 spheroids showed that long-term treatment (two weeks) with DEX (10 and 100 μM) significantly reduced spheroids size, compared to controls, in a dose-dependent manner. (D) Representative spheroids from each group. (E) Quantitative PCR analysis and (F) proteome profiler array from treated spheroids revealed impaired expression of CA IX and HIF-1α also in this 3D culture model. (A, B, C and E) The results represent the mean from three independent biological experiments. T-tests were performed among DEX-treated samples and their appropriate untreated controls. P<0.05 was considered significant. *P<0.05, **P<0.01 and ***P<0.001.

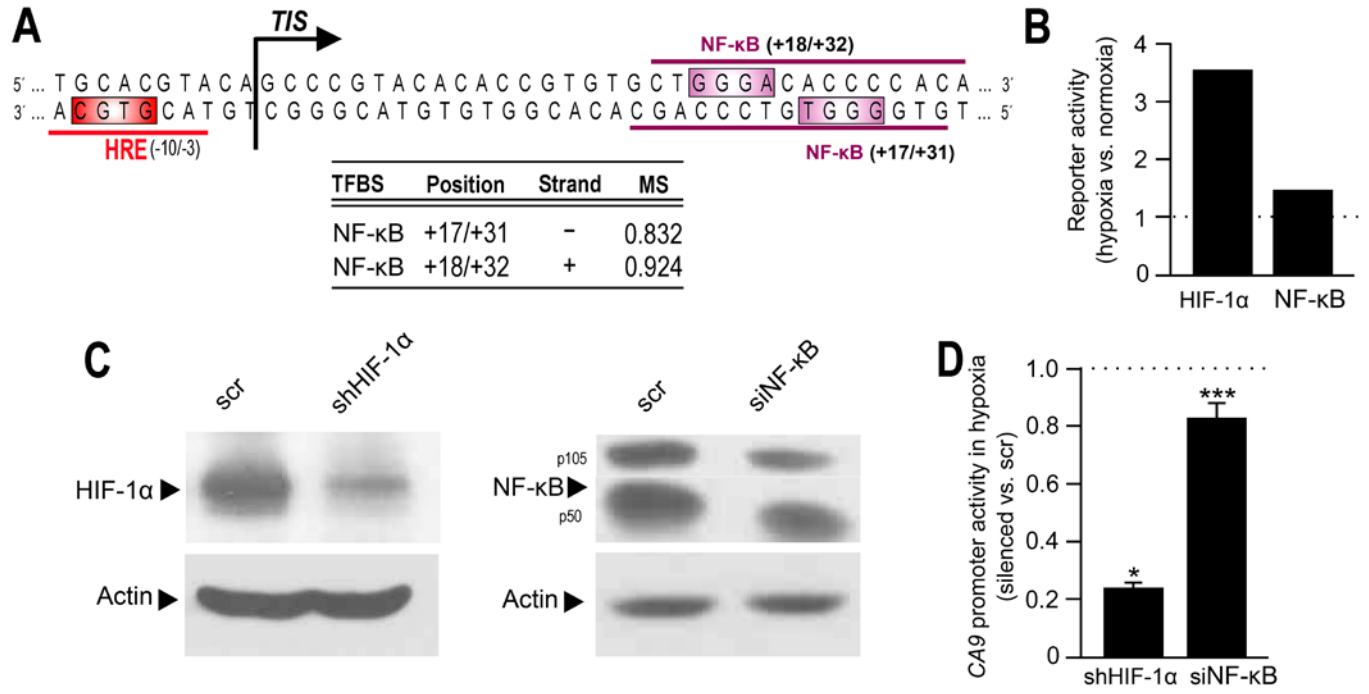

Figure 5. Promoter analysis and transcriptional response of CA9 to NF-κB knockdown. (A) Schematic representation of potential transcription factor binding sites (TFBS) in -174/+37 promoter region of *CA9* gene. MatInspector analysis predicted two NF-κB binding sites. Core sequences are highlighted in color frames. The table summarizes predicted positions relative to the transcription iniciation site (TIS), corresponding strand as well as matrix similarity (MS). (B) Cell-based Dual-luciferase reporter array of normoxic (control samples set to 1) vs. hypoxic RKO cells (without DEX treatment). Results show that hypoxia increased transactivation of HIF-1α and NF-κB. (C) Western blot analysis of hypoxic RKO cells demonstrates the impact of transient silencing on the protein levels of both transcription factors. (D) Analysis of the *CA9* promoter activity in hypoxic RKO cells with suppressed expression of HIF-1α and NF-κB. RKO cells were co-transfected with pGL3-*CA9* (-174/+37), pRL-TK plasmids and si/shRNA or scrambled control (scr), incubated in hypoxia (without DEX treatment) and analyzed by Dual-luciferase assay. The data show that transient silencing of HIF-1α caused 5-fold reduction of *CA9* promoter activity. Suppression of NF-κB diminished activity by almost 20%. Results indicate that NF-κB contributes to *CA9* upregulation during hypoxia, without presence of DEX. (B and D) The results represent the mean from three independent biological experiments, all data are related to appropriate control samples, set to 1 (B, normoxia; D, scrambled control). T-tests in D were performed between silenced samples and their appropriate scrambled controls. P<0.05 was considered significant. *P<0.05,**P<0.01 and ***P<0.001.

scription factor is the major transcriptional regulator of CA IX during tumor hypoxia. HIF-1 binds to an HRE sequence localized immediately before the transcription initiation site (7) and represents a critical component of the core promoter of the *CA9* gene. Luciferase assay (Fig. 3A and B) showed that dexamethasone acts equally on the activity of both *CA9* promoter constructs: longer (-1500/+37) and shorter (-174/+37) one. These data lead to the conclusion that the reduction of *CA9* transcription by dexamethasone could be mediated mainly by a lowered binding activity of HIF-1α to the core promoter region of *CA9*.

However, *CA9* promoter contains additional *cis*-elements that may have modulatory effects on *CA9* transcription (28). Detailed *in silico* analysis of the *CA9* regulatory region revealed several transcription factor binding sites in the close proximity to the HRE, the most interesting of them were those for NF-κB (Fig. 5A). NF-κB has been shown as hypoxia-responsive (26,38,39). Therefore, we examined their possible participation in the basal hypoxic induction of CA IX. First, we tested NF-κB transcriptional activity during hypoxia, in the absence of dexamethasone treatment (Fig. 5B). Results indicated that also in colorectal RKO cells hypoxia could trigger the transactivation of NF-κB. Next, we analyzed the activity of the *CA9* promoter in hypoxic nontreated cells after transiently silencing the NF-κB expression (Fig. 5C and D). Interestingly, NF-κB suppression led to a downregulation of the *CA9* promoter activity by almost 20% under hypoxic conditions (in the absence of DEX).

Contribution of NF-κB to dexamethasone-mediated reduction of CA IX expression. As synthetic glucocorticoids are often used during chemotherapy of various tumor patients, we examined the effects of dexamethasone on cancer-related signaling pathways in *in vitro* colorectal carcinoma model. Cignal finder reporter array (Fig. 6A) showed that dexamethasone treatment of hypoxic RKO cells caused an additional activation of NF-κB signaling in hypoxia in a concentration-dependent manner, which is in agreement with higher protein level of NF-κB detected in DEX-treated MCF-7 spheroids (Fig. 4F). Besides gene transactivation, glucocorticoids can also act as repressors. This inhibitory effect of GCs can result from the interaction between ligand-activated GR and another transcription factor e.g. AP-1, thus, preventing them from binding to their specific site. Dexamethasone decreased the transactivation ability of AP-1 (Fig. 6A), as well as other proteins involved in GC mediated downregulation (SMADs and STATs).

As a next step we wanted to investigate whether different mechanisms beyond diminished HIF-1α function could participate in the observed dexamethasone-triggered down-

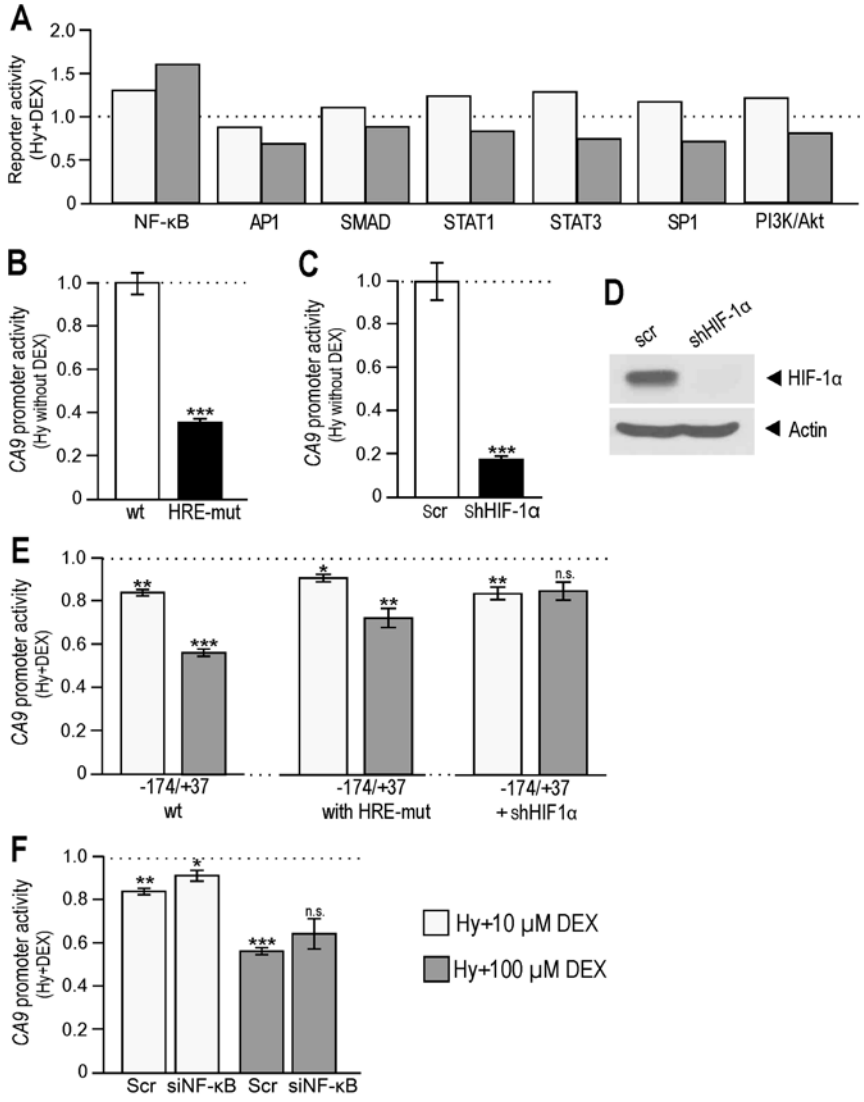

Figure 6. Participation of NF-κB in the regulation of *CA9* gene (A) Molecular response of RKO cells to hypoxia and DEX. Cell-based dual luciferase reporter array of hypoxic RKO cells (control, ethanol 10 and 100) vs. hypoxic RKO cells treated with DEX (10 and 100 μM) revealed alterations of signal transduction pathways leading to changes in transactivation activities of NF-κB and other signaling pathways, such as SP1, STAT3, SMAD and PI3K/Akt. (B) Graph gives the activity of pGL3-*CA9* (-174/+37) promoter construct with mutated HRE vs. wild-type construct (set to 1) transfected into RKO cells and cultured in hypoxia (without DEX). The results of HRE-mut samples were compared to wild-type counterparts (***P<0.001 significant difference). (C) Graph shows the activity of the wild-type pGL3-*CA9* (-174/+37) promoter construct in RKO stable transfectants without HIF-1α expression vs. scrambled control (set to 1) cultured in hypoxia (wihout DEX). The results of silenced samples were compared to scrambled counterparts (***P<0.001 significant difference). (D) Western blot analysis of hypoxic RKO cells stable-transfected with shHIF-1α. (E) Left part represents the analysis of the pGL3-*CA9* (-174/+37_wt) in hypoxic RKO cells treated with DEX. Middle part represents the analysis of the pGL3-*CA9* with mutated HRE sequence (-174/+37_HRE-mut) in hypoxic RKO cells treated with DEX. Right part gives the analysis of the pGL3-*CA9* (-174/+37_wt) in hypoxic RKO cells with HIF-1α stable knock-down, all after DEX treatment. The data show that DEX reduced *CA9* promoter activity also in the conditions when HIF-1α was unable to bind to its HRE. The results in E were related to their control, untreated, samples (containing ethanol) which were set to 1. T-tests were performed among DEX-treated samples (-174/+37 wt, -174/+37 with HRE-mut, -174/+37 +shHIF-1α) and their appropriate untreated controls. P<0.05 was considered significant. *P<0.05, **P<0.01 and ***P<0.001. (F) Analysis of the *CA9* promoter activity in hypoxic RKO cells with suppressed expression of NF-κB after DEX treatment. RKO cells were co-transfected with pGL3-*CA9* (-174/+37), pRL-TK plasmids and siNF-κB, treated with DEX, incubated in hypoxia and finally analyzed by Dual-luciferase assay. The data show that transient silencing of NF-κB caused a smaller reduction of *CA9* promoter activity indicating that NF-κB contributes to *CA9* downregulation in the presence of DEX. Differences of DEX treated samples with silenced NF-κB in F were evaluated against their corresponding DEX-treated scrambled controls. P<0.05 was considered significant. *P<0.05, **P<0.01 and ***P<0.001.

regulation of CA IX. Therefore, we created the situation when HIF-1α was knocked-down or unable to bind to the *CA9* promoter. This was achieved by transfection of -174/+37 construct containing a mutation in the corresponding HRE (Fig. 6B) or by the transfection of -174/+37 wild-type construct into cells in which HIF-1α was completely knocked-down by a stable transfection of pSuper_shHIF-1α (Fig. 6C and D). Our results showed (middle and right graph in Fig. 6E) that dexamethasone was capable of reducing the activity of *CA9* promoter in both conditions when compared to control samples of HRE-mutant and silenced cells not treated by DEX. These findings confirm that an additional repressive mechanism (mediated by other trasncription factors than HIF-1α) was involved in CA IX regulation during the treatment. Moreover, simultaneous suppression of NF-κB led to an increase in *CA9* promoter activity when compared to the

scrambled dexamethasone group (Fig. 6F). Our data indicate that NF-κB contributes to dexamethasone-mediated modulation of the hypoxic expression of CA IX.

Discussion

Natural glucocorticoids are steroid hormones synthesized and secreted by the adrenal cortex. In man, they are involved in the regulation of a variety of physiologic functions such as growth, development, metabolism, immune response and in the maintenance of the basal and stress-related homeostasis. One of the main effects of glucocorticoids (GCs) is their ability to reduce inflammatory responses. Therefore, synthetic GCs belong to the most used agents in the treatment of different diseases which are typically accompanied by inflammation, including cancer. Dexamethasone (DEX) is a frequently prescribed synthetic glucocorticoid often used in cancer treatment in a number of different ways. Its beneficial properties include mainly prevention of allergic reactions, nausea and vomiting caused by certain chemotherapy drugs, and reduction of swelling, especially for tumors in the brain, spinal cord or bones. Several studies reported a decreased tumor volume as the effect of dexamethasone treatment (23,35,40). DEX was also shown to affect angiogenesis *in vitro* as well as *in vivo* cancer models by means of reduced VEGF expression (21-23). Recently, the involvement of HIF-1 in glucocorticoid-mediated downregulation of VEGF was shown (24).

In the present study, we also showed DEX-mediated downregulation of hypoxia-induced VEGF as well as other hypoxia-induced targets, including CA IX, Glut1 and LDHa. In accordance with the known data, DEX caused a decrease in HIF-1 transcriptional activity and reduced protein levels of HIF-1α oxygen-sensitive subunit in both 2D colorectal carcinoma and 3D breast cancer models. However, no downregulation of HIF-1α was detected at the transcriptional level after DEX treatment of RKO cell monolayer, 10 μM concentration even caused an elevation of HIF-1α mRNA, indicating post-transcriptional action of DEX on HIF-1α. A wide spectrum of modulators regulates transcription of the gene encoding HIF-1α. Although hypoxia regulates HIF-1α mainly at the post-translational level, several hypoxia-responsive factors initiate its transcription. Among them NF-κB contributes to its upregulation through a binding element located at -197/-188 bp upstream from the transcription initiation site (TIS) of the HIF-1α gene (41). Another hypoxia-increased protein SP1 was defined as important for maintaining HIF-1α transcription. Multiple SP1 binding sites have been described at -85/-65 bp region from the TIS (42) and their deletion resulted in a decrease in HIF-1α promoter activity (43). Furthermore, JAK/STAT signaling pathway, which is implicated in mediating the inflammatory responses, was linked to the expression of HIF-1α. Particularly STAT3 was shown to bind to a DNA-binding motif placed at -363/-355 bp (44). In this study, we showed that DEX changed cellular signaling during hypoxia and that these effects varied depending on DEX amount. In line with known transcriptional regulation of HIF-1α gene, the activation of NF-κB, SP1 and STAT3 signaling caused by 10 μM DEX (Fig. 6A) could participate in the observed increase of HIF-1α at transcriptional level.

Since we did not detect any downregulation in the HIF-1α transcription, we propose that DEX probably affects its degradation and/or translation. Besides the negative regulation of HIF-1α by proteasomal degradation, a number of other mechanisms contribute to the reduction of HIF-1α protein level or activity. Among them Cited2, activated in hypoxia, was found to downregulate HIF-1-mediated transactivation (45). However, our analysis of MCF-7 spheroids showed DEX-induced reduction of Cited2 at the protein level (Fig. 4D). Stabilization of HIF-1α can also be modulated by other signaling pathways including PI3K/Akt kinase cascade, which is known to operate via positive as well as negative regulation, depending on downstream effectors. Downstream target of Akt implicated in the positive regulation of HIF-1α is the mammalian target of rapamycin, mTOR, which likely upregulates HIF-1α through a translation-dependent pathway (46,47). DEX was shown to repress protein synthesis through inhibition of mTOR signaling by reducing phosphorylation of the downstream targets S6K1 and 4E-BP1 (48). Heat shock protein expression also contributes to the stabilization of HIF-1α (49,50). In hypoxia, Hsp90 binding protects HIF-1α from degradation by non-VHL mediated ubiquitination (50) and its inhibition results in decreased HIF-1α accumulation and activation. Maheshwari *et al* (51) detected significant reduction of Hsp90 in HD150Q cells and HD mice cortex after DEX treatment. Recently, glucocorticoid-induced leucine zipper (GILZ) was shown to suppress the expression of HIF-1α at the protein level via the proteasomal pathway (52). It has also been demonstrated that DEX directly reduced phosphorylation of the serine/threonine kinase Akt (53) which, in turn, can lead to the activation of downstream target GSK3β, thus, preventing further HIF1α accumulation (54,55). All these data support our conclusions denoting a key role of the synthetic glucocorticoid dexamethasone in HIF-1α destabilization and subsequent reduction of HIF-1 transcriptional activation.

HIF-1 activates transcription of many genes mediating adaptive responses to hypoxia. Among them the gene encoding carbonic anhydrase IX is one of the most strongly activated. Within the regulatory region of *CA9* gene a hypoxia-responsive element (HRE) is localized at -10/-3 bp next to the transcription initiation site (7). In line with DEX-mediated reduction of HIF-1α expression and activity it is not surprising that DEX reduced the *CA9* promoter activation, transcription and CA IX protein level in both RKO and MCF-7 cancer models. However, our data indicate also the existence of an additional mechanism contributing to the decreased CA IX expression after DEX treatment besides impaired HIF-1 function (Fig. 6E).

At the cellular level, the mechanism of GCs actions is mediated through glucocorticoid receptors (GRs) which function as ligand-dependent transcription factors. Basic genomic regulation involves a direct receptor binding to DNA on its specific sequences termed glucocorticoid response elements (GREs) providing gene transactivation. However, regulation mediated by GRs can lead also to the transrepression depending on a promoter context and GRE sequence present (56). Direct negative regulation by GCs occurs via the interaction of GR with a negative GRE site the actual sequence of which is poorly defined. *In silico* analysis of the regulatory region of *CA9* gene revealed two potential GREs (-8/+10 and +3/+21), both local-

ized in a close proximity to the TIS (data not shown). A precise regulatory action of these GREs remains undetermined and requires further study. Alternatively, ligand-activated GR can mediate a transrepression independently of GRE by means of a protein-protein interaction with another transcription factor, thus preventing them from binding to their specific site. Most of anti-inflammatory effects of GCs are mediated through this pathway, especially via GR cross-talk with pro-inflammatory NF-κB or AP-1. AP-1-responsive element was described in CA9 promoter up-stream of HRE by Kaluz *et al* (57). This element functionally contributes to *CA9* transcription and, therefore, DEX could repress it via GR and AP-1 interaction. This conclusion is also supported by decreased transactivation ability of AP-1 after DEX treatment as determined by Cignal reporter array (Fig. 6A). Moreover, we also identified potential NF-κB-binding sites (Fig. 5A) and suggested their involvement in DEX-mediated inhibition of *CA9* transcription as a knock-down of NF-κB resulted in a weaker effect of DEX on *CA9* promoter activity (Fig. 6F). However, DEX treatment increased protein level and subsequent transcriptional activity of NF-κB in the Cignal array (Figs. 4F and 6A). Although NF-κB is primarily considered a transcriptional activator, recently its role in downregulating gene expression has been reported (58-60). Thus, we propose that NF-κB could act on *CA9* promoter in DEX treated cells as a transcriptional repressor, however, precise mechanism of NF-κB action on *CA9* regulation during hypoxia and in GCs presence remains to be elucidated.

Our results showed a dose-dependent reduction of mRNA and protein levels of CA IX in spheroids after dexamethasone treatment (Fig. 4E and F). The size of treated spheroids was significantly decreased in a concentration-dependent manner (Fig. 4C and D). This reduction could be at least partially caused by lowered levels of CA IX after DEX treatment which can lead to impaired pH regulation in the hypoxic core, resulting in slower proliferation and worsened cell survival. Bladder carcinoma RT112 spheroids expressing surface CA IX activity were more successful in removing surface-to-core difference in intracellular pH (61). Spheroids formed from HCT116 colorectal cancer cells transfected with CA IX were shown to better suppress intracellular acidity in the core than their control counterparts lacking CA IX (62). The use of membrane-impermeant CA inhibitors linked this effect to CA IX activity. Research of tumor cell clusters loaded with pH sensitive dye carboxy-SNARF-1 demonstrated that hypoxia-insensitive Na-dependent bicarbonate ion transport is important for setting and stabilizing of resting pH at a mildly alkaline level, promoting growth (63). CA IX facilitates this process by its cooperation with bicarbonate transporters NBC1 and AE2 by forming a bicarbonate transport metabolon (5). Experiments with carnosine which disrupts bicarbonate transport metabolon and thus impairs CA IX activity also proved the importance of CA IX for spheroid growth (64). After a hypoxic gradient was established *CA9* silencing in LS174 colon adenocarcinoma spheroids led to a diminished proliferation (65). CA IX expression was linked with increased growth rate in spheroids formed from colorectal tumor cells (66). Silencing of *CA9* significantly decreased number of spheroid-forming cells in various breast cancer cell lines in hypoxia (67). Above mentioned data obtained from different tumor 3D models point to the role of CA IX in promoting 3D cell growth.

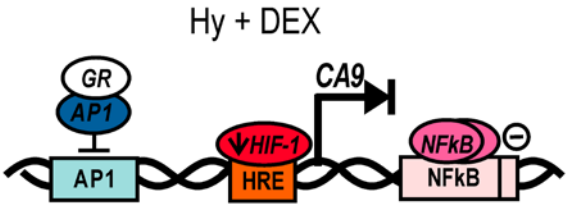

Figure 7. Proposed modes of DEX-mediated transcriptional regulation of the *CA9* gene. In the present study, we showed that dexamethasone treatment causes reduction of hypoxia-induced HIF-1α, which in turn leads to a decreased expression of tumor-associated carbonic anhydrase IX. However, our data indicate also the existence of other regulatory mechanisms with the repressive mode of action on *CA9* transcription. In response to GCs, glucocorticoid receptors function as main transcriptional mediators with ability to mediate gene transactivation as well as transrepression, depending on target promoter arrangement. This mechanism mostly includes the interaction of GR with pro-inflammatory AP-1 and NF-κB, which prevents binding to their respective sites, both present in *CA9* promoter, or results in masking of their transactivation domain. As the suppression of NF-κB resulted in a smaller effect of DEX on the CA9 promoter activity we assume a possible binding of NF-κB on the *CA9* promoter and its role in *CA9* regulation. Although NF-κB is primarily considered a transcriptional activator, several studies reported a direct down-regulation of gene expression by NF-κB. We also propose that NF-κB could act on the *CA9* promoter as a transcriptional repressor, however, a precise mechanism of NF-κB action on *CA9* regulation during basal hypoxia and in GCs presence remains to be elucidated.

However, dexamethasone action must also be taken into account when assessing spheroid growth. Glucocorticoids were shown to exert anti-proliferative effects mediated by a G1-block in cell cycle progression (68). Dexamethasone inhibited proliferation of tumor cells and suppressed the activity of transcription factor NF-κB, which controls the expression of genes such as cyclooxygenase COX-2 and cyclin D1, involved in proliferation control of tumor cells (69). Anti-proliferative action of GC/GR is correlated to high concentration of GC (31), and our results from the proliferation assay in hypoxia confirmed a bimodal effect of dexamethasone on RKO cell monolayer (Fig. 1B). Interestingly, in MCF-7 cells even higher DEX dose did not inhibit proliferation (data not shown). Our exploration of hypoxia related pathways (data not shown) showed that 100 μM dexamethasone dose led to a decrease of c-myc, Nanog and E2F that play a role in the cycle progression, apoptosis, and regulation of genes involved in cell proliferation (70-72). Downregulation of proto-oncogenes cyclin D1 and c-myc was also demonstrated in head and neck tumor cells after the inhibition of NF-κB signaling by 10 μM dexamethasone (73). Akt kinase is also implicated in cancer progression as it stimulates proliferation and suppresses apoptosis. This is in agreement with our findings showing reduced activation of PI3K signaling in the presence of 100 μM DEX (Fig. 6A). Taken together, the effect of dexamethasone on 3D growth seems to be an interplay between impaired pH-regulation in the hypoxic core due to lower expression of CA IX and the influence of dexamethasone itself on hypoxic signaling pathways.

By its catalytical as well as PG-domain related action, carbonic anhydrase IX is involved in various processes leading to tumor cell survival and dissemination. Clinical data show that strong CA IX expression often correlates with high tumor

grade, advanced stage, necrosis or poor prognosis in various types of tumors [including glioblastoma (74), breast carcinoma (75-77), lung carcinoma (78), head and neck cancer (79), brain tumors (80), gastric cancer (81), and non-small cell lung cancer (82)]. CA IX expression seems to relate to resistance to antitumor therapy, including chemotherapy, radiotherapy and anti-angiogenic treatment. Studies of *in vivo* xenograft models of various human tumor cells showed that high CA IX expression was linked with worse radio- and chemo-sensitivity, but the response to therapy was improved after the catalytic activity of CA IX was inhibited (33,83). Similar results were obtained for anti-angiogenic therapy aimed against VEGF (66). These data are in accordance with the observation that extracellular acidosis impairs intake of chemotherapeutic agents and affects radiation damage (84). At present, the association between CA IX level in tumor tissue and worse response to therapy has been proved also in patients suffering from various types of carcinoma [e.g. head and neck (85), rectal (85,86), breast (87,88) and non-small cell lung cancer (82,89)]. Synthetic glucocorticoid dexamethasone is often prescribed in cancer-related clinical settings. In the present study, we confirmed that dexamethasone decreases HIF-1α level and transcriptional activity by post-transcriptional action. For the first time, we demonstrated that dexamethasone treatment can reduce mRNA and protein levels of CA IX in 2D and 3D tumor models. Following dexamethasone dose, *CA9* promoter activity was inhibited by several mechanisms: as a consequence of decreased activity and level of HIF-1α and also directly by signaling of activated glucocorticoid receptors. We propose an additional way of transcriptional downregulation of *CA9*: via protein-protein interactions between activated GR and transcriptional factors (such as AP-1 or NF-κB) (Fig. 7). These interactions could lead to sequestration of transcriptional factors or to their action in a repressive mode but the elucidation of precise mechanisms requires further study. However, as the important fact remains that the final effect of dexamethasone treatment is the reduction of CA IX level. As CA IX represents an important component of pro-survival and pro-migratory machinery of tumors, linked to chemo- and radiotherapy resistance, our research generated important knowledge with possible implications for dexamethasone treatment regimens in cancer patients.

Acknowledgements

The present study was supported by the grants APVV-0893-11, Biomed ITMS 26240220087, VEGA 2/0081/14 and VEGA 2/0122/16.

References

1. Semenza GL: Targeting HIF-1 for cancer therapy. Nat Rev Cancer 3: 721-732, 2003.
2. Lendahl U, Lee KL, Yang H and Poellinger L: Generating specificity and diversity in the transcriptional response to hypoxia. Nat Rev Genet 10: 821-832, 2009.
3. Pastorek J and Pastorekova S: Hypoxia-induced carbonic anhydrase IX as a target for cancer therapy: From biology to clinical use. Semin Cancer Biol 31: 52-64, 2015.
4. Ditte P, Dequiedt F, Svastova E, Hulikova A, Ohradanova-Repic A, Zatovicova M, Csaderova L, Kopacek J, Supuran CT, Pastorekova S, *et al*: Phosphorylation of carbonic anhydrase IX controls its ability to mediate extracellular acidification in hypoxic tumors. Cancer Res 71: 7558-7567, 2011.
5. Svastova E, Witarski W, Csaderova L, Kosik I, Skvarkova L, Hulikova A, Zatovicova M, Barathova M, Kopacek J, Pastorek J, *et al*: Carbonic anhydrase IX interacts with bicarbonate transporters in lamellipodia and increases cell migration via its catalytic domain. J Biol Chem 287: 3392-3402, 2012.
6. Csaderova L, Debreova M, Radvak P, Stano M, Vrestiakova M, Kopacek J, Pastorekova S and Svastova E: The effect of carbonic anhydrase IX on focal contacts during cell spreading and migration. Front Physiol 4: 271, 2013.
7. Wykoff CC, Beasley NJ, Watson PH, Turner KJ, Pastorek J, Sibtain A, Wilson GD, Turley H, Talks KL, Maxwell PH, *et al*: Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer Res 60: 7075-7083, 2000.
8. Zatovicova M, Jelenska L, Hulikova A, Csaderova L, Ditte Z, Ditte P, Goliasova T, Pastorek J and Pastorekova S: Carbonic anhydrase IX as an anticancer therapy target: Preclinical evaluation of internalizing monoclonal antibody directed to catalytic domain. Curr Pharm Des 16: 3255-3263, 2010.
9. Dehne N and Brüne B: HIF-1 in the inflammatory microenvironment. Exp Cell Res 315: 1791-1797, 2009.
10. Eltzschig HK and Carmeliet P: Hypoxia and inflammation. N Engl J Med 364: 656-665, 2011.
11. Barnes PJ and Karin M: Nuclear factor-kappaB: A pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 336: 1066-1071, 1997.
12. Taylor CT: Interdependent roles for hypoxia inducible factor and nuclear factor-kappaB in hypoxic inflammation. J Physiol 586: 4055-4059, 2008.
13. Chen ZJ, Parent L and Maniatis T: Site-specific phosphorylation of IkappaBalpha by a novel ubiquitination-dependent protein kinase activity. Cell 84: 853-862, 1996.
14. Israël A: The IKK complex: An integrator of all signals that activate NF-kappaB? Trends Cell Biol 10: 129-133, 2000.
15. Karin M: Nuclear factor-kappaB in cancer development and progression. Nature 441: 431-436, 2006.
16. Rutz HP: Effects of corticosteroid use on treatment of solid tumours. Lancet 360: 1969-1970, 2002.
17. Rutz HP and Herr I: Interference of glucocorticoids with apoptosis signaling and host-tumor interactions. Cancer Biol Ther 3: 715-718, 2004.
18. Schaaf MJ and Cidlowski JA: Molecular mechanisms of glucocorticoid action and resistance. J Steroid Biochem Mol Biol 83: 37-48, 2002.
19. Kim H, Lee JM, Park JS, Jo SA, Kim YO, Kim CW and Jo I: Dexamethasone coordinately regulates angiopoietin-1 and VEGF: A mechanism of glucocorticoid-induced stabilization of blood-brain barrier. Biochem Biophys Res Commun 372: 243-248, 2008.
20. Hata Y, Sassa Y, Kita T, Miura M, Kano K, Kawahara S, Arita R, Nakao S, Shih JL and Ishibashi T: Vascular endothelial growth factor expression by hyalocytes and its regulation by glucocorticoid. Br J Ophthalmol 92: 1540-1544, 2008.
21. Iwai A, Fujii Y, Kawakami S, Takazawa R, Kageyama Y, Yoshida MA and Kihara K: Down-regulation of vascular endothelial growth factor in renal cell carcinoma cells by glucocorticoids. Mol Cell Endocrinol 226: 11-17, 2004.
22. Machein MR, Kullmer J, Rönicke V, Machein U, Krieg M, Damert A, Breier G, Risau W and Plate KH: Differential downregulation of vascular endothelial growth factor by dexamethasone in normoxic and hypoxic rat glioma cells. Neuropathol Appl Neurobiol 25: 104-112, 1999.
23. Yano A, Fujii Y, Iwai A, Kageyama Y and Kihara K: Glucocorticoids suppress tumor angiogenesis and in vivo growth of prostate cancer cells. Clin Cancer Res 12: 3003-3009, 2006.
24. Wu Y, Lucia K, Lange M, Kuhlen D, Stalla GK and Renner U: Hypoxia inducible factor-1 is involved in growth factor, glucocorticoid and hypoxia mediated regulation of vascular endothelial growth factor-A in human meningiomas. J Neurooncol 119: 263-273, 2014.
25. Bao Y, Lv F and Ma Y: Effect of dexamethasone on expression of hypoxia inducible factor-1α and vascular endothelial growth factor in hypoxic mice. Zhongguo Fei Ai Za Zhi 9: 143-146, 2006 (In Chinese).
26. Leonard MO, Godson C, Brady HR and Taylor CT: Potentiation of glucocorticoid activity in hypoxia through induction of the glucocorticoid receptor. J Immunol 174: 2250-2257, 2005.
27. Kopacek J, Barathova M, Dequiedt F, Sepelakova J, Kettmann R, Pastorek J and Pastorekova S: MAPK pathway contributes to density- and hypoxia-induced expression of the tumor-associated carbonic anhydrase IX. Biochim Biophys Acta 1729: 41-49, 2005.

28. Kaluz S, Kaluzová M, Chrastina A, Olive PL, Pastoreková S, Pastorek J, Lerman MI and Stanbridge EJ: Lowered oxygen tension induces expression of the hypoxia marker MN/carbonic anhydrase IX in the absence of hypoxia-inducible factor 1 alpha stabilization: A role for phosphatidylinositol 3'-kinase. Cancer Res 62: 4469-4477, 2002.
29. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M and Werner T: MatInspector and beyond: Promoter analysis based on transcription factor binding sites. Bioinformatics 21: 2933-2942, 2005.
30. Quandt K, Frech K, Karas H, Wingender E and Werner T: MatInd and MatInspector: New fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res 23: 4878-4884, 1995.
31. Abrahám IM, Meerlo P and Luiten PG: Concentration dependent actions of glucocorticoids on neuronal viability and survival. Dose Response 4: 38-54, 2006.
32. Cianchi F, Vinci MC, Supuran CT, Peruzzi B, De Giuli P, Fasolis G, Perigli G, Pastorekova S, Papucci L, Pini A, *et al*: Selective inhibition of carbonic anhydrase IX decreases cell proliferation and induces ceramide-mediated apoptosis in human cancer cells. J Pharmacol Exp Ther 334: 710-719, 2010.
33. Dubois L, Peeters S, Lieuwes NG, Geusens N, Thiry A, Wigfield S, Carta F, McIntyre A, Scozzafava A, Dogné JM, *et al*: Specific inhibition of carbonic anhydrase IX activity enhances the in vivo therapeutic effect of tumor irradiation. Radiother Oncol 99: 424-431, 2011.
34. Morris JC, Chiche J, Grellier C, Lopez M, Bornaghi LF, Maresca A, Supuran CT, Pouysségur J and Poulsen SA: Targeting hypoxic tumor cell viability with carbohydrate-based carbonic anhydrase IX and XII inhibitors. J Med Chem 54: 6905-6918, 2011.
35. Ikeda Y, Carson BS and Long DM: The effects of topical dexamethasone on experimental brain tumors and peritumoral brain edema. Acta Neurochir Suppl (Wien) 60: 397-399, 1994.
36. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER and Ratcliffe PJ: The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 399: 271-275, 1999.
37. Wiesener MS, Turley H, Allen WE, Willam C, Eckardt KU, Talks KL, Wood SM, Gatter KC, Harris AL, Pugh CW, *et al*: Induction of endothelial PAS domain protein-1 by hypoxia: Characterization and comparison with hypoxia-inducible factor-1alpha. Blood 92: 2260-2268, 1998.
38. Culver C, Sundqvist A, Mudie S, Melvin A, Xirodimas D and Rocha S: Mechanism of hypoxia-induced NF-kappaB. Mol Cell Biol 30: 4901-4921, 2010.
39. Koong AC, Chen EY and Giaccia AJ: Hypoxia causes the activation of nuclear factor kappa B through the phosphorylation of I kappa B alpha on tyrosine residues. Cancer Res 54: 1425-1430, 1994.
40. Badruddoja MA, Krouwer HG, Rand SD, Rebro KJ, Pathak AP and Schmainda KM: Antiangiogenic effects of dexamethasone in 9L gliosarcoma assessed by MRI cerebral blood volume maps. Neuro Oncol 5: 235-243, 2003.
41. van Uden P, Kenneth NS and Rocha S: Regulation of hypoxia-inducible factor-1alpha by NF-kappaB. Biochem J 412: 477-484, 2008.
42. Iyer NV, Leung SW and Semenza GL: The human hypoxia-inducible factor 1alpha gene: HIF1A structure and evolutionary conservation. Genomics 52: 159-165, 1998.
43. Minet E, Ernest I, Michel G, Roland I, Remacle J, Raes M and Michiels C: HIF1A gene transcription is dependent on a core promoter sequence encompassing activating and inhibiting sequences located upstream from the transcription initiation site and cis elements located within the 5'UTR. Biochem Biophys Res Commun 261: 534-540, 1999.
44. Niu G, Briggs J, Deng J, Ma Y, Lee H, Kortylewski M, Kujawski M, Kay H, Cress WD, Jove R, *et al*: Signal transducer and activator of transcription 3 is required for hypoxia-inducible factor-1alpha RNA expression in both tumor cells and tumor-associated myeloid cells. Mol Cancer Res 6: 1099-1105, 2008.
45. Bhattacharya S, Michels CL, Leung MK, Arany ZP, Kung AL and Livingston DM: Functional role of p35srj, a novel p300/CBP binding protein, during transactivation by HIF-1. Genes Dev 13: 64-75, 1999.
46. Hudson CC, Liu M, Chiang GG, Otterness DM, Loomis DC, Kaper F, Giaccia AJ and Abraham RT: Regulation of hypoxia-inducible factor 1alpha expression and function by the mammalian target of rapamycin. Mol Cell Biol 22: 7004-7014, 2002.
47. Treins C, Giorgetti-Peraldi S, Murdaca J, Semenza GL and Van Obberghen E: Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. J Biol Chem 277: 27975-27981, 2002.
48. Wang H, Kubica N, Ellisen LW, Jefferson LS and Kimball SR: Dexamethasone represses signaling through the mammalian target of rapamycin in muscle cells by enhancing expression of REDD1. J Biol Chem 281: 39128-39134, 2006.
49. Zhou J, Schmid T, Frank R and Brüne B: PI3K/Akt is required for heat shock proteins to protect hypoxia-inducible factor 1alpha from pVHL-independent degradation. J Biol Chem 279: 13506-13513, 2004.
50. Katschinski DM, Le L, Schindler SG, Thomas T, Voss AK and Wenger RH: Interaction of the PAS B domain with HSP90 accelerates hypoxia-inducible factor-1alpha stabilization. Cell Physiol Biochem 14: 351-360, 2004.
51. Maheshwari M, Bhutani S, Das A, Mukherjee R, Sharma A, Kino Y, Nukina N and Jana NR: Dexamethasone induces heat shock response and slows down disease progression in mouse and fly models of Huntington's disease. Hum Mol Genet 23: 2737-2751, 2014.
52. Lim W, Park C, Shim MK, Lee YH, Lee YM and Lee Y: Glucocorticoids suppress hypoxia-induced COX-2 and hypoxia inducible factor-1α expression through the induction of glucocorticoid-induced leucine zipper. Br J Pharmacol 171: 735-745, 2014.
53. Leis H, Page A, Ramírez A, Bravo A, Segrelles C, Paramio J, Barettino D, Jorcano JL and Pérez P: Glucocorticoid receptor counteracts tumorigenic activity of Akt in skin through interference with the phosphatidylinositol 3-kinase signaling pathway. Mol Endocrinol 18: 303-311, 2004.
54. Box AH and Demetrick DJ: Cell cycle kinase inhibitor expression and hypoxia-induced cell cycle arrest in human cancer cell lines. Carcinogenesis 25: 2325-2335, 2004.
55. Mottet D, Dumont V, Deccache Y, Demazy C, Ninane N, Raes M and Michiels C: Regulation of hypoxia-inducible factor-1alpha protein level during hypoxic conditions by the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3beta pathway in HepG2 cells. J Biol Chem 278: 31277-31285, 2003.
56. De Bosscher K, Vanden Berghe W and Haegeman G: The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: Molecular mechanisms for gene repression. Endocr Rev 24: 488-522, 2003.
57. Kaluz S, Kaluzová M, Opavský R, Pastoreková S, Gibadulinová A, Dequiedt F, Kettmann R and Pastorek J: Transcriptional regulation of the MN/CA 9 gene coding for the tumor-associated carbonic anhydrase IX. Identification and characterization of a proximal silencer element. J Biol Chem 274: 32588-32595, 1999.
58. Ashburner BP, Westerheide SD and Baldwin AS Jr: The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. Mol Cell Biol 21: 7065-7077, 2001.
59. Baetz D, Regula KM, Ens K, Shaw J, Kothari S, Yurkova N and Kirshenbaum LA: Nuclear factor-kappaB-mediated cell survival involves transcriptional silencing of the mitochondrial death gene BNIP3 in ventricular myocytes. Circulation 112: 3777-3785, 2005.
60. Datta De D, Datta A, Bhattacharjya S and Roychoudhury S: NF-kappaB mediated transcriptional repression of acid modifying hormone gastrin. PLoS One 8: e73409, 2013.
61. Swietach P, Wigfield S, Cobden P, Supuran CT, Harris AL and Vaughan-Jones RD: Tumor-associated carbonic anhydrase 9 spatially coordinates intracellular pH in three-dimensional multicellular growths. J Biol Chem 283: 20473-20483, 2008.
62. Swietach P, Patiar S, Supuran CT, Harris AL and Vaughan-Jones RD: The role of carbonic anhydrase 9 in regulating extracellular and intracellular ph in three-dimensional tumor cell growths. J Biol Chem 284: 20299-20310, 2009.
63. Hulikova A, Harris AL, Vaughan-Jones RD and Swietach P: Regulation of intracellular pH in cancer cell lines under normoxia and hypoxia. J Cell Physiol 228: 743-752, 2013.
64. Ditte Z, Ditte P, Labudova M, Simko V, Iuliano F, Zatovicova M, Csaderova L, Pastorekova S and Pastorek J: Carnosine inhibits carbonic anhydrase IX-mediated extracellular acidosis and suppresses growth of HeLa tumor xenografts. BMC Cancer 14: 358, 2014.

65. Chiche J, Ilc K, Laferrière J, Trottier E, Dayan F, Mazure NM, Brahimi-Horn MC and Pouysségur J: Hypoxia-inducible carbonic anhydrase IX and XII promote tumor cell growth by counteracting acidosis through the regulation of the intracellular pH. Cancer Res 69: 358-368, 2009.
66. McIntyre A, Patiar S, Wigfield S, Li JL, Ledaki I, Turley H, Leek R, Snell C, Gatter K, Sly WS, *et al*: Carbonic anhydrase IX promotes tumor growth and necrosis in vivo and inhibition enhances anti-VEGF therapy. Clin Cancer Res 18: 3100-3111, 2012.
67. Ivanova L, Zandberga E, Siliņa K, Kalniņa Z, Ābols A, Endzeliņš E, Vendina I, Romanchikova N, Hegmane A, Trapencieris P, *et al*: Prognostic relevance of carbonic anhydrase IX expression is distinct in various subtypes of breast cancer and its silencing suppresses self-renewal capacity of breast cancer cells. Cancer Chemother Pharmacol 75: 235-246, 2015.
68. Mattern J, Büchler MW and Herr I: Cell cycle arrest by glucocorticoids may protect normal tissue and solid tumors from cancer therapy. Cancer Biol Ther 6: 1345-1354, 2007.
69. Takada Y, Bhardwaj A, Potdar P and Aggarwal BB: Nonsteroidal anti-inflammatory agents differ in their ability to suppress NF-kappaB activation, inhibition of expression of cyclooxygenase-2 and cyclin D1, and abrogation of tumor cell proliferation. Oncogene 23: 9247-9258, 2004.
70. Wang ML, Chiou SH and Wu CW: Targeting cancer stem cells: Emerging role of Nanog transcription factor. Onco Targets Ther 6: 1207-1220, 2013.
71. Dang CV: c-Myc target genes involved in cell growth, apoptosis, and metabolism. Mol Cell Biol 19: 1-11, 1999.
72. Lavia P and Jansen-Dürr P: E2F target genes and cell-cycle checkpoint control. BioEssays 21: 221-230, 1999.
73. Meyer C, Pries R and Wollenberg B: Established and novel NF-κB inhibitors lead to downregulation of TLR3 and the proliferation and cytokine secretion in HNSCC. Oral Oncol 47: 818-826, 2011.
74. Mayer A, Schneider F, Vaupel P, Sommer C and Schmidberger H: Differential expression of HIF-1 in glioblastoma multiforme and anaplastic astrocytoma. Int J Oncol 41: 1260-1270, 2012.
75. Hussain SA, Ganesan R, Reynolds G, Gross L, Stevens A, Pastorek J, Murray PG, Perunovic B, Anwar MS, Billingham L, *et al*: Hypoxia-regulated carbonic anhydrase IX expression is associated with poor survival in patients with invasive breast cancer. Br J Cancer 96: 104-109, 2007.
76. Wykoff CC, Beasley N, Watson PH, Campo L, Chia SK, English R, Pastorek J, Sly WS, Ratcliffe P and Harris AL: Expression of the hypoxia-inducible and tumor-associated carbonic anhydrases in ductal carcinoma in situ of the breast. Am J Pathol 158: 1011-1019, 2001.
77. Chu CY, Jin YT, Zhang W, Yu J, Yang HP, Wang HY, Zhang ZJ, Liu XP and Zou Q: CA IX is upregulated in $CoCl_2$-induced hypoxia and associated with cell invasive potential and a poor prognosis of breast cancer. Int J Oncol 48: 271-280, 2016.
78. Konno H, Ishii G, Nagai K, Yoshida J, Nishimura M, Nara M, Fujii T, Murata Y, Miyamoto H and Ochiai A: Carbonic anhydrase IX expression is associated with tumor progression and a poor prognosis of lung adenocarcinoma. Lung Cancer 54: 409-418, 2006.
79. Le QT, Kong C, Lavori PW, O'byrne K, Erler JT, Huang X, Chen Y, Cao H, Tibshirani R, Denko N, *et al*: Expression and prognostic significance of a panel of tissue hypoxia markers in head-and-neck squamous cell carcinomas. Int J Radiat Oncol Biol Phys 69: 167-175, 2007.
80. Haapasalo J, Hilvo M, Nordfors K, Haapasalo H, Parkkila S, Hyrskyluoto A, Rantala I, Waheed A, Sly WS, Pastorekova S, *et al*: Identification of an alternatively spliced isoform of carbonic anhydrase XII in diffusely infiltrating astrocytic gliomas. Neuro Oncol 10: 131-138, 2008.
81. Chen J, Röcken C, Hoffmann J, Krüger S, Lendeckel U, Rocco A, Pastorekova S, Malfertheiner P and Ebert MP: Expression of carbonic anhydrase 9 at the invasion front of gastric cancers. Gut 54: 920-927, 2005.
82. Stewart DJ, Nunez MI, Behrens C, Liu D, Lin YH, Lee JJ, Roth J, Heymach J, Swisher SG, Hong WK, *et al*: Membrane carbonic anhydrase IX expression and relapse risk in resected stage I-II non-small-cell lung cancer. J Thorac Oncol 9: 675-684, 2014.
83. Dubois L, Peeters SG, van Kuijk SJ, Yaromina A, Lieuwes NG, Saraya R, Biemans R, Rami M, Parvathaneni NK, Vullo D, *et al*: Targeting carbonic anhydrase IX by nitroimidazole based sulfamides enhances the therapeutic effect of tumor irradiation: A new concept of dual targeting drugs. Radiother Oncol 108: 523-528, 2013.
84. Wojtkowiak JW, Verduzco D, Schramm KJ and Gillies RJ: Drug resistance and cellular adaptation to tumor acidic pH microenvironment. Mol Pharm 8: 2032-2038, 2011.
85. Koukourakis MI, Giatromanolaki A, Danielidis V and Sivridis E: Hypoxia inducible factor (HIf1alpha and HIF2alpha) and carbonic anhydrase 9 (CA9) expression and response of head-neck cancer to hypofractionated and accelerated radiotherapy. Int J Radiat Biol 84: 47-52, 2008.
86. Lee-Kong SA, Ruby JA, Chessin DB, Pucciarelli S, Shia J, Riedel ER, Nitti D and Guillem JG: Hypoxia-related proteins in patients with rectal cancer undergoing neoadjuvant combined modality therapy. Dis Colon Rectum 55: 990-995, 2012.
87. Span PN, Bussink J, Manders P, Beex LV and Sweep CG: Carbonic anhydrase-9 expression levels and prognosis in human breast cancer: Association with treatment outcome. Br J Cancer 89: 271-276, 2003.
88. Tan EY, Yan M, Campo L, Han C, Takano E, Turley H, Candiloro I, Pezzella F, Gatter KC, Millar EK, *et al*: The key hypoxia regulated gene CAIX is upregulated in basal-like breast tumours and is associated with resistance to chemotherapy. Br J Cancer 100: 405-411, 2009.
89. Giatromanolaki A, Koukourakis MI, Sivridis E, Pastorek J, Wykoff CC, Gatter KC and Harris AL: Expression of hypoxia-inducible carbonic anhydrase-9 relates to angiogenic pathways and independently to poor outcome in non-small cell lung cancer. Cancer Res 61: 7992-7998, 2001.