High cell density-mediated pericellular hypoxia is a crucial factor inducing expression of the intrinsic hypoxia marker CA IX *in vitro* in HeLa cells*

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Received October 30, 2002

Oxygen plays a central role in respiration of the cells and thus in generation of energy by aerobic metabolism. The cells precisely detect oxygen level and changes in oxygen perfusion leads to induction of various responses enabling to adapt to unfavorable conditions.

CA IX carbonic anhydrase is a hypoxia-inducible tumor-associated antigen which is overexpressed in dense HeLa cells. Presented study investigates the effects of oxygen tension on CA IX expression in HeLa cell culture. Using of an immunoradiometric assay to quantify CA IX protein, it was revealed that expression of CA IX correlates with increasing cell density, lactate production and medium acidification under normoxic conditions. These observations and hypoxia-inducibility of CA IX suggested a possible role of pericellular hypoxia in density-induced CA IX expression. To test this hypothesis, HeLa cells were incubated in normobaric hyperoxia (50% O_2) or cell culture medium was convected to disturb oxygen deprivation. Both approaches completely abrogated CA IX expression in dense HeLa cell cultures and therefore confirmed the importance of decreased oxygen tension in high cell density-induced CA IX expression. In addition, HeLa cells exposed to hyperoxia retained inducibility of CA IX expression by transition metals and iron chelators, suggesting that they act independently of cell density mediated-pO_2-gradient or at a downstream site from oxygen sensor.

Observed data indicate that high cell density-lowered pericellular pO_2 is a crucial factor inducing CA IX expression and influencing composition of metabolic micromilieu surrounding the dense HeLa cells.

Key words: CA IX, hypoxia, hyperoxia.

The microenvironmental physiology of tumors is quite different from that of normal tissues. Considerable differences are between the vascularity of tumor and normal tissue. Tumor blood vessels are highly irregular, torturous, have arterio-venous shunts, blind ends, high intercapillary distances etc. These structural and physiological abnormalities result in low oxygen perfusion of some areas of tumor tissue, thereby becoming hypoxic and eventually necrotic. And thus hypoxia is a common feature of human and animal tumors [16]. Hypoxia as a factor altering behaviour of tumor cells is associated with malignant aggressiveness of tumors due to increased invasivity, metastatic potential and resistance to radiation and chemotherapy [4, 9].

Molecular oxygen, O_2, as a terminal acceptor of electrons from respiratory chain is essential for cellular respiration and thus for production of ATP. Whereas low oxygen tension has significant impact on energy metabolism of the cells, hypoxic cells induce variety of defense and rescue mechanisms to compensate hypoxic stress [8]. Changes in oxygen tension results in alteration of metabolic activity, membrane electrochemical potentials and fluidity, ion permeability etc. Hypoxia up-regulates the expression of genes

*Financial support for this work was provided by grants from the Slovak Scientific Grant Agency (No. 2/2025/22) and Bayer Corporation.

Abbreviations: Akt – protein kinase B; CA IX – human carbonic anhydrase IX protein; *CA9* – human carbonic anhydrase 9 gene; DFO – desferrioxamine; DNP – 2,4-dinitrophenol; HEPES – N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HIF-1 – hypoxia inducible factor 1 transcriptional complex; HIF-1α – hypoxia-inducible factor 1α; HRE – hypoxia response element; mAb – monoclonal antibody; IRMA – immunoradiometric assay; M75 – CA IX specific monoclonal antibody; oPE – o-phenanthroline; PBS – phosphate buffered saline; pH_e – extracellular pH; pH_i – intracellular pH; PI3-K – phosphatidylinositol 3-kinase; PMSF – phenylmethylsulfonyl fluoride; PR1 – protected region 1; pVHL – protein product of von Hippel-Lindau gene; ROS – reactive oxygen species; SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

involved in adaptation to decreased O_2. The expression of these genes has a key function in angiogenesis, vascular reactivity and remodeling capacity, cell proliferation and survival, glucose and energy metabolism [22]. Hypoxia-accelerated anaerobic glycolysis leads to excessive accumulation of lactate. The lactic acid and impaired efflux of acidic catabolites from tumor's interstitium are supposed to be major causes of low extracellular pH of solid tumors [13, 30]. Acidic pH environment stimulates expression of IL-8 proangiogenic factor [29] and is connected with tumor invasion [14].

CA IX is a cell surface glycoprotein which is ectopicaly expressed in a number of human malignant tumors. In some recent studies has been described that the intensity of expression of CA IX is connected with lowered oxygen tension [10, 28] and therefore *CA9* was proposed as an intrinsic marker of intratumoral hypoxia [3, 12, 17]. The promoter of *CA9* contains HIF-1 dependent hypoxia response element (HRE) and hypoxia-induced expression of *CA9* is tightly regulated by HRE-dependent oxygen controlled HIF-1α/pVHL mechanism [28]. CA IX is fully enzymatically active and thus is able to catalyse reversible hydration of carbon dioxide [31]. Enzymatic activity of CA IX could be involved in the acidification of extracellular milieu and in the pH homeostasis of tumor cells under hypoxic conditions and so it could provide selective advantage for tumor growth and malignant progression.

CA IX is overexpressed in dense HeLa cells, whereas it is not expressed in sparse cell culture [7, 11, 19, 32]. This density-dependent expression of CA IX could be probably caused by cell-to-cell interactions through cell-adhesion molecules or by density-mediated lowered oxygen tension (pericellular hypoxia). The major focus of this study was to analyze the possible role of pericellular hypoxia in the density-induced CA IX expression in HeLa cells. Obtained results indicate that high density-mediated pericellular hypoxia is a decisive factor inducing CA IX expression in dense HeLa cells under normoxic conditions.

Material and methods

Cells and culture conditions. HeLa cervical adenocarcinoma cells (ATCC CCL-2) were grown in DMEM medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, supplemented by 2mM L-glutamine and 160 μg gentamicin/ml. Cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. For experiments, cells were seeded at a low density ($1.5x10^5$ cells per 3.5 cm dish), high density ($1.5x10^6$ cells per 3.5 cm dish) or various density ($15–70x10^3$ cells per 96-well plate), 1 day before hypoxic, normoxic or hyperoxic exposures. HeLa cells used for plating were maintained at least two passages at sparse density. Hypoxic and hyperoxic conditions were achieved in a Napco 7000 incubator, which maintained an humidified atmosphere with 5% CO_2, 0.1% or 50% O_2 and the balance made up of nitrogen.

Expression of CA IX, pH and lactate content were determined by methods described below.

Immunoradiometric assay. The murine mAb M75 specific for CA IX [20] was labeled with iodine-125 to specific activity 5.5 $\mu Ci.\mu g^{-1}$ and used for immunoradiometric assay (IRMA) as was described previously [7]. Briefly, the cells were incubated with ^{125}I-M75 mAb $1x10^6$ cpm.ml^{-1} (1 ml per 3.5 cm dish and 0.1 ml per 96-well plate) at 37 °C for 2 h and then washed. The cultures were then extracted by RIPA lysis buffer (1% Triton X-100, 0.1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml trypsin inhibitor, 1 mM ethylenediaminetetraacetic acid in PBS) for 30 min at room temperature. Extracts were centrifuged and concentration of proteins in supernatants was determined by bicinchoninic acid protein assay kit (Pierce) according to the manufacturer's instructions. Radioactivity in cell extracts was measured by Clinigamma counter (Pharmacia LKB) at 60 s counting intervals. Radioactivity of samples was normalized to protein amount and results were expressed as means with corresponding standard deviations.

Determination of lactate and pH. The concentration of lactate in culture medium was determined enzymatically by the glucose oxidase method using the LACTATE REAGENT (Sigma, USA) according to the manufacturer's instructions.

pH of larger volumes of cell culture medium was measured by PHM63 digital pH meter (Radiometer, Copenhagen, Denmark). For determination of pH of samples with small volume (100–200 μl) a simple, rapid and reproducible sphectroforometric method was used. Absorbance of samples at 540 nm, at proximity to local absorbtion maximum of fenol red (phenolsulfonphtalein, acidobasic indicator presented in DMEM medium) was measured by microplate reader (Labsystems Multiskan MS, USA) and pH was interpolated by linear regression from calibration curve using a series of standards with different pH (medium buffered with 25 mM HEPES).

Western blotting. The cell monolayers were washed twice with PBS and then extracted by RIPA lysis buffer (1% Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF, 20 μg/ml trypsin inhibitor, 1 mM ethylenediaminetetraacetic acid in PBS) for 30 min at room temperature. The extracts were then centrifuged (15 min at 13 000 rpm) and concentration of proteins was determined by BCA assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Equal amounts of protein extracts (40 μg) were fractionated by SDS-PAGE gel electrophoresis and transferred to PVDF membrane (Immobilon^{TM}-P, Millipore, Bedford, U.K.). The membrane was incubated with ^{125}I-labeled M75 mAb and then exposed to X-ray film.

Results

Increasing density of HeLa cells leads to induction of CA IX expression, lactate overproduction and medium acidification. Increasing number of HeLa cells covering whole range of densities (from sparse to dense) were plated and grown overnight in normoxia and then analyzed for CA IX expression, pH of culture medium and lactate content (Fig. 1). CA IX was not expressed in sparse cells and its expression exponentially increased with growning cell density. The density-dependent exponential increase of CA IX expression was accompanied by an increase in lactate production with corresponding medium acidification. This suggests that in certain density of HeLa cells, pericellular oxygen tension reaches a level which may turn on a transcription machinery of CA IX expression.

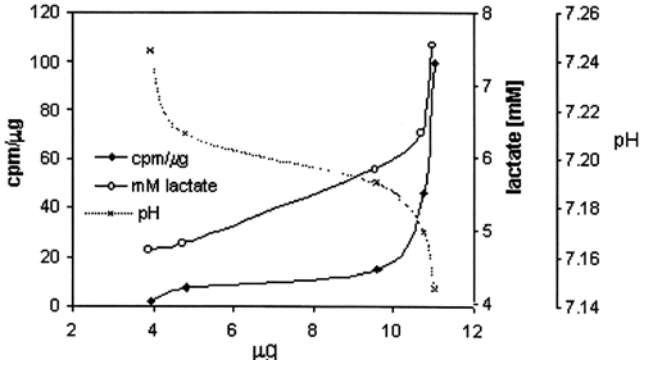

Figure 1. CA IX expression, pH of medium and lactate production by HeLa cells as a function of cell density in the normoxia. Increasing number of HeLa cells were seeded in 96-well plates in 100 µl of medium (n=3) and grown for 16 h in normoxia. Then CA IX expression was analyzed by immunoradiometric assay, pH was measured spectrophotometrically and lactate concentration was determined enzymatically using the LACTATE REAGENT. Cell density-related protein amount was determined by BCA assay. Error bars are omitted for clarity.

Hyperoxia and medium convection abrogates density induced CA IX expression. To evaluate possible role of pericellular hypoxia in induction of CA IX expression, densely plated HeLa cells were exposed in parallel both to hyperoxia (50% O_2) and normoxia. Cell cultures displayed increasing acidification of culture medium with decrease of oxygen tension (Fig. 2b), probably due to enhanced rate of anaerobic glycolysis and related overproduction of lactic acid. Observed CA IX expression was approximately 29% of its level in the normoxic culture (Fig. 2a). Also simple convection of culture medium in normoxia had similar effect (Fig. 2a). Remaining CA IX level in dense culture either incubated in hyperoxia or shaked could be the result of accumulation before the experimental incubation or cell-to-cell interaction induced expression. To differentiate between the role of density-mediated cell-to-cell interactions and pericellular hypoxia (also caused by high density) in CA IX induction in dense culture, sparsely plated HeLa cells (CA IX negative) were left to grown to high density in hyperoxia, normoxia and normoxia with shaking (Fig. 3). Expression of CA IX in HeLa cells incubated in normoxia with shaking and hyperoxia was completely diminished despite the comparable confluent density in control normoxic culture (Fig. 3a–3c). IRMA showed background signal on the level of sparse HeLa cells.

CA IX expression is abrogated in hyperoxia but remains inducible by hypoxia mimics. HeLa cells incubated in hyperoxia for prolongated time preserved inducibility of CA IX expression by transition metals (Ni^{2+} and Co^{2+}) and iron chelators (desferrioxamine and o-phenanthroline), but lost inducibility of CA IX expression by density under normoxic conditions (Fig. 4). This loss of CA IX expression in dense culture in normoxia after hyperoxic exposure may be probably caused by inactivation of mitochondrial enzyme com-

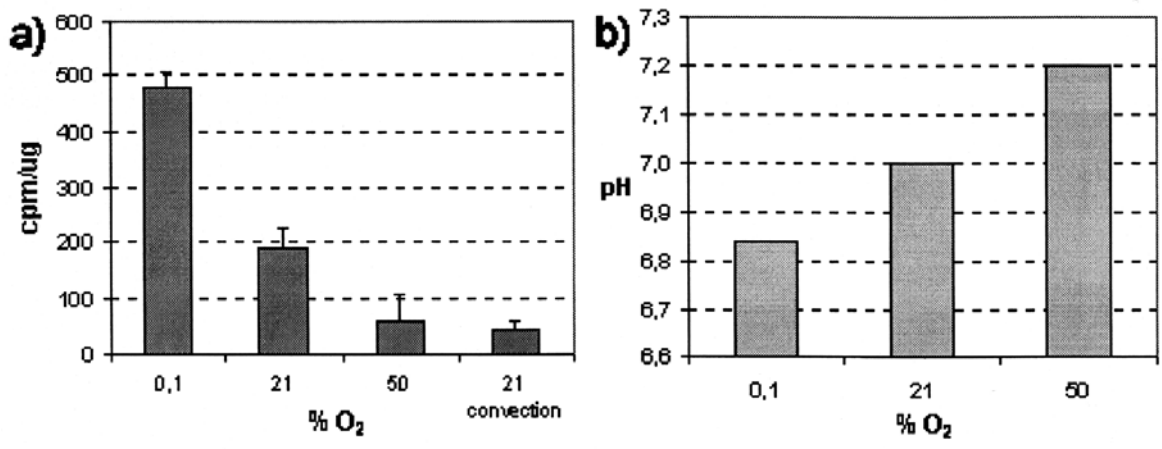

Figure 2. Influence of O_2 concentration on CA IX expression (a) and acidification of cell culture medium (b) in dense HeLa cell culture. Dense HeLa cell cultures were incubated in hypoxia (0.1% O_2), normoxia (21% O_2), hyperoxia (50% O_2) or were shaked in normoxic conditions (70 rpm) for 16 h. CA IX expression was analyzed by immunoradiometric assay, n=3. pH of cell culture medium was measured by PHM63 digital pH meter.

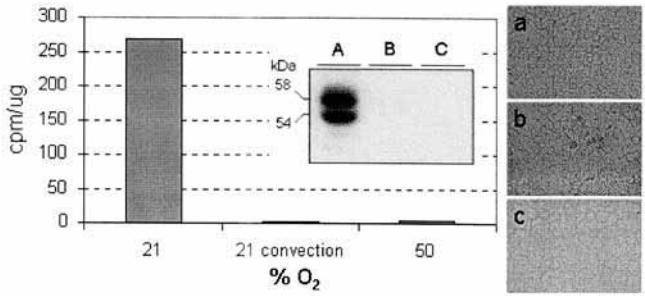

Figure 3. Expression of CA IX in dense HeLa cell culture grown under indicated conditions from sparse plated culture. Sparse HeLa cell cultures were grown in normoxia (21% O_2) with or without shaking (70 rpm), and hyperoxia (50% O_2) until they reach confluency (96 h). CA IX expression was than analyzed by immunoradiometric assay and western blotting. Inset: western blotting, 40 μg protein extract from HeLa cells loaded per lane (A – normoxia; B – normoxia with shaking; C – hyperoxia) extracts were loaded per line. Visible light microscopy indicates comparable high density of HeLa cells (a – normoxia; b – normoxia with shaking; c – hyperoxia).

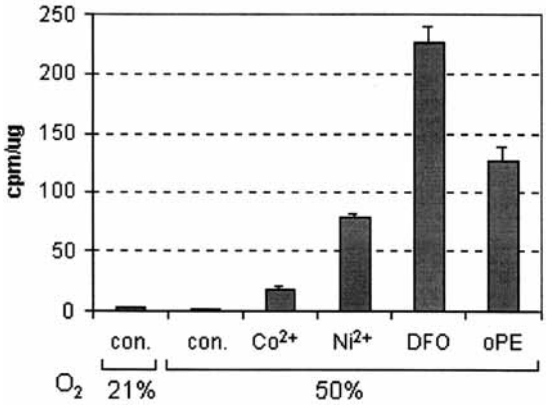

Figure 4. Expression of CA IX in dense HeLa cells exposed under hyperoxia to the indicated agents. Sparse plated HeLa cells were grown in hyperoxia (50% O_2) until they reach confluency (96 h). The cells were than incubated in normoxic conditions or exposed to $NiCl_2$ (300 μM), $CoCl_2$ (200 μM), DFO (200 μM), oPE (150 μM) under hyperoxic atmosphere for 16 h (con. – control without addition of a hypoxia mimic). CA IX expression was analyzed by immunoradiometric assay, n=3.

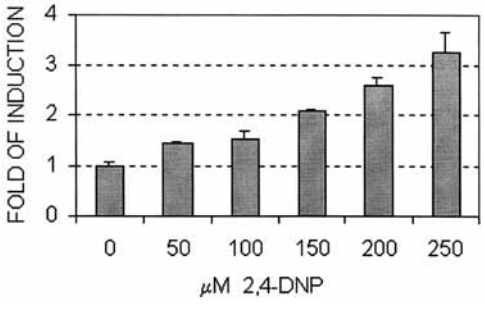

Figure 5. Stimulatory effect of 2,4-dinitrophenol on hypoxia induced CA IX expression. Sparse HeLa cells were incubated with increasing concentration of DNP in hypoxic atmosphere (0.1% O_2) for 16 h, n=3. CA IX expression was analyzed by immunoradiometric assay. Fold induction was calculaded with respect to cells without addition of DNP.

plexes (I and II) involved in generation of reactive oxygen species (ROS) by mitochondria. In this coherence when mitochondrial respiration was stimulated by 2,4-dinitrophenol (DNP) in HeLa cells exposed to hypoxia, CA IX expression was upregulated approximately 3.5 times at the highest tested concentration (Fig. 5).

Discussion

It has been reported that CA IX expression positively correlates with increasing density of HeLa cells [19, 32]. This phenomenon is anchorage and serum independent [11]. It appears that density induced expression of CA IX is not mediated by soluble factor and is not affected by medium acidification or glucose concentration [11]. Cell density-induced CA IX expression requires minimal HIF-1α activity and phosphatidylinositol 3-kinase (PI3-K) pathway activation [11].

The major aim of this study was to evalute possible role of pericellular hypoxia in density-induced expression of CA IX in HeLa cells *in vitro*. In the preliminary experiments the cell density-dependent alteration of lactic acid production and pH of culture medium was investigated. HeLa cells displayed exponential increase of lactate content and related decrease of medium pH in relationship with increasing cell density from sparse to high dense culture. These data confirm that elevated production of lactic acid causes acidification of culture medium [30]. However lactate is not the only determinant of low interstitial pH_e of solid tumors [30] and transmembrane tumor-associated carbonic anhydrases such as CA IX and CA XII could contribute to this effect by catalyzed hydration of CO_2. The increase in lactate production under hypoxic conditions was recently reported [18] proving that lactate concentration reflects oxygen tension. Exponential (but not proportional) increase of lactate concentration as a function of cell density suggested on a discontinuous decrease of pO_2 at certain density of HeLa cells.

Early study of Stevns (1965) predicted that oxygen requirements of a monolayer culture under the standard medium overlay could not be adequately supplied by atmospheric pO_2 [24]. Voltamperometric measurements of Werrlein and Glinos (1974) suggested that oxygen availability and microenvironmental pO_2 gradients are directly related to the cell density and respiratory activity of the whole cell population in cell culture [26]. Also another studies confirmed that confluent cells are exposed to pericellular hypoxia under the normoxic conditions due to diffusion-limited oxygen delivery [15, 23, 27]. The oxidative metabolism of proliferative active cells intensely consumpts oxygen dissolved in the nearest extracellular milieu and therefore around densely growing cells arises spatial gradient of O_2 concentration determined by limited microregio-

nal delivery and high oxygen uptake. Under these conditions, when respiration is restricted by diffusion-limited O_2 supply from extracellular space pericellular hypoxia arise and cells when trying to adapt to these instances partially switch from aerobic to anaerobic metabolism in concordance with pO_2 concentration. Enhanced rate of anaerobic glycolysis leads to excessive production of lactic acid. This may probably lead to intracellular metabolic acidosis. Living cells maintain their intracellular pH_i within a limited range of values (neutral to slightly alkaline) to provide favorable environment for cellular metabolic functions. Moderately elevated pH_i is considered to be permissive for cell growth. And therefore lactate is exported from cells by H^+-monocarboxylate cotransporter. Even other mechanisms how to maintain neutral or relatively elevated pH_i may be employed such as amiloride-sensitive Na^+/H^+ exchanger, DIDS-sensitive Na^+-dependent HCO_3^-/Cl^- antiporter or vacuolar type H^+ ATPase in the plasma membrane [25]. Here, CA IX and CA XII could be potentially employed in the regulation of pH_i. Its intrinsic enzymatic activity of extracellular carbonic anhydrase domain (CA) could provide HCO_3^- anion for import into the intracellular space in cooperation with HCO_3^-/Cl^- antiporter. Remaining H^+ may contribute to further acidification of extracellular space. Altogether, carboanhydrase activity of CA IX may probably help to maintain intracellular acidobasic homeostasis with corresponding decrease of extracellular pH_e under the conditions of lowered oxygen tension, thereby it could be helpful for survival, growth and metastatic spread of cancer cells in the adverse microenvironments.

To elucidate the possible role of high cell density-lowered pO_2 in density-induced CA IX expression, local pericellular pO_2 gradient was reduced by incubation in normobaric hyperoxia (50% O_2) and simple convection. The concentration, respectively molar ratio x of oxygen dissolved in its aquaeous solution (culture medium) is proportional to the partial pressure pO_2 in an air phase as given by Henry's law (1803): $p=Hx$, were H is the Henry's constant. Thus in open system with higher partial pressure of oxygen pO_2 in the hyperoxic atmosphere (50% O_2) also concentration of O_2 dissolved in culture medium is proportionally higher. Incubation in hyperoxic atmosphere completely suppressed density-induced CA IX expression. Diffusion-limited transfer of oxygen from culture medium to medium-cells surface interface was also by-passed by agitation of medium. Both, hyperoxia and shaking of culture medium diminished density induced CA IX expression and thus confirmed the importance of pericellular hypoxia in cell density induced CA IX expression in HeLa cells. Results of medium shaking is in agreement with parallel independent observation [11]. However stirring of culture medium disturbs concentration gradient of another low molecular weight metabolites, growth factors and nutrients, and therefore experiments with hyperoxia-abrogated CA IX expression provide definite and direct evidence of relationship between the high cell density-mediated pO_2 gradient and CA IX expression in dense HeLa cells.

The loss of CA IX expression observed in normoxic dense culture after incubation in hyperoxia may be probably consequence of inactivation of NADH-ubiquinone reductase (complex I) and succinate-ubiquinone reductase (complex II) observed in prolonged hyperoxia [21]. Both enzyme complexes are involved in generation of reactive oxygen species (ROS) by mitochondria. Recent data suggest that ROS produced by mitochondria are involved in signal transduction process of oxygen sensing [5, 6]. Furthermore, inhibition of complex I by mitochondrial inhibitors attenuates the accumulation of HIF-1α during hypoxia [1, 2]. Also stimulatory effect of oxidative phosphorylation uncupler (DNP) suggests on involvement of mitochondrial respiration in oxygen sensing. DNP as a weak acid lipid-soluble aromatic protonophore discharges the protonmotive force generated by proton pumps of respiratory chain and by this way uncouples oxidative phosphorylation and accelerates respiration. This could probably lead to further decrease of pericellular oxygen tension and to increased production of ROS with accompanying stimulation of CA IX expression, but certainly precise mechanism remains to be elucidated.

At this level of knowledge, it is not possible to explain whether high cell-density caused moderate pO_2 changes directly affect oxygen sensor or secondarily induced adaptive responses and metabolic changes stimulate signal transduction leading to activation of PI3-K/Akt pathway acting on the HRE and PR1 elements of CA IX promoter. Generally, these observations suggest that high cell density-lowered pericellular oxygen tension in standard normoxic culture significantly influences the gene expression and composition of microenvironment of the tumor cells.

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