Three-dimensional culture promotes reconstitution of the tumor-specific hypoxic microenvironment under TGFβ stimulation

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Abstract. *In vitro* tumor growth in a three-dimensional (3D) architecture has been demonstrated to play an important role in biology not only for developmental organogenesis and carcinogenesis, but also for analyses on reconstitution and maintenance in a variety of biological environments surrounding the cells. In addition to providing architectural similarity to living organisms, 3D culture with a radial flow bioreactor (RFB) can also closely mimic the living hypoxic microenvironment under which specific organogenesis or carcinogenesis occurs. The findings of the present study under the RFB culture conditions show that cancer cells underwent a shift from aerobic to hypoxic energy metabolism, in addition to protein expression to maintain the 3D structure. In RFB-cultured cells, protein stability of hypoxia-inducible factor 1 (HIF1) α, a subunit of HIF1, was increased without upregulation of its mRNA. Under these conditions, PHD2, HIF-prolyl-4-hydroxylase 2 and a HIF1 downstream enzyme, were stabilized without affecting the mRNA levels via downregulation of FK506-binding protein 8. PHD2 accumulation, which occurred concomitant with HIF1 stabilization, may have compensated for the lack of oxygen under hypoxic conditions to regulate the HIF levels. 3D-culture-induced overexpression of carbonic anhydrase (another representative HIF downstream enzyme) was found to occur independently of cell density in RFB-cultured cells, suggesting that the RFB provided an adequately hypoxic microenvironment for the cultured cells. From these results, it was hypothesized that the key factors are regulatory molecules, which stabilize and degrade HIF molecules, thereby activating the HIF1 pathway under a hypoxic milieu.

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Introduction

For the past few decades, cancer chemotherapy has gradually improved with the development of novel antitumor agents and is having a profound impact on many hematological malignancies and some specific solid tumors. However, the most common solid tumors, like hematopoietic malignancies, are often resistant to chemotherapy and frequently spread by metastasis. As a result, many patients have died from cancer even though they were treated properly. This feature of solid tumors, the so-called 'malignant progression', is one of the principal problems in treating these tumors. There is a specific mechanism by which solid tumors acquire drug resistance and metastatic properties (1-3). Malignant progression is traditionally considered to be due to genetic alterations and involves changes in gene expression or increased activity of membrane-associated drug transport molecules (such as P-glycoprotein or multidrug resistance protein), anti-apoptotic and survival signaling molecules and cell motility-related molecules. On the other hand, malignant progression may also be influenced by abnormal microenvironmental changes occurring within the tumors, such as growth factors, hormones, physiological stresses (e.g., glucose starvation, hypoxia, low pH, and other nutrient deprivation), physical stresses (e.g., high cell densities and shear stress) and limited penetration or diffusion of drugs into the deeper layers of a tumor mass (4-11). Conversely, cancer cells actively re-construct their microenviroment to survive and maintain their viability (1-3). Because of the serious clinical outcome, the mechanisms and functions by which solid tumors acquire malignant potential are now undergoing intensive investigation. However, the molecular mechanisms of common solid tumors are not fully understood. Currently, the mainstream approach to evaluating and determining the characteristics of cancer cells or drug efficacy against cancer cells, is through the use of two-dimensional, so-called monolayer (2D) culture. However, 2D culture systems cannot fully replicate the biologically meaningful pathways that link the cell-cell adhesion, interaction, and growth that occur in the three-dimensional (3D) tissues of living organisms (12-20), because of changes in the character of the cells when they are isolated from their original tissues for *in vitro* culture. By contrast, animal models may yield

more accurate morphologic and biochemical insights, but they are not only considerably less amenable to large-scale genetic studies, but also have the potential for unknown biological effects of the animal status or tumor behavior. A 3D cell culture system combines the virtues of animal models and 2D cell culture as previously described and the 3D culture model allows cells to develop into structures similar to those in living organisms (12-20).

Examination of the molecular and biological mechanisms of the cancer cell can be carried out in the environment using a 3D culture system that mimics the living organism, mainly through a spheroid culture system. Recent progress has been demonstrated with many types of bioreactor for 3D and high-density cell culture systems (20,36,37). In our laboratory, a radial flow type bioreactor (RFB) was developed and several characteristics of RFB-cultured cells were determined. In our recent study, the similarities and differences between the gene expression profiles were clarified in A431 tumor cells grown under 2D and 3D conditions in RFB culture with different microenvironments (20). Moreover, under RFB culture conditions, growing tumor cells re-exhibited their true malignant character that was not evident during and after the adaptation procedure for *in vitro* 2D culture conditions.

In general, tumor oxygenation has been considered one of the primary regulators in providing an optimum cellular micro-environment for tumor cell survival, invasion, and metastasis. Most solid tumors develop regions of hypoxia as they grow rapidly and outstrip the blood supply. In order to survive under hypoxic stress, tumor cells have developed a coordinated set of responses that orchestrate their adaptation to such conditions. The surviving adapted tumor cells consequently gain the features of aggressiveness and resistance to therapy. Many studies have been conducted to examine tumor cell behavior and interaction between the tumor cell and the extra-cellular matrix under a hypoxic microenvironment (21-35). The present study demonstrated that the RFB induces a hypoxic microenvironment surrounding the cultured cells, thereby mimicking the living body; the study also focused on protein expression under hypoxic regulation and the resultant energy metabolism.

Materials and methods

Monolayer (2D conditions) culture and spheroid culture using conventional devices. The A431 cell line was derived from a human head and neck squamous cell carcinoma and routinely grown in conventional monolayer cultures using culture dishes (BD Falcon, Japan) in RPMI-1640 (Invitrogen, Carlsbad, CA) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, Japan). Conditioned media from static culture were collected from the culture dishes and stored at -80˚C. For treatment with transforming growth factor (TGF)β in spheroid culture, the A431 cells ($1x10^5$ cells) were seeded in 2 different devices (VECELL-3D Insert, Asahi Glass, Tokyo, Japan and 3D-Ha with nonwoven fabric, AC Biotechnologies, Inc., Yokohama, Japan) according to the instructions from each manufacturer. After 3 days of culture, cells were treated with or without transforming growth factor (TGF)β1 at 0.5 ng/ml for 24 h under the conventional culture conditions in a CO_2 incubator.

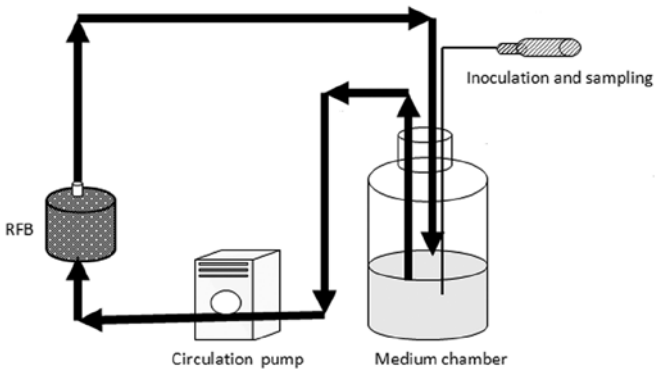

Figure 1. RFB 3D cell culture system. Schematic of RFB cell culture system flow. A medium flows back by a circulation pump between RFB and medium chamber.

3D culture in the RFB. Culture conditions were employed as reported previously (20,36,37). Briefly, A431 cells ($1x10^7$ cells) that had been injected in the reservoir of the RFB system were trapped by adherence to the matrix and then continuously cultured with 150 ml of RPMI-1640 medium supplemented 10% FBS in a CO_2 incubator (Fig. 1). Concentrations of glucose, lactate and pH in the culture media were measured through the experiment. The culture medium was changed properly and the conditioned media were collected and stored at -80˚C. After 14 days of culture, the cell culture reached confluence and the hydroxyl apatite beads with cells attached in the RFB were collected and kept in liquid nitrogen until use.

mRNA isolation and DNA microarray and RT-PCR analyses. After culturing the A431 cells in 2D and RFB culture systems, total RNA was extracted using the RNeasy kit (Qiagen, Tokyo, Japan) as described previously (17,20). For gene expression analysis, a Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA) was used according to the manufacturer's instructions as reported previously. Two separate hybridizations were performed for each sample and the images obtained were analyzed using the GeneChip® Operating Software (Affymetrix). Semiquantitative reverse transcription-PCR (RT-PCR) experiments were carried out as previously reported. PCR reactions were optimized for the number of cycles to ensure product intensity within the logarithmic phase of amplification. The following primer sequences in each gene were used (Table I).

Analysis of mRNA sequence of von Hippel-Lindau (VHL) tumor suppressor. Total RNA of A431 cells was extracted using the RNeasy kit (Qiagen). The cDNA was prepared by Prime Script reverse transcriptase (Takara Bio. Inc., Tokyo, Japan) using the total RNA, and the cDNA of VHL, containing the open reading flame (214-855), was amplified by PCR using the cDNA that had been obtained as the template. The primer for VHL used was as follows: A: GAAGACTACGGAGGTCG ACT; B: GGAACCAGTCCTGTATCTAG. After separation of the PCR reaction mixture by agarose gel electrophoresis, the PCR product was purified by extraction from the agarose gel using Wizard SV Gel and the PCR Clean-Up System (Promega KK., Tokyo, Japan). The sequence of the PCR product was analyzed using the Big Dye Terminator v3.1

Table I. Primers used.

Gene symbols/Gene names	bp	5'	3'
CDH1/E-cadherin	567	ggttcaagctgctgaccttc	agccagttggcagtgtctct
ITGA5/integrin α5	405	cagcctcagaaggaggagga	ctcccgctgcaagaaagtct
VIM/vimentin	170	gagaactttgccgttgaagc	tccagcagcttcctgtaggt
PAI1/plasminogen activator inhibitor 1	192	ctctctctgccctcaccaac	gtggagaggctcttggtctg
CIP1/Waf1, p21	320	gattcgtaggctaaccctg	ctgggatgtccttttddaa
GLUT1/glucose transporter 1	230	cttcactgtcgtgtcgctgt	tgaagagttcagccacgatg
HK2/hexokinase 2	358	ccacctttgtgaggtccact	tctctgccttccactccact
MCT1/monocarboxylate transporter 1	163	tccagctctgacatgattg	gcccccaagaattagaaagc
MCT4/monocarboxylate transporter 4	164	gcacccacaagttctccagt	caaaatcagggaggaggtga
PDK1/pyruvate dehydrogenase kinase 1	243	cacgctgggtaatgaggatt	actgcatctgtcccgtaacc
FURIN/furin	463	tgtggtgtaggtgtggccta	tgtgagactccgtgcacttc
TGFA/transforming growth factor α	168	tgtgtctgccattctgggta	gacctggcagcagtgtatca
LOX/lysyl oxidase	223	cagaggagagtggctgaagg	ccaggtagctggggtttaca
LOXL2/lysyl oxidase-like 2	470	cccgagtacttccagcaacc	gctcgaatccgaatgtcctc
PGK1/phosphoglycerate kinase 1	226	catcggtctgggaaccttcg	tgacttgggcctgactttga
GAPDH/glyceraldehyde-3-phosphate dehydrogenase	326	catcaccatcttccaggagc	tgatggcatggactgtggtc
CA9/carbonic anhydrase IX	250	tctcatctgcacaaggaacg	taagcagctccacaccctct
PLAUR/plasminogen activator, urokinase receptor	386	ggaaggtgtcgttgttgtgg	gctggagctggtggagaaaa
MMP2/MMP2	369	gtgcgaccacagccaactac	tcacagtccgccaaatgaac
VEGFA/vascular endothelial growth factor A	442	cgaaaccatgaactttctgc	cggcttgtcacatttttctt
HIF1A/hypoxia-inducible factor 1α	424	ccgtgtgtgtgtgtgtgt	ccaccagttgccctgactat
FIH/factor of inhibitor HIF	245	gtgggcaggaagattgtcat	gggtgatgaacagggtatgg
PHD2/prolyl-4-hydroxylase 2	381	gaaagccatggttgcttgtt	ttaccgaccgaatctgaagg
FKBP8/FK506-binding protein 8	230	ggctgttgaggaagaagacg	cttggagtcagcagtgacca
HSP90/heat shock protein 90	191	atgaaactgcgctcctgtct	ttcttccatgcgtgatgtgt
HSP70/heat shock protein 70	352	gaaaatgaggagccaatgga	ccactgcgttcttagcatca

Cycle Sequencing kit (Applied Biosystems, Tokyo, Japan). The sequencing primer used was the PCR primer mentioned above.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Whole cell lysates from either 2D- or 3D cultured cells were prepared using lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 0.1% SDS, 1 mM EDTA, 1% Triton X-100, 1% deoxycholate, 1 mM Na_3VO_4, 1 mM NaF and protease inhibitor cocktail (Pierce, Rockford, IL), as reported previously (20,38). After SDS-PAGE and blotting, the filters were blocked and then incubated with the corresponding primary antibodies followed by alkaline phosphatase-labeled anti-mouse IgG or anti-rabbit IgG and the signals were developed with an alkaline phosphatase substrate (Pierce). Horseradish peroxidase-labeled second antibodies (Pierce) were also used for ECL detection (Immunostar, Wako Chemicals, Tokyo, Japan). ECL signals were detected by a cooled CCD camera system (ATTO light-capture II type AE-6981, ATTO Co., Tokyo, Japan). The primary antibodies used were murine monoclonal antibodies against human hypoxia inducible factor 1α (HIF1α), vimentin (VIM), E-cadherin (CDH1), von Hippel-Lindau (VHL), heat shock protein (HSP)70, HSP90, integrin α5 (ITGA5), plasminogen activator inhibitor 1 (PAI1), $p21^{Cip1/WAF1}$ (Cip1), (BD Biosciences, Tokyo, Japan), β actin (Sigma-Aldrich Japan), rabbit monoclonal antibodies against factor of inhibitor HIF (FIH), pyruvate dehydrogenase kinase 2 (PDK2) (Epitomics Inc., Burlingame, CA), rabbit polyclonal antibodies against prolyl-4-hydroxylase 2 (PHD2), carbonic anhydrase 9 (CA9), FK506-binding protein 8 (38) (FKBP8), glucose transporter isoform 1 (GLUT1) , PDK1 (Abcam, Tokyo, Japan), monocarboxylate transporter isoform 1 (MCT1) (Abgent, San Diego, CA), MCT4 (Sigma-Aldrich Japan), and hexokinase 2 (HK2) (Santa Cruz Biotechnol Inc., Santa Cruz, CA). Digital images of electrophoretic bands were captured by Adobe Photoshop CS and were performed using the NIH Image J, 1.36b software. All data were expressed as the mean ± SD of at least triplicate measurements. The significance of the effect of each experiment ($p<0.05$) was determined by the Student's t-test analysis using StatView software.

In vivo tumor models of A431 cells transplanted to nude mice. Xenotransplantation was carried out as described in a previous report (20,38). In brief, four weeks after transplantation of 431 tumor cells ($5x10^6$) to nude mice (5-weeks-old, male), both total RNA and protein were isolated from transplanted tumors

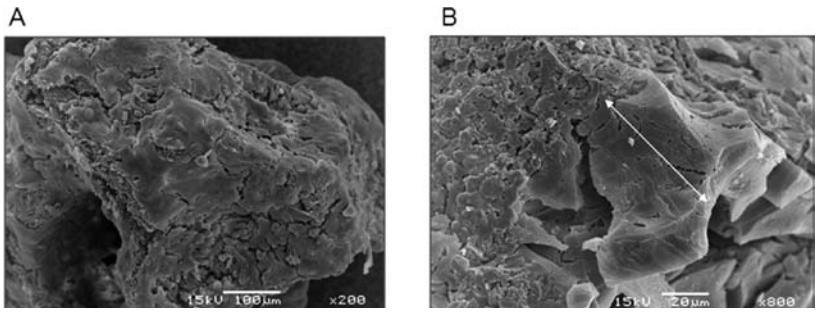

Figure 2. Fine structural observation of A431 cells cultured in a radial flow bioreactor under an SEM. (A) A431 cells cover the surface of the hydroxyapatite beads. Bar, 100 μm. (B) The cells have formed thick layers (white arrow) on the surface of the hydroxyapatite beads within the pores. Bar, 20 μm.

as described in the above sections. All animal studies were performed in conformity with the Guidelines on Animal Experimentation of Jikei University containing ethical instructions on animal experimentation.

Fine morphology. For the scanning electron microscopy, cultured cells obtained from 2D or 3D conditions were fixed with 1.2% glutaraldehyde in 0.1 M Na-phosphate buffer, pH 7.4 and postfixed with 1% OsO_4 in the same buffer (39). The fixed cells were rinsed, and then dehydrated in ascending concentrations of ethanol. After critical point drying with carbon dioxide, the specimens were coated by vacuum-evaporated carbon and ion-spattered gold, and were observed by the JSM-35 (JEOL, Tokyo, Japan) at an accelerated voltage of 10 kV.

Results and Discussion

Fine structural morphology. A431 cells grew well and were distributed tightly on the surface of the hydroxyl apatite beads (Fig. 2A). The cell surface had short, but well-defined microvillous projections as shown in the normal squamous epithelium. In the cross-sectional view, the flattened cells were irregular in shape and formed thick, approximately five to six layers. It is interesting that these cells grew and overlapped at a high density in the pores of the hydroxyl apatite matrices, indicating that a 3D culture in the RFB had been well-established (Fig. 2B).

Gene expression profiles of A431 cells cultured in 2D and 3D under RFB conditions. In the DNA chip used, over 25,000 genes were found to be expressed in A431 cells. Cells cultured in the RFB displayed significant modulation in the expression of a number of genes, as compared with those cultured in 2D counterparts.

As demonstrated by a previous report on the autocrined TGFβ enforced epithelial-mesenchymal transition (20), to construct and maintain the specific architecture under the 3D culture environment, the tumor cells showed significantly upregulated expression of genes encoding a number of proteases related materials, such as matrix metalloproteinase 9 (MMP9, 177.7-fold), PAI1 (34.8-fold), protease inhibitor 3 (SKALP, 17-fold), matrix metalloproteinase 2 (MMP2, 3.5-fold), urinary-type plasminogen activator (uPA, 2.7-fold) and its receptor (uPAR, 2.2-fold), as compared with the cells cultured in a 2D culture environment. Additionally, the expression of a large number of genes encoding extracellular matrix proteins, such as laminin α3, β3 and γ2 (9.6-, 9.2- and 4.3-fold, respectively), fibronectin (FN1, 5.4-fold), collagen type IV α1, V α1 and XVII α1 (2.6-, 7.5-and 5.9-fold, respectively) also was up-regulated in cells cultured in the RFB, as compared to those cultured under 2D culture conditions. Furthermore, to maintain the organoid structure in the RFB, various intracellular intermediate filaments appear to be important to protect the cells against physical and shear stress (18,19,40,41). Under such conditions, increased gene expression of small protein rich protein 1B (SPRR1B), which is known as a material maintaining the strength and flexibility of stratified squamous epithelia (42), keratin 16, keratin 1 and keratin 6A (68.2-, 10.6-, 7.1- and 5.8-fold, respectively) were found in the RFB, as compared to cells cultured in a 2D environment. Some RT-PCR and Western blot analyses of the representative molecules also demonstrated the existence of the 3D architecture (Fig. 3A) as reported previously (20).

It is also important to determine whether 3D culture with the RFB can not only reproduce cellular structures similar to those in living organisms, but also closely mimic the living microenvironment in which cancer pathogenesis occurs, namely a hypoxic environment.

The tumor cell's regulatory mechanisms that adapt it to the hypoxic environment have been reported by many investigators (11,21,24,27,30-35,43). In our microarray study, in order to overcome the hypoxic environment in 3D culture, the cells expressed genes encoding angiogenesis such as Jagged 1 (4.4-fold), as well as the tumor necrosis factor receptor superfamily 12A (3.9-fold), angiopoietin-like 4 (ANGPTL4, 66.4-fold) and vascular endothelial growth factor (VEGF, 3.6-fold), all of which were noted to be obviously upregulated in cells cultured under 3D in comparison with those in a 2D environment. Additionally, as shown in Fig. 3B, fluctuating expression of factors related to energy metabolism was noted in the transition to a hypoxic environment. In 3D cultured cells, there was an increased level of expression of GLUT1, HK2 which catalyzes the initial step of glycolysis, and MCT1 that acts in co-transportation of H^+ with monocarboxylate anions such as lactate. In contrast, the level of MCT4, another monocarbxylate transporter, was found to have decreased. HIF1 is known to stimulate the conversion of glucose to pyruvate and lactate by upregulating GLUT1, HK1 and HK2, and lactate dehydrogenase A, as well as the lactate-extruding MCT4 (44,45). In addition, HIF1 decreases the conversion of pyruvate to acetyl-CoA by pyruvate dehydrogenase (PDH) via

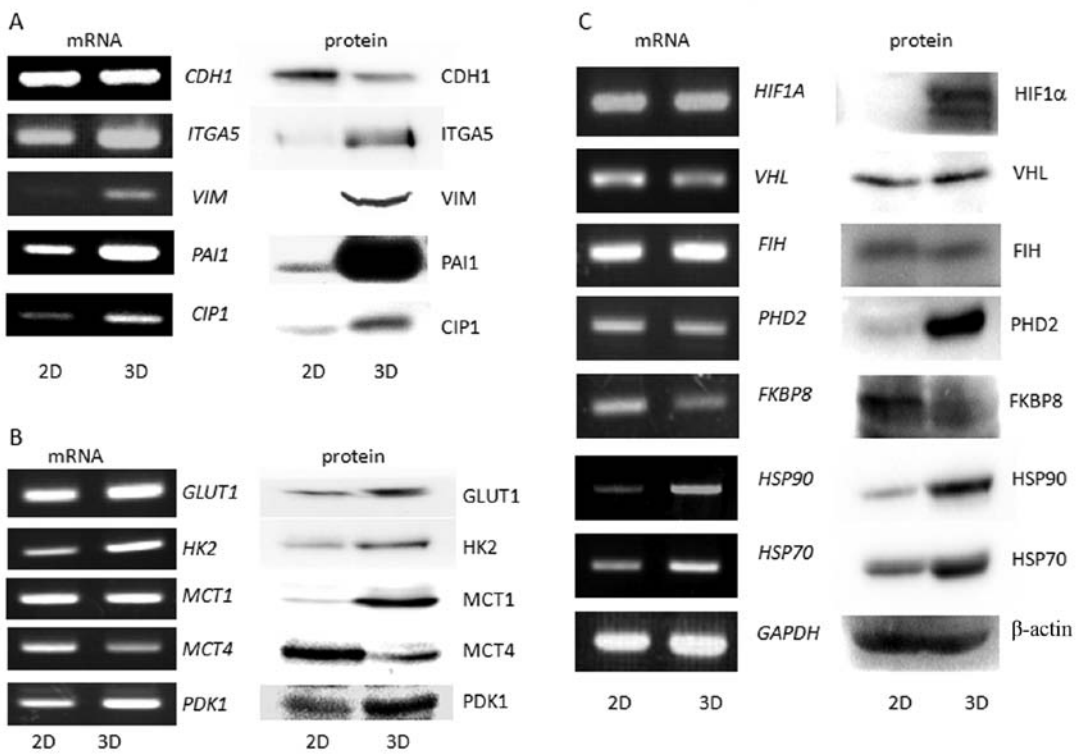

Figure 3. Representative protein expressions in cells cultured with 2D, monolayer, or 3D, RFB, condition. (A) Typical fluctuating expression of representative proteins in cells cultured with RFB. (B) Expression of pivotal proteins relating to energy metabolism in cancer cells. (C) HIF1α stabilization and profiles of several related proteins.

HIF1 transactivation of the gene encoding PDH kinase 1, 2 (PDK1, 2) (46,47). In general, tumor tissues consist of aerobic, well-oxygenated and anaerobic or hypoxic, poorly oxygenated regions, which have been thought to utilize glucose for oxidative and glycolytic metabolism, respectively. There have been several significant descriptions indicating that under hypoxic conditions, the cultured tumor cells convert glucose to lactate and extrude it mainly via MCT4, whereas aerobic tumor cells take up lactate via MCT1 and utilize it for oxidative phosphorylation, thus demonstrating the existence of this give and take of lactate between tumor cells as a 'metabolic symbiosis' between hypoxic and aerobic cancer cells (10,11,35,43,48). In tumor tissue, lactate produced by hypoxic cells is taken up by aerobic cells and they use it as their principal substrate for oxidative phosphorylation. As a result, the limited glucose available to the tumor is used most efficiently to maintain redox homeostasis and to maintain energy homeostasis (46,49,50,51). Reports have also emphasized that a key player in this symbiotic relationship is MCT1, which differs from MCT4 in two respects: the expression of MCT1 is hypoxia-repressed rather than hypoxia-induced, and it transports lactate into, rather than out of, cancer cells (11,33,43,48,52,53). These previous results cited above and our present result suggest that the A431 tumor cell line used in the present study might have been established from cells isolated at operation from the part of the tumor tissues growing under a slight hypoxic environment rather than under aerobic conditions.

Expression of hypoxia inducible factor 1 and transcriptional activation of its downstream genes. An investigation was conducted into whether hypoxia-related inducible genes were upregulated if the hypoxic condition is actually induced and exists in the RFB under 3D culture conditions. Both microarray and RT-PCR analyses showed that many hypoxia inducible genes were upregulated under 3D culture conditions in comparison with 2D culture (Fig. 4 and Table II). These results suggested that the hypoxic condition can be acquired

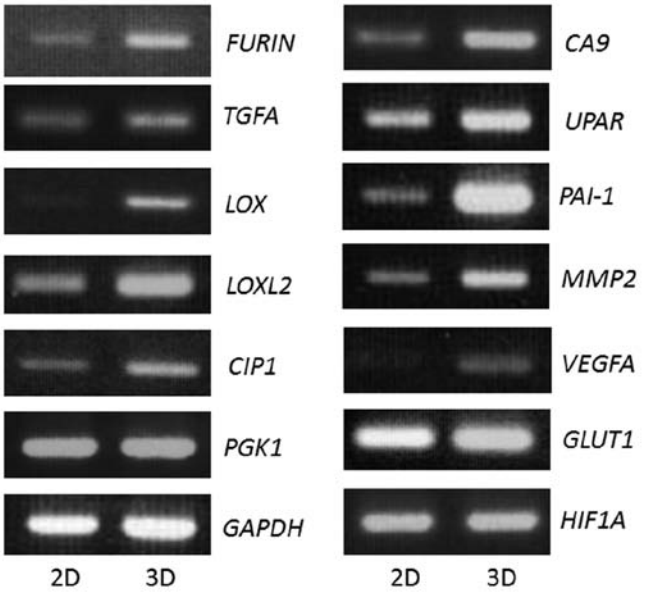

Figure 4. RT-PCR shows hypoxia inducible genes up-regulated under 3D culture conditions in comparison with 2D culture.

Table II. Upregulated HIF1 downstream genes.

Probe set ID	Gene name	Gene symbol	Fold expression
223333_s_at	Angiopoietin-like 4	ARP4	66.43
202628_s_at	Plasminogen activator inhibitor 1	PAI1	34.77
201426_s_at	Vimentin	VIM	18.74
204508_s_at	Carbonic anhydrase 12	CA12	14.4
211003_x_at	Transglutaminase 2	TG2	9.151
215446_s_at	Human lysyl oxidase	LOX	8.795
203325_s_at	Collagen type V, α1	COL5A1	7.543
204200_s_at	Platelet-derived growth factor β	PDGFB	6.447
205199_at	Carbonic anhydrase 9	CA9	6.222
209652_s_at	Placental growth factor	PGF	5.979
212464_s_at	Fibronectin 1	FN	5.432
203939_at	Ecto-5'-nucleotidase	CD73	5.062
201249_at	Glucose transporter 1	GLUT1	4.460
217279_x_at	Matrix metallopeptidase 14	MMP14	4.446
201945_at	Furin	FRN	4.091
202284_s_at	Cyclin-dependent kinase inhibitor 1A	WAF1	3.747
211527_x_at	Vascular endothelial growth factor	VEGF	3.615
201069_at	Matrix metalloproteinase 2	MMP2	3.548
204995_at	Cyclin-dependent kinase 5, regulatory subunit 1	P35	3.537
202998_s_at	Lysyl oxidase-like 2	LOXL2	3.178
202934_at	Hexokinase 2	HK2	2.979
226452_at	Pyruvate dehydrogenase kinase 1	PK1	2.815
205016_at	Transforming growth factor-α	TGFA	2.633

easily and naturally in RFB culture without any specific treatment to induce the hypoxic condition.

All organisms possess mechanisms to maintain oxygen homeostasis for survival. The HIF1 is central to adaptation to low oxygen availability. HIF1 in turn, regulates the transcription of many genes involved in cellular and systemic responses to hypoxia, including breathing, vasodilation, anaerobic metabolism, erythropoiesis and angiogenesis as described in Materials and methods. TGFβ is the essential cytokine that stimulates HIF1α protein expression (1-3) and our previous report (20) described the finding that increased secretion and the consequent autocrine loop of TGFbeta was established more easily in RFB culture. In the present study, a discrepancy in HIF1α expression between the mRNA and protein levels was recognized at the initial array determination. While tumor cells from 2D and 3D culture did not show any difference in the level of mRNA expression of HIF1α, of particular interest was the observation that upregulated HIF1α protein was clearly detectable in cells from RFB culture, but was not detected in the 2D sample (Fig. 3C). Tumor cells derived from tissue xenotransplanted into nude mice yielded similar results to those from RFB culture (data not shown). Comparison of VHL, PHD2 and FIH, which are mainly related to degradation and functional stabilization of HIF1α in the cells, did not reveal any difference in the protein expression levels of VHL and FIH between 2D and 3D culture conditions, but the PHD2 was extremely elevated in the 3D cultured cells (Fig. 3C). Since the VHL sequence of A431 cells corresponded perfectly to that of VHL transcript variant 2 (NM_198156), the A431 cells used in the present experiment did not manifest any genetic mutations in the DNA sequence of the VHL gene, showing a normal function. These findings suggest that, assuming VHL exhibits complete functionality, HIF1 is able to induce PHD2-expression in a VHL-dependent manner in 3D culture under hypoxic conditions.

As PHD2 is the main member of the PHD enzyme family that is involved in oxygen-sensing prolyl hydroxylation of the oxygen-dependent degradation domain in the HIF1 molecule (54,55), elevation and activation of PHD2 may have the compensatory effect of maintaining the HIF1 molecule as a mechanism leading to the hypoxic condition. In spite of equal levels of expression of mRNA between 2D and RFB cultured cells, there was a difference in the levels of PHD2 protein. In the present study, an increased level of expression of the PHD2 protein and stabilization might have been induced by HIF1 stabilization under 3D hypoxic culture conditions in the RFB and the HIF1 might have further enhanced not only PHD2 expression, but also the expression of other HIF downstream genes, such as CA9. Many previous reports demonstrated that the transcript level of PHD2 was regulated by HIF, and its induction under hypoxic conditions led to attenuated HIF1α levels (53,56-59). Under such a hypoxic

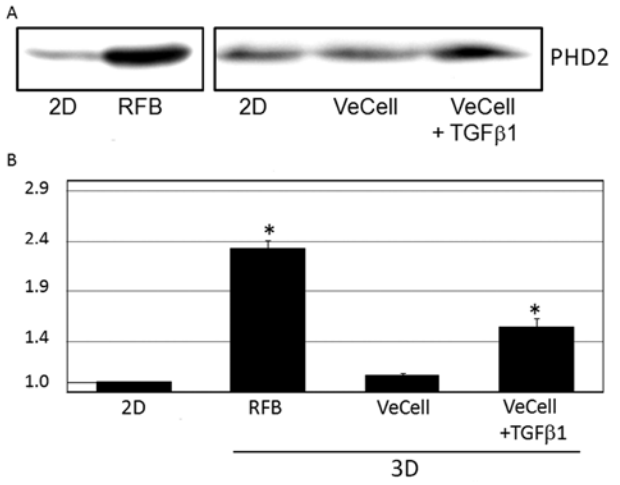

Figure 5. PHD2, main HIF-prolyl hydroxylation enzyme upregulation in cells cultured in RFB with/without TGFβ1 stimulation. Densitometric analysis was conducted according to the method described in Materials and methods. *p<0.05, significant difference from 2D.

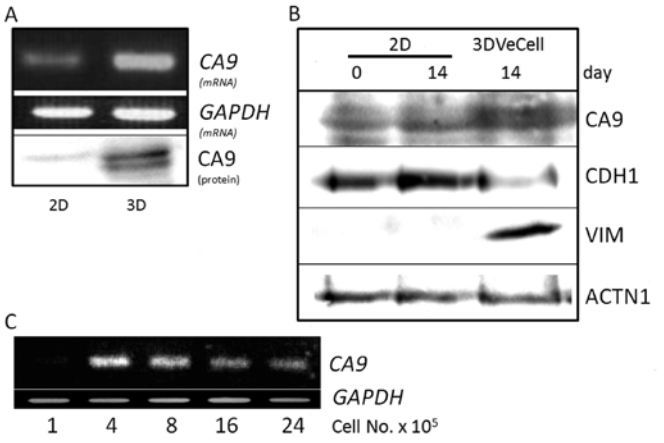

Figure 6. CA9 upregulation independent of cell density, in cells cultured under 3D conditions. (A) CA9 expression in 2D and RFB culture conditions. (B) Increase in CA9 protein expressions during under construction of 3D architecture using VECELL device, with subconfluent (50%) cell density. (C) Cell density is independent of CA9 mRNA expression in 2D. Cells (1-24x10^5) were plated in culture dishes (6 cm diameter) and after culture the extracted mRNAs were determined. For detail, see Materials and methods.

environment, the elevation of intracellular PHD-derived hydroxylation enzyme activity was also demonstrated (54,55). However, our present study showed that the PHD2 protein level elevated concomitantly with the increased level of HIF1α stabilization. Additionally, it is unlikely that HSP70/HSP90 axis regulation for the VHL-independent HIF1α degradation pathway was disrupted (61,62), because of the several-fold increase in the expression of both HSP proteins (Fig. 3C). As compared with 2D culture, the PHD2 mRNA level showed neither a significant increase nor decrease under 3D conditions, suggesting that PHD2 protein stability might have been prolonged via regulatory interaction with FK506-binding protein 8 (FKBP8) or some other unknown factor(s). In the 3D cultured A431 cells in our experiment, the mRNA and protein levels of FKBP8 decreased as compared with those in 2D cells (Fig. 3C). We predicted that the induced, stabilized and increased level of PHD2 may compensate for the lack of oxygen in the newly constructed architecture under hypoxic 3D conditions. Bath and co-workers described their findings that PHD2 protein stability under hypoxia is regulated by interaction with FKBP8 via a ubiquitin-independent proteasomal pathway (54,55). Our result supports this phenomenon. Moreover, in our present RFB culture experiment, PHD2 levels may have been regulated rapidly by the elevated proteasomal enzyme activity (an approximately 3-fold increase as previously reported (20) in the RFB under 3D conditions via the FKBP8 regulatory mechanism (54,55). It is of interest that the PHD2 protein levels in cells cultured with VECELL device (another 3D-culture device) and with the same device in the presence of TGFβ1 (0.5 ng/ml) were elevated approximately 1.07 and 1.54 times, respectively, as compared to those grown in 2D culture (Fig. 5A). This result indicated that TGFβ1 stimulated the PHD2 expression even under spheroid, conventional 3D-culture condition. Furthermore, the level of PHD2 protein was elevated up to 2.33-fold in RFB cultured cells (Fig. 5B). Indeed our present experiment at 14 days of culture found that the TGFβ1 levels of the culture media from A431 cells in RFB reached a significant 2-fold level above (556±124 pg/ml media) that at the initiation of culture (264±97 pg/ml media. By contrast, the cells cultured under 2D conditions, did not detect the increased accumulation of TGFβ1 level during the same period of time (approximately 227±41 pg/ml media). The A431 cells secreted TGFβ1 extensively into the surrounding culture medium during culture in the RFB under 3D conditions, in the same way as a former experiment result (20). TGFβ1 was found to facilitate an increased level of PHD2, just as BMP2 (a member of the TGFβ superfamily) mediated PHD2 stabilizing action via FKBP8 regulatory pathway, which was reported previously (60). These results suggested that tumor cells overcame the hypoxic condition not only by enhancing the expression of HIF1α, but also by maintaining the level of protein via every possible metabolic and regulatory means related to HIF1 protein stabilization and degradation pathways, while TGFβ stimulation pathway to FKBP8 regulation is not known. Further study is necessary to clear this matter.

Increased expression of CA9 in cultured cells under 3D conditions was caused by HIF1α stabilization, independent of cell density. In the microarray analysis, mRNA expression of CA9 (an isoenzyme of the carbonic anhydrase family) was remarkably upregulated (6.2-fold) under RFB culture conditions. RT-PCR and Western blot analyses also confirmed the upregulation of CA9 expression in RFB as compared to 2D culture (Fig. 6A). Among many downstream expression genes of HIF1, the genes that lead to an efficient metabolic response from normoxia to hypoxia constitute one of the essential gene groups enabling cells to adapt and survive under a stressful hypoxic microenvironment. Under such extracellular conditions, HIF1 induces the expression of proteins that are glucose transporters, glycolytic enzymes, proton and lactate efflux pumps, to maintain the energy supply and resultant pH balance in both the intra- and extracellular milieu due to the hypoxic, anaerobic glycolysis (30,33). Moreover, aerobic glycolysis causes

enhanced acidification of the extracellular milieu due to the action of multi proton-extruding molecules, because of the mixed mosaic population of the tumor cells. CA9, similar to CA12, is induced by transcriptional activation of HIF1 and thereby generates membrane-impermeable H^+ and HCO_3^- (63,34). CA9 is known as a main functional molecule involved in rearranging the construction of the tumor-induced micro-environment (63,64). It is also well known that CA9 is induced under hypoxic conditions in cultured cells and that the expression of CA9 in hypoxic regions of human tumors suggests the potentially important role of CA9 in tumor adaptation to hypoxic conditions (67,68). As described in many previous reports, the expression of CA9 has been identified in a large number of human tumors but not in the corresponding normal tissue (67-72). Moreover, many investigators have reported a high CA9 correlation with a poor prognosis in patients with various tissue-derived carcinomas (69,73-77), because hypoxia and acidosis lead to genomic instability, which potentiates further tumor progression. In contrast to CA9 protein expression under hypoxia in the living body, *in vitro* regulation of the protein expression is reported to be positively related to dense culture (78,79). In order to determine whether or not CA9 molecular induction in 3D cultured cells was dependent on the increasing cell density, the effects of cell density on CA9 protein expression levels were analyzed using a 2D, monolayer culture dish, the RFB and other culture devices to provide spheroid culture conditions, since 3D culture in the RFB has been known to culture cells at a high density while mimicking the *in vivo* architecture (37,80). Results showed that the increased protein levels of CA9 at various degrees under 3D culture conditions in all devices yielded cell densities at lower than 50% confluence and showed non-significant cell density-dependent induction of CA9 mRNA expression during the period of the experiment in the 2D cultured cells (Fig. 6B and C).

Our present results indicated that HIF1α-induced elevations of the CA9 mRNA and protein expression in the cells cultured under RFB culture conditions occurred because of the stabilization of HIF1 activation under the hypoxic milieu and not due to the increase in cell density. This in turn strongly suggests that the hypoxic micro-environment surrounding the cultured cells must be acquired easily in the RFB culture enabling the growing tumor cells to manifest their characteristic property and ability similar to that *in vivo* without any treatment to create the hypoxic condition. Since the hypoxia and acidosis create a nurturing environment for tumor progression and the evolution of metastatic, drug-resistant cells, the ability to target the hypoxic compartment should allow the overall survival of patients bearing solid tumor malignancies, hence the RFB system is a powerful tool for examining and exploring these targets.

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References

1. Nguyen DX and Massagué J: Genetic determinants of cancer metastasis. Nat Rev Genet 8: 341-352, 2007.
2. Huber MA, Kraut N and Beug H: Molecular requirements for epithelial-mesenchymal transition during tumor progression. Curr Opin Cell Biol 17: 548-558, 2005.
3. Tarin D, Thompson EW and Newgreen DF: The fallacy of epithelial mesenchymal transition in neoplasia. Cancer Res 65: 5996-6000, 2005.
4. Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H, Swartz M, Fukumura D, Jain RK and Alitalo K: Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science 276: 1423-1425, 1997.
5. Sutherland RM: Cell and environment interactions in tumor microregions: the multicell spheroid model. Science 40: 177-184, 1988.
6. Brown JM and Giaccia AJ: The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. Cancer Res 58: 1408-1416, 1998.
7. Tomida A and Tsuruo T: Drug resistance mediated by cellular stress response to the microenvironment of solid tumors. Anticancer Drug Des 14: 169-177, 1999.
8. Gupta GP and Massagué J: Cancer metastasis: building a framework. Cell 17: 679-695, 2006.
9. Gogvadze V, Orrenius S and Zhivotovsky B: Mitochondria in cancer cells: what is so special about them? Trends Cell Biol 18: 165-173, 2008.
10. Kroemer G and Pouyssegur J: Tumor cell metabolism: cancer's Achilles' heel. Cancer Cell 13: 472-482, 2008.
11. Semenza GL: Tumor metabolism: cancer cells give and take lactate. J Clin Invest 118: 3835-3837, 2008.
12. Margulis A, Zhang W, Alt-Holland A, Crawford HC, Fusenig NE and Garlick JA: E-cadherin suppression accelerates squamous cell carcinoma progression in th170 ree-dimensional, human tissue constructs. Cancer Res 65: 1783-1791, 2005.
13. Debnath J and Brugge JS: Modelling glandular epithelial cancers in three-dimensional cultures. Nat Rev Cancer 5: 675-688, 2005.
14. Friedrich MJ: Studying cancer in 3 dimensions. 3-D models foster new insights into tumorigenesis. JAMA 18: 1977-1979, 2007.
15. Muthuswamy SK, Li D, Lelievre S, Bissell MJ and Brugge JS: ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. Nat Cell Biol 3: 785-792, 2001.
16. Ghosh S, Spagnoli GC, Martin I, Ploegert S, Demougin P, Heberer M and Reschner A: Three-dimensional culture of melanoma cells profoundly affects gene expression profile: a high density oligonucleotide array study. J Cell Physiol 204: 522-531, 2005.
17. Hongo T, Kajikawa M, Ishida S, Ozawa S, Ohno Y, Sawada J, Ishikawa Y and Honda H: Gene expression property of high-density three-dimensional tissue of HepG2 cells formed in radial-flow bioreactor. J Biosci Bioeng 101: 243-250, 2006.
18. Debnath J, Mills KR, Collins NL, Reginato MJ, Muthuswamy SK and Brugge JS: The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. Cell 111: 29-40, 2002.
19. Weaver VM, Lelièvre S, Lakins JN, Chrenek MA, Jones JC, Giancotti F, Werb Z and Bissell MJ: Beta4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. Cancer Cell 2: 205-216, 2002.
20. Shibata S, Marushima H, Asakura T, Matsuura T, Eda H, Aoki K, Matsudaira H, Ueda K and Ohkawa K: Three-dimensional culture using a radial flow bioreactor induces matrix metalloprotease 7-mediated EMT-like process in tumor cells via TGFbeta1/Smad pathway. Int J Oncol 34: 1433-1448, 2009.
21. Choi KS, Bae MK, Jeong JW, Moon HE and Kim KW: Hypoxia-induced angiogenesis during carcinogenesis. J Biochem Mol Biol 36: 120-127, 2003.
22. Ke Q and Costa M: Hypoxia-inducible factor-1 (HIF-1). Mol Pharmacol 70: 1469-1480, 2006.
23. De Clerck K and Elble RC: The role of hypoxia and acidosis in promoting metastasis and resistance to chemotherapy. Front Biosci 15: 213-225, 2010.
24. Mizukami Y, Kohgo Y and Chung DC: Hypoxia inducible factor-1 independent pathways in tumor angiogenesis. Clin Cancer Res 13: 5670-5674, 2007.

25. Calzada MJ and del Peso L: Hypoxia-inducible factors and cancer. Clin Transl Oncol 9: 278-289, 2007.
26. Sahlgren C, Gustafsson MV, Jin S, Poellinger L and Lendahl U: Notch signaling mediates hypoxia-induced tumor cell migration and invasion. Proc Natl Acad Sci USA 105: 6392-6397, 2008.
27. Liao D and Johnson RS: Hypoxia: a key regulator of angiogenesis in cancer. Cancer Metastasis Rev 26: 281-290, 2007.
28. Chaudary N and Hill RP: Hypoxia and metastasis in breast cancer. Breast Dis 26: 55-64, 2007.
29. Bristow RG and Hill RP: Hypoxia and metabolism. Hypoxia, DNA repair and genetic instability. Nat Rev Cancer 8: 180-192, 2008.
30. Semenza GL: Hypoxia-inducible factor 1: regulator of mitochondrial metabolism and mediator of ischemic preconditioning. Biochim Biophys Acta doi:10.1016/j.bbamcr.2010.08.006, 2010.
31. Semenza GL: Targeting HIF-1 for cancer therapy. Nat Rev Cancer 3: 721-732, 2003.
32. Semenza GL: Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene 29: 625-634, 2010.
33. Semenza GL: HIF-1: upstream and downstream of cancer metabolism. Curr Opin Genet Dev 20: 51-56, 2010.
34. Semenza GL: Regulation of oxygen homeostasis by hypoxia-inducible factor 1. Physiology 24: 97-106, 2009.
35. Semenza GL: Regulation of cancer cell metabolism by hypoxia-inducible factor 1. Semin Cancer Biol 19: 12-16, 2009.
36. Aizaki H, Nagamori S, Matsuda M, Kawakami H, Hashimoto O, Ishiko H, Kawada M, Matsuura T, Hasumura S, Matsuura Y, Suzuki T and Miyamura T: Production and release of infectious hepatitis C virus from human liver cell cultures in the three-dimensional radial-flow bioreactor. Virology 15: 16-25, 2003.
37. Kanai H, Marushima H, Kimura N, Iwaki T, Saito M, Maehashi H, Shimizu K, Muto M, Masaki T, Ohkawa K, Yokoyama K, Nakayama M, Harada T, Hano H, Hataba Y, Fukuda T, Nakamura M, Totsuka N, Ishikawa S, Unemura Y, Ishii Y, Yanaga K and Matsuura T: Extracorporeal bioartificial liver using the radial-flow bioreactor in treatment of fatal experimental hepatic encephalopat. Artif Organs 31: 148-151, 2007.
38. Ohkawa K, Hatano T, Yamada K, Joh K, Takada K, Tsukada Y and Matsuda M: Bovine serum albumin-doxorubicin conjugate overcomes multidrug resistance in a rat hepatoma. Cancer Res 53: 4238-4242, 1993.
39. Ohkawa K, Amasaki H, Terashima Y, Aizawa S and Ishikawa E: Clear cell carcinoma of the ovary, light and electron microscopic studies. Cancer 40: 3019-3029, 1977.
40. Gaedtke L, Thoenes L, Culmsee C, Mayer B and Wagner E: Proteomic analysis reveals differences in protein expression in spheroid versus monolayer cultures of low-passage colon carcinoma cells. J Proteome Res 6: 4111-4118, 2007.
41. Morales J and Alpaugh ML: Gain in cellular organization of inflammatory breast cancer: a 3D *in vitro* model that mimics the *in vivo* metastasis. BMC Cancer 9: 462, 2009.
42. Candi E, Tarcsa E, Idler WW, Kartasova T, Marekov LN and Steinert PM: Transglutaminase cross-linking properties of the small proline-rich 1 family of cornified cell envelope proteins. integration with loricrin. J Biol Chem 274: 7226-7237, 1999.
43. Feron O: Pyruvate into lactate and back: from the Warburg effect to symbiotic energy fuel exchange in cancer cells. Radiother Oncol 92: 329-333, 2009.
44. Pouysségur J, Dayan F and Mazure NM: Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature 441: 437-443, 2006.
45. Semenza GL: Hypoxia-inducible factor 1 (HIF-1) pathway. Sci STKE 2007: cm8, 2007.
46. Kim JW, Tchernyshyov I, Semenza GL and Dang CV: HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab 3: 177-185, 2006.
47. Papandreou RA, Cairns L, Fontana AL, Lim and Denko NC: HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab 3: 187-197, 2006.
48. Sonveaux P, Végran F, Schroeder T, Wergin MC, Verrax J, Rabbani ZN, De Saedeleer CJ, Kennedy KM, Diepart C, Jordan BF, Kelley MJ, Gallez B, Wahl ML, Feron O and Dewhirst MW: Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. J Clin Invest 118: 3930-3942, 2008.
49. Zhang H, Bosch-Marce M, Shimoda LA, Tan YS, Baek JH, Wesley JB, Gonzalez FJ and Semenza GL: Mitochondrial autophagy is a HIF-1-dependent adaptive metabolic response to hypoxia. J Biol Chem 283: 10892-10903, 2008.
50. Ullah MS, Davies AJ and Halestrap AP: The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. J Biol Chem 281: 9030-9037, 2006.
51. Seagroves TN, Ryan HE, Lu H, Wouters BG, Knapp M, Thibault P, Laderoute K and Johnson RS: Transcription factor HIF-1 is a necessary mediator of the pasteur effect in mammalian cells. Mol Cell Biol 21: 3436-3444, 2001.
52. Marxsen JH, Stengel P, Doege K, Heikkinen P, Jokilehto T, Wagner T, Jelkmann W, Jaakkola P and Metzen E: Hypoxia-inducible factor-1 (HIF-1) promotes its degradation by induction of HIF-alpha-prolyl-4-hydroxylases. Biochem J 381: 761-767, 2004.
53. Berra E, Benizri E, Ginouvès A, Volmat V, Roux D and Pouysségur J: HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. EMBO J 22: 4082-4090, 2003.
54. Barth S, Nesper J, Hasgall PA, Wirthner R, Nytko KJ, Edlich F, Katschinski DM, Stiehl DP, Wenger RH and Camenisch G: The peptidyl prolyl cis/trans isomerase FKBP38 determines hypoxia-inducible transcription factor prolyl-4-hydroxylase PHD2 protein stability. Mol Cell Biol 27: 3758-3768, 2007.
55. Barth S, Edlich F, Berchner-Pfannschmidt U, Gneuss S, Jahreis G, Hasgall PA, Fandrey J, Wenger RH and Camenisch G: Hypoxia-inducible factor prolyl-4-hydroxylase PHD2 protein abundance depends on integral membrane anchoring of FKBP38. J Biol Chem 284: 23046-23058, 2009.
56. Metzen E, Stiehl DP, Doege K, Marxsen JH, Hellwig-Bürgel T and Jelkmann W: Regulation of the prolyl hydroxylase domain protein 2 (phd2/egln-1) gene: identification of a functional hypoxia-responsive element. Biochem J 387: 711-717, 2005.
57. Nakayama K, Frew IJ, Hagensen M, Skals M, Habelhah H, Bhoumik A, Kadoya T, Erdjument-Bromage H, Tempst P, Frappell PB, Bowtell DD and Ronai Z: Siah2 regulates stability of prolyl-hydroxylases, controls HIF1alpha abundance, and modulates physiological responses to hypoxia. Cell 117: 941-952, 2004.
58. Pescador N, Cuevas Y, Naranjo S, Alcaide M, Villar D, Landázuri MO and Del Peso L: Identification of a functional hypoxia-responsive element that regulates the expression of the egl nine homologue 3 (egln3/phd3) gene. Biochem J 390: 189-197, 2005.
59. Stiehl DP, Wirthner R, Köditz J, Spielmann P, Camenisch G and Wenger RH: Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels. Evidence for an auto-regulatory oxygen-sensing system. J Biol Chem 281: 23482-23491, 2006.
60. Pistollato F, Rampazzo E, Abbadi S, Della Puppa A, Scienza R, D'Avella D, Denaro L, Te Kronnie G, Panchision DM and Basso G: Molecular mechanisms of HIF-1alpha modulation induced by oxygen tension and BMP2 in glioblastoma derived cells. PLoS One 4: e6206, 2009.
61. Kong X, Lin Z, Liang D, Fath D, Sang N and Caro J: Histone deacetylase inhibitors induce VHL and ubiquitin-independent proteasomal degradation of hypoxia-inducible factor 1alpha. Mol Cell Biol 26: 2019-2028, 2006.
62. Isaacs JS, Jung YJ, Mimnaugh EG, Martinez A, Cuttitta F and Neckers LM: Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1 alpha-degradative pathway. J Biol Chem 277: 29936-29944, 2002.
63. De Simone G and Supuran CT: Carbonic anhydrase IX: biochemical and crystallographic characterization of a novel antitumor target. Biochim Biophys Acta 1804: 404-409, 2010.
64. 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.
65. Thiry A, Dogné JM, Masereel B and Supuran CT: Targeting tumor-associated carbonic anhydrase IX in cancer therapy. Trends Pharmacol Sci 27: 566-573, 2006.
66. Supuran CT: Carbonic anhydrases: catalytic and inhibition mechanisms, distribution and physiological roles. In: Carbonic Anhydrase. Its Inhibitors and Activators. Supuran CT, *et al* (eds). CRC Press, ppl-23, 2004.
67. Wykoff CC, Beasley NJP, Watson PH, Turner KJ, Pastorek J, Sibtain A, Wilson GD, Turley H, Talks KL, Maxwell PH, Pugh CW and Ratcliffe PJ: Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer Res 60: 7075-7083, 2000.

68. Pastoreková S and Pastorek J: Cancer-related carbonic anhydrase isozymes and their inhibition. In: Carbonic Anhydrase. Its Inhibitors and Activators. Supuran CT, *et al* (eds). CRC Press, pp255-281, 2004.
69. Ivanov S, Liao SY, Ivanova A, Danilkovitch-Miagkova A, Tarasova N, Weirich G, Merrill MJ, Proescholdt MA, Oldfield EH, Lee J, Zavada J, Waheed A, Sly W, Lerman MI and Stanbridge EJ: Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. Am J Pathol 158: 905-919, 2001.
70. Höckel M and Vaupel P: Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 93: 266-276, 2001.
71. Pastorekova S, Parkkila S, Pastorek J and Supuran CT: Carbonic anhydrases: current state of the art, therapeutic applications and future prospects. J Enzyme Inhib Med Chem 19: 199-229, 2004.
72. Generali D, Berruti A, Brizzi MP, Campo L, Bonardi S, Wigfield S, Bersiga A, Allevi G, Milani M, Aguggini S, Gandolfi V, Dogliotti L, Bottini A, Harris AL and Fox SB: Hypoxia-inducible factor-1alpha expression predicts a poor response to primary chemoendocrine therapy and disease-free survival in primary human breast cancer. Clin Cancer Res 12: 4562-4568, 2006.
73. De Schutter H, Landuyt W, Verbeken E, Goethals L, Hermans R and Nuyts S: The prognostic value of the hypoxia markers CA IX and GLUT 1 and the cytokines VEGF and IL 6 in head and neck squamous cell carcinoma treated by radiotherapy +/- chemotherapy. BMC Cancer 5: 42, 2005.
74. Liao SY, Darcy KM, Randall LM, Tian C, Monk BJ, Burger RA, Fruehauf JP, Peters WA, Stock RJ and Stanbridge EJ: Prognostic relevance of carbonic anhydrase-IX in high-risk, early-stage cervical cancer: a Gynecologic Oncology Group study. Gynecol Oncol 116: 452-458, 2010.
75. Woelber L, Mueller V, Eulenburg C, Schwarz J, Carney W, Jaenicke F, Milde-Langosch K and Mahner S: Serum carbonic anhydrase IX during first-line therapy of ovarian cancer. Gynecol Oncol 117: 183-188, 2010.
76. Hyrsl L, Zavada J, Zavadova Z, Kawaciuk I, Vesely S and Skapa P: Soluble form of carbonic anhydrase IX (CAIX) in transitional cell carcinoma of urinary tract. Neoplasma 56: 298-302 2009.
77. Tan EY, Yan M, Campo L, Han C, Takano E, Turley H, Candiloro I, Pezzella F, Gatter KC, Millar EK, O'Toole SA, McNeil CM, Crea P, Segara D, Sutherland RL, Harris AL and Fox SB: 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.
78. Pastorek J, Pastoreková S, Callebaut I, Mornon JP, Zelník V, Opavský R, Zat'ovicová M, Liao S, Portetelle D, Stanbridge EJ, *et al*: Cloning and characterization of MN, a human tumor-associated protein with a domain homologous to carbonic anhydrase and a putative helix-loop-helix DNA binding segment. Oncogene 9: 2877-2888, 1994.
79. 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.
80. Sussman NL, Chong MG, Koussayer T, He DE, Shang TA, Whisennand HH and Kelly JH: Reversal of fulminant hepatic failure using an extracorporeal liver assist device. Hepatology 16: 60-65, 1992.