

Constitutive Expression of Hypoxia-inducible Factor-1 α Renders Pancreatic Cancer Cells Resistant to Apoptosis Induced by Hypoxia and Nutrient Deprivation¹

Nobuaki Akakura, Masanobu Kobayashi,² Iori Horiuchi, Akiko Suzuki, Jingxin Wang,² Jian Chen, Hiroto Niizeki, Ken-ichi Kawamura, Masuo Hosokawa, and Masahiro Asaka

Division of Cancer Pathobiology, Institute for Genetic Medicine, Hokkaido University [N. A., M. K., I. H., A. S., J. W., J. C., H. N., M. H.]; Departments of Gastroenterology and Hematology [N. A., A. S., M. A.] and Surgical Oncology [H. N.], Hokkaido University Graduate School of Medicine; and Sapporo Posts and Telecommunications Hospital [K. K.], Sapporo, 060-8638 Japan

ABSTRACT

Hypovasculature is an outstanding characteristic of pancreatic cancers in imaging diagnosis, suggesting that blood supply is poor in pancreatic cancer tissues. Despite poor blood supply, pancreatic cancer cells survive and proliferate in severe hypoxia and nutrient deprivation. To demonstrate how pancreatic cancer cells adapt themselves to hypoxia and nutrient deprivation, we investigated the expression of hypoxia-inducible factor 1 α (HIF-1 α) protein and HIF-1-inducible genes in human pancreatic cancer cell lines in comparison with other cancer cell lines. We found that HIF-1 α protein was constitutively expressed in 15 of 20 pancreatic cancer cell lines (75%) but in none of other cancer cell lines tested in this study. The cells with constitutive expression of HIF-1 α were more resistant to apoptosis induced by hypoxia and glucose deprivation than those without constitutive expression of HIF-1 α . Transfection with HIF-1 α transformed the latter cells resistant to apoptosis and increased *in vivo* tumorigenicity. Furthermore, anaerobic metabolism-associated genes, *Glut1* and *aldolase A*, were more highly expressed in the cells with constitutive expression of HIF-1 α than in the cells without it. These results suggest that constitutive expression of HIF-1 α contributes to the survival and proliferation of pancreatic cancer cells in hypoxia and glucose deprivation through the activation of anaerobic metabolism.

INTRODUCTION

Pancreatic cancers, 90% of which are ductal adenocarcinomas, are highly aggressive and one of the dominant causes of cancer death. Currently, there is no effective therapy for this cancer. Surgical resection is available only to a small fraction of patients and has only a marginal effect on overall survival rates. Chemotherapy and radiotherapy have very limited effects on patients' survival. Furthermore, even in patients with curatively resected pancreatic cancers, relapses involving hepatic metastases frequently result in death within a short period. To establish new approaches to such an aggressive disease, we need to understand detailed molecular basis of the pathogenesis.

Aggressive tumors often have insufficient blood supply, partly because tumor cells grow faster than endothelial cells and partly because a newly formed vascular supply is disorganized (1–3). When the tumor cells are exposed to hypoxia, HIF-1,³ which is a transcription factor composed of HIF-1 α and HIF-1 β subunits (4, 5), is stabilized and activated to promote the transcription of several genes such as glucose transporters, glycolytic enzymes, and angiogenic factors (6–9). Thus, HIF-1 α plays an important role in solid tumor formation *in vivo* by promoting angiogenesis and anaerobic metabolism (10, 11). In accordance with these reports, overexpression of

HIF-1 α protein has been reported recently (12) in human common cancers *in vivo*.

Whereas angiogenesis is required for the growth of solid tumors over several mm³ theoretically (13), hypovasculature is an outstanding characteristic of pancreatic cancers in imaging diagnosis, especially ductal adenocarcinomas (14). Angiography demonstrates that most of the pancreatic cancers are avascular and that tumor vessels are noted only in a small number of cases (15). Sonography and computed tomography demonstrate that vasculature is very poor in most of the pancreatic cancers (16, 17). In contrast to the macrovessels, microvessel density in pancreatic cancers is somewhat controversial. Several reports (18–20) demonstrated that high microvessel density and high angiogenic factor expression in pancreatic cancers were correlated with poor prognosis and that antiangiogenic drugs were effective in experimental pancreatic cancer models. Conversely, it has been reported recently (21) that microvessel density in pancreatic cancer tissues implanted in rats was significantly less than in normal pancreatic tissues. According to the imaging diagnosis demonstrating that macrovessels were poor in pancreatic cancers, we hypothesized that blood supply is poor in pancreatic cancer tissues and that pancreatic cancer cells are exposed to severe hypoxia and nutrient deprivation continuously compared with other well-vascularized tumor cells. Then, how do pancreatic cancer cells acquire the potential to proliferate in severe hypoxia and nutrient deprivation? Recently (22), it has been reported that HIF-1 α affects tumor growth in the ways unrelated to its regulation of VEGF expression. Therefore, we hypothesized that expression of HIF-1 α protein and/or HIF-1-inducible genes other than angiogenesis-related genes might be differently regulated in pancreatic cancers from that in other well-vascularized solid tumor cells.

In this study, we first examined the expression of HIF-1 α protein under normoxic and hypoxic conditions in pancreatic cancer cell lines and other solid tumor cell lines. Next, we examined the growth and survival of pancreatic cancer cell lines under hypoxic and/or glucose-deprived conditions, and we examined the effects of transfection with HIF-1 α on the growth and survival of pancreatic cancer cell lines *in vitro* and *in vivo*. To define the mechanisms by which pancreatic cancer cells acquire the resistance to apoptosis induced by hypoxia and nutrient deprivation, we examined the expression of HIF-1-inducible genes other than angiogenesis-related genes in pancreatic cancer cell lines including HIF-1 α -transfectants after exposure to hypoxia and glucose deprivation *in vitro*.

MATERIALS AND METHODS

Cell Lines. Pancreatic ductal adenocarcinoma cell lines (PCI-10, -19, -35, -43, -55, -66, -68, -72, and -79 cells) were kindly supplied by Dr. Hiroshi Ishikura (The First Department of Pathology, Hokkaido University School of Medicine, Sapporo, Japan). Pancreatic cancer cell lines KMP-2, -3, -4, -5, -7, and -8 were kindly supplied by Dr. Yutaka Shimada (Department of Surgery and Surgical Basic Science Graduate School of Medicine, Kyoto University, Kyoto, Japan). Pancreatic cancer cell lines PSN-1, Miapaca-2, Panc-1, Bxpc-3, and ASPC-1 and the cell lines described above were maintained in DMEM: Ham's F-12 medium or DMEM medium supplemented with 10% FCS. TTOV

Received 1/30/01; accepted 6/21/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹Supported in part by grants from the Japanese Ministry of Education, Science and Culture.

²To whom requests for reprints should be addressed, at Division of Cancer Pathobiology, Institute for Genetic Medicine, Hokkaido University, Kita-15, Nishi-7, Kita-ku, Sapporo, 060-8638 Japan. Phone: 81-11-706-5070; Fax: 81-11-706-7238; E-mail: mkobaya@med.hokudai.ac.jp.

³The abbreviations used are: HIF-1, hypoxia-inducible factor-1; VEGF, vascular endothelial growth factor; vHL, von Hippel-Lindau.

and TAOV (ovarian cancer cell line), HepG2 (hepatoma cell line), KM-12 (colon cancer cell line), and PC-6 (lung cancer cell line) were also maintained in DMEM medium supplemented with 10% FCS. p53 status in pancreatic cancer cell lines was examined by a yeast functional assay (23). Briefly, yeast functional assay was done as follows. p53 cDNAs synthesized from total RNA of the cell lines were amplified by PCR and then used for the transformation of the yeast reporter strain, yIG397, which contains an integrated plasmid with the *ADE2* open reading frame under the control of a p53-responsive promoter. When the yIG397 is transformed with mutated p53, it fails to express *ADE2* and forms red colony because of the accumulation of an intermediate in adenine metabolism. In contrast, it forms white colony when transformed by wild-type p53. All of the pancreatic cancer cell lines tested in this study had mutated p53 except KMP-8.

Reagents. Anti-HIF-1 α antibody was purchased from Transduction Laboratories (Lexington, KY). Annexin-V-FLUOS staining kit was purchased from Japan Roche Diagnostic Co. Ltd. (Tokyo, Japan). Topo TA cloning kit was purchased from Invitrogen (Carlsbad, CA). Lactacystin, cobalt chloride, and cycloheximide were purchased from Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan) and Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Rhodamine 123 was purchased from Cosmo Bio Co. Ltd. (Tokyo, Japan).

Fluorescence-activated Cell Sorter Analysis. After staining with propidium iodide and FITC-conjugated anti-annexin V by the use of annexin-V-FLUOS according to the manufacturer's instruction, the cells were analyzed with a FACScalibur (Becton Dickinson, Mountain View, CA).

Extraction of Cytoplasmic Proteins and Nuclear Proteins. The cells were recovered from the cultures, washed twice with PBS, resuspended in buffer A [10 mM HEPES-KOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA (pH 8.0), and 0.1% NP40], and then vigorously mixed. After centrifugation at 1300 rpm for 5 min, the supernatants were recovered as cytoplasmic proteins. The pellets were resuspended in buffer C [50 mM HEPES-KOH (pH 7.8), 420 mM KCl, 0.1 mM EDTA (pH 8.0), 5 mM MgCl₂, and 20% glycerol] and gently mixed at 4°C for 30 min. After centrifugation at 15,000 rpm at 4°C for 30 min, the supernatants were recovered as nuclear proteins.

Western Blot Analysis. The samples were electrophoresed under reducing conditions on 7.5% or 12% polyacrylamide gels in Tris-glycin buffer and transferred to 0.45- μ m nitrocellulose membranes. The membrane was then blocked for 30 min in blocking buffer (5% skim milk in 1% Tween-PBS) and probed with first antibody for 1 h. After being washed, the membrane was incubated with a peroxidase-conjugated goat antimouse IgG and developed with the use of an enhanced chemiluminescence detection kit (Amersham, Tokyo, Japan).

Northern Blot Analysis. Total RNA (25 μ g) was separated by electrophoresis in 1% denaturing formaldehyde-agarose gels. The RNA was transferred to nylon membrane (Hybond N⁺; Amersham) by capillary elution overnight and UV cross-linked. After prehybridization of blots for 1–2 h at 42°C in prehybridization buffer (5 \times saline-sodium phosphate-EDTA, 5 \times Denhardt's solution, 1% SDS, 50% formamide, and 0.1 mg/ml of denatured salmon sperm DNA), the membrane was hybridized overnight at 42°C with the cDNA probe labeled with ³²P by a random primer DNA labeling kit (Takara Biomedicals, Tokyo, Japan) for Glut 1 and aldolase A. The probed membrane was then washed and exposed to Bas-III Imaging Plate, and the images were scanned by Bas-2000 Image Scanning System (Fuji Film Co. Ltd.). Probes for Glut 1 and aldolase A were obtained by PCR amplification with the use of PCR primers as follows: Glut 1 forward, atgaaggaagagagtvg-gca; reverse, tgaagagttcagccagatg; aldolase A forward, cactgggatcactctctgt; reverse, aagacaccacacaccactgt.

Establishment of HIF-1 α -transfectants. Full-length cDNA for HIF-1 α was amplified from reverse transcription products of mRNAs purified from the colon cancer cell line KM-12 and then cloned into PCR4-TOPO. Plasmids were recovered, purified, and sequenced with a DyeDeoxy Terminator kit (Perkin-Elmer, Urayasu, Japan) on an ABI 377 automated sequencer (Applied Biosystems, Urayasu, Japan) under the conditions according to the manufacturer's protocol. Cloned fragments were recovered from vectors and ligated into PcDNA3.1+ (Invitrogen). PCI-10 cells were transfected with an expression vector with the use of Lipofectamine (Life Technologies, Inc., Tokyo, Japan). Transfectants were cloned by a limiting dilution method after the selection with G-418 at 800 μ g/ml. The transfectants were maintained in the presence of 400 μ g/ml of G-418. PCR primers for HIF-1 α were as follows: forward, cgggatccggggaccgattaccat; reverse, gcatcaggtctctcttaagttt.

Growth of the Cell Lines in Hypoxia and Low Glucose. Two \times 10⁴/ml (5×10^5 /ml in some experiments) cells were plated in 5 ml of DMEM medium supplemented with 10% FCS or glucose-free DMEM medium (Life Technologies, Inc.) supplemented with 10% FCS in the wells of 6-well plates. After 24-, 48-, 72-, and 96-h incubation under 20% O₂/5% CO₂ or 1% O₂/5% CO₂, they were harvested and counted by a trypan blue dye exclusion test. Incubation under hypoxic conditions (1% O₂) was done in a hypoxic chamber gassed with 95% N₂ and 5% CO₂ (Wakenyaku Co. Ltd., Tokyo, Japan). The data were presented as mean \pm SD of three different experiments.

In Vivo Tumorigenicity. Five \times 10⁵, 5 \times 10⁶, and 10⁷ cells were inoculated s.c. into the right flanks of nude mice. Tumor formation was observed every 3 other days and determined 3 weeks after inoculation. Mice with tumors that were larger than 10 mm in major and minor axes were defined as tumor positive. Immunohistochemical determination of microvessel density in xenografts of the transfectants was done with the use of an ABC staining kit and antimouse CD31 antibody (Southern Biotechnology Associates Inc., Birmingham, AL).

RESULTS

Constitutive Expression of HIF-1 α Protein in Pancreatic Cancer Cells. As shown in Fig. 1A, none of ovarian cancer, lung cancer, hepatoma, and colon cancer cell lines expressed HIF-1 α protein in normoxia. This result was consistent with the previous report (19) that HIF-1 α protein was usually degraded through the ubiquitin/proteasome system in normoxia and was undetectable in normoxic cells. However, 15 of 20 pancreatic cancer cell lines (75%) expressed various but significantly high levels of HIF-1 α protein even in normoxia (Fig. 1B).

Resistance to Apoptosis Induced by Hypoxia and Glucose Deprivation in Pancreatic Cancer Cells. Among the cell lines that expressed or did not express HIF-1 α proteins under normoxic conditions, we selected the cell lines of which growth was similar under normoxic conditions. As shown in Fig. 2A, the cells with constitutive expression of HIF-1 α protein (PCI-35, PCI-43, and ASPC-1) proliferated

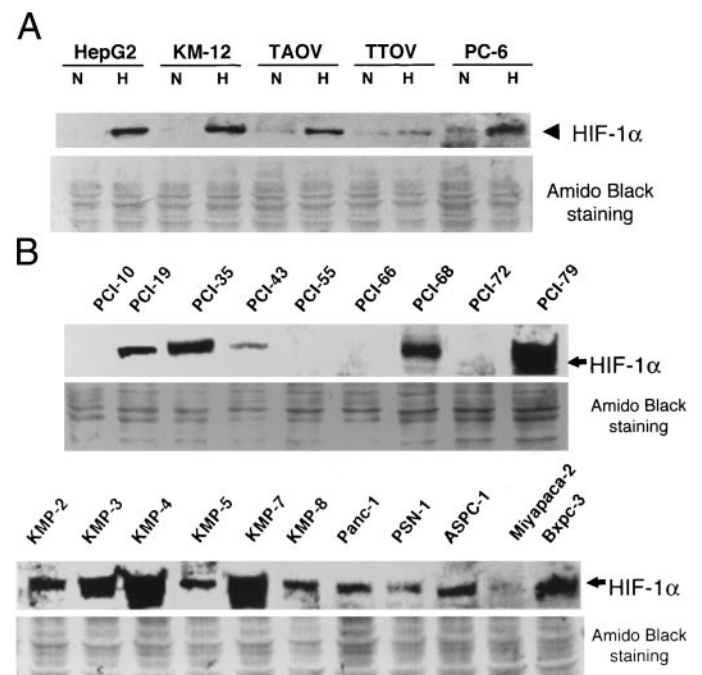


Fig. 1. Expression of HIF-1 α protein. Immunoblot of HIF-1 α protein and total nuclear protein stained with Amido Black are shown. A, HIF-1 α protein expression in ovarian cancer (TAOV and TTOV), colon cancer (KM-12), and hepatoma (HepG2) cell lines under normoxic (N) and hypoxic conditions (H). B, HIF-1 α protein in pancreatic cancer cell lines under normoxic conditions. Representative results of three different experiments were shown.

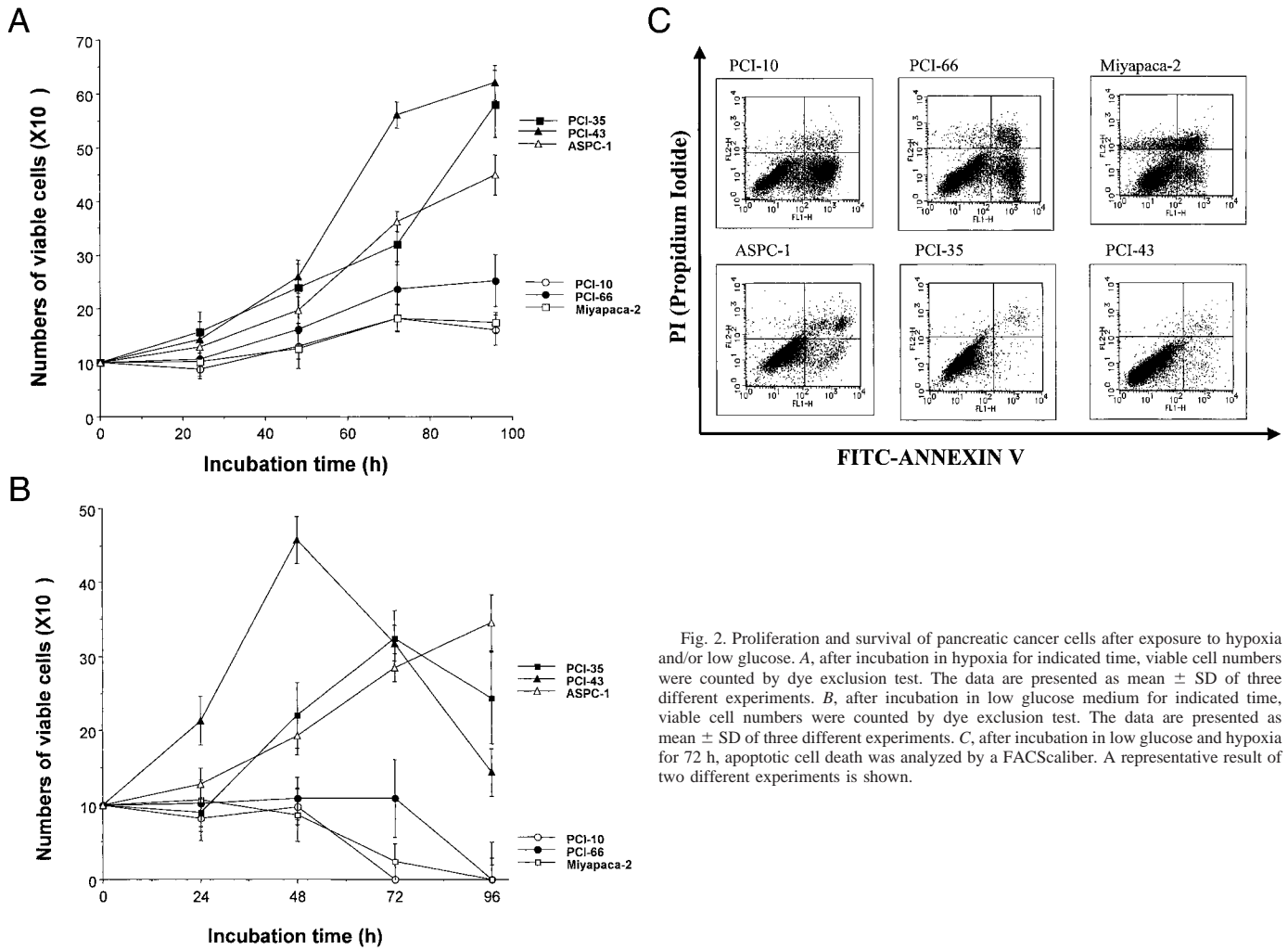


Fig. 2. Proliferation and survival of pancreatic cancer cells after exposure to hypoxia and/or low glucose. **A**, after incubation in hypoxia for indicated time, viable cell numbers were counted by dye exclusion test. The data are presented as mean \pm SD of three different experiments. **B**, after incubation in low glucose medium for indicated time, viable cell numbers were counted by dye exclusion test. The data are presented as mean \pm SD of three different experiments. **C**, after incubation in low glucose and hypoxia for 72 h, apoptotic cell death was analyzed by a FACS caliber. A representative result of two different experiments is shown.

erated faster under hypoxic conditions than those without constitutive expression of HIF-1 α protein (PCI-10, PCI-66, and Miyapaca-2). Hypoxia itself induced growth inhibition but not cell death of the pancreatic cancer cells (data not shown). However, glucose deprivation induced the cell death of PCI-10, PCI-66, and Miyapaca-2 cells within 72-h and 48-h incubation, respectively (Fig. 2B). PCI-35, PCI-43, and ASPC-1 cells were less affected by glucose deprivation than PCI-10, PCI-66, and Miyapaca-2 cells (Fig. 2B). Furthermore, PCI-10, PCI-66, and Miyapaca-2 cells, but none of PCI-35, PCI-43, and ASPC-1 cells, underwent apoptosis when cultured under hypoxic and glucose-deprived conditions (Fig. 2C).

Increased Expression of HIF-1 α Protein, Glut1, and Aldolase A mRNAs after Exposure to Hypoxia and Glucose Deprivation in Pancreatic Cancer Cells. To determine the mechanisms responsible for the different responses of the cells with or without constitutive expression of HIF-1 α protein to hypoxia and glucose deprivation, we then examined the protein levels of HIF-1 α after exposure to hypoxia and glucose deprivation. HIF-1 α protein expression was induced in PCI-10 cells 4 h after exposure to hypoxia and glucose deprivation, but the protein levels were lower than those in PCI-35 cells (Fig. 3A). In contrast, HIF-1 α protein was already expressed before exposure to hypoxia and glucose deprivation, and the protein levels remained higher up to 24 h after the exposure in PCI-35 cells than those in PCI-10 cells.

Because glucose deprivation was more cytotoxic than hypoxia, an increase of glucose metabolism seemed to be essential for pancreatic

cancer cells to adapt themselves to hypoxia and glucose deprivation. Therefore, we examined the mRNA expression of one of the glucose transporters, Glut1, and one of the glycolytic enzymes, aldolase A, chronologically after exposure to hypoxia and glucose deprivation.

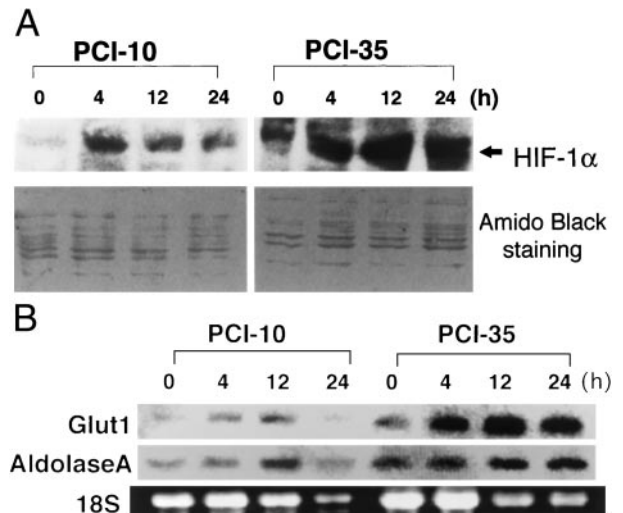


Fig. 3. HIF-1 α protein expression and Glut1 and aldolase A mRNA expression after exposure to hypoxia and low glucose for indicated time. **A**, immunoblot of HIF-1 α protein and total nuclear protein stained with Amido Black are shown. **B**, Glut1 and aldolase A mRNA expression. Representative results of two different experiments are shown.

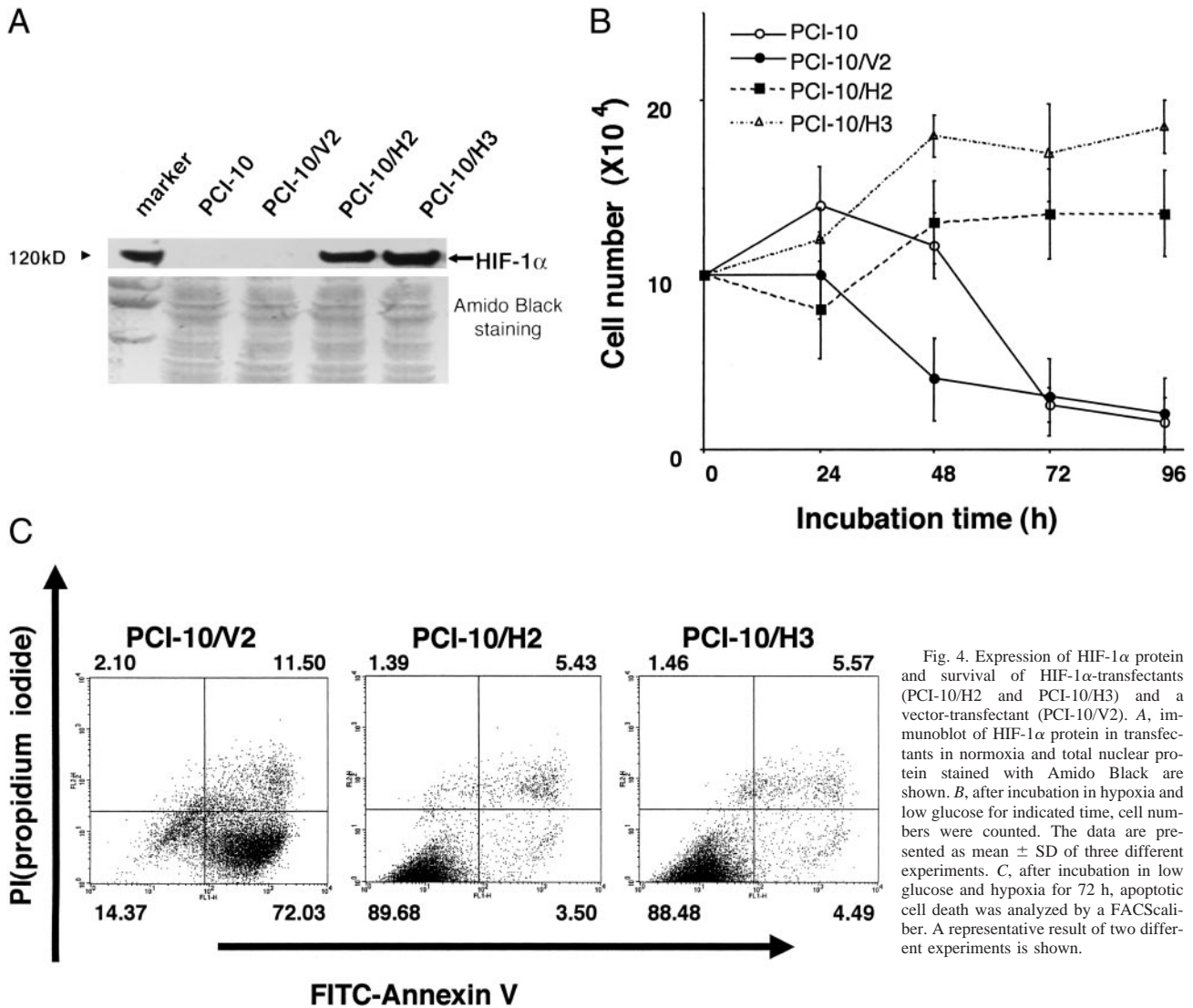


Fig. 4. Expression of HIF-1 α protein and survival of HIF-1 α -transfectants (PCI-10/H2 and PCI-10/H3) and a vector-transfectant (PCI-10/V2). *A*, immunoblot of HIF-1 α protein in transfectants in normoxia and total nuclear protein stained with Amido Black are shown. *B*, after incubation in hypoxia and low glucose for indicated time, cell numbers were counted. The data are presented as mean \pm SD of three different experiments. *C*, after incubation in low glucose and hypoxia for 72 h, apoptotic cell death was analyzed by a FACS-caliber. A representative result of two different experiments is shown.

Glut1 and aldolase A mRNAs were expressed at higher levels in PCI-35 cells than in PCI-10 cells under normoxic conditions (Fig. 3*B*). After exposure to hypoxia and glucose deprivation, expression of Glut1 and aldolase A mRNAs increased to higher levels in PCI-35 cells than that in PCI-10 cells (Fig. 3*B*). These results suggested that Glut1 and aldolase A mRNAs were more efficiently expressed in the cells expressing HIF-1 α protein in advance to the exposure to hypoxia and glucose deprivation than in the cells without constitutive expression of HIF-1 α and that the increased anaerobic metabolism might render the pancreatic cancer cells with constitutive expression of HIF-1 α protein resistant to apoptosis induced by hypoxia and glucose deprivation.

Resistance to Apoptosis Induced by Hypoxia and Glucose Deprivation in HIF-1 α -transfectants. Because we had speculated that overexpression of HIF-1 α protein might potentiate the growth and survival of pancreatic cancer cells even under hypoxic and glucose-deprived conditions, we then established HIF-1 α -transfectants from the PCI-10 cells. Fig. 4*A* shows the expression of HIF-1 α protein in the transfectants under normoxic conditions. The HIF-1 α -transfectants (PCI-10/H2 and PCI-10/H3) expressed HIF-1 α protein intensely in normoxia, whereas a vector-transfectant (PCI-10/V2) did not. These results indicated that the overproduction of HIF-1 α protein

overcame the degradation of HIF-1 α protein in normoxia. As shown in Fig. 4, *B* and *C*, PCI-10/H2 and PCI-10/H3 cells could survive under hypoxic and glucose-deprived conditions, whereas PCI-10/V2 cells underwent apoptosis. These results confirmed that the constitutive expression of HIF-1 α protein potentiated the growth and survival of pancreatic cancer cells even under hypoxic and glucose-deprived conditions.

Expression of HIF-1 α Protein, Glut1, and Aldolase A mRNAs in the Transfectants after Exposure to Hypoxia and Glucose Deprivation. HIF-1 α protein expression was induced in the vector-transfectant 4 h after exposure to hypoxia and glucose deprivation, whereas HIF-1 α protein was already expressed before exposure to hypoxia and glucose deprivation, and the protein levels remained higher up to 24 h after the exposure in the HIF-1 α -transfectant than those in the vector-transfectant (Fig. 5*A*). Fig. 5*B* shows that Glut1 and aldolase mRNA expression increased to higher levels after exposure to hypoxia and glucose deprivation in the PCI-10/H2 and PCI-10/H3 cells than in the PCI-10/V2 cells. These results also suggested that Glut1 and aldolase A mRNAs were more efficiently expressed in the cells expressing HIF-1 α proteins in advance to the exposure than in the cells without constitutive expression of HIF-1 α and that the increased anaerobic metabolism contributed to the resistance to ap-

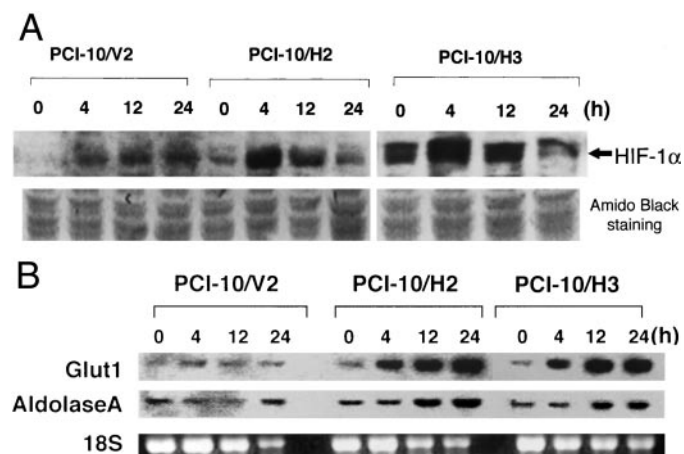


Fig. 5. Expression of HIF-1 α proteins, Glut1, and aldolase A mRNAs in HIF-1 α -transfectants (PCI-10/H2 and PCI-10/H3) and a vector-transfectant (PCI-10/V2) after exposure to hypoxia and low glucose for indicated time. A, immunoblot of HIF-1 α protein in transfectants and total nuclear protein stained with Amido Black are shown. B, Glut1 and aldolase A mRNA expression. A representative result of three different experiments is shown.

optosis in the pancreatic cancer cells expressing HIF-1 α constitutively.

In Vivo Growth of the HIF-1 α -transfectants. Table 1 shows the tumorigenicity of the HIF-1 α -transfectant and the vector-transfectant inoculated in nude mice. The PCI-10/H2 cells formed s.c. tumors in one of five mice and five of five mice when inoculated at 5×10^6 /mouse and 1×10^7 /mouse, respectively, whereas the PCI-10/V2 and PCI-10 cells formed s.c. tumors in only one of five mice even when inoculated at 1×10^7 /mouse.

DISCUSSION

In this study, we demonstrated that most pancreatic cancer cell lines (75%) expressed HIF-1 α protein even under nonhypoxic conditions, whereas other cancer cell lines tested in this study expressed HIF-1 α protein only under hypoxic conditions. Our results appear to be inconsistent with the recent report (12) demonstrating that common cancer cells including pancreatic cancer cells express HIF-1 α protein *in vivo*. However, because most cancer cells are exposed to hypoxia and hence express HIF-1 α protein *in vivo*, only *in vitro* examination can demonstrate the difference of HIF-1 α protein expression between pancreatic cancer cells and other cancer cells under normoxic conditions. Previous reports (5) demonstrated that HIF-1 α protein was degraded by the ubiquitin/proteasome mechanism and was undetectable under normoxic conditions. It has been reported recently (24) that vHL tumor suppressor gene product is required for the degradation of HIF-1 α protein by the ubiquitin/proteasome mechanism. In accordance with these reports, constitutive expression of HIF-1 α has been detected in the cancer cells related with vHL disease and the sporadic renal carcinoma cells caused by loss of vHL function (25, 26). Besides the vHL-related cancers, human prostate cancer cells have been reported (27) to overexpress HIF-1 α mRNA and protein under nonhypoxic conditions. However, this report showed that HIF-1 α protein was expressed under normoxic conditions in only one prostate cancer cell line, PC-3. Our finding is the first evidence that HIF-1 α protein is expressed under normoxic conditions even in vHL-unrelated pancreatic ductal adenocarcinoma cells at high frequency. We sequenced vHL gene in all of the pancreatic cancer cell lines and found a missense mutation of vHL gene in 2 of 15 cell lines with constitutive expression of HIF-1 α protein (data not shown), indicating that constitutive expression of HIF-1 α protein in pancreatic cancer cells is

caused by unknown mechanisms other than vHL mutation. Although details are now under examination, we now focus on the role of a phosphatidylinositol 3'-kinase/Akt pathway because this pathway has been reported to activate HIF-1 α expression in prostate cancer cells and to be involved in the resistance of pancreatic cancer cells to apoptosis induced by nutrient deprivation (28, 29).

Expression of HIF-1 α protein induces the production of pro-angiogenic factors resulting in the neovascularization for most cancer cells to adapt themselves to severe hypoxia (6–9). In fact, vHL-related cancers and sporadic renal carcinomas caused by loss of vHL function are well vascularized (30, 31). Therefore, increased tumorigenicity of HIF-1 α -transfectants in our study seemed to be caused by increased angiogenesis as well as by anaerobic metabolism. However, intratumoral microvessel density in the xenografts of the vector-transfectant and that in the HIF-1 α -transfectants were not significantly different (data not shown). Because HIF-1 α -transfectants expressed higher levels of VEGF mRNA than vector control (data not shown), it was somewhat surprising. A possible explanation is that angiogenesis may be compensated in the established tumors of the vector-transfectant. Alternatively, VEGF production by a small number of the vector-transfectants under severe hypoxic conditions may be able to compensate for generally low expression of VEGF. This possible explanation was consistent with the recent report by Ryan *et al.* (22) demonstrating that loss of HIF-1 α did not alter tumor vascularization. As the third possible explanation, we are now examining whether pancreatic cancer cells may secrete elastase that converts plasminogen into an angiogenesis inhibitor, angiostatin, because angiostatin is generated by incubating plasminogen with pancreas-derived elastase *in vitro*, and serum elastase levels are frequently high in patients with pancreatic cancers (32). Consistent with our hypothesis, it has been reported recently (33) that angiostatin was generated by pancreatic cancer cells.

Because solid tumor cells are exposed to not only chronic hypoxia but also acute hypoxia because of the fluctuation of blood flows as frequently observed in tumors *in vivo* (34), pancreatic cancer cells should be exposed to severe acute hypoxia as well as to severe chronic hypoxia. Our results demonstrated that the cells with constitutive expression of HIF-1 α protein were more resistant to apoptosis induced by hypoxia and glucose deprivation than those without constitutive expression of HIF-1 α protein. As our experiments mimic the acute hypoxia, such constitutive expression of HIF-1 α protein should contribute to the survival of pancreatic cancer cells exposed to acute hypoxia *in vivo*. Recently (35), it has been reported that disruption of hypoxia-inducible transcription suppresses tumor growth *in vitro* and *in vivo*. Taken together, our results suggest that the expression of hypoxia-inducible genes may be a new target of the therapy for pancreatic cancers. Therefore, we are now examining the effect of dominant negative HIF-1 α on the growth of pancreatic cancer cells *in vitro* and *in vivo*.

Glut1 is the most primitive type of glucose transporters expressed in most types of tissues and cell lines (36). It is expressed in a dominant form in most of the human fetal pancreatic tissues (37). Aldolase A is one of the key regulatory glycolytic enzymes; it has been reported to increase in the sera of patients with several cancers including pancreatic cancers (38). Therefore, we examined the ex-

Table 1 *In vivo* tumorigenicity^a

Cell	10^7	5×10^6	5×10^5
PCI-10	1/5	0/5	0/5
PCI-10/V2	1/5	0/5	0/5
PCI-10/H2	5/5	1/5	0/5

^a Indicated numbers of cells were inoculated s.c. Tumor formation was observed every 3 other days after inoculation and then determined 3 weeks after inoculation.

pression of Glut1 and aldolase A as a representative glucose transporter and a glycolytic enzyme in this study. We demonstrated that Glut1 and aldolase A mRNAs were expressed at higher levels in the cells with constitutive expression of HIF-1 α protein than in those without it before and after exposure to hypoxia and glucose deprivation. These results suggest that most pancreatic cancer cells with constitutive expression of HIF-1 α protein adapt themselves to hypoxia and glucose deprivation by increased glucose uptake and anaerobic metabolism. However, as the increased glucose uptake and anaerobic metabolism have been documented in common cancer cells since the early observations by Warburg (39), the increased anaerobic metabolism is not specific to pancreatic cancer cells. We propose that anaerobic metabolism plays a more important role in pancreatic cancers than in other well-vascularized cancers so that pancreatic cancer cells can adapt themselves to hypoxia and nutrient deprivation. Glut1 protein in pancreatic cancer cells *in vivo* and aldolase A protein in the sera of patients with pancreatic cancers are reported to increase (38, 40). The increased anaerobic metabolism may also play an important role in the growth and survival of pancreatic cancer cells *in vivo*.

In addition to well-known HIF-1-inducible genes, several other genes such as transferrin receptor and ceruloplasmin are also regulated by HIF-1 (41, 42). Because several studies (43, 44) demonstrated that patients with hypoxic tumors had worse prognoses and that hypoxic tumors were resistant to radiation therapy, we now speculate that HIF-1 could induce some genes other than anaerobic metabolism-associated genes to function as antiapoptotic factors that could play important roles in the resistance to apoptosis induced by hypoxia and glucose deprivation. To seek those genes, we are now comparing mRNA expression in a pancreatic cancer cell line cultured in hypoxia and normoxia with the use of a DNA microarray system.

ACKNOWLEDGMENTS

We thank Dr. Hiroshi Ishikura (The First Department of Pathology, Hokkaido University School of Medicine, Sapporo, Japan) and Dr. Yutaka Shimada (Department of Surgery and Surgical Basic Science Graduate School of Medicine, Kyoto University, Kyoto, Japan) for providing us with pancreatic cancer cell lines. We thank Masako Yanome for assistance in preparing the manuscript.

REFERENCES

- Vaupel, P., Kallinowski, F., and Okunieff, P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. *Cancer Res.*, **49**: 6449–6465, 1989.
- Dachs, G. U., Patterson, A. V., Firth, J. D., Ratcliffe, P. J., Stuart Townsend, K. M., Stratford, I. J., and Harris, A. L. Targeting gene expression of hypoxic cells. *Nat. Med.*, **3**: 515–520, 1997.
- Richard, D. E., Berra, E., and Pouyssegur, J. Angiogenesis. How a tumor adapts to hypoxia. *Biochem. Biophys. Res. Commun.*, **266**: 718–722, 1999.
- Wang, G. L., Jiang, B. H., Rue, E. A., and Semenza, G. L. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc. Natl. Acad. Sci. USA*, **92**: 5510–5514, 1995.
- Salceda, S., and Caro, J. Hypoxia-inducible factor 1 α (HIF-1 α) protein is rapidly degraded by the ubiquitin/proteasome system under normoxic conditions. *J. Biol. Chem.*, **272**: 22642–22647, 1997.
- Guillemin, K., and Krasnow, M. A. The hypoxic response: Huffing and HIFing. *Cell*, **89**: 9–12, 1997.
- Blancher, C., and Harris, A. L. The molecular basis of hypoxia response pathway: tumor hypoxia as a therapy target. *Cancer Metastasis Rev.*, **17**: 187–194, 1998.
- Dang, C. V., and Semenza, G. L. Oncogenic alterations of metabolism. *Trends Biochem. Sci.*, **24**: 68–72, 1999.
- Semenza, G. L. Expression of hypoxia-inducible factor 1: mechanisms and consequences. *Biochem. Pharmacol.*, **59**: 47–53, 2000.
- Ryan, H. E., Lo, J., and Johnson, R. S. HIF-1 α is required for solid tumor formation and embryonic vascularization. *EMBO J.*, **17**: 3005–3015, 1998.
- Maxwell, P. H., Dachs, G. U., Gleadle, J. M., Nicholls, L. G., Harris, A. L., Stratford, I. J., Hankinson, O., Pugh, C. W., and Ratcliffe, P. J. Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. *Proc. Natl. Acad. Sci. USA*, **94**: 8104–8109, 1997.
- Zhong, H., Marzo, A. M. D., Laughner, E., Lim, M., Hilton, D. A., Zagzag, D., Buechler, P., Isaacs, W. B., Semenza, G. L., and Simons, J. W. Overexpression of hypoxia-inducible factor 1 α in common human cancers and their metastasis. *Cancer Res.*, **59**: 5830–5835, 1999.
- Hanahan, D., and Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*, **86**: 353–364, 1996.
- Raijman, I., and Levin, B. Exocrine Tumors of Pancreas *In: W. S. Haubrich and F. Schaffner (eds.), Bockus Gastroenterology*, 5th ed., pp. 2984–3001. Philadelphia: W. B. Saunders Co., 1995.
- Rannigber, K., and Saldino, R. M. Arteriographic diagnosis of pancreas lesion. *Radiology*, **86**: 470–474, 1966.
- Yassa, N. A., Yang, J., Stein, S., Johnson, M., and Ralls, P. Gray-scale and color flow sonography of pancreatic ductal adenocarcinoma. *J. Clin. Ultrasound*, **25**: 473–480, 1997.
- Megibow, A. J. Pancreatic adenocarcinoma: designing the examination to evaluate the clinical questions. *Radiology*, **183**: 297–303, 1992.
- Ikeda, N., Adachi, M., Taki, T., Huang, C., Hashida, H., Takabayashi, A., Sho, M., Nakajima, Y., Kanehiro, H., Hisanaga, M., Nakano, H., and Miyake, M. Prognostic significance of angiogenesis in human pancreatic cancer. *Br. J. Cancer*, **79**: 1553–1563, 1999.
- Seo, Y., Baba, H., Fukuda, T., Takashima, M., and Sugimachi, K. High expression of vascular endothelial growth factor is associated with liver metastasis and a poor prognosis for patients with ductal pancreatic adenocarcinoma. *Cancer (Phila.)*, **88**: 2239–2245.
- Bruns, C. J., Solorzano, C. C., Harbison, M. T., Ozawa, S., Tsan, R., Fan, D., Abbruzzese, J., Traxler, P., Buchdunger, E., Radinsky, R., and Fidler, I. J. Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. *Cancer Res.*, **60**: 2926–2935, 2000.
- Schmidt, J., Ryschich, E., Daniel, V., Herzog, L., Werner, J., Herfarth, C., Longnecker, D. S., Gebhard, M. M., and Klar, E. Vascular structure and microcirculation of experimental pancreatic carcinoma in rats. *Eur. J. Surg.*, **166**: 328–335, 2000.
- Ryan, H. E., Poloni, M., McNulty, W., Elson, D., Gassmann, M., Arbeit, J. M., and Johnson, R. S. Hypoxia-inducible factor-1 α is a positive factor in solid tumor growth. *Cancer Res.*, **60**: 4010–4015, 2000.
- Kashiwazaki, H., Tonoki, H., Tada, M., Chiba, I., Shindo, M., Totsuka, Y., Iggo, R., and Moriuchi, T. High frequency of p53 mutations in human oral epithelial dysplasia and primary squamous cell carcinoma detected by yeast functional assay. *Oncogene*, **15**: 2667–2674, 1997.
- Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R., and Ratcliffe, P. J. The tumor suppressor VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature (Lond.)*, **399**: 271–275, 1999.
- Ohh, M., Park, C. W., Ivan, M., Hoffman, M. A., Kim, T. Y., Huang, L. E., Pavletich, N., Chau, V., and Kaelin, W. G. Ubiquitination of hypoxia-inducible factor requires direct binding to the β -domain of the von Hippel-Lindau protein. *Nat. Cell Biol.*, **2**: 423–427, 2000.
- Krieg, M., Haas, R., Brauch, H., Acker, T., Flamme, I., and Plate, K. H. Up-regulation of hypoxia-inducible factors HIF-1 α and HIF-2 α under normoxic conditions in renal carcinoma cells by von Hippel-Lindau tumor suppressor gene loss of function. *Oncogene*, **19**: 5435–5443, 2000.
- Zhong, H., Agani, F., Baccala, A. A., Laughner, E., Comacho, N. R., Issacs, W. B., Simons, J. W., and Semenza, G. L. Increased expression of hypoxia inducible factor-1 α in rat and human prostate cancer. *Cancer Res.*, **58**: 5280–5284, 1998.
- Zhong, H., Chiles, K., Feldser, D., Laughner, E., Hanrahan, C., Georgescu, M. M., Simons, J. W., and Semenza, G. L. Modulation of hypoxia-inducible factor 1 α expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTE/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res.*, **60**: 1541–1545, 2000.
- Izuishi, K., Kato, K., Ogura, T., Kinoshita, T., and Esumi, H. Remarkable tolerance of tumor cells to nutrient deprivation: possible new biochemical target for cancer therapy. *Cancer Res.*, **60**: 6201–6207, 2000.
- Wizigmann-Voos, S., Breier, G., Risau, W., and Plate, K. Up-regulation of vascular endothelial growth factor and its receptors in von Hippel-Lindau disease-associated and sporadic hemangioblastomas. *Cancer Res.*, **55**: 1358–1364, 1995.
- Takahashi, A., Sasaki, H., Kim, S. J., Tobisu, K., Kakizoe, T., Tsukamoto, T., Kuramoto, Y., Sugimura, T., and Terada, M. Markedly increased amounts of messenger RNAs for vascular endothelial growth factor and placenta growth factor in renal cell carcinoma associated with angiogenesis. *Cancer Res.*, **54**: 4233–4237, 1994.
- Yamamura, H., Tatsuta, M., Ito, M., Iishi, H., Noguchi, S., Okuda, S., Yoshida, M. Effectiveness of discriminant analysis of serum CA 19–9 and elastase 1 in diagnosis of pancreatic carcinoma. *Pancreas*, **4**: 401–405, 1989.
- O'Mahony, C. A., Seidel, A., Albo, D., Chang, H., Tuszynski, G. P., and Berger, D. H. Angiostatin generation by human pancreatic cancer. *J. Surg. Res.*, **77**: 55–58, 1998.
- Brown, J. M. The hypoxic cell: a target for selective cancer therapy—eighteenth Bruce F. Cain memorial award lecture. *Cancer Res.*, **59**: 5863–5870, 1999.
- Kung, A. L., Wang, S., Klco, J. M., Kaelin, W. G., Jr., and Livingston, D. M. Suppression of tumor growth through disruption of hypoxia-inducible transcription. *Nat. Med.*, **6**: 1335–1340, 2000.

36. Merral, N. W., Plevin, R., and Gould, G. W. Growth factors, mitogens, oncogenes and the regulation of glucose transport. *Cell. Signal.*, 5: 667–675, 1993.
37. Mally, M. I., Otonkoski, T., Lopez, A. D., and Hayek, A. Developmental gene expression in the human fetal pancreas. *Pediatr. Res.*, 36: 537–544, 1994.
38. Asaka, M., Nagase, K., Miyazaki, T., and Alpert, E. Radioimmunoassay of aldolase A. Determination of normal serum levels and increased serum concentration in cancer patients. *Cancer (Phila.)*, 51: 1873–1878, 1983.
39. Warburg, O. *The Metabolism of Tumors*, pp. 129–169. New York: Richard R. Smith, Inc., 1930.
40. Higashi, T., Tamaki, N., Honda, T., Torizuka, T., Kimura, T., Inokuma, T., Ohshiro, G., Hosotani, R., Imamura, M., and Konishi, J. Expression of glucose transporters in human pancreatic tumors compared with increased FDG accumulation in PET study. *J. Nucl. Med.*, 38: 1337–1344, 1997.
41. Tacchini, L., Bianchi, L., Zazzera, A. B., and Cairo, G. Transferrin receptor induction by hypoxia: HIF-1-mediated transcriptional activation and cell-specific post-translational regulation. *J. Biol. Chem.*, 274: 24142–24146, 1999.
42. Mukhopadhyay, C. K., Mazumder, B., and Fox, P. L. Role of hypoxia-inducible factor-1 in transcriptional activation of ceruloplasmin by iron deficiency. *J. Biol. Chem.*, 275: 21084–21054, 2000.
43. Gatenby, R. A., Kessler, H. B., Rosenblum, J. S., Coia, L. R., Moldofsky, P. J., Hartz, W. H., and Broder, G. J. Oxygen distribution in squamous cell carcinoma metastases and its relationship to outcome of radiation therapy. *Int. J. Radiat. Oncol. Biol. Phys.*, 14: 831–838, 1988.
44. Hockel, M., Schlenger, K., Hockel, S., and Vaupel, P. Hypoxic cervical cancers with low apoptotic index are highly aggressive. *Cancer Res.*, 59: 4525–4528, 1999.