The American Journal of Pathology, Vol. 170, No. 2, February 2007

DOI: 10.2353/ajpath.2007.060489

Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Hypoxia Inducible Factor-1 Mediates Effects of Insulin on Pancreatic Cancer Cells and Disturbs Host Energy Homeostasis

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Intratumoral hypoxia and paracrine insulin stimulate the expression of hypoxia inducible factor-1α (HIF-1α) in pancreatic cancer cells. In the present studies, we investigated whether insulin-induced HIF-1α expression is a prerequisite for insulin to induce other trophic effects in MiaPaCa2 human pancreatic cancer cells and whether inhibition of HIF-1α expression would decrease tumor glycolysis and improve host energy homeostasis. We found that hypoxia was a prerequisite for induction of HIF-1α mRNA expression by insulin in MiaPaCa2 cells. Under hypoxic conditions, insulin stimulated glycolysis, cell proliferation, and the secretion of vascular endothelial growth factor in regular MiaPaCa2 cells but not in a MiaPaCa2 variant (si-MiaPaCa2) that expressed specific short interfering RNA for HIF-1α and therefore lacked HIF-1α protein. This suggests that HIF-1α expression is required for insulin to induce other trophic effects. When si-MiaPaCa2 cells were transplanted into the pancreas of athymic mice, they were less tumorigenic and expressed less hexokinase than regular MiaPaCa2 cells. Body weight gain was attenuated in mice hosting tumors composed of regular MiaPaCa2 but not si-MiaPaCa2 cells. These results suggest that an interaction between insulin and HIF-1α helps sustain pancreatic cancer cells and disturbs host energy homeostasis. ***(Am J Pathol 2007, 170:469–477; DOI: 10.2353/ajpath.2007.060489)***

Exocrine pancreatic cancer cells migrate easily, resist therapy, and tolerate nutrient scarcity. The presence of pancreatic cancer cells in the body has a profound impact on the energy homeostasis of the host and causes metabolic disorders such as body weight decrease, diabetes, and cachexia.[1,2] Because of the aggressiveness of pancreatic cancer cells, the disease has a dismal prognosis.[3]

In the pancreas, the blood leaving endocrine islets passes through permeable vessels and delivers insular products (eg, insulin) into the interstitial fluid.[4] At high concentrations, interstitial insulin nourishes exocrine pancreatic cells.[5,6] When pancreatic cancer occurs, insulin may stimulate the growth of the cancer cells as well. *In vitro*, insulin has been found to stimulate proliferation and glycolysis in pancreatic cancer cells.[7,8] Thus, the interstitial insulin in the pancreas may be a driving force behind the aggressiveness of pancreatic cancer cells.

Hypoxia inducible factor-1 (HIF-1) is a heterodimeric (α/β) transcription factor.[9–12] In normoxia, newly produced HIF-1α protein is quickly hydroxylated at Pro402 and Pro564 by oxygen-activated HIF-1 prolyl hydroxylase (PHD) and degraded by the proteasomal pathway. Thus, mammalian cells are normally devoid of HIF-1α *in vivo*. During hypoxia, however, oxygen-stimulated PHD is inactivated. Consequently, HIF-1α accumulates and associates with HIF-1β. The mature HIF-1 binds to several dozen genes whose products include glycolytic enzymes, angiogenic factors (eg, vascular endothelial growth factor, VEGF), and proteins involved in cell viability. As a result, cells expressing HIF-1α become well prepared for hypoxic stress.

HIF-1α is also expressed in different malignant tumor cells including pancreatic cancer cells.[13] Because of the biological alterations after HIF-1 expression, tumor cells expressing HIF-1α are more aggressive than similar tumor cells lacking HIF-1α. Clinically, HIF-1α-positive tu-

Supported by the Swedish Cancer Foundation (grant 4871-B0503XAC to F.W. and J.P.), the Swedish Research Council (to J.H.), the Swedish Diabetes Association (to J.H.), and the Tobias and the Lars-Eric Gelin Foundations (to J.H.).

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Accepted for publication October 31, 2006.

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mors have a poorer prognosis than the same type of tumors devoid of HIF-1α.[13,14] Several mechanisms have been proposed for the expression of HIF-1α in malignant tumor cells. One hypothesis is that the poor microcirculation frequently found in malignant tumors causes intratumoral hypoxia and, therefore, stabilizes HIF-1α. On the other hand, cancer cells may accumulate metabolites that directly inhibit PHD and thereby stabilize HIF-1α independent of hypoxia.[15] Cancer cells may also accelerate HIF-1α production spontaneously or in response to stimuli such as insulin, insulin-like growth factors (IGFs), and epidermal growth factor (EGF).[16–18] When more HIF-1α is produced than the cells degrade, HIF-1α is expressed.

Severe intratumoral hypoxia is present in pancreatic cancer patients,[19] suggesting that hypoxia plays a role in the expression of HIF-1α in pancreatic cancer cells. Because pancreatic cancer cells are exposed to high concentrations of insulin, insulin may induce HIF-1α expression in these cancer cells as well. The relative contributions of hypoxia and insulin to the expression of HIF-1α in pancreatic cancer cells are unclear. It is also unclear whether there is an interaction between hypoxia and insulin as stimuli of HIF-1α in pancreatic cancer cells.

In the present study, we studied the effects of hypoxia and insulin on the expression of HIF-1α mRNA and protein in MiaPaCa2 human pancreatic cancer cells. We also investigated whether insulin-induced HIF-1α expression was necessary for the induction of other trophic effects by insulin in the same cancer cells. We used a MiaPaCa2 variant (si-MiaPaCa2) that stably expressed a specific short interfering RNA targeting HIF-1α (siRNA$_{\text{HIF-1}\alpha}$) and control MiaPaCa2 cells with intact HIF-1α transcripts. We also transplanted the different MiaPaCa2 cells into the pancreas of athymic mice to compare the survival of the cells and their impact on energy homeostasis in the host.

Materials and Methods

Lentiviruses and cv-MiaPaCa2 and si-MiaPaCa2 Cells

The HIV-based lentiviral transfer vector, pHOX-GFP-SIN, carrying the gene encoding the green fluorescent protein (GFP) downstream of the EF1α promoter, was a kind gift of Dr. D. Trono (Geneva University, Geneva, Switzerland).[20] The H1 human RNA polymerase III promoter was amplified by polymerase chain reaction (PCR) from human placenta genomic DNA, using 5′-cgcggcatcgataattcgaacgctgacgt-3′ and 5′-cgcggcatcgattgatcagtcgacactagtgtggtctcatacagaacttataag-3′ as the forward and reverse primers. The pHOX-GFP-SIN vector was digested by *Cla*I, and the H1 promoter was incorporated upstream of the EF1α promoter, giving a pHSER-EF1α-GFP-SIN vector. Restriction analysis determined that the insert was correctly oriented. A pair of nucleotide oligos (sense: 5′-ctagtcccatggaacatgatggttcacTTCAAGAGAgtgaaccatcatgttccatttttttg-3′ and anti-sense: 5′-tcgacaaaaaaagttaacatgatggttcacTCTCTTGAAgtgaaccatcatgttccatggga-3′) was synthesized (Cybergene, Stockholm, Sweden) that contained a 19-nucleotide sequence of the human HIF-1α gene (2192-2210; Medline, U22431). The oligos were annealed and subcloned into the pHSER-EF1α-GFP-SIN vector digested by *Spe*I and *Sal*I, giving the vector pHSER-siRNA-EF1α-GFP-SIN. Both the pHSER-EF1α-GFP-SIN and pHSER-siRNA-EF1α-GFP-SIN vectors had intact GFP genes.

Human embryonic kidney 293T cells were cultured in two 75-cm^2 flasks using 10 ml of Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Stockholm, Sweden) with 10% fetal calf serum (FCS). The flasks were incubated at 37°C in 95% air and 5% CO_2. When the cells were 90% confluent, 20 μg of either pHSER-EF1α-GFP-SIN or pHSER-siRNA-EF1α-GFP-SIN was mixed with 15 μg of the packaging plasmid pCMVΔ8.91, 5 μg of the envelope plasmid pMD.G, 20% glucose, and polyethylenimine. The resulting mixtures were transferred to the two flasks. The media were replaced the next day by fresh DMEM containing 2% FCS. The medium was renewed daily for the next 4 days. Media collected from the same flask on the different days were pooled and filtered (pore size, 0.45 μm). The filtrates with the pHSER-EF1α-GFP-SIN- or pHSER-siRNA-EF1α-GFP-SIN-based viruses were used as transducing media to create cv-MiaPaCa2 and si-MiaPaCa2 cells.

Wild-type MiaPaCa2 cells (wt-MiaPaCa2) were bought from the American Type Culture Collection (Rockville, MD) and incubated in a 24-well plate at 37°C in 95% air and 5% CO_2 using DMEM containing 10% FCS. When the cells were 80 to 90% confluent, they were incubated with one of the transducing media (1 ml/well) for a day and then cultured in fresh DMEM containing 10% FCS. Fluorescence microscopy was used to identify transduced cells expressing GFP. Ten days after transduction, fluorescent cells accounted for 10 to 20% of the cell populations. The cells were trypsinized and suspended in phosphate-buffered saline (PBS) (pH 7.2). A FACS Vantage flow cytometer (Becton Dickinson, Franklin Lakes, NJ) was used to isolate fluorescent cells from nontransduced cells, and a purity of more than 94% was achieved. The cells were grown in DMEM containing 10% FCS. Before the experiments, the purity of fluorescent cells was double-checked and found to be unchanged.

Hypoxic Incubation

Wt-, cv-, and si-MiaPaCa2 cells were incubated at 37°C in 95% air and 5% CO_2 (normoxic conditions) in DMEM containing 10% FCS. When the cells were 90% confluent, the medium was replaced with DMEM containing 0.1% bovine serum albumin. Human insulin (Novo Nordisk, Bagsværd, Denmark) was added to designated media with the final concentrations ranging from 0.1 to 1000 nmol/L. Culture vessels were placed in a hypoxic chamber (Billups-Rothenberg, Del Mar, CA). A probe of the PBI dual-channel (O_2/CO_2) gas meter (Dansensor, Ringstad, Denmark) was inserted into the chamber. The air inside the chamber was replaced with mixed N_2 (95%) and CO_2 (5%). When the gas meter indicated that the oxygen concentration had reached 1% in association with 4 to

Table 1. Antibodies in Western Blotting

Antigens	Primary antibodies			Secondary antibodies	
	Manufacturer	Number	Type	Manufacturer	Article number
HIF-1α	BD Biosciences	610958	Monoclonal	Amersham	NA931
Topo1	TopoGen	2012-2	Polyclonal	Amersham	NA934
Hexokinase	Santa Cruz	sc-6521	Polyclonal	Chemicom	AP106P
Aldolase	Chemicom	AB1809	Polyclonal	Chemicom	AP106P
β-Actin	Abcam	8227	Polyclonal	Amersham	NA934

6% CO_2, the air replacement was stopped. The chamber was sealed and kept at 37°C. In some experiments, MiaPaCa2 cells were prepared in duplicate for simultaneous incubation under hypoxia and normoxia.

Orthotopic Transplantation

The Southern Stockholm Animal Research Committee approved the present study. Male athymic C57BL/6 nude mice were purchased from Taconic Europe (Ry, Denmark) and kept in a room with a 12-hour light/dark cycle. The mice had free access to food and water, unless otherwise described. The mean body weight of the mice was 21.7 ± 0.4 g (n = 33) before the experiment. The mice were randomly allocated to a control group (n = 8) and three MiaPaCa2 recipient groups (n = 8, 8, and 9) that received wt-, cv-, and si-MiaPaCa2 cells, respectively. One million wt-, cv-, or si-MiaPaCa2 cells were suspended in 10 μl of PBS and injected into the duodenal lobe of the pancreas of each mouse.[21] Mice in the control group were untreated. All mice were followed for 15 weeks. On weeks 4, 8, 12, and 15, the mice were fasted for 6 hours and then underwent an intraperitoneal glucose tolerance test (glucose/body weight, 2 mg/g). Blood was taken from the tail, and glucose levels were determined using a glucose meter.

Survivors of the experiment were sacrificed at the end of week 15 after a 6-hour fast. Thirty minutes before sacrifice, mice were injected with glucose (2 mg/g, i.p.) to stimulate insulin release. Mice were sacrificed under anesthesia. The heart was punctured with a thick needle, and 0.8 ml of blood was withdrawn. The pancreas was checked and any visible tumors were resected, weighed, and stored at −80°C. Plasma was separated from the blood. The concentration of plasma insulin was determined using an RI-13K RIA kit (Linco, St. Charles, MO), and the concentrations of glucose and lactate were measured using a biochemical analyzer (Yellow Springs Instruments, Yellow Springs, OH).

Real-Time PCR

Wt-, cv-, and si-MiaPaCa2 cells were incubated in serum-free DMEM for 4 hours under normoxic or hypoxic conditions. Total RNA was extracted using the RNAqueous-4PCR kit (Ambion, Austin, TX). For extraction of total RNA from tumor grafts, the frozen tumors were ground physically and suspended in lysis buffer. The preparation was plunged through a 20-gauge needle several times and then processed like the cultured cells. For a HIF-1α/18S duplex real-time PCR, cDNAs were synthesized by incubating the following together for 60 minutes at 42°C: 1 μg of RNA, 0.5 μg of oligo(dT), the primers for 18S rRNA (400 pmol/L), and a master mix containing concentrated reaction buffer, AMV reverse transcriptase (0.8 U), $MgCl_2$, and RNasin (Promega, Madison, WI). The resulting cDNA (50 ng) was mixed with the human HIF-1α TaqMan gene expression mix (assay ID, Hs00153153_m1) and the probe (500 nmol/L) and the forward/reverse primers (100 nmol/L) for 18S rRNA (Applied Biosystems, Foster City, CA). The reagents went through a phase of 50°C (2 minutes) and 95°C (10 minutes) in an ABI Prism 7000 sequence detection system and were then cycled 40 times between 95°C (15 seconds) and 60°C (1 minute). The resulting HIF-1α data were analyzed in relation to the concurrent 18S data.

Western Blot

Wt-, cv-, and si-MiaPaCa2 cells were incubated in serum-free DMEM for 6 hours under hypoxic or normoxic conditions. Nuclear and cytosolic proteins were extracted sequentially.[22] When tumor grafts were handled, tumor tissues were first homogenized in ice-cold lysis buffer with a Heidolph homogenizer and then processed as cultured cells. Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL). In different assays, nuclear or cytosolic proteins (30 μg) were separated on 6 to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, transferred to polyvinylidene difluoride membranes, and incubated with antibodies (Table 1). In general, a membrane was incubated with the primary antibody at 4°C overnight and the secondary antibody at room temperature for 1 hour. The membrane was treated with enhanced chemiluminescence detection reagents (Amersham Biosciences, Buckinghamshire, UK). Specific blotting was recorded on film in a dark room. When necessary, the blotting was scanned and digitalized, and the data were analyzed using ImageJ software (version 1.62; National Institutes of Health, Bethesda, MD).

Assays for Other Trophic Effects of Insulin in Vitro

Wt-, cv-, and si-MiaPaCa2 cells were seeded in 96-well culture plates (5×10^4/well) and were cultured in DMEM

(10% FCS) for 3 days. The cells were incubated for 6 hours under hypoxic conditions in serum-free DMEM. The test media contained 0.1% bovine serum albumin, insulin (0 to 1000 nmol/L), and [^{3}H]thymidine (1 μCi/ml; Amersham). Afterward, the cells were transferred to Filtermat-A papers (Perkin Elmer, Boston, MA), and radioactivity was measured. The concentrations of VEGF in the media were determined using a human VEGF immunoassay kit (R&D Systems, Stockholm, Sweden), and the concentrations of glucose and lactate were determined as in mouse plasma.

Immunohistochemistry

Cryosections were prepared from frozen tumors and fixed in acetone. The sections (10 μm thick) were stained with hematoxylin and eosin (H&E). Before H&E staining, some sections were also stained by immunohistochemistry to show cells that expressed proliferating cell nuclear antigen (PCNA) or the endothelial cell marker CD31. A mouse monoclonal anti-PCNA antibody (Abcam 912, 1:100; Cambridge, UK), a rabbit anti-CD31 antibody (IHC-03300P, 1:200; Bethyl Laboratories, Montgomery, TX), and Vector ABC kits (Vector Laboratories, Burlingame, CA) were used in the immunostaining.

Statistics

Statistical analysis was done with Instat software (version 1.12). Tumorigenicity data were assessed by χ^2 analysis. Other data are expressed as means ± SEM. When three or more groups were involved, the data were analyzed using analysis of variance followed by the Bonferroni posttest. When fewer groups were involved, the Student's *t*-test was used. $P < 0.05$ was considered significant.

Results

HIF-1α Expression

Under normoxic conditions, similar HIF-1α mRNA levels were seen in wt- and cv-MiaPaCa2 cells (Figure 1A), suggesting that the control virus did not affect HIF-1α mRNA levels. In normoxic si-MiaPaCa2 cells, HIF-1α mRNA levels were reduced to 42 ± 3% of the control values ($P < 0.01$, Figure 1A), suggesting that these cells formed siRNA$_{\text{HIF-1}\alpha}$-based RNA-induced silencing complexes that degraded the HIF-1α mRNA. Moderate decreases (20 to 30%) in HIF-1α mRNA were found in hypoxic wt-, cv-, and si-MiaPaCa2 cells compared to the HIF-1α mRNA in the same cells under normoxia (Figure 1A). During hypoxia, insulin increased HIF-1α mRNA in wt- and cv-MiaPaCa2 cells in a dose-dependent manner but had no effects on HIF-1α mRNA in si-MiaPaCa2 cells (Figure 1B). Insulin had no significant effects on HIF-1α mRNA in normoxic wt-MiaPaCa2 cells (Figure 1B, inset).

Normoxic wt-MiaPaCa2 cells frequently showed low levels of HIF-1α protein, and the HIF-1α expression was moderately enhanced when insulin was added to the culture medium (Figure 2A). In some individual experiments, however, normoxic MiaPaCa2 cells failed to show any HIF-1α protein, and insulin induced hardly any HIF-1α expression in these cells (data not shown). Under normoxic conditions, cv-MiaPaCa2 cells expressed HIF-1α protein as wt-MiaPaCa2 cells, and si-MiaPaCa2 cells did not express detectable HIF-1α protein (data not shown). After a 6-hour hypoxic incubation, wt-MiaPaCa2 cells showed HIF-1α protein expression (Figure 2A). Hypoxia-induced HIF-1α expression was further enhanced when insulin was added to culture medium (Figure 2, A and D). In hypoxic cv-MiaPaCa2 cells, basal HIF-1α protein levels and insulin-induced HIF-1α protein expression were similar to those seen in hypoxic wt-MiaPaCa2 cells (Figure 2, B and D). Hypoxic si-MiaPaCa2 cells showed low levels of HIF-1α protein expression in both the presence and absence of insulin (Figure 2, C and D).

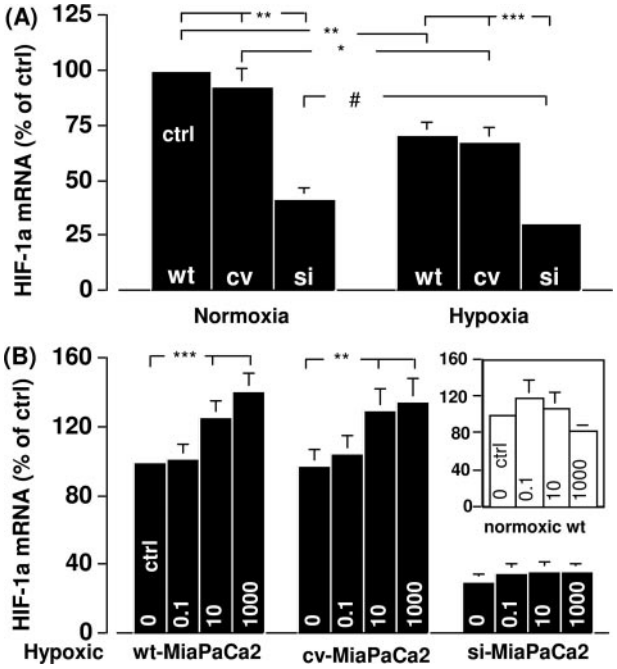

Figure 1. HIF-1α mRNA expression. Wt-MiaPaCa2 (wt) and cv-MiaPaCa2 (cv) (which both have intact HIF-1α expression) and si-MiaPaCa2 (si) cells, which express a specific siRNA targeting HIF-1α, were incubated for 4 hours under normoxic or hypoxic (1% O_2) conditions in serum-free culture medium. RNA was isolated, and duplex real-time PCR was conducted to determine HIF-1α and 18S mRNAs. HIF-1α data were corrected with concomitant 18S data. HIF-1α mRNA in the wild-type cells under normoxic conditions (ctrl) was used as the baseline to which the results in other cells were related. $n = 6$ to 8; $^{\#}P = 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. **A:** Wt, cv, and si cells under normoxia and hypoxia. **B:** Hypoxic wt, cv, and si cells and normoxic wt cells (**inset**) in media containing different concentrations (nmol/L) of insulin as indicated in the bars.

Trophic Effects of HIF-1α and Insulin on MiaPaCa2 Cells

Glucose consumption, lactate production, VEGF secretion, and thymidine uptake were decreased in si-MiaPaCa2 cells after a 6-hour hypoxic incubation compared with the values seen in wt- and cv-MiaPaCa2 cells (Figure 3). Under hypoxic conditions, insulin increased glucose consumption, lactate production, VEGF secretion, and thymidine uptake in wt- and cv-MiaPaCa2 cells

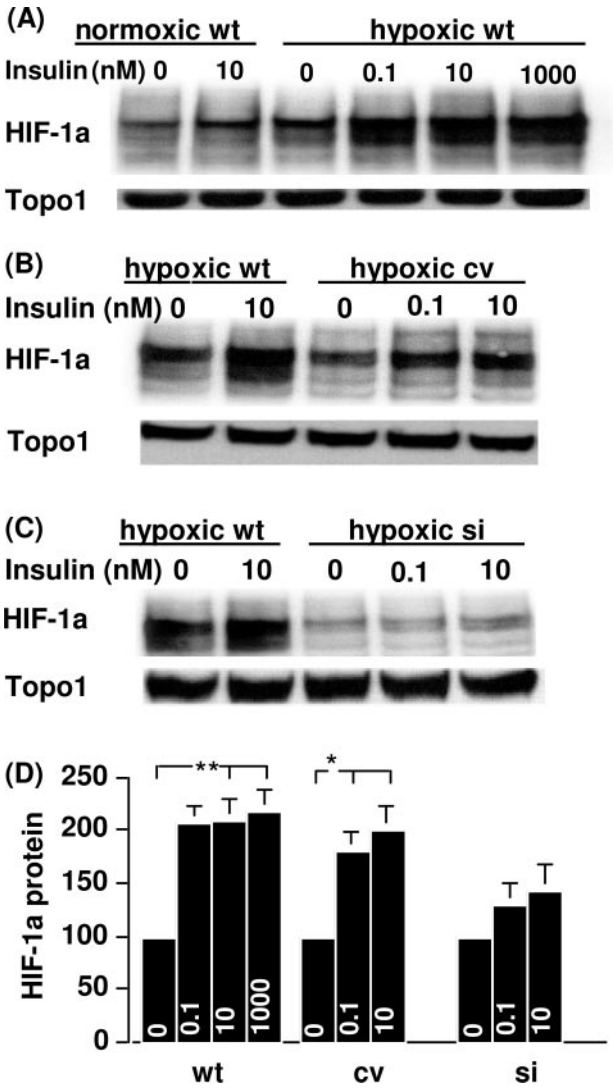

Figure 2. HIF-1α protein expression. Wt-, cv-, and si-MiaPaCa2 cells were incubated in serum-free culture medium for 6 hours under normoxia or hypoxia. Human insulin was added to test media as indicated. HIF-1α protein expression was assessed by Western blot, using Topo-1 as the loading control. **A:** The expression of HIF-1α in wt-MiaPaCa2 cells (wt) under normoxia or hypoxia. **B** and **C:** Basal and insulin-stimulated HIF-1α expression in hypoxic cv-MiaPaCa2 (cv) and si-MiaPaCa2 (si) cells, using HIF-1α expressions in hypoxic wt cells as controls. **D:** The intensity of HIF-1α blotting in relation to that of Topo-1 blotting in hypoxic wt, cv, and si cells in media containing different concentrations (nmol/L) of insulin as indicated in the bars. Results are derived from four experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

but not si-MiaPaCa2 cells (Figure 4). Under hypoxic conditions, the expression of hexokinase was similar between wt- and cv-MiaPaCa2 cells but reduced in si-MiaPaCa2 cells (Figure 5A). Insulin increased hexokinase and/or aldolase expression in hypoxic wt- and cv-MiaPaCa2 but not si-MiaPaCa2 cells (Figure 5, B and C).

Tumorigenicity and Graft Analysis

During weeks 12 to 15, three wt-MiaPaCa2 recipients and one cv-MiaPaCa2 recipient died of pancreatic cancer. The other mice were sacrificed at the end of week 15. Taken together, six of eight wt-MiaPaCa2 recipients and

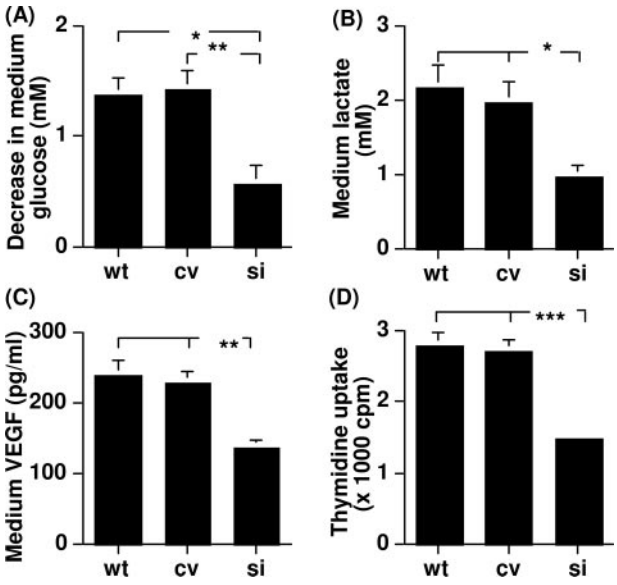

Figure 3. Involvement of HIF-1α in the biology of MiaPaCa2 cells in the absence of insulin. Wt-MiaPaCa2 (wt), cv-MiaPaCa2 (cv), and si-MiaPaCa2 (si) cells were plated in different wells (5×10^4/well) and cultured in serum-containing medium for 3 days. The different MiaPaCa2 cells were incubated under hypoxic conditions for 6 hours. Test media contained radioactive thymidine. After incubation, culture media were analyzed to determine decrease in glucose concentration (compared to original glucose concentration, **A**) and the accumulation of lactate (**B**) and VEGF (**C**). **D:** Thymidine uptake was measured in cell homogenates. Results are derived from six experiments. In each experiment, each test condition involved three wells of cells. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

seven of eight cv-MiaPaCa2 recipients had pancreatic tumors (collectively, 13 of 16, 81%), whereas three of nine (33%) si-MiaPaCa2 recipients had pancreatic tumors ($P < 0.05$, si-MiaPaCa2 recipients versus wt- and cv-MiaPaCa2 recipients combined). In further analyses, the results from wt- and cv-MiaPaCa2 recipients were combined.

No significant difference was found in the weights of pancreatic tumors composed of wt/cv-MiaPaCa2 cells (1.4 ± 0.3 g, $n = 13$) or si-MiaPaCa2 cells (1.6 ± 0.5 g, $n = 3$). HIF-1α mRNA and protein contents in wt/cv-MiaPaCa2 tumors were significantly greater than in si-MiaPaCa2 tumors (Figure 6A). Wt/cv-MiaPaCa2 tumors also had greater hexokinase expression than si-MiaPaCa2 tumors (Figure 6B). All three si-MiaPaCa2 tumors had central necrosis as shown in Figure 7A. Of three wt/cv-MiaPaCa2 tumors examined, two tumors had no central necrosis (Figure 7B) and one had minor central necrosis. Blood vessels were more numerous in wt/cv-MiaPaCa2 tumors than in si-MiaPaCa2 tumors (Figure 7, A and B). In si-MiaPaCa2 tumors, proliferating cells (PCNA-positive) were usually found as close to the periphery of the tumor or to intratumoral vessels (Figure 7, C and D). The relation of PCNA-positive cells to the periphery of the tumor or to intratumoral vasculature was also true for tumors composed of wt/cv-MiaPaCa2 cells (data not shown). In addition, sporadic PCNA-positive cells were found in the inner regions of the wt/cv-MiaPaCa2 tumors (Figure 7E). The inner regions of wt/cv-MiaPaCa2 tumors also had blood vessels (Figure 7F).

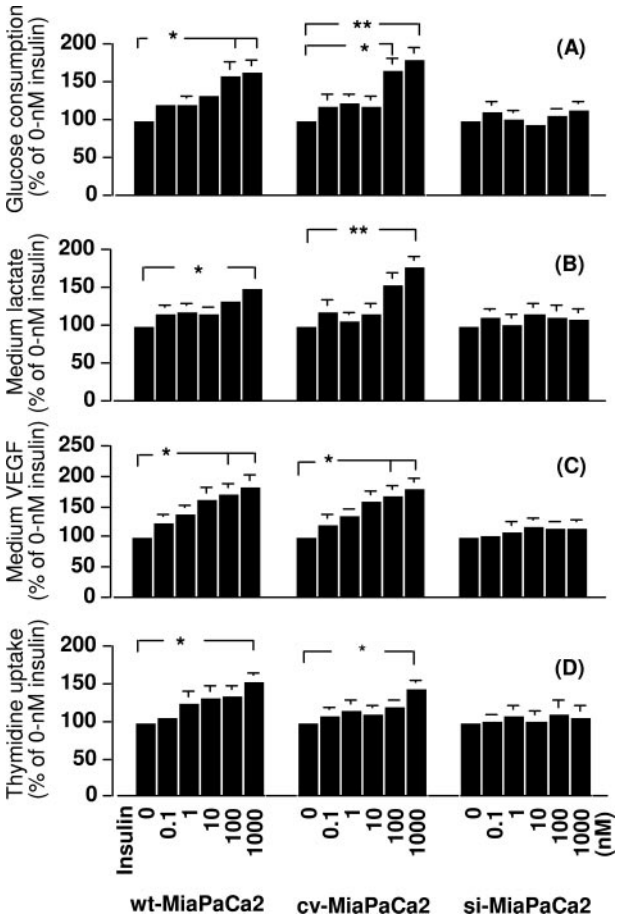

Figure 4. Involvement of HIF-1α in the biology of MiaPaCa2 cells in the presence of insulin. Cells were cultured as described in Figure 3, but insulin was added to the test media in concentrations of 0.1 to 1000 nmol/L. After incubation, test media and cell homogenates were examined as described in Figure 3. The present figure shows the effects of insulin on glucose consumption (ie, the difference between original and measured glucose concentrations, **A**), lactate production (**B**), VEGF secretion (**C**), and thymidine uptake (**D**) in each cell line, using the values in the absence of insulin as 100%. Results are derived from six individual experiments. In each experiment, each condition was tested in three wells of cells. $^{*}P < 0.05$ and $^{**}P < 0.01$.

Host Energy Homeostasis

Normal glucose tolerance was seen in all groups of mice between weeks 4 to 15 (data not shown). When the week 15 intraperitoneal glucose tolerance test data were analyzed after nontumor-bearing MiaPaCa2 recipients were excluded, the same result was obtained (Figure 8A). However, the glucose excursion appeared to be exaggerated in wt/cv-MiaPaCa2 hosts compared to the other groups, although the difference was not statistically significant. At the time of sacrifice, plasma concentrations of insulin, glucose, and lactate were not significantly different between normal mice and any of the MiaPaCa2 recipient groups (data not shown).

No significant difference was found in body weight gains between normal control mice and any MiaPaCa2 recipient group during the 15-week experiment (Figure 8B). From week 4, the normal mice and the wt/cv-MiaPaCa2 recipients showed the biggest and the smallest values, respectively, although the differences were not statistically significant (Figure 8B). After nontumor-bearing MiaPaCa2 recipients were excluded, a significant difference was seen in final body weight gains (last body weight minus first body weight minus tumor weight) between mice with wt/cv-MiaPaCa2 tumors and normal mice ($P < 0.01$), but body weight gains were not significantly different between the mice with si-MiaPaCa2 tumors and the controls (Figure 8C).

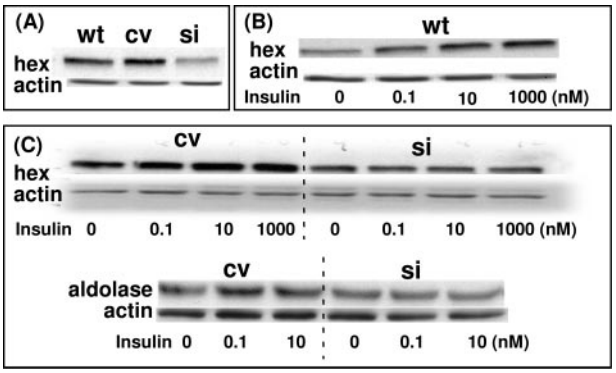

Figure 5. Expression of hexokinase and aldolase in wt-, cv-, and si-MiaPaCa2 cells. Wt-MiaPaCa2 (wt), cv-MiaPaCa2 (cv), and si-MiaPaCa2 (si) cells were incubated under hypoxic conditions for 6 hours. Cytosolic proteins were extracted and examined by Western blotting assays. β-Actin staining was used as the loading control. **A:** Expression of hexokinase (hex) in wt, cv, and si cells. **B:** Expression of hexokinase in wt cells in the presence of insulin as indicated. **C:** Expression of hexokinase and aldolase in cv and si cells in the presence of insulin as indicated.

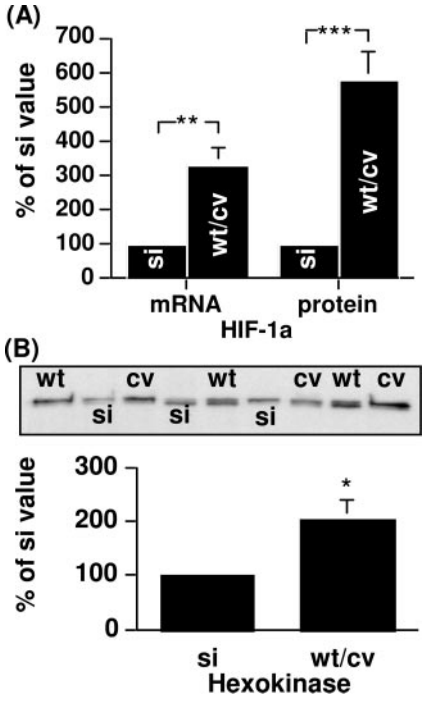

Figure 6. Expression of HIF-1α and hexokinase in tumor xenografts. Wt-MiaPaCa2 (wt), cv-MiaPaCa2 (cv), and si-MiaPaCa2 (si) cells were transplanted in the pancreas of athymic mice for 15 weeks. HIF-1α and hexokinase expression was examined in three si tumors and nine wt/cv tumors. **A:** HIF-1α mRNA and protein were determined by real-time PCR and Western blotting. The results in three si tumors were used as references to which the results in nine wt/cv tumors were related. $^{**}P < 0.01$, $^{***}P < 0.001$. **B:** The expression of hexokinase in tumors composed of wt, cv, and si cells (top) and relative hexokinase expression in these tumors (bottom). $^{*}P < 0.05$.

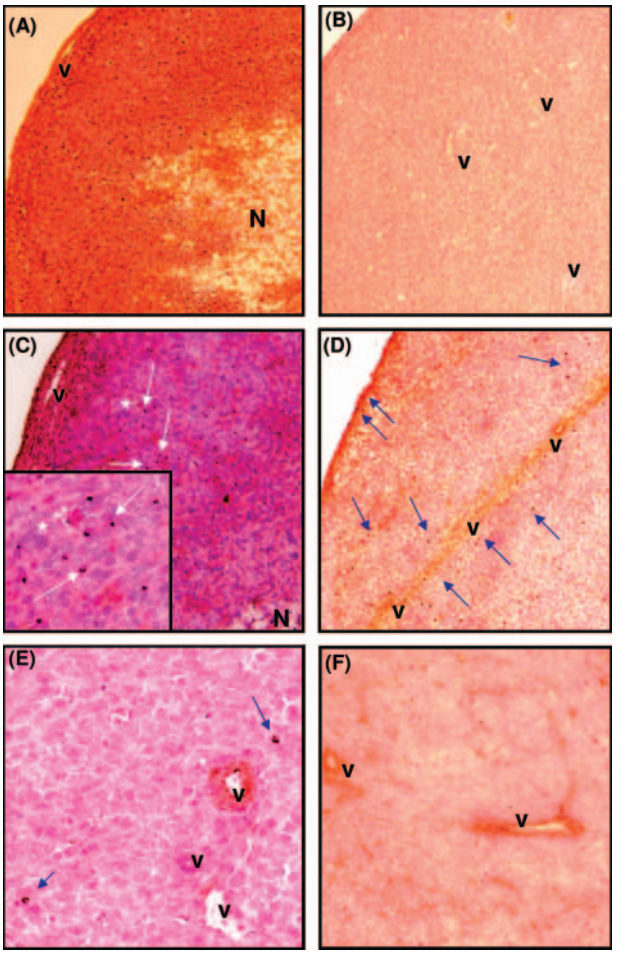

Figure 7. Morphology of tumor xenografts. Cryosections were prepared from three si-MiaPaCa2 tumors and three wt/cv-MiaPaCa2 tumors. All sections were stained with H&E. Some sections were also stained by immunohistochemistry to show the cells that expressed the proliferating cell nuclear antigen (PCNA, **C–E**) or the endothelial cell marker CD31 (**F**). N, necrosis; v, vessel. **Arrows** indicate typical PCNA-positive cells. **A:** A representative section of a si-MiaPaCa2 tumor. **B:** A representative section of a wt/cv-MiaPaCa2 tumor. **C** and **D:** PCNA-positive cells in a si-MiaPaCa2 tumor. A landmark (*) indicates the origin of the **inset** in **C**. **E:** Sporadic PCNA-positive cells in inner region of a wt/cv-MiaPaCa2 tumor. **F:** A section of a wt/cv-MiaPaCa2 tumor stained with anti-CD31 antiserum. Original magnifications: ×40 (**A**, **B**); ×100 (**C**, **D**, **F**); ×400 [**C** (**inset**), **E**].

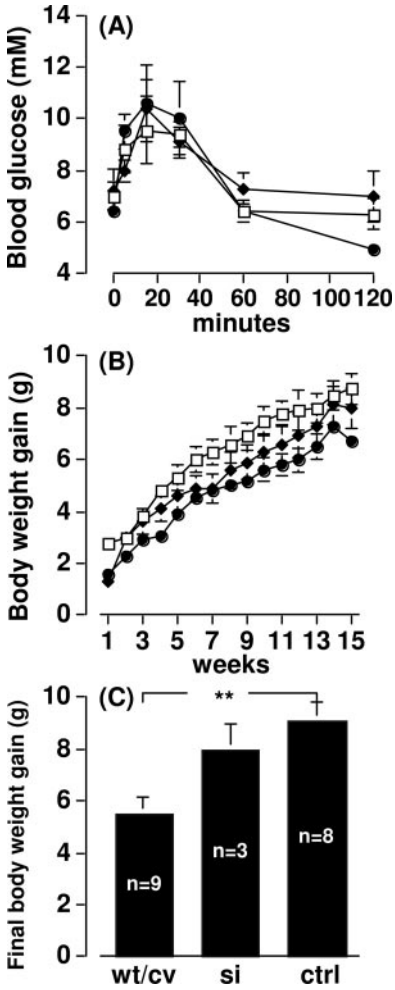

Figure 8. Energy homeostasis in athymic mice hosting pancreatic tumors. **A:** Intraperitoneal glucose tolerance test in normal mice (**open squares**, $n = 8$) and their littermates carrying pancreatic tumors composed of wt/cv-MiaPaCa2 (**filled circles**, $n = 9$) or si-MiaPaCa2 cells (**filled diamonds**, $n = 3$). **B:** Cumulative body weight gains in 16 wt/cv-MiaPaCa2 cell recipients (**filled circles**), nine si-MiaPaCa2 cell recipients (**filled diamonds**), and eight normal mice (**open squares**). Four wt/cv-MiaPaCa2 recipients died during weeks 12 to 15. **C:** Final body weight gains (last body weight minus first body weight minus tumor weight) in nine wt/cv-MiaPaCa2 tumor carriers (wt/cv), three si-MiaPaCa2 tumor carriers (si), and eight normal controls (ctrl). $^{**}P < 0.01$.

Discussion

Previous studies have reported stimulatory, inhibitory, or no effects of hypoxia on HIF-1α mRNA in different cells.[9,23,24] In the present study, hypoxia moderately decreased HIF-1α mRNA in MiaPaCa2 cells. In a recent study, similar inhibition of HIF-1α mRNA by hypoxia was found in human A549 cells.[24] Because HIF-1 is thought to help cells resist hypoxic stress, the inhibitory effect of hypoxia on HIF-1α mRNA is intriguing. The decreased HIF-1α mRNA may result from suppression in gene transcription that occurs when cells are subjected to hypoxic stress.[25] In the present study, hypoxia was found to induce HIF-1α protein expression in wt- and cv-MiaPaCa2 cells despite the concomitant decrease in HIF-1α mRNA. This suggests that the HIF-1α protein expression induced by hypoxia was so efficient that it overwhelmed any negative effects associated with reduced HIF-1α mRNA.

In vitro, insulin and growth factors increase HIF-1α protein in both benign and cancer cells.[16–18,26–28] It is known that these HIF-1α stimuli motivate intracellular signal cascades leading to HIF-1α biosynthesis.[17,18] The present results suggest that intratumoral hypoxia is a prerequisite for interstitial insulin to have a significant effect on HIF-1α expression. Because intratumoral hypoxia is common in pancreatic cancer,[19] pancreatic cancer cells are subjected to a hypoxic environment *in vivo*. The present hypoxia/insulin cooperation seen in MiaPaCa2 cells *in vitro* may reflect a hypoxia/insulin interaction that affects HIF-1α expression in pancreatic cancer cells *in vivo*.

In a previous study, insulin exposure did not change HIF-1α mRNA in DU145 human prostate cancer cells

during normoxia.[29] Likewise, insulin did not increase HIF-1α mRNA in normoxic wt-MiaPaCa2 cells in the present study. Under hypoxic conditions, however, insulin increased HIF-1α mRNA significantly in wt- and cv-MiaPaCa2 cells. The effect of insulin on HIF-1α mRNA was not as strong as the effect of insulin on HIF-1α protein. In addition, the lowest insulin concentration required to increase HIF-1α mRNA was higher than that needed to increase HIF-1α protein. Therefore, the effects of insulin on HIF-1α mRNA and HIF-1α protein appear to involve different pathways.

In the present study, inhibition of HIF-1α expression was associated with decreases in cell proliferation, glycolysis, and VEGF production in MiaPaCa2 cells under hypoxic conditions. These results are in line with data suggesting that HIF-1 plays a master role in cell survival. In addition, we found that the stimulating effects of insulin on cell proliferation, glycolysis, and VEGF production were also dependent on the expression of HIF-1α in hypoxic MiaPaCa2 cells. Thus, insulin induces HIF-1 expression in MiaPaCa2 cells, and HIF-1 in turn enhances other biological effects of insulin in the same tumor cells. The interaction between HIF-1 and insulin is comparable with the interaction found between HIF-1 and IGFs produced by the same tumor cells.[30,31]

In the present study, si-MiaPaCa2 cells formed tumor xenografts less frequently than wt/cv-MiaPaCa2 cells. Given that si-MiaPaCa2 cells were unresponsive to insulin *in vitro*, it is conceivable that interstitial insulin in the mouse pancreas did not have as strong an effect on these cells as on the wt/cv-MiaPaCa2 cells. Without the stimulation of insulin on cell glycolysis and VEGF production, the chance for si-MiaPaCa2 cells to survive *in vivo* was reduced. The finding that si-MiaPaCa2 tumors had central necrosis was in keeping with the knowledge that HIF-1 plays a key role in cell viability and survival. The central necrosis may also be related to the shortage of blood vessels in the si-MiaPaCa2 tumors. In both si-MiaPaCa2 and wt/cv-MiaPaCa2 tumors, proliferating cells were usually seen adjacent to tumor surface or to blood vessels, suggesting that the access to microcirculation was necessary for the growth of the cells.

Tumors composed of si-MiaPaCa2 cells expressed less hexokinase than wt/cv-MiaPaCa2 tumors, suggesting that glycolysis in si-MiaPaCa2 cells was reduced *in vivo* as well as *in vitro*. If so, si-MiaPaCa2 tumors may have consumed less glucose and released less lactate than the wt/cv-MiaPaCa2 tumors. Therefore, the negative effect of the si-MiaPaCa2 tumors on glucose turnover and energy homeostasis may have been less than that of the wt/cv-MiaPaCa2 tumors. The decrease in body weight gain compared with normal mice that was seen in mice with wt/cv-MiaPaCa2 tumors but not in mice with si-MiaPaCa2 tumors supports this hypothesis. Unlike human pancreatic cancer that usually occurs in adults, pancreatic cancer was induced in growing mice in the present study. This may explain why the effect of disturbed energy homeostasis on body weight was seen in this study as a smaller increase in body weight, rather than an absolute decrease as is usually seen in pancreatic cancer patients.[2]

In the current study, glucose tolerance and glucose-stimulated insulin secretion were not altered in mice with tumors composed of MiaPaCa2 cells. This result is not consistent with the prevalence of glucose intolerance, impaired insulin release, and diabetes in human pancreatic cancer patients.[1,32] Therefore, in terms of glucose homeostasis, the mice tolerated human pancreatic cancer cells better than people do. The mechanisms underlying this species difference are unknown. However, human patients are usually exposed to high-fat diet, obesity, cigarette smoking, and insulin resistance before developing pancreatic cancer.[33,34] These risk factors for pancreatic cancer also strain glucose homeostasis and may make it susceptible to further insult. When pancreatic cancer develops, glucose homeostasis may succumb easily to tumor-derived influences.

In summary, we have shown that insulin increased HIF-1α mRNA and protein in MiaPaCa2 cells under hypoxic conditions, and the resultant HIF-1 mediated the trophic effects of insulin on the cells. In particular, HIF-1-mediated glycolysis in the tumor cells appeared to accelerate glucose turnover in the host and thereby participate in disarranged energy homeostasis in the host. The present results may not be reproduced in human pancreatic cancer cells that do not express HIF-1. In other HIF-1-expressing pancreatic cancer cells, however, HIF-1 may regulate cell survival and glycolysis, as it did in MiaPaCa2 cells in the present study.

Acknowledgments

We thank Iréne Engström, Annika Wagman, and Beth Hagman for technical assistance; Dr. Rene Henig (Heidelberg University, Heidelberg, Germany) for demonstration of cell transplantation into the pancreas of athymic mice; and Professor Margery K. Herrington (Adams State College, Alamosa, CO) for scientific and linguistic comments when we prepared the manuscript.

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