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Author(s)	Natsuizaka, Mitsuteru; Ozasa, Mariko; Darmanin, Stephanie; Miyamoto, Masaki; Kondo, Satoshi; Kamada, Suguru; Shindoh, Masanobu; Higashino, Fumihiro; Suhara, Wakako; Koide, Hideyuki; Aita, Kiyoshi; Nakagawa, Koji; Kondo, Takeshi; Asaka, Masahiro; Okada, Futoshi; Kobayashi, Masanobu
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Synergistic up-regulation of Hexokinase 2, glucose transporters and angiogenic factors  
in pancreatic cancer cells by glucose-deprivation and hypoxia

Mitsuteru Natsuizaka <sup>1,2</sup>, Mariko Ozasa <sup>1,2</sup>, Stephanie Darmanin <sup>3</sup>, Masaki Miyamoto <sup>5</sup>,  
Satoshi Kondo <sup>5</sup>, Satoru Kamada <sup>6</sup>, Masanobu Shindoh <sup>7</sup>, Fumihiro Higashino <sup>7</sup>,  
Wakako Suhara <sup>8</sup>, Hideyuki Koide <sup>8</sup>, Kiyoshi Aita <sup>8</sup>, Koji Nakagawa <sup>8</sup>, Takeshi Kondo <sup>2</sup>,  
Masahiro Asaka <sup>2</sup>, Futoshi Okada <sup>9</sup> and Masanobu Kobayashi <sup>1,4,8</sup>

<sup>1</sup>Department of Pathological Oncology, Division of Cancer Medicine, <sup>2</sup>Department of  
Gastroenterology and Hematology, <sup>5</sup> Department of Surgical Oncology, Hokkaido  
University Graduate School of Medicine, <sup>3</sup> Division of Cancer-Related Gene, <sup>4</sup> Division  
of Cancer Biology, Institute for Genetic Medicine, Hokkaido University, <sup>6</sup> Department  
of Oral and Maxillofacial Surgery, <sup>7</sup> Department of Oral Pathobiological Science,  
Hokkaido University Graduate Dental School, <sup>8</sup> Oncorex Inc., <sup>9</sup> Department of  
Biomolecular Function, Yamagata Graduate School of Medical Science.

Running title: Adaptation responses to hypoxia and glucose deprivation

Correspondence: M Kobayashi MD & PhD, Division of Cancer Biology, Institute for  
Genetic Medicine, Hokkaido University. Kita-15, Nishi-7, Kita-Ku, Sapporo 060-0815  
Japan. TEL: 81-11-706-4770, FAX; 81-11-706-4770, e-mail:  
mkobaya@igm.hokudai.ac.jp

## ABSTRACT

There is accumulating evidence demonstrating that HIF-1 functions as a key regulator of the adaptation responses to hypoxia in cancer tissues. To this evidence, we add that adaptation responses to glucose-deprivation plus hypoxia are also necessary for the survival of tumor cells in the tumor microenvironment, as cancer tissues are exposed to glucose-deprivation as well as hypoxia. We found that adrenomedullin (AM), VEGF, Glut-1, Glut-3, and Hexokinase-2 among 45 hypoxia-inducible genes investigated were expressed at higher levels under glucose-deprived hypoxic conditions than under hypoxic conditions. Glucose-deprivation activated the AMPK under normoxia and hypoxia. Compound C, an inhibitor of AMPK, suppressed the expressions of AM and VEGF which had already been enhanced under glucose-deprived hypoxic conditions. SiRNAs for both AMPK $\alpha$ 1 and AMPK $\alpha$ 2 suppressed the expressions of AM and VEGF. HIF-1 $\alpha$  protein level and the transcriptional activity of HIF-1 under glucose-deprived hypoxic conditions were thus found to be similar to those under hypoxic conditions. Furthermore, tumor cells in 15 out of 20 human pancreatic cancer tissue specimens were stained by anti-phospho-AMPK $\alpha$  antibody. Our results thus suggest that the enhanced expressions of those genes mediated by the activation of AMPK and HIF-1 therefore

play a pivotal role in the tumor formation of pancreatic cancers.

**Key words:** Hypoxia, HIF, AMPK, and glucose-deprivation

## INTRODUCTION

As tumors increase in size, the cancer cells are exposed to heterogeneous microenvironments, with some regions displaying a significant lack of critical metabolites including oxygen, glucose, other nutrients and growth factors [1-4]. Therefore, angiogenesis is necessary for malignant growth of solid tumor cells and its involvement has been confirmed in various clinical cancers [5-7]. Tissue hypoxic response is critical for maintaining the microenvironmental homeostasis and is sophisticatedly regulated by complex mechanisms. Hypoxia-inducible factor-1 (HIF-1) is a central regulator of these hypoxic responses [1-3]. Once it has been activated, HIF-1 stimulates transcription of a series of genes for adaptation to hypoxia including angiogenic factors, glycolytic enzymes and glucose transporters. These responses explain very well how tumor cells survive under hypoxic conditions; however, these responses are insufficient for survival of the tumor cells in the tumor tissues, which are simultaneously exposed to glucose deprivation as well as to hypoxia. Glucose deprivation has been reported to disrupt protein folding in the endoplasmic reticulum (ER), resulting in the activation of the unfolded protein response (UPR), which enhances cell survival by limiting accumulation of unfolded or misfolded proteins in the ER and activating the transcription of the ER-resident molecular

chaperones [8-11]. Glucose deprivation has also been reported to activate an AMPK pathway [12], which enhances transcription of genes involved in lipid and glucose metabolism [13]. We have recently reported that glucose deprivation enhances the expressions of more than 60 genes including asparagine synthetase to protect the cells from apoptosis induced by glucose deprivation [14]. These reports suggest that the expressions of several genes are up-regulated under glucose-deprived conditions. We aimed to determine further whether the expressions of hypoxia-inducible genes essential for the survival of cancer cells under hypoxic conditions are up-regulated under glucose-deprived hypoxic conditions (HL conditions). As pancreatic cancers are continuously exposed to such severe conditions because of their hypovasculature as demonstrated by imaging diagnosis (15, 16), we selected pancreatic cancer cell lines in this study. We hypothesized that pancreatic cancer cells should acquire potential adaptation machineries responding to hypoxia plus glucose-deprivation for their growth and survival and thus they over-express some hypoxia-inducible genes essential for their growth and survival also under HL conditions.

To test the above hypothesis, we sought the genes whose expressions were enhanced in HL conditions by a DNA microarray analysis of a pancreatic cancer cell line. We found that 9 out of 45 known hypoxia-inducible genes were expressed under

HL conditions at 2-fold higher than under normoxic normal glucose conditions (NN conditions). We then found that five out of the nine hypoxia-inducible genes were expressed at higher levels under HL conditions than under hypoxic conditions in five cancer cell lines including three pancreatic cancer cell lines. To explore the mechanisms responsible for the enhanced expressions of those genes under HL conditions, we first examined the AMPK activity and the effects of an AMPK inhibitor and AMPK siRNAs respectively, on the expression of those genes, as AMPK has been reported to be an energy sensor [12]. We next measured the HIF-1 $\alpha$  protein levels and the transcriptional activity of HIF-1 under different conditions. Finally, we examined the expression of phosphorylated AMPK in the tumor cells of 20 human pancreatic cancer tissue specimens to explore the possible roles of phosphorylated AMPK in human pancreatic cancers.

## Materials and Methods

### *Cell culture*

HeLa (cervical cancer), HepG2 (hepatoma), BxPC3 (pancreatic cancer), PCI-43 (pancreatic cancer) and MiaPaCa2 (pancreatic cancer) were cultured in Dulbecco's modified Eagle medium (glucose 1000mg/l, Nissui Pharmaceutical Co., Ltd., Japan) supplemented with 10% fetal bovine serum (FBS, Japan Bioserum Co., Ltd., Japan), penicillin (25U/ml) and streptomycin (25 $\mu$ g/ml). According to the previous definition of tumor hypoxia (median pO<sub>2</sub> < 10mmHg, approximately 1.25% O<sub>2</sub>), the cells in the hypoxic group were incubated at 1 % O<sub>2</sub> in a hypoxic chamber (Wakenyaku Co. Ltd., Tokyo) gassed with 1% O<sub>2</sub>, 94% N<sub>2</sub> and 5% CO<sub>2</sub>. For the cultures under glucose-deprived condition, glucose-free Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and an appropriate amount of glucose (final concentration of glucose: 50mg/dl) was used, unless otherwise specified. The cells used for the experiments were mainly MiaPaCa2 cells, unless otherwise specified.

### *Antibodies and chemical reagents*

Anti-HIF-1 $\alpha$ , anti-aldolase A, and anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-adrenomedullin antibody was from

Alpha Diagnostic International (San Antonio, TX). Anti-AMPK $\alpha$  and anti-phospho-AMPK $\alpha$  antibodies were purchased from Cell Signaling Technology (Danvers, MA) and anti-AMPK $\alpha$ 1 and anti-AMPK $\alpha$ 2 antibodies were from Upstate (Chicago, IL). Peroxidase-conjugated goat and rabbit antibodies to anti-mouse IgG, anti-rabbit IgG, and anti-goat IgG, respectively, were from Jackson Immuno Research Laboratories (West Grove, PA). Phosphatase inhibitor cocktails 1 and 2 were purchased from SIGMA (St. Louis, MO), and a protease inhibitor cocktail (Complete, Mini, EDTA-free) was obtained from Roche (Basel, Switzerland). Actinomycin D was purchased from Wako Pure Chemical Industries (Tokyo, Japan), and AMPK inhibitor, compound C, was from Calbiochem (Darmstadt, Germany).

***DNA microarray analysis:***

Total RNA was extracted with the use of TRIZOL Reagent (LIFE TECHNOLOGIES, Tokyo, Japan) from the MiaPaca2 cells that had been incubated for 16 hours under glucose-deprived hypoxic conditions and normal-glucose normoxic condition. Incubation under hypoxic conditions (1 % O<sub>2</sub>) was done in a hypoxic chamber gassed with 94 % N<sub>2</sub> and 5 % CO<sub>2</sub> (Wakenyaku Co. Ltd., Tokyo). The glucose concentrations were 10 mg/dl and 100 mg/dl. mRNA was purified from the total RNA with the use of a Quickprep mRNA purification kit (Amersham Pharmacia

Biotech, Tokyo, Japan). The differentially expressed genes were screened by using a DNA microarray system (Hokkaido System Science, Sapporo, Japan). We defined the genes which were expressed at more than 2-fold intensity under HL conditions than under normal-glucose normoxic conditions as possible glucose-deprivation- and hypoxia-inducible genes in this study.

### ***Real-time PCR***

Total RNA was prepared by using the RNeasy® mini kit and RNase-free DNase Set (Qiagen, Valencia, CA). cDNA was synthesized by the use of M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Each cDNA (10 ng) was amplified in triplicate with the use of Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) and then it was detected on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The condition of real-time PCR was as follows: 50 °C for 2 min and 95 °C for 2min for the initial incubation, 95 °C for 15 sec for denaturing, 60 °C for 30 sec for annealing, 72 °C for 1 min for extension for 45 cycles, and for the last dissociation 95 °C for 15 sec and 60 °C for 15 sec and 95 °C for 15 sec. mRNA of  $\beta$ -actin was used to standardize the total amount of cDNA in real-time PCR. The primer pairs for 19 genes (adrenomedullin, VEGF, Glut-1, Glut-3, Hexokinase-1, Hexokinase-2, Enolase 1,

Aldolase A, Aldolase C, Heme oxygenase 1, Transferrin, Ceruloplasmin, Adenylate kinase, Carbonic anhydrase 9, plasminogen activator inhibitor, prolylhydroxylase 2, IGF2, NIP3, Endothelin 1) and  $\beta$ -actin used for real-time PCR are shown in Table 1.

### ***siRNA Preparation and Transfection***

SiRNAs for AMPK $\alpha$ 1 and AMPK $\alpha$ 2 were purchased from B-Bridge International, Inc (Sunnyvale, CA). The antisense sequences of siRNAs were as follows: uaucaaaauagcucuccTT, uuuccuuacaccuugguguTT and uugcucuacauacuucugcTT for AMPK $\alpha$ 1, and uuuuucacacacuucuuucTT, uaugcauucacuaccuuccTT and uuuaccuacaccaaacagcTT for AMPK $\alpha$ 2. A non-silencing control siRNA was purchased from Qiagen (Valencia, CA). SiRNA transfection was performed with Lipofectamine<sup>TM</sup> 2000 (Invitrogen, Carlsbad, CA) at 30% to 40% cell confluence in an antibiotic-free medium.

### ***Western Blotting***

The cells were lysed with cell lysis buffer (Cell Signaling, Danvers, MA) supplemented with 1mM PMSF and a protease inhibitor cocktail (Roche, Basel, Switzerland). Phosphatase inhibitor cocktails (SIGMA, St. Louis, MO) were used with the cell lysis buffer when a phospho-specific antibody was used. The samples were run on Tris-HCl or Bis-Tris gels and electro-transferred to PVDF membranes.

The membranes were blocked by the blocking buffer (5% skimmed milk or 1% bovine serum albumin in 0.1% Tween-PBS) for 1 hour, and then incubated with primary antibodies diluted in Can Get Signal<sup>TM</sup> (TOYOBO CO., LTD., Tokyo, Japan) overnight at 4 °C. After washing the membranes with Tween-PBS, the membranes were then incubated for 1 hour with secondary antibodies at room temperature. Development was done with the ECL detection kit (Amersham, Piscataway, NJ).

### ***Luciferase Assay***

The cells were seeded in 24-well plates at  $7 \times 10^4$  cells per well for 24 hours before transfection. Reporter vectors were transfected with Lipofectamine<sup>TM</sup> 2000. Five tandem repeats of the sequence 5'-CGCCCTACGTGCTGTCTCACACAGCCTGTCTGA-3', including a hypoxia-response element (HRE) from the erythropoietin gene or the sequence (4kbp) corresponding to the VEGF promoter from -1.5 kb to +1.5 kb, were inserted into the Rapid Response<sup>TM</sup> pGL3-basic vector (Promega, Madison, WI). The Rapid Response<sup>TM</sup> pRL-CMV Vector (Promega, Madison, WI) was transfected simultaneously and used as an internal transfection control. One hundred nanograms of the reporter vector pGL3-5xHRE or pGL3-proVEGF and 5 ng of pRL-CMV vector were transfected. The culture medium was changed 6 hours after the transfection, and then the cells were

cultured under different conditions for 24 hours. The luciferase activity was measured by using the Dual-Luciferase® Reporter assay system (Promega, Madison, WI) and ARVO™ MX 1420 multilabel counter (Perkin Elmer, Wellesley, MA) according to the manufacturer's instructions.

***Immunohistochemical staining:***

After deparaffinization and rehydration, antigen was unmasked by boiling the samples in 10mM sodium citrate buffer (pH 6.0) for 10 minutes. Next, the samples were incubated in the 0.3% hydrogen peroxide to quench the endogenous peroxidase activity. Antibody for phosphorylated AMPK $\alpha$  (Cell Signaling Technology, Danvers, MA) was diluted in DakoCytomation Antibody Diluent (Dako, Glostrup, Denmark) at 1:50. The expression of phosphorylated AMPK $\alpha$  was analyzed by immunocytochemical staining using Histofine SAB-PO kit (Nichirei, Tokyo) according to the manufacturer's instructions. DAB was used as a chromogen to visualize the reaction products. Finally, all the slides were counter-stained with Meyer's hematoxylin.

***In vivo tumor model:***

Five million MiaPaca cells were inoculated subcutaneously into the right flanks of SCID mice (n=5), and then the mice were sacrificed 45 days after the inoculation. For

hypoxia staining, 60 mg/kg intra-peritoneal pimonidazole (Hypoxyprobe-1 Plus Kit, Chemicon International, Temecula, CA) was injected into the mice 3 hours before sacrifice. After the sacrifice, the tumor was fixed in formalin and embedded in a paraffin block. Paraffin-embedded slides were incubated with an FITC-labeled anti-pimonidazole antibody for 45 min, thereafter with an HRP-labelled anti-FITC antibody for 30 min and then with Diaminobenzidine solution (Dako, Glostrup, Denmark). Finally, the slides were counter-stained with Meyer's hematoxylin.

***Statistical analysis:***

The statistical analysis was done by using Student's *t* test.

## RESULTS

### *Expression levels of hypoxia-inducible genes:*

DNA microarray analysis demonstrated that expressions of nine (adrenomedullin (AM), VEGF, Glut-1, Glut-3, Hexokinase-2, Heme oxidase-1, Plasminogen activator inhibitor 1, Prolylhydroxylase 2, and Hexokinase-1) out of forty-five known hypoxia-inducible genes were enhanced under glucose-deprived hypoxic (HL) conditions compared with under normal-glucose normoxic (NN) conditions (Table 2). We then confirmed the expressions of the nineteen genes (9 genes enhanced under HL conditions and 10 genes whose expressions were not enhanced under HL conditions in the DNA microarray analysis) in five cancer cell lines, including three pancreatic cancer cell lines, under four different conditions by real-time PCR. Real-time PCR demonstrated that expressions of AM, VEGF, Glut-1, Glut-3, and Hexokinase-2 were enhanced and thus reached higher levels under HL conditions than those under other conditions (Fig. 1a). Although the expressions of the five genes under HL conditions were not enhanced uniformly to similar levels in all cell lines, the expressions were enhanced at least in all of the three pancreatic cancer cell lines. We showed the expression levels of aldolase A, celluloplasmin, aldolase C, carbonic anhydrase 9 and adenylate kinase as representative genes whose expressions were not enhanced under

HL conditions (Fig. 1b). The expressions of these genes were enhanced under hypoxic conditions but not further enhanced under HL conditions. We then examined the expression levels of AM protein. It was expressed at the highest levels in the cells cultured under HL conditions (Fig. 1c), in accordance with the results obtained by real-time PCR.

To confirm the effects of glucose-deprivation on the expression levels of hypoxia-inducible genes, we examined the expression levels of AM under hypoxia and normoxia in the medium supplemented with different concentrations of glucose in MiaPaca2. Glucose-deprivation enhanced the expression of AM mRNA under hypoxia and normoxia (Fig. 1d). We next examined the expression levels of AM at the indicated time periods after incubation under different conditions. While hypoxia alone enhanced the expression of AM mRNA already at 6 hours after incubation, glucose-deprivation in addition to hypoxia enhanced the expression of AM mRNA at 12 to 16 hours after incubation (Fig. 1e).

*Activity of AMPK under HL conditions and the effects of an inhibitor of AMPK and siRNAs for AMPKs on the expressions of AM and VEGF:*

As AMPK has been reported to be an energy sensor [12] and be critical for the expression of VEGF [17], we first examined the activity of AMPK under normoxia and

hypoxia at different concentrations of glucose. Both glucose-deprivation and hypoxia increased the phosphorylation of AMPK and glucose deprivation plus hypoxia further increased the phosphorylation of AMPK (Fig. 2a). These results are consistent with the recent report [18]. Compound C, an inhibitor of AMPK, suppressed the enhanced expressions of AM and VEGF under HL conditions but not under HN conditions (Fig. 2b). Thereafter, we examined the effects of siRNAs for AMPK $\alpha$ 1 and AMPK $\alpha$ 2 on their expressions. The siRNAs for AMPK $\alpha$ 1 and AMPK $\alpha$ 2 efficiently suppressed the expressions of AMPK $\alpha$ 1 and AMPK $\alpha$ 2, respectively (Fig. 2c). They almost completely suppressed the enhanced expressions of AM and VEGF induced under HL conditions, whereas they did not show any effect on the expression of aldolase A (Fig. 2d). These results suggest that both AMPK $\alpha$  isoforms are implicated in the signal transduction pathway to enhanced expression of AM and VEGF under HL conditions.

*Protein levels and transcriptional activity of HIF-1:*

Although it has been reported that AMPK did not affect the HIF-1 $\alpha$  protein expression or nuclear translocation [19], expression levels of HIF-1 $\alpha$  protein under HL conditions have not been demonstrated. Therefore, we examined the protein levels of HIF-1 $\alpha$  protein under different conditions. HIF-1 $\alpha$  protein was expressed obviously under hypoxic conditions; however, the expression was not further enhanced under HL

conditions. (Fig. 3a). Its expression was rather suppressed under HL conditions compared with that under hypoxic conditions. Furthermore, siRNAs for AMPK did not show any significant effect on the expression levels of HIF-1 $\alpha$  protein under hypoxic conditions (Fig. 3b). In consistent with the HIF-1 $\alpha$  protein expression, luciferase activities increased under hypoxic conditions, but not further increased under HL conditions, when a pGL4 5xHRE-Luc vector containing hypoxia-responsive elements was used (Fig. 3c); however, the luciferase activities increased under hypoxic conditions and further increased under HL conditions, when a pGL3-proVEGF reporter vector was used (Fig. 3d). Furthermore, siRNAs for AMPK suppressed the luciferase activities increased under HL conditions, when a pGL3-proVEGF reporter vector was used (Fig. 3e). These results suggest that the enhanced expression of at least VEGF under HL conditions may not be explained by the enhanced transcription of VEGF through the activation of HIF-1 $\alpha$  alone and that some transcription factor(s) activated by the AMPK pathway may be involved in the enhanced expression of VEGF. As recent reports have demonstrated that glucose-deprivation enhances the mRNA stability of VEGF through the activation of AMPK [20], this mechanism may explain the enhanced expressions of AM and VEGF under HL conditions. We then examined the mRNA stability of AM, VEGF and Glut-1. The stability of mRNAs was not affected

under HL conditions (Fig. 4). The half-lives of mRNAs were found to be substantially shorter under HL conditions than those under other conditions.

*Expression of activated AMPK in human pancreatic cancer tissues:*

As our *in vitro* results suggest that AMPK is implicated in the signal transduction system to enhance certain hypoxia-inducible factors under HL conditions in pancreatic cancer cells, and a recent report demonstrated that AMPK was activated in hypoxic regions in experimental tumors [18], we examined the expression of phosphorylated AMPK $\alpha$  in 20 human pancreatic cancer tissue specimens to explore the roles of AMPK in human pancreatic cancers. First we examined the hypoxic areas and the expression of activated-AMPK in the tumor tissues of SCID mouse xenograft model, using pimonidazole and anti-phospho-AMPK $\alpha$  antibody to confirm the validity of anti-phospho-AMPK $\alpha$  antibody. The similar areas were stained with pimonidazole and anti-phosphorylated AMPK antibody (Fig. 5a). The cells near necrotic tissue were stained with anti-phospho-AMPK $\alpha$  antibody, but those near blood vessels were not (Fig. 5b). These results suggested that anti-phospho-AMPK $\alpha$  antibody could be used for the immunohistochemical staining of phosphorylated AMPK in the tumor tissues. Then we examined the expression of phosphorylated AMPK $\alpha$  in human pancreatic cancer tissue specimens. We found that 15 out of 20 pancreatic cancer tissue

specimens were positive for phosphorylated AMPK $\alpha$  by an immunohistochemical examination. A representative pancreatic cancer tissue specimen that was positive for anti-phosphorylated-AMPK $\alpha$  antibody is shown in Fig. 5c.

## DISCUSSION

In this study, we for the first time demonstrated that some, though not all, hypoxia-inducible genes were expressed at higher levels under HL conditions than under hypoxic conditions. Up to now, more than 60 hypoxia-inducible genes have been reported, and we know that their protein products play important roles in angiogenesis, vascular reactivity and remodeling, energy metabolism, erythropoiesis, cell proliferation, and survival [21]. Although it is unknown at this point whether the expression of every hypoxia-inducible gene is up-regulated under HL conditions, the current study led us to hypothesize that certain genes up-regulated by hypoxia plus glucose-deprivation may play more critical roles in tumor formation *in vivo* than other genes whose expressions are not enhanced by hypoxia plus glucose-deprivation.

We first examined the roles of AMPK in the enhancement of expressions of those genes under HL conditions, as AMPK is an energy sensor [12, 22, 23]. We herein clearly demonstrated that glucose-deprivation activated AMPK under both hypoxia and normoxia and that the blockade of AMPK activation almost completely suppressed the expressions of those genes enhanced under HL conditions, which thus suggests that AMPK contributes to the signal transduction for enhancing the expressions of those five genes under HL conditions. AMPK is a heterotrimer that

contains  $\alpha$ -,  $\beta$ -, and  $\gamma$ -subunits, each of which has at least two isoforms [12]. Decreases in the energy state cause conformational changes in AMPK, which makes it susceptible to phosphorylation and activation by AMPK kinase. Once activated, AMPK phosphorylates several down-stream substrates, thus inducing an overall effect, including the switching off of ATP-consuming pathways that are not acutely necessary for survival, and the switching on of ATP-generating pathways such as glycolysis and fatty acid oxidation necessary for survival [12]. The relationship between AMPK and cancer remains intriguing enough to study because of numerous reasons [22]. One is that the tumor suppressor LKB1 has been identified to be an upstream activator of AMPK and the second tumor suppressor, TSC2, is a downstream effector of AMPK [24, 25]. The second reason is that AMPK activation inhibits the growth of some cancer cells *in vitro* through the activation of wild type p53 [26]. The third one is that an AMPK activator, 5-amino-4-imidazolecarboxamide ribose (AICAR), disrupts the survival of prostate cancer cells [27]. These reports demonstrate that AMPK activation tends to suppress the growth and survival of tumor cells. In contrast to these reports, some reports have demonstrated that AMPK activation inhibited apoptosis in several settings [28-30]. A recent study has also demonstrated that full activation of AMPK following glucose deprivation rescued cell from apoptosis [31]. In this study, we

clearly showed that HL conditions induced the activation of AMPK and enhanced expressions of the five hypoxia-inducible genes essential for glycolysis and angiogenesis without inducing apoptosis. Further, we demonstrated for the first time that AMPK was activated in human pancreatic cancer cells *in vivo* in accordance with the findings of previous reports demonstrating that AMPK contributed to the tolerance of nutrient deprivation in pancreatic cancer cell lines [29]. Therefore, our results suggest that AMPK activation supports the survival of pancreatic cancer cells through the enhancement of transcription of certain hypoxia-inducible genes essential for adaptation responses to hypoxia and glucose-deprivation *in vivo*.

In our previous reports, we demonstrated that HIF-1 $\alpha$  was essential for the survival and proliferation of pancreatic cancer cells *in vivo* [32, 33]. In this study, we demonstrated that HIF-1 $\alpha$  protein expression was not affected by glucose-deprivation, thus suggesting that HIF-1 $\alpha$  alone could not account for the enhanced expressions of those five genes under HL conditions. VEGF promoter activities increased under hypoxic conditions and further increased under HL conditions, whereas the HIF-1 transcriptional activity substantially decreased under HL conditions in comparison to those under hypoxic conditions. Further we demonstrated that siRNAs for AMPK suppressed the VEGF promoter activities enhanced by HL conditions. Then, mRNA

stabilization by glucose deprivation was not observed in our experiments. These results suggest that some transcription factor(s) other than HIF-1 may therefore play an important role in enhancing the mRNA expression of certain genes under HL conditions. We are now examining the possible functions of several transcription factors identified by a two-hybrid analysis, for identifying the proteins bound to AMPK, of their ability to enhance the mRNA expressions of some genes under HL conditions.

Collectively, our results suggest that the expressions of certain hypoxia-inducible genes become enhanced under HL conditions, which thus mimics the tumor microenvironment of pancreatic cancers, that such expressions may play a critical role in the tumor formation, and that the enhanced expressions of those genes under HL conditions were mediated through the activation of AMPK as well as HIF-1.

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## FIGURE LEGENDS

Figure 1. Expressions of hypoxia-inducible genes under different conditions.

a: MiaPaCa2, PCI-43, BxPC-3, HepG2 and HeLa cells were incubated under normoxic normal-glucose conditions (NN), normoxic glucose-deprived conditions (NL), hypoxic normal-glucose conditions (HN), and hypoxic glucose-deprived conditions (HL). The glucose concentration of the normal-glucose group was 100mg/dl, and that of the glucose-deprived group was 50mg/dl. After incubation for 24 hours, total RNA was extracted and cDNA was synthesized. The mRNA levels of adrenomedullin, VEGF, GLUT-1, GLUT-3, and hexokinase 2 were analyzed by real-time PCR, and the results of the representative genes are shown. All experiments were done in triplicate and the vertical bars indicate standard deviations. b: MiaPaCa2 cells were incubated under the four different conditions. After incubation for 24 hours, total RNA was extracted and cDNA was synthesized. The mRNA levels for aldolase A, aldolase C, ceruloplasmin carbonic anhydrase 9, and adenylate kinase were analyzed by real-time PCR, and the results of the representative genes are shown. All experiments were done in triplicate and the vertical bars indicate standard deviations. c: HeLa, MiaPaCa2, and HepG2 cells were incubated under the four different conditions. After incubation for 48 hours, the protein was prepared. The protein levels of adrenomedullin as determined by a

Western blot analysis are shown. d: MiaPaCa2 cells were incubated under the indicated concentrations of glucose, and normoxia and hypoxia. After incubation for 24 hours, the total RNA was extracted and cDNA was synthesized. The mRNA levels of adrenomedullin were analyzed by real-time PCR. e: The cells were incubated for the indicated time periods under the four different conditions. The time courses of mRNA induction of adrenomedullin are shown. All experiments were done in triplicate and the vertical bars indicate standard deviations.

Figure 2. AMPK activity under different conditions and the effects of an AMPK inhibitor and siRNAs for AMPK.

a: MiaPaCa2 cells were incubated at the indicated concentrations of glucose, and normoxia and hypoxia for 6 hours. Western blot analyses of phospho-AMPK, AMPK, and actin are shown. b: After the pre-incubation with an AMPK inhibitor, Compound C, for 60min, the cells were incubated under the four different conditions for 12 hours. mRNAs of VEGF and adrenomedullin were analyzed by real-time PCR. c: MiaPaCa2 cells were transfected with 5nM siRNA, and total RNA and protein were prepared 48 hours after the transfection. mRNAs of AMPK $\alpha$ 1 and AMPK $\alpha$ 2 were analyzed by real time PCR. The protein levels of total AMPK, AMPK $\alpha$ 1, AMPK $\alpha$ 2, and actin are also shown. d: MiaPaCa2 cells were transfected with 5nM siRNA at day 0 and day

2, and then the cells were incubated under the four different conditions for 24 hours. Total RNA was extracted after the incubation, and mRNAs of adrenomedullin, aldolase A and VEGF were analyzed by real-time PCR. Experiments were done in triplicate and vertical bars indicate the standard deviations. \*\*:  $p < 0.01$ ,

Figure 3. Expression levels and transcriptional activities of HIF-1 under different conditions.

a: The expression of HIF-1 $\alpha$  protein under indicated conditions are shown. MiaPaCa2 cells were incubated for 20 hours under the indicated conditions. b. The expression of HIF-1 $\alpha$  protein under indicated conditions are shown. MiaPaCa2 cells were transfected with 5nM siRNA at day 0 and day 2, and then the cells were incubated under the four different conditions for 24 hours. c: The culture medium was changed 6 hours after the reporter vector (pGL3-5HRE) and the internal control vector were transfected, and then the cells were incubated under the four different conditions. After incubation for 24 hours, the cells were lysed and the luciferase activity was measured. The luciferase activity was determined by normalization to the corresponding renilla luciferase activity of the internal control vector. The vertical bars indicate the standard deviations of three independent experiments. d: The culture medium was changed 6 hours after the reporter vector (pGL3-proVEGF) and the internal control vector were

transfected, and then the cells were incubated under the four different conditions. After incubation for 24 hours, the luciferase activity was measured as described in (c).

e. The culture medium was changed 6 hours after siRNA transfection reporter vector (pGL3-proVEGF) and the internal control vector were transfected. Then the cells were incubated under NL or HL condition. The luciferase activity was measured after 24 hours incubation as described in (c).

Fig. 4. mRNA stability of AM, VEGF and Glut-1 under different conditions.

After MiaPaCa2 cells were incubated under the indicated conditions for 24 hours, 2µg/ml of actinomycin D (ActD) was added into the medium. Then, total RNA was extracted after the indicated time period, and mRNA of VEGF was analyzed by real-time PCR. Percentages of the remaining mRNA after the treatment are shown. Real-time PCR was done in triplicate and vertical bars indicate standard deviations.

(a: adrenomedullin, b: VEGF, c: GLUT1)

Figure 5. Expression of activated AMPK in human pancreatic cancer cells.

SCID mice were sacrificed at 45 days after the transplantation with  $5 \times 10^6$  MiaPaCa2 cells (human pancreatic cancer cells). a: Tumor tissues stained by anti-pimonidazole antibody (upper panel) and stained by anti-phospho-AMPK $\alpha$  antibody (lower panel) are shown. The hypoxic area was stained with pimonidazole. Phospho-AMPK $\alpha$  was

detected around the area of pimonidazole staining. b. Tumor tissues near the necrotic tissue (upper panel) and blood vessel (lower panel) stained with phospho-AMPK antibody. Phospho-AMPK $\alpha$  was strongly stained near the necrotic tissue, whereas it not detected near the blood vessel. c: A representative human pancreatic cancer tissue specimen was stained with anti-phosphorylated AMPK $\alpha$  antibody (upper panel: low power view, lower panel: high power view).

Table 1. Primer sets for Real-time PCR

<b>Gene names</b>	<b>Forward primers</b>	<b>Reverse primers</b>
VEGF	AGCCTTGCCTTGCTGCTCTAC	GCTGCGCTGATAGACATCCAT
Adrenomedullin	GGAAGAGGGAACTGCGGATGT	GGCATCCGGACTGCTGTCT
GLUT-1	CTTCTCTGTGGGCCTTTTCGT	CAAAGGACTTGCCCAGTTTCG
GLUT-3	TATTTTCCGTCGGGGGTATGA	CCATAAAGCAGCCACCAGTGA
Aldolase A	GGGTGTCATCCTCTTCCATGA	GACCACGCCCTTGTCTACCTT
Aldolase C	CTGGCTGCGGATGAGTCTGTA	CACACGGTCATCAGCACTGAA
Enolase 1	AAAGCTGGTGCCGTTGAGAAG	AGCATGAGAACCGCCATTGAT
Hexokinase 1	GGAAGCAGACGCACAACAATG	CACGGAAATTGGTTCCTCCAA
Hexokinase 2	ATGAGGGGCGGATGTGTATCA	GGTTCAGTGAGCCCATGTCAA
Heme oxygenase 1	CCAGTCTTCGCCCTGTCTAC	GGCTGGTGTGTAGGGGATGAC
Trasnferrin	TGAATGCAAGCCTGTGAAGTG	TCTTCGGTGGTCTCTGCTGAT
Ceruloplasmin	AGTTGATGTGCACGCAGCTTT	GGGTTCTGGGCCACCATATAA
Adenylate kinase	CCGAAGCCCTGGACAAAATCT	CCTTCCGCTAGGAGGGTGAAT
Carbonic anhydrase9	GCTGAGCCAGTCCAGCTGAAT	GCTGCCTTCTCATCTGCACAA
Plasminogen activator inhibitor	GCTCATCAGCCACTGGAAAGG	TCGGTCATTCCCAGGTTCTCT
IGF2	TGGTGGACACCCTCCAGTTC	GGTCACAGCTGCGGAAACA

Prolylhydroxylase 2	CCAGGATGGGAGTGGAGAGTT	CCAAAACCGTCCCGAAGAG
NIP3	GCACTTCAGCAATGGGAATGG	GAGCTCTTGGAGCTGCTTCGT
Endothelin 1	CTGCCACCTGGACATCATTG	TCACGGTCTGTTGCCTTTGTG
Beta actin	TTGCCGACAGGATGCAGAA	GGCGATCCACACGGAGTACT

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Table 2. Expression levels of hypoxia -inducible genes under glucose-deprived hypoxic conditions by a DNA microarray analysis

Gene	H+L	Hypoxia
Adrenomedullin	10.7	5.3
Hexokinase2	8.62	2.0
Glut-1	6.37	2.2
Plasminogen activator inhibitor 1	5.2	2.8
VEGF	3.99	1.4
Heme oxygenase-1	3.34	1.2
Glut-3	3.18	1.8
Prolylhydroxylase 2	2.49	1.6
Hexokinase1	2.26	1.7
Lactate dehydrogenase A (LDHA)	1.83	2.1
Prolyl –4-hydroxylase a1	1.63	1.9
Transferrin receptor	1.63	1.8
Adenylate kinase 3	1.47	1.7

a1b-adrenergic receptor	1.39	1.6
Aldolase C	1.39	1.7
Carbonic anhydrase 9	1.29	1.9
Insulin-like growth factor 2	1.27	1.6
IGF binding protein 3	1.19	1.7
Aldolase A	1.18	2.0
Transferrin	1.17	1.3
Prolylhydroxylase 3	1.16	1.5
Nitric oxide synthase 2	1.15	1.4
Glyceraldehyde-3- phosphate dehydrogenase	1.10	1.4
Intestinal trefoil factor	1.08	1.5
Flt-1	1.07	1.4
IGF binding protein 1	1.07	1.6
6-phosphofructose-2-kinase/fructose-2, 6-biphosphatase-3	1.05	1.4
Erythropoietin	1.05	1.7
Aminopeptidase A	1.04	1.4
ETS-1	1.02	1.5
NIP3	1.02	1.6

LDL receptor-related protein 1	1.01	1.4
Transforming growth factor b3	1.01	1.4
Transforming factor a	0.99	1.6
Carbonic anhydrase 12	0.95	1.4
Triosephosphate isomerase	0.94	1.5
Pyruvate kinase M	0.9	1.4
IGF binding protein 2	0.89	1.6
Ceruloplasmin	0.87	1.4
Endothelin 1	0.84	1.5
Phosphofructokinase L (PFKL)	0.81	1.7
Transglutaminase 2	0.79	1.6
Enolase 1	0.78	1.5
<u>PDGF-B</u>	<u>0.62</u>	<u>1.4</u>

Fold change is the ratio of the mean gene expression values of the cancer cells under H+L conditions or hypoxic conditions to the mean gene expression values of the cancer cells under normoxic normal glucose conditions.

Fig. 1

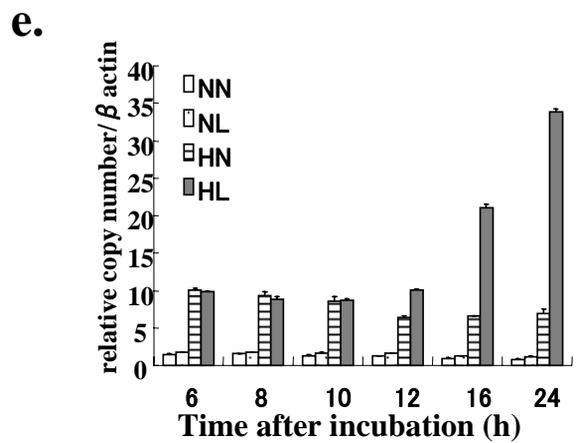
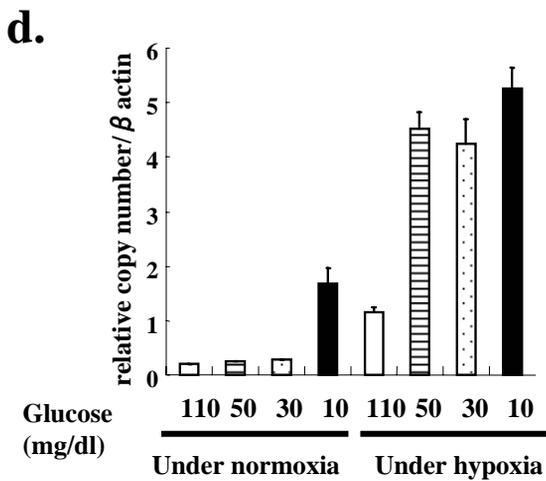
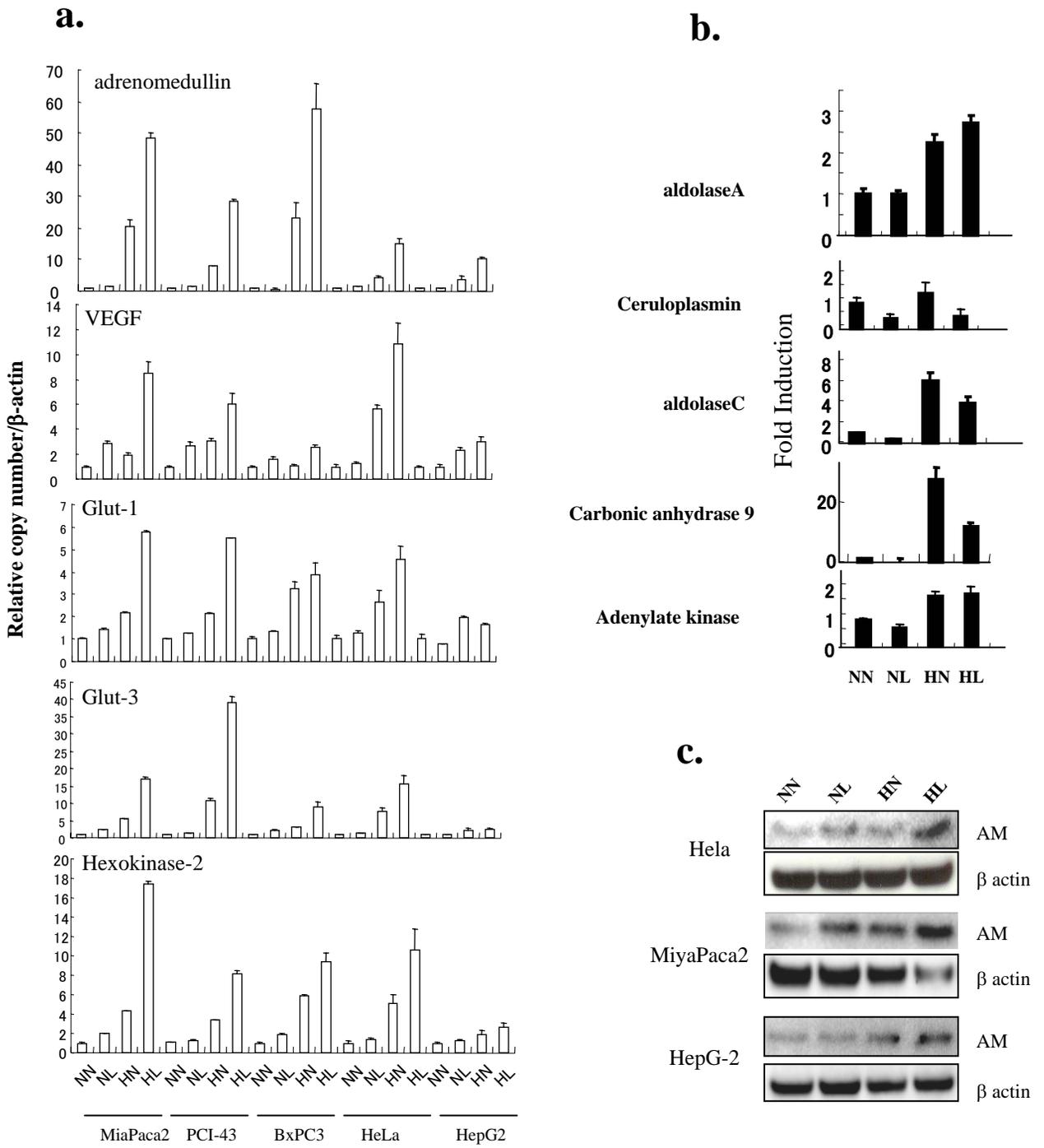


Fig. 2

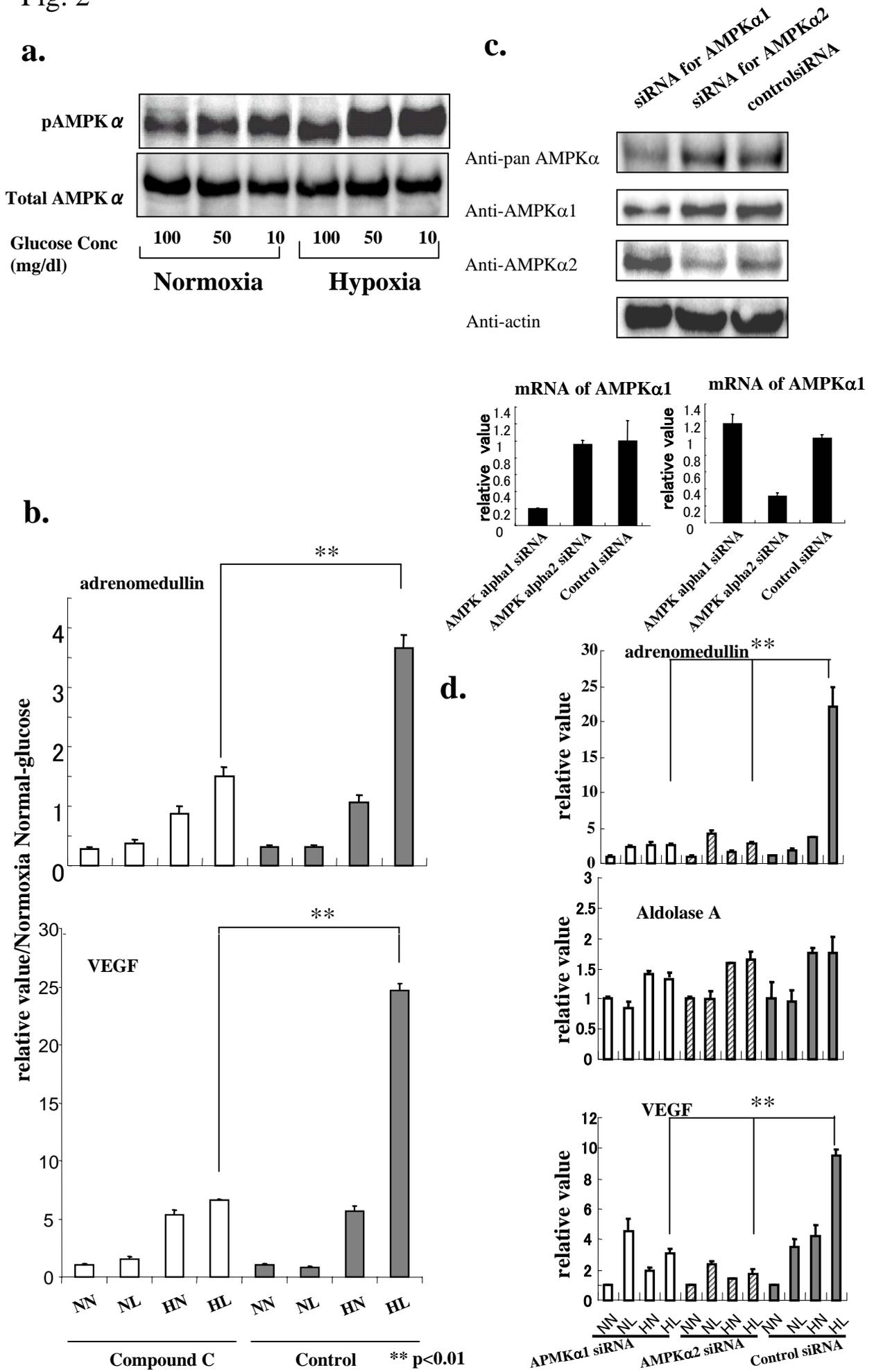


Fig. 3

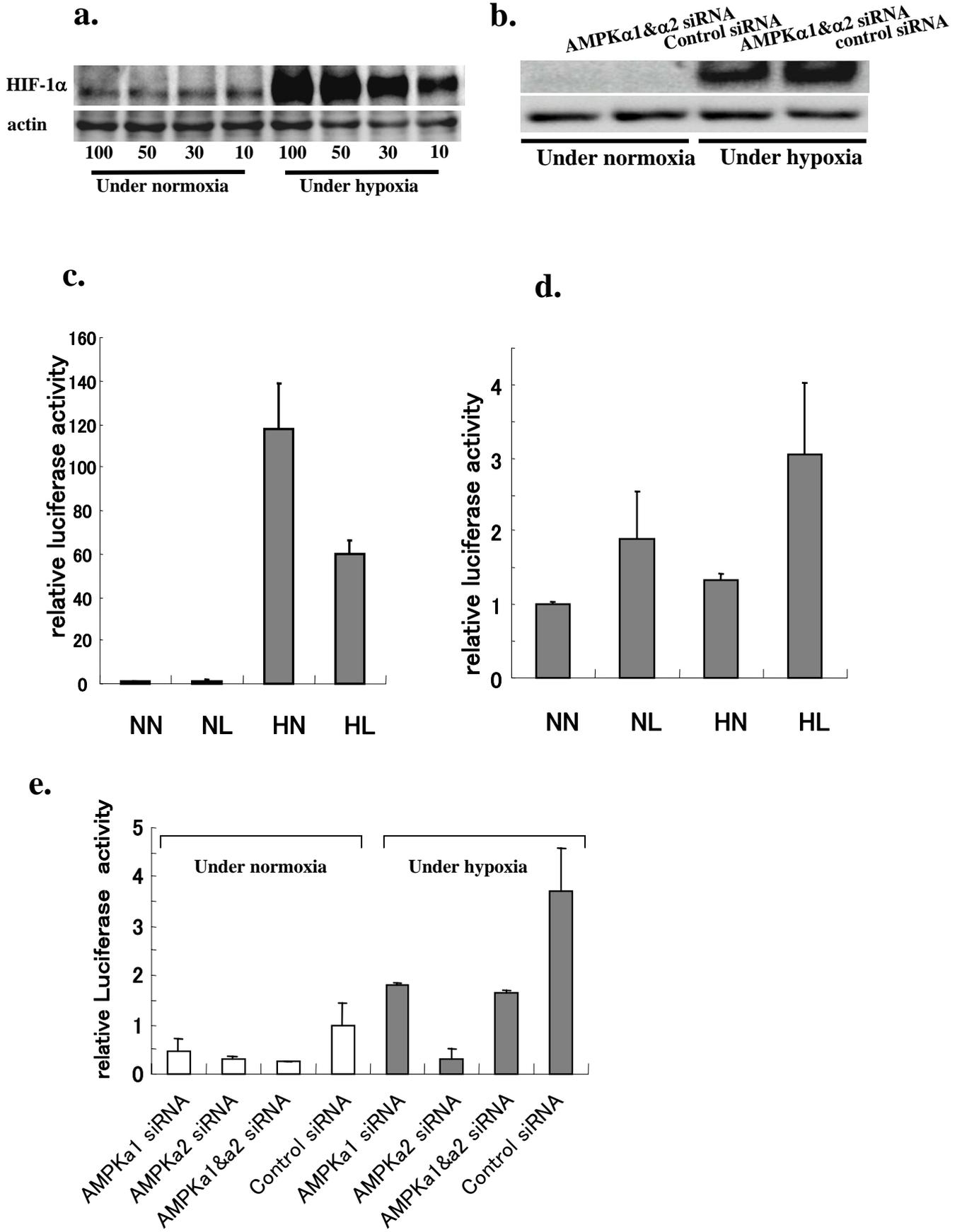


Fig. 4

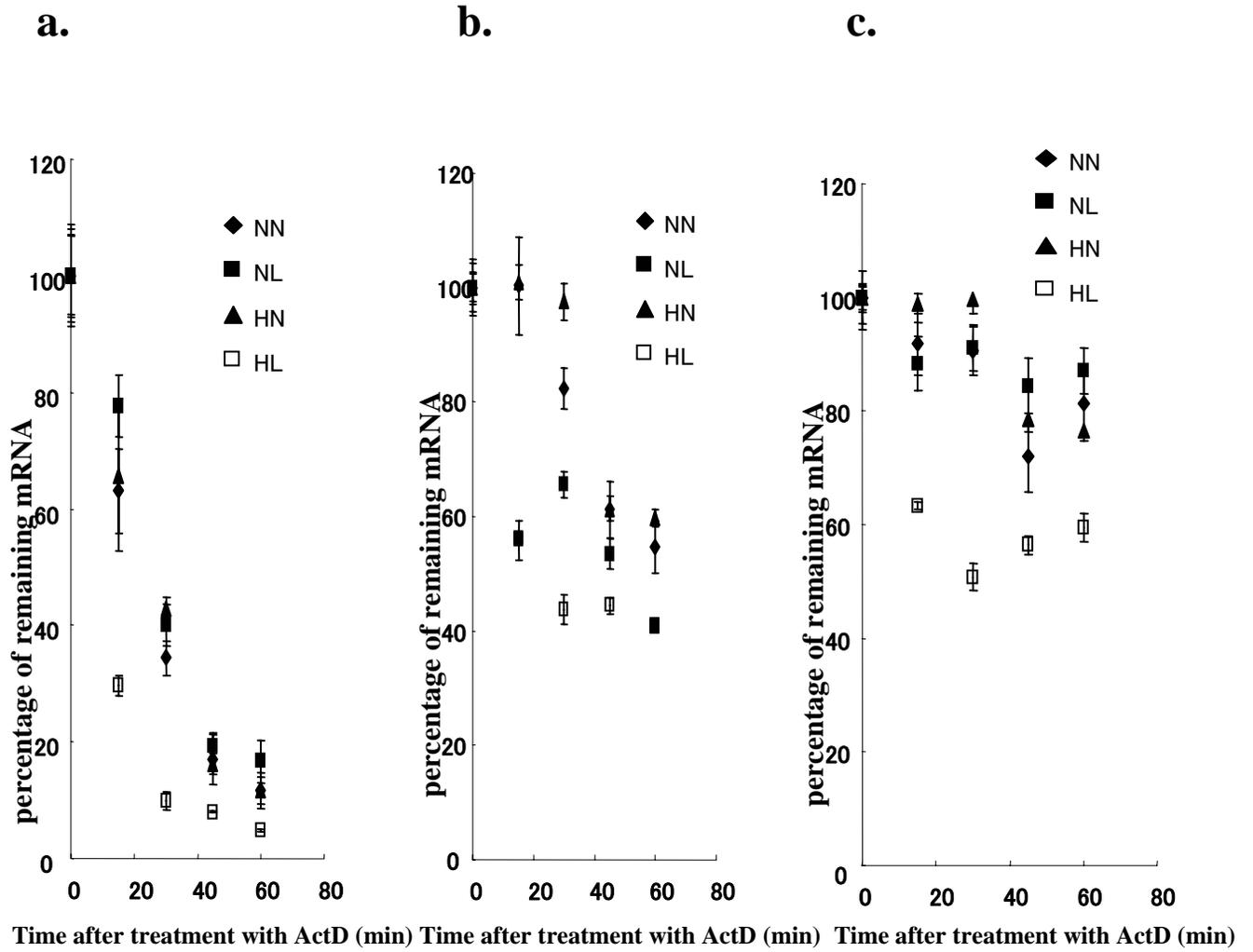


Fig. 5

