The Journal of Applied Pharmacology, 14, 132-136(2006)

Aryl Hydrocarbon Receptor Nuclear Translocator is Involved in ATP Homeostasis in Both Normoxic and Hypoxic Monolayer Mouse Hepatoma Cells

Su Mi CHOI, and Hyunsung PARK*

Department of Life Science, University of Seoul, Seoul 130-743, Korea

(Received September 11, 2006; Accepted September 20, 2006)

Abstract – Aryl hydrocarbon receptor nuclear translocator (Arnt) belongs to bHLH-PAS protein family. Here, we study the role of Arnt in both cell growth and glucose metabolism. Our results demonstrated that the absence of Arnt does affect ATP homeostasis but not cell growth in monolayer-cultured mouse hepatoma cells. ATP level of Arnt defective BpRc1 hepatoma cells is less than that of wild type hepatoma cells in both normoxia and hypoxia. BpRc1 cells also fail to increase the expression of glycolytic enzymes in response to hypoxia. Our results suggest that Arnt is essential for glucose metabolism and ATP production but not for cell growth.

Key words □ Aryl hydrocarbon receptor nuclear translocator (Arnt), ATP, Cell growth, Hypoxia, Glucose metabolism

INTRODUCTION

Cellular oxygen is an important regulatory stimulus for many physiological and pathological processes. Under low oxygen tension, cells adapt by up-regulating the transcription of specific genes that are involved in angiogenesis, erythropoiesis, and glycolysis. The genes upregulated during hypoxia include vascular endothelial growth factor (VEGF), erythropoietin (EPO), and several glycolytic enzymes (Iyer *et al.*, 1998). These diverse target genes are induced by a heterodimeric transcription factor HIF-1α/Arnt which belongs to a member of the basic helix-loop-helix bHLH-PAS superfamily (Semenza and Wang, 1992). Beside of HIF-1α, Arnt makes heterodimers with several other bHLH-PAS proteins including Aryl hydrocarbon receptor (AhR), and single-minded (Sim), thereby Arnt plays a central role in the function of the bHLH-PAS family (Crews *et al.*, 1999; Semenza, 2001). These various Arnt containing heterodimeric transactivators serve to activate several target genes in response to specific stimuli for its partner proteins. Arnt plays a role in the regulation of gene expression under a variety of conditions, including exposure to dioxin, hypoxia, hypoglycemia and development (Maltepe *et al.*, 1997). Here, we used mouse Hepa1c1c7 hepatoma cells and its variant cells BpRc1. BpRc1 cells were isolated by using benzo(*a*)pyrene selection and fluorescence-activated cell sorting (Miller and Whitlock, 1981). They loose responsiveness to both dioxin and hypoxia (Li *et al.* 1997). Screening of genomic library found a gene that can complement the defect of BpRc1, and the gene reveals Arnt.

Here, we investigated the function of Arnt in cell growth and ATP homeostasis by comparing BpRc1 cells with its wild type cells.

*Corresponding author
Tel: +82-2-2210-2622, Fax: +82-2-2210-2888
E-mail: hspark@uos.ac.kr

MATERIALS AND METHODS

Cell cultures and treatments

Wild-type mouse Hepa1c1c7 cells and Arnt-defective BpRc1 cells were cultured in α-MEM supplemented with 10% fetal bovine serum (Invitrogen) in humidified air containing 5 % CO_2 at 37°C. For treatment of glucose deprivation, high glucose medium (25 mM glucose) was used DMEM (Life Technologies Gibco BRL) to culture wild type mouse Hepa1c1c7 cells and Arnt-defective BpRc1 cells and low glucose medium (2 mM glucose) was prepared by mixing with glucose-free DMEM (Life Technologies Gibco BRL) and DMEM containing 25 mM glucose in ratio of 23:2. Cells were exposed to low glucose medium (each of 2 mM or 0 mM glucose) for 8 hours. The cells were made hypoxic by incubation in an anaerobic incubator (Model 1029, Forma Scientific, Inc.) in 5% CO_2, 10% H_2, and 85% N_2 at 37°C.

Measurement of cell viability and cell growth

Cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Wild type mouse Hepa1c1c7 cells and Arnt defective BpRc1 cells were maintained in α-MEM (5 mM glucose). 1×10^4 cells were plated in an each well of 96-well plates. Media was removed at indicating time and 50 μl of MTT (5 mg/ml in phosphate-buffered saline pH7.4) was added to each well. Plate was incubated for 4 hours under humidified air containing 5 % CO_2 at 37°C. After incubation, MTT solution was carefully removed and added 50 μl of DMSO to dissolve MTT-formazan. Absorbance means cell viability and recorded absorbance at 570 nm with 655 nm as reference by ELISA reader. The values are representative of average duplicated culture experiment ± the standard error of the mean (SEM). Cell growth was determined by counting. 5×10^4 cells were plated in a 35-mm plate. After 16 hours, the cells were exposed to the hypoxia. Growth assay described were repeated at least four times, unless otherwise noted, and the values described in the figures are representative of average three each culture experiments and counted fourth every each plate ± SEM.

Measurement of ATP

A constant-light signal luciferase assay developed by Boehringer-Mannheim (ATP Bioluminescence Assay Kit CLS II) was utilized to determine levels of ATP production during normoxia and hypoxia. Wild type mouse Hepa1c1c7 cells and Arnt defective BpRc1 cells were plated in duplicate at 5×10^4 cells in 35-mm tissue culture plate and allowed to incubate overnight. After 16 hours, the cells were exposed to the hypoxia for indicating time. Cells were harvested. Cell extracts was used for analysis according to the manufacturer's instructions. Briefly, 25 μl of luciferase reagent provided in the CLS II kit was added to 25 μl of diluted whole-cell extract and read in a luminometer (Berthold Lumat LB9501) at a 2-s integration. The molar amount of ATP corresponding to each sample was determined based on a log-log plot of the ATP standards (10^{-4} to 10^{-11} M ATP) versus the relative luciferase units. Next, the molar amount of ATP produced by each cell was determined for each duplicate plate of cells for each experiment. But the values described in the figures are relative values of log plot of the ATP concentration. That graph represents the results of the average of two experiments (± SEM).

Northern Analysis

Cells were grown to 70~80% confluence on a 100-mm tissue culture plate serum-starved for 16~24 h with 0.5% fetal bovine serum prior to exposure to hypoxia or glucose deprivation. Total RNA was isolated using an RNeasy spin column (Qiagen Inc.). For Northern Analysis, total RNA was resolved by electrophoresis through an 1% formaldehyde-agarose gel and transferred to a Nytran membrane. Blots were hybridized with α-^{32}P-labeled cDNA, washed, dried, and autoradiographed with Hyperfilm MP (Amersham Biosciences) as described previously (Yim *et al.* 2001).

RESULTS

Morphology of mouse Hepa1c1c7 cells and BpRc1 cells

Western analysis showed that Arnt is not expressed in Arnt defective BpRc1 cells. The morphologies of two cells are different (Fig. 1). Hepa1c1c7 cells are well spread out and exhibit epithelial morphology with cell-cell contact, which produces a cobblestone pattern, whereas Arnt defective BpRc1 cells form foci more typical of fibroblasts (Sadek and Allen-Hoffmann, 1994). BpRc1 cells reconstituted with full length Arnt recover the expression of Arnt, but partly recover the morphological changes (Ko *et al.* 1996). In addition, it should be noted that BpRc1 cells do not form confluent monolayers at high density. There are always characteristics gaps found distributed throughout the monolayer.

Arnt does not significantly affect cell survival in monolayer culture.

We investigated the effect of Arnt on cell growth in both normoxia and hypoxia. We seeded 5×10^4 cells in a 35-mm plate, incubated them in hypoxic condition (0.1% O_2) for indicated hours, and then counted the survival cells by adding tryphan blue. Statistic analysis showed that the growth rate of BpRc1 is not significantly different from that of wild type Hepa1c1c7 cells in both normoxic and hypoxic condition (Fig. 2).

We also compared the viability of two cells in other way by using MTT assay. The reduced form of MTT-formazan is measured spectrophotometrically. This reduction takes place only when mitochondrial reductase is active, and thus conversion is directly related to the number of viable cells. This finding suggests that Arnt does not affect viability and growth of monolayered cells in both normoxic and hypoxic condition (Fig. 3). As a result, Arnt does not significantly affect the growth and viability of cells in monolayer culture.

Lack of Arnt reduces ATP contents.

Since Arnt/HIF-1α heterodimer is a transactivator for glyco-

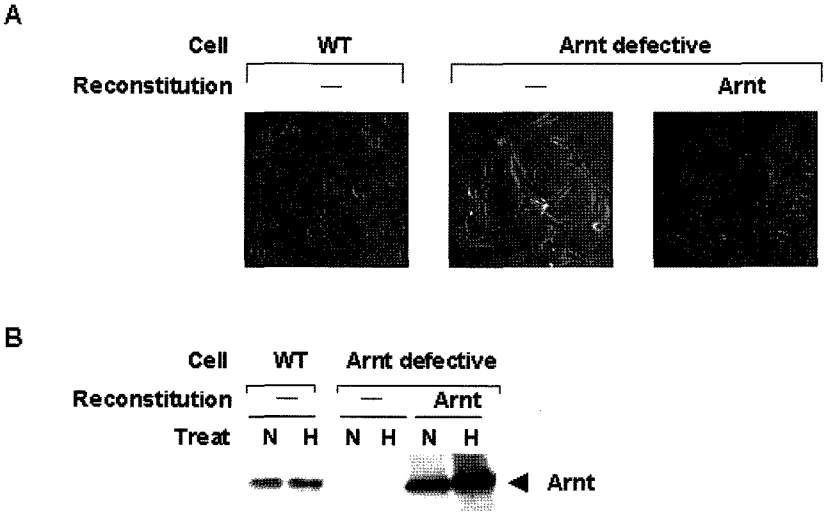

Fig. 1. Arnt expression and the morphologies of Hepa1c1c7 cells and its variant, BpRc1 cells. (A) The morphologies of wild type mouse Hepa1c1c7 cells, its variant BpRc1 cells, and BpRc1 cells reconstituted with Arnt were visualized by using microscopy and magnification is ×50. BpRc1 were infected by retrovirus encoding mouse full length Arnt (Yim *et al.* 2001). (B) Arnt protein levels in Hepa1c1c7 cells and its variants. Cells were serum-starved with medium containing 0.5 % fetal bovine serum for 36-48 hours and then treated with hypoxia for 6 hours. 30 μg of whole cell lysates were separated on 8 % SDS-PAGE and transferred to nytran membrane. Immunoblot analysis was performed using anti-HIF-1β (ARNT) antibody and visualized by using a chemiluminescence-based system.

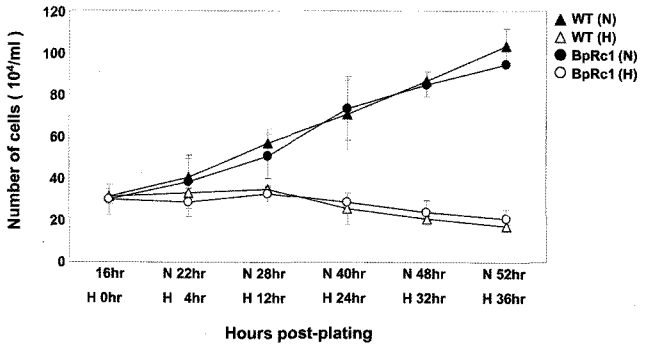

Fig. 2. Growth rate of Hapa1c1c7 cells and BpRc1 cells in monolayer cultures. 5×10^4 cells were plated in a 35-mm tissue culture plate. After 16 hours cells were exposed to hypoxic condition (1% O_2, 5% CO_2, 84% N_2, 10% H_2) for indicated hours. Cells of each plate were counted. The value represents average and the standard deviation of at least three experiments. The p values of two sets of data, WT (N) and BpRc1 (N) at each time point were calculated by Student's t test: $p_{N\,22h}$ = 0.280, $p_{N\,28h}$ = 0.135, $p_{N\,40h}$ = 0.094, $p_{N\,48h}$ = 0.369, and $p_{N\,52h}$ = 0.190, respectively. The p values of two sets of data, WT (H) and BpRc1 (H) at each time point were calculated by Student's t test: $p_{H\,4h}$ = 0.087, $p_{H\,12h}$ = 0.233, $p_{H\,24h}$ = 0.259, $p_{H\,32h}$ = 0.329, and $p_{H\,36h}$ = 0.204, respectively.

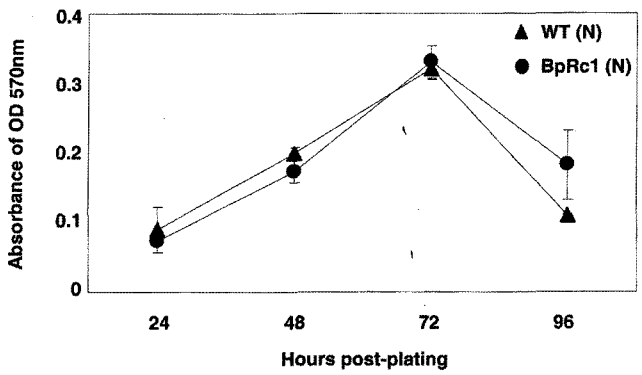

Fig. 3. Arnt have no effect on cell survival in normoxia. Hepa1c1c7 cells and its variant BpRc1 cells were maintained in α-MEM (5 mM glucose) medium. 1 x 10^4 cells were plated in each well of 96-well plates and incubated for indicated hours. Cell viability was determined by MTT assay. Absorbance means cell viability and recorded absorbance at 570 nm with 655 nm as reference by ELISA reader. The value represents average and the standard deviation of at least three experiments. The p values of two sets of data, WT (N) and BpRc1 (N) at each time point were calculated by Student's t test: p_{24h} = 0.158, p_{48h} = 0.010, p_{72h} = 0.062, and p_{96h} = 0.030, respectively.

lytic enzymes and glucose transporters, we investigated whether Arnt affects energy production of cells. We measured ATP contents of both wild type and Arnt defective BpRc1 cells. As shown Fig. 4, we found that ATP contents of both wild type and Arnt defective cells gradually increased as cells grew. However, statistic analysis indicated that ATP content of BpRc1 cells was less than that of wild type Hepa1c1c7 cells in both normoxia and hypoxia. These results strongly suggest that Arnt is required for adequate production of ATP not only in hypoxic

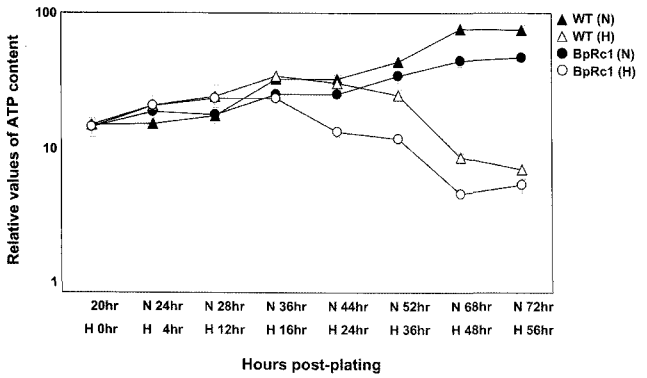

Fig. 4. ATP contents of Hepaclc17 cells and its variant BpRc1 cells. 5×10^4 cells were plated in a 35-mm tissue culture plate. After 16 hours cells were exposed to hypoxia for indicated hours. ATP content was measured by using ATP Bioluminescence Assay (Boehringer-Mannheim). The amount of ATP was determined based on a log-log plot of the ATP standards (10^{-4} to 10^{-11} M ATP). The value represents the average and standard deviation of at least three experiments. The p values of two sets of data, WT (N) and BpRc1 (N) at each time point were calculated by Student's t test: $p_{N\,20h} = 0.415$, $p_{N\,24h} = 0.218$, $p_{N\,28h} = 0.452$, $p_{N\,36h} = 0.001$, $p_{N\,44h} = 0.006$, $p_{N\,52h} = 0.195$, $p_{N\,68h} = 0.009$, and $p_{N\,72h} = 0.075$, respectively. The p values of two sets of data, WT (H) and BpRc1 (H) at each time point were calculated by Student's t test: $p_{H\,4h} = 0.482$, $p_{H\,12h} = 0.385$, $p_{H\,16h} = 0.138$, $p_{H\,24h} = 0.047$, $p_{H\,36h} = 0.097$, $p_{H\,48h} = 0.044$, and $p_{H\,56h} = 0.073$, respectively.

cells but also in normoxic cells.

Arnt is required for induction of glycolytic enzymes in response to hypoxia.

We investigated whether the lack of Arnt decreases the transcription of glycolytic enzymes and glucose transporters, by using Northern analysis. The results in Fig. 5 showed that mRNA levels of glucose transporter-1 (Glut-1), lactate dehydrogenase-A (LDH-A) and phosphoglycerate kinase (PGK-1) are not significantly changed by the absence of Arnt, while hypoxic induction of Glut-1, PGK-1 and LDH-A is not detected in Arnt defective BpRc1 cells. The additional upper band of Glut-1 mRNA was observed in hypoxia- treated sample. In contrast, these genes are not induced in BpRc1 cells. Interestingly BpRc1 cells reconstituted with full-length Arnt (Ko *et al.*, 1996) restored the hypoxia-induced gene expression of these genes. Therefore, these results clearly indicated that Arnt is required for hypoxia-induced expression of Glut-1, LDH-A and PGK-1. However we could not find that the absence of Arnt causes any significant decreases in basal level expression of these genes. We could not found that lack of glucose causes any significant changes both in wild type and BpRc1 cells.

DISCUSSION

Our findings showed that Arnt does not affect growth rate and viability of monolayer cultured hepatoma cells, whereas its absence reduces the ATP content. The absence of Arnt does not reduce the basal level expressions of Glut-1, PGK-1 and LDH-A, however its absence reduces the induction of Glut-1, PGK-1 and LDH-A in response to hypoxia. It remains to investigate how Arnt is involved in maintain ATP homeostasis. We could not detect hypoglycemic induction of these genes in both Hepa1c1c7 cells and its variant BpRc1 cells.

Due to lack of oxygen and mitochondria injury, glycolysis

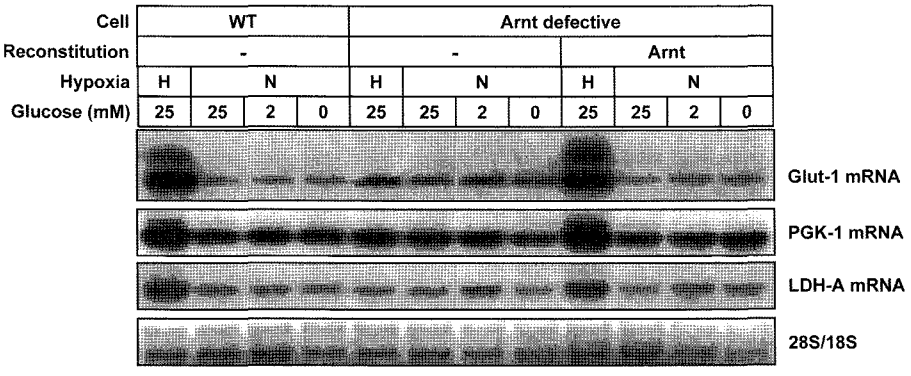

Fig. 5. The expression of Glut-1, PGK-1 and LDH-A of Hepa1c1c7 cells and BpRc1 cells. Cells were cultured in high-glucose DMEM (25 mM glucose). 70% confluent cells were exposed to either hypoxia glucose deprivation (2 mM and 0 mM) for 8 hours. Total RNA was isolated and 20 μg of total RNA was separated on the 1 % formaldehyde agarose gel. RNA was transferred onto nitrocellulose membrane. Blots were hybridized with [α-^{32}P]-labeled cDNA, washed, dried, and autoradiographed with Hyperfilm MP (Amersham Pharmacia Biotech).

for ATP generation is increased in cancer cells (Segroves *et al.* 2001; Xu *et al.* 2005). This phenomenon is known as the Warburg effect which is associated with resistance to therapeutic agent (Pelicano *et al.* 2006). Our finding that Arnt is involved in ATP homeostasis in both normoxia and hypoxia by maintaining the expression of glycolytic enzymes suggests that Arnt is a good target for reducing the Warburg effect.

Griffiths *et al.* (2002) used magnetic resonance (MR) methods and complementary techniques to monitor metabolic changes in tumors derived from mouse hepatoma lines that were either wild type Hepac1c17 cells or Arnt deficient c4 cells. c4 tumors grew significantly more slowly than the WT tumors, but were examined at a similar size. They also found that the ATP content of the c4 tumor was less than in the WT tumor. Here we confirmed that ATP content is reduced in BpRc1 as like in c4 cells, but BpRc1 does not grow more slowly than wild type Hepa1c1c7 cells. We speculated that Arnt regulates cell growth only in three dimensional cell mass, but not in monolayered cells.

Recently, it was demonstrated that Arnt expression is reduced in diabetic human islets and that pancreatic beta cell-specific Arnt knockout mice show the impaired glucose tolerance and abnormal insulin secretion that are characteristic of type 2 diabetes (Gunton *et al.* 2005; Levisetti and Polonsky 2005; Yim et al. 2006). Therefore Arnt plays important and novel in function of glucose metabolism. It remains to investigate the mechanism that Arnt regulated the glucose metabolism and ATP homeostasis.

ACKNOWLEDGMENTS

This study was supported by a grant (2003) from University of Seoul to H. Park.

REFERENCES

Crews, S.T. and Fan, C.M. (1999) Remembrance of things PAS: regulation of development by bHLH-PAS proteins. *Curr Opin Genet Dev.* **9,** 580-587.

Griffiths, J.R., McSheehy, P.M.J., Robinson, S.P., Tryo, H., Chung, Y., Leek, R.D., Williams, R.J., Stratford, I.J., Harris, A.L. and Stubbs, M. (2002) Metabolic changes detected by *in vivo* magnetic resonance studies of HEPA-1 wild-type tumors and tumors deficient in Hypoxia-inducible Fector-1β (HIF-1β): evidence of an anabolic role for the HIF-1 Pathway. *Cancer Res.* **62,** 688-695.

Gunton, J.E., Kulkarni, R.N., Yim, S., Okada, T., Hawthorne, W.J., Tseng, Y.H., Roberson, R.S., Ricordi, C., O'Conne, P.J., Gonzalez, F.J., and Kahn, C.R. (2005) Loss of ARNT/HIF1beta mediates altered gene expression and pancreatic-islet dysfunction in human type 2 diabetes. *Cell.* **122,** 337-49.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., Yu, A.Y. and Semenza, G.L. (1998) Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev,* **12,** 149-162.

Ko, H.P., Okino, S.T., Ma, Q., and Whitlock, J.P.Jr. (1996) Dioxin-induced CYP1A1 transcription *in vivo*: the aromatic hydrocarbon receptor mediates transactivation, enhancer-promoter communication, and changes in chromatin structure. *Mol Cell Biol.* **16,** 430-436.

Levisetti, M.G., Polonsky, K.S., (2005) A complex network of interacting transcription factors plays a critical role in normal pancreatic beta cell function, with mutations in certain transcription factor genes known to cause diabetes. *Cell Metab.* **2,** 78-80.

Li, H., Ko, H.P., and Whitlock, J.P. Jr. (1997) Induction of phosphoglycerate kinase 1 gene expression by hypoxia. Roles of Arnt and HIF1alpha. *J Biol Chem.* **271,** 21262-21267.

Maltepe, E., Schmidt, J.V., Baunoch, D., Bradfield, C.A., and Simon, M.C. (1997) Abnormal angiogenesis and response to glucose and oxygen deprivation in mice lacking the protein ARNT. *Nature.* **386,** 403-407.

Miller, A.G. and Whitlock, J.P. Jr., (1981) Novel variants in benzo(*a*)pyrene metabolism. *J Biol Chem,* **256,** 2433-2437.

Pelicano, H., Martin, D.S., Xu, R.H., and Huang, P. (2006) Glycolysis inhibition for anticancer treatment. *Oncogene.* **25,** 4633-4646.

Sadek, C.M. and Allen-Hoffmann, B.L. (1994) Suspension-mediated induction of Hepa1c1c7 cyp1a1 expression is dependent on the Ah receptor signal-transduction pathway. *J Biol Chem.* **269,** 31505-31509.

Segroves, T.N., Ryan H.E., Lu, H., Wouters, B.G., Knapp, M., Thibault, P., Laderoute, K. and Johnson, R.S. (2001) Transcription Factor HIF-1 is a necessary mediator of the pasteur effect in mammalian cells. *Mol Cell Biol.* **21,** 3436-3444.

Semenza, G.L. and Wang, G.L. (1992) A nuclear factor induced by hypoxia via *de novo* protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol Cell Biol.* **12,** 5447-5454.

Semenza, G.L. (2001) Hypoxia-inducible factor-1: oxygen homeostasis and disease pathophysiology. *Trends Mol Med.* **7,** 345-350.

Xu, R.H., Pelicano, H., Zhou, Y., Carew, J.S., Feng, L., Bhalla, K.N., Keating, M.J., Huang, P. (2005) Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. *Cancer Res.* **65,** 613-621.

Yim, S.H., Shah, Y., Tomita, S., Morris, H.D., Gavrilova, O., Lambert, G., Ward, J.M,, Gonzalez, F.J. (2006) Disruption of the Arnt gene in endothelial cells causes hepatic vascular defects and partial embryonic lethality in mice. *Hepatology.* **44,** 550-560.

Yim, S., Choi, S.M., Choi, Y., Lee, N., Chung, J., and Park, H. (2003) Insulin and hypoxia share common target genes but not the hypoxia-inducible factor-1alpha. *J Biol Chem.* **278,** 38260-38268.y