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ORIGINAL ARTICLE

Cobalt chloride, a chemical inducer of hypoxia-inducible factor-1α in U251 human glioblastoma cell line

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Abstract Tumor hypoxia has been described to increase the resistance of cancer cells to radiation therapy and chemotherapy. Hypoxia-inducible factor-1α (HIF-1α) is the main transcriptional factor activated by hypoxia and it plays a key role in reprogramming tumor growth. We examined in this study whether cobalt chloride induce HIF-1α in different concentrations. U251 human glioblastoma cell line was incubated at 16 h under normoxia with or without $CoCl_2$ at 1, 5, 10, 20, 25, 50, 100, 150 and 200 μM treatments. In proliferation assay, $CoCl_2$ have shown an increase in cellular induction between 50 and 200 μM, proportionally. $CoCl_2$ have also shown at 50 μM the maximum induction effect. In addition, $CoCl_2$ at 50 μM displayed maximum response at 20,000, 30,000 and 40,000 U251 cells, respectively. In HIF-1 expression assay, $CoCl_2$ increases HIF-1α gene expression between 50 and 200 μM. Western analysis revealed sharp protein band at 118 KDa which represented the HIF-1α protein with high band density at 50 μM $CoCl_2$. The present paper reports the adaptive response of human glioblastoma cells to $CoCl_2$, a chemical hypoxia-mimicking agent. The effects of the treatment were evaluated on cell proliferation, and HIF-1α gene expression.

1. Introduction

HIF is a heterodimeric protein that consists of a constitutively nuclear-located β-subunit, also termed aryl hydrocarbon nuclear translocator (ARNT) and a highly regulated α-subunit (Wang et al., 1995; Jaakkola et al., 2001). Both, α- and β-subunits belong to the family of proteins that contain basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) motifs that are crucial for heterodimerisation between the HIF-α and HIF-β subunits (Wang et al., 1995). To date, three HIF-α subunits with specific tissue expression patterns have been identified (HIF-1α, HIF-2α, and HIF-3α) (Hogenesch et al., 1997).

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HIF-1α contains oxygen-dependent degradation domain (ODDD) that is central to the oxygen-regulated stability of this protein (Pugh et al., 1997). HIF-1α also contains in its C-terminal region two transactivation domains, N-terminal (N-TAD), and C-terminal (C-TAD), respectively (Ruas et al., 2002). These transactivation domains mediate interaction of the HIF transcription factor with other co-activators of gene transcription, such as p300/CBP, that confer target gene specificity (Lando et al., 2002; Hu et al., 2007).

In a normoxic condition, HIF-1α is hydroxylated on specific proline or asparagine residues by enzymes that have an absolute requirement for dioxygen (O_2), Fe (II) and 2-oxoglutarate (McNeil et al., 2002). Four hydroxylases have been described to act on HIF-1α: (i) three prolyl-4-hydroxylase domain-containing enzymes (PHD1-3), which modify the proline residues Pro^{402} and Pro^{564} and (ii) FIH (factor inhibiting HIF) which modifies an asparagine residue Asn^{803} (Walmsley et al., 2008) (Fig. 1). Both, Pro^{402} and Pro^{564} are located in the ODDD, while Asn^{803} is located in the C-TAD of HIF-1α. This novel group of hydroxylase enzymes (PHDs and FIH) show an absolute necessity for dioxygen, Fe (II) and 2-oxoglutarate. The proteosomal degradation of HIF is dependent on the targeting of prolyl hydroxylated α-subunits to the ubiquitin pathway by high-affinity binding to the von Hippel Lindau E3 ubiquitin ligase (VHL) (Jaakkola et al., 2001). Thus, reduced PHD and FIH hydroxylase activity at oxygen tension site permits stabilization and transcriptional activation of HIF resulting in the modulation of multiple HIF effector genes which contain hypoxia response elements (HRE), to facilitate the cellular adaptive responses to hypoxia (Wenger, 2002).

Furthermore, HIF-1α plays a central role in tumor progression and angiogenesis in vivo. Oncogenic activation or loss of tumor suppressor function is associated with HIF-1-mediated tumor progression (Bárdos et al., 2004). Exposure to a variety of growth factors has also been shown to increase HIF-1α activity in normoxic and hypoxic conditions. HIF-1α is overexpressed in many human cancers and has been associated with tumor aggressiveness, vascularity, treatment failure and mortality (Birner et al., 2000; Giaccia et al., 2003). All of these activities make the HIF-1α transcription factor an attractive target for the development of new anticancer therapeutics (Belozerov and Meir, 2005).

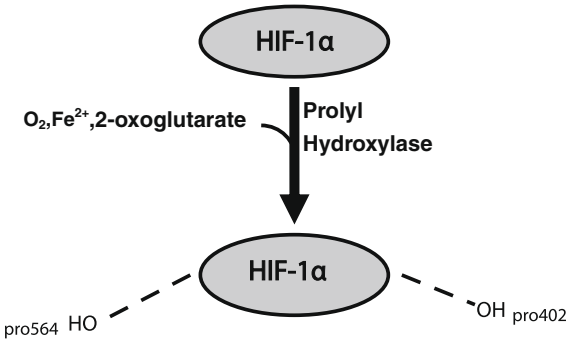

Figure 1 Under normal oxygen tensions, HIF-1α is hydroxylated by prolyl hydroxylases, which require oxygen, ferrous ion, and 2-oxoglutarate for activity. Prolyl-4-hydroxylases (PHD1-3) modify the proline residues Pro^{402} and Pro^{564}.

Some metals are known as hypoxic mimicking agents including cobalt chloride, nickel chloride and desferrioxamine (Goldberg et al., 1998). Epstein et al. (2001) reported that specific prolyl hydroxylases have an iron-binding centre and that iron is critical for their enzymatic activities. It is proposed that iron chelators can remove iron from the iron-binding centre of the enzyme and that the iron can be replaced with cobalt at this site, which will inactivate the hydroxylation activity. It was also reported that $CoCl_2$ treatment induced HIF-1α expression by binding to the PAS domain resulting in blockage of HIF-1α pVHL binding and thereby HIF-1α stability (Yuan et al., 2003).

The purpose of this study is to investigate the role of $CoCl_2$ in U251 human glioblastoma cell proliferation and to determine potential interactions between $CoCl_2$ and HIF-1α in these cells in normoxia conditions. Therefore, we applied different $CoCl_2$ concentrations to investigate the cellular induction, as well as the effect of $CoCl_2$ in U251 cell density, and studied the effects of $CoCl_2$ on HIF-1α gene expression at protein levels, in U251 human glioblastoma cells.

2. Materials and methods

2.1. Cell line and reagents

U251 human glioma cell line obtained from ECCAC (UK) and maintained in RPMI 1640 (Whittacker Bioproducts, Walkersville, USA) supplemented with 5% heat-inactivated fetal bovine serum (Imperial Laboratories, Andover, UK), penicillin (50 IU/ml), streptomycin (50 μg/ml) and 2 mM glutamine (all purchased from Sigma, UK). Cells were maintained at 37 °C in a humidified incubator containing 21% O_2, 5% CO_2 in air (referred to as normoxic conditions).

2.2. Hypoxic treatment

To prepare $CoCl_2$ stock solutions in RPMI 1640 tissue culture medium, usually as 10% w/v solutions, the chemicals were dissolved directly in culture medium (800 μM/10 ml RPMI). The stock solutions were filter-sterilized (0.22 μm). The resultant solutions were kept at 4 °C and used within 24 h for the assay. Nine separate serial $CoCl_2$ (Sigma, UK) concentrations in culture medium were prepared for addition to cell culture (1, 5, 10, 20, 25, 50, 100, 150 and 200 μM).

U251 cell culture medium was removed from the flask and the cells were rinsed with Trypsin EDTA solution and Trypsin EDTA solution (sigma) were then added to the flask and incubated at 37 °C for 2 min. Cells were then resuspended with RPMI 1640 and cultured at a concentration of 5000 cells/well in tissue culture 96 wells plates. The cells were left 48 h to adhere at 37 °C in a humidified atmosphere tissue culture incubator containing 5% CO_2. After 48 h, cells were treated with serial $CoCl_2$ concentrations (1, 5, 10, 20, 25, 50, 100, 150 and 200 μM) with 20% O_2 at 37 °C for 16 h.

2.3. Cell density experiment

U251 cell suspensions were prepared in a standard manner. Culture medium was removed from the flask and the cells were rinsed with Trypsin EDTA solution and Trypsin EDTA solu-

tion (Sigma) were then added to the flask and incubated at 37 °C for 2 min. In order to monitor the cellular sensitivity of $CoCl_2$, U251 suspensions were seeded in a standard manner at 5000, 10,000, 15,000, 20,000, 30,000 or 40,000 cells per 75 cm^2 tissue culture flasks.

2.4. Cell proliferation assay

The U251 human cell line were grown and treated with $CoCl_2$ as mentioned above. The Promega CellTiter 96® AQueous non-radioactive cell proliferation assay has been applied here for in vitro cytotoxicity assessment. This assay uses the novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and the electron coupling reagent, phenazine methosulfate (PMS). MTS is chemically reduced by cells into formazan, which is soluble in tissue culture medium (Malich et al., 1997). The measurement of the absorbance of the formazan can be carried out using 96 microplates at 492 nm. The assay measures dehydrogenase enzyme activity found in metabolically active cells. Since the production of formazan is proportional to the number of living cells, the intensity of the produced color is a good indication of the viability of the cells. MTS solutions were prepared according to the manufacturer's instructions. The solutions were then stored in light-protected tubes at −20 °C.

A sample of culture medium was used as a ''medium-only'' control. The same cell suspension as that prepared for the assay was also used as a ''cell-only'' control as untreated. Promega CellTiter 96® AQueous. The final solutions were added, in duplicates (100 μl in each well) using a multichannel pipettor, then one solution reagent was added directly at 10 μl to culture wells then the 96 microwell plates were returned to the tissue culture incubator for 1–4 h/37 °C to allow the colorimetric reaction to occur then measured at 490 nm. The microplate was incubated for another 4 h under identical conditions.

2.5. Nuclear extract preparation

Nuclear extracts were prepared from U251 cells as described (Jiang et al., 1996). We routinely collected the U251 cells after 72 h in which they usually reached 70% confluent then cells washed twice with ice-cold Dulbecco's PBS 1× (PBS) and pelleted by centrifugation at 1200 rpm at 4 °C. The cell pellet was subsequently washed once in a hypotonic buffer (10 mM Tris–HCl (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 2 mM DTT, 1 mM Pefabloc, 2 mM sodium vanadate, 4 μg/ml pepstatin, 4 μg/ml leupeptin, and 4 μg/ml aprotinin), resuspended in the same buffer, and incubated for 10 min on ice. The cell suspension was homogenized with 18–20 strokes in a glass Dounce homogenizer. The nuclear pellet was obtained after centrifugation at 1000*g* at 4 °C for 10 min and resuspended in a hypertonic buffer (20 mM Tris–HCl (pH 7.5), 1.5 mM $MgCl_2$, 0.42 M KCl, 20% glycerol, 2 mM DTT, 1 mM Pefabloc, 2 mM sodium vanadate, 4 μg/ml pepstatin, 4 μg/ml leupeptin, and 4 μg/ml aprotinin) to obtain nuclear extracts. The nuclear suspension was rotated at 4 °C for 30 min, and nuclear debris were pelleted by centrifugation at 15,000 *g* for 30 min at 4 °C. The protein concentration was determined and standardized using the Bradford protein assay (Bradford, 1976) with bovine serum albumin (Sigma, UK) as the standard.

2.6. SDS–PAGE and Western blot analysis

Extracts of U251 cell line were processed for Western blot according to previously published methods (Alokail, 2005). Twenty micrograms of protein was resolved by electrophoresis on 12% polyacrylamide gels and electrotransferred on an Immobilon-P membrane. After blocking with 5% non-fat dry milk in PBS(−) containing 0.01% Tween-20 (T-PBS), the membrane was blotted overnight at 4 °C in rotation with primary anti-HIF-1α monoclonal antibody (diluted 1:400 in blocking buffer) (Santa Cruz Biotechnology, USA). The blots were then washed in T-PBS (1× PBS and 0.1% Tween-20) three times for 5 min each time at room temperature with rotation. Membranes were incubated for 2 h at room temperature with a horseradish peroxidase-conjugated secondary anti-mouse Ig monoclonal antibody (diluted 1:2000 in blocking buffer) (Santa Cruz Biotechnology, USA). Membranes were then washed three times in T-PBS buffer, and chemiluminescence detection was performed using an enhanced chemiluminescence kit according to the manufacturer's protocol (ECL

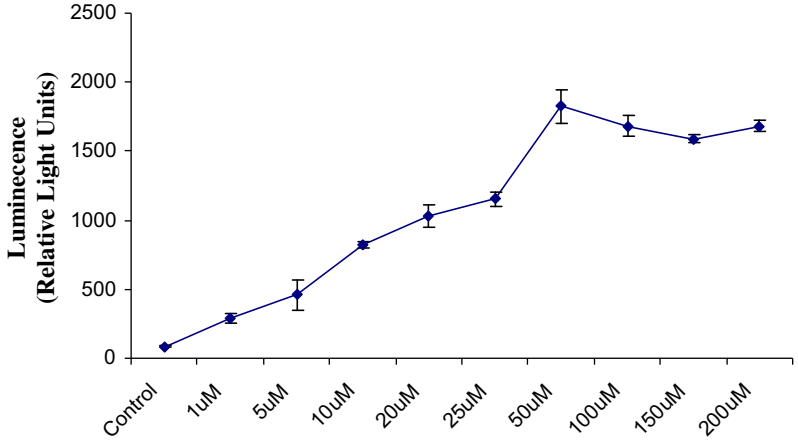

Figure 2 Cobalt chloride dose response concentration in cell proliferation. U251 cells were treated with serial $CoCl_2$ concentrations (1, 5, 10, 20, 25, 50, 100, 150 and 200 μM) at 37 °C under 20% O_2 for 16 h as mentioned in Section 2. Results represent means ± SE of three independent experiments.

Western blotting analysis system, Amersham Pharmacia Biotech, Buckinghamshire, UK). HIF-1α protein band signals were detected by Bio-Rad Imager system (Bio-Rad, UK).

3. Results

In order to reach maximum HIF-1α stimulation, different working $CoCl_2$ concentrations (1, 5, 10, 20, 25, 50, 100, 150 and 200 μM) were prepared and applied as final $CoCl_2$ treatment in U251 cell culture. In $CoCl_2$ concentrations under 20% O_2 at 37 °C for 16 h, the cell proliferation assay have shown that the $CoCl_2$ treatment for 16 h induced the U251 cells in a dose-dependent manner until 50 μM (Fig. 2) then decreased and become steady between 100 and 200 μM. The treatment duration was deduced from several pre-experiments (data not shown) to maximize the effect of $CoCl_2$ on the U251 cell line and minimize the possible confounding effects of $CoCl_2$ itself on the cell culture.

In order to determine the cellular density of U251 cell culture and the effect of 50 μM $CoCl_2$ sensitivity, U251 suspensions were prepared in a standard manner (5000, 10,000, 15,000, 20,000, 30,000 and 40,000) cells were grown in 75 cm^2 tissue culture flasks as mentioned in Section 2. The data have shown that 50 μM $CoCl_2$ have shown a maximum stimulation in a high cell density (30,000 and 40,000 cells/well) compared with weak response in low cell density (5000, 10,000, 15,000 and 20,000), (Fig. 3).

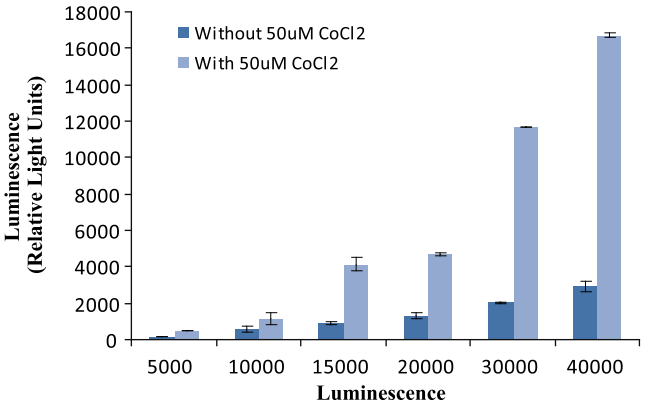

Figure 3 Effect of cobalt chloride in cellular proliferation. The U251 human cell line were seeded at 5000, 10,000, 15,000, 20,000, 30,000 or 40,000 cells per 75 cm^2 tissue culture flasks then all cells were treated for 16 h with $CoCl_2$ (50 μM) as mentioned in Section 2. Results represent means ± SE of three independent experiments.

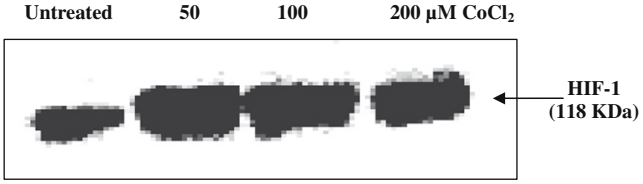

Figure 4 Induction of HIF-1α protein expression by cobalt chloride. Effect of PEITC on proteasome inhibitor-induced HIF-1α activity. U251 human cells were left untreated, or treated with 50,100, or 200 μM $CoCl_2$ for 1 h. Expression of HIF-1α determined by Western blot analysis. HIF-1α band detected 118 kDa. Results represent one of three independent experiments.

Moreover, in HIF-1α gene expression, $CoCl_2$ treatment for 16 h induced the expression of HIF-1α in a dose-dependent manner until 200 μM (Fig. 4). Again, 50 μM $CoCl_2$ have shown a higher HIF-1α expression compared with other concentrations. The HIF-1α protein expression have also reveled in Western blot analysis one sharp protein band at 118 kilo Dalton which represented the HIF-1α band.

4. Discussion

When the tumor grows, it develops extensive regions of poor oxygenation and high acidity due to the discrepancy between the rapid rate of tumor growth and the capacity of existing blood vessels to supply oxygen (Hockel and Vaupel, 2001). At a cellular level, a hypoxic stress generates an adaptive response that is mediated by the HIF-1α (Semenza, 1998). Thus a positive correlation between HIF-1α over expression and poor prognosis has been reported (Sohda et al., 2004; Theodoropoulos et al., 2004).

HIF-1α induces synthesis of proteins that promote metabolic changes in the cells of hypoxic tissues and, induces neovascularization to re-establish an adequate oxygen supply. Consequently, cells and tissues adapt to a broad range of oxygen concentrations, from normoxia (~21% O_2) to hypoxia (~1–5% O_2) Wang et al., 1995. HIF-1α is central to regulation of apoptosis and cell survival, cell adhesion and extracellular matrix turnover, cytoskeletal structure, cell motility, epithelial homeostasis, vascular tonus, erythropoiesis and iron metabolism, metabolic homeostasis, and pH regulation (Brahimi-Horn et al., 2005).

In mammalian systems, $CoCl_2$ has been used as a chemical agent that reportedly induces a biochemical and molecular response similar to that observed under low-oxygen conditions (Grasselli et al., 2005). Many reports have shown that both cobalt and hypoxia regulate a similar group of genes on a global gene expression level (Lee et al., 2001; Vengellur et al., 2003). In addition to this, $CoCl_2$ has been widely used as a hypoxia-mimicking agent in both in vivo (Badr et al., 1999) and in vitro studies (Wang and Semenza, 1993). Cobalt is essential for human health because of its critical role in the synthesis of vitamin B_{12} (Roessner et al., 2001), however, excess exposure of cobalt can lead to tissue and cellular toxicity.

In this study, we investigate the effects of the hypoxia-mimetic $CoCl_2$ in U251 human glioblastoma cells. We demonstrate that $CoCl_2$ treatment leads to increase HIF-1α gene expression and cellular induction of these cells. In comparison with previous experiments on gene expression (Chang et al., 2007), found limited similarity in the transcriptional response to $CoCl_2$, demonstrating that these treatments have distinct effects on cell physiology. We therefore propose that in U251 human glioblastoma cells the response to $CoCl_2$ mimics certain aspects of normoxia by targeting HIF-1α gene and various proteins that promote metabolic changes in the cells. Due to this, $CoCl_2$ appears to be an effective chemical to screen for all pathways involved in oxygen sensing, and may be useful in HIF-1α as central in regulation of apoptosis and cell sur-

vival. Other group, however, suggested that $CoCl_2$ appears to be not an effective in pathways involved in oxygen sensing, but may be useful in uncovering factors involved in the Sre1p-mediated response (Lee et al., 2007).

As for all proteins, the balance between the rate of synthesis and the rate of catabolism determines the HIF-1α protein level. While HIF-1α protein synthesis is regulated by O_2-independent mechanisms, HIF-1α protein degradation is primarily regulated through O_2-dependent mechanisms. Under normoxic conditions the catabolic rate of the α-subunit is very high and thus the protein is generally not undetectable, while under hypoxic conditions the α-subunit is stable and available for binding to the β-subunit to form an active transcriptional heterodimer. In addition, both O_2-independent and O_2-dependent mechanisms mediate posttranslational modifications that alter protein stability and transcriptional activity. Furthermore, the expression of HIF-1α protein is modulated through different pathways that alter HIF-1-dependent transcriptional activity and makes the HIF-1α transcription factor an attractive target for new drug development. For example, a number of anticancer drugs have been shown to inhibit HIF-1α, but none of these drugs target HIF-1α directly and specifically (Semenza, 2003; Generali et al., 2006; Powis and Kirkpatrick, 2004). However, most inhibitors affect multiple signaling pathways and only indirectly target the HIF-1α transcription factor. Therefore, the ability to identify, visualize and validate changes in the dynamics and stability of the HIF-1α protein is likely to be useful as a pharmacodynamic end point and read-out of inhibition in the development of new anticancer drugs that target HIF-1α in the future.

The present paper reports the adaptive response of U251 human glioblastoma cells to cobalt chloride $CoCl_2$, a chemical hypoxia-mimicking agent. The effects of the treatment were evaluated on cell proliferation and HIF-1α gene expression. In addition, because HIF-1α plays a central role in tumor progression, angiogenesis in vivo and oncogenic activation or loss of tumor suppressor function is associated with HIF-1α mediated tumor progression, HIF-1α gene expression levels were investigated. In the present study, $CoCl_2$ had U251 cellular induction and stimulated HIF-1α gene expression. Thus, we suggested here the more experiments are needed to define HIF-1α since we are interested in brain cancer and its cell death-related mechanisms.

References

Alokail, M.S., 2005. Cell Biochem. Funct. 23, 157.

Badr, G.A., Zhang, J.Z., Tang, J., Kern, T.S., Ismail-Beigi, F., 1999. Mol. Brain Res. 64, 24.

Bárdos, J.I., Chau, N.M., Ashcroft, M., 2004. Mol. Cell Biol. 24, 2905.

Belozerov, V.E., Meir, Van, 2005. Anticancer Drug 16, 901.

Birner, P., Schindl, M., Obermair, A., Plank, C., Breitenecker, G., 2000. Cancer Res. 60, 4693.

Bradford, M.M., 1976. Anal. Biochem. 72, 248.

Brahimi-Horn, C., Mazure, N., Pouyssegur, J., 2005. Cell Sign 17, 1.

Chang, E.I., Thangarajah, H., Hamou, C., Gurtner, G.C., 2007. Lymphat. Res. Biol. 5, 237.

Epstein, A.C., Gleadle, J.M., McNeill, L.A., Dhanda, A., Tian, Y.M., Masson, N., Hamilton, D.L., Jaakkola, P., Basrtead, R., Hodgkin, J., Maxwell, P.H., Pugh, C.W., Schoofield, P.J., Ratcliffe, P.J., 2001. Cell 107, 43.

Generali, D., Fox, S.B., Berruti, A., Brizzi, M.P., Campo, L., Bonardi, S., Wigfield, S.M., Bruzzi, P., Bersiga, A., Allevi, G., Milani, M., Aguggini, S., Dogliotti, L., Bottini, A., Harris, A.L., 2006. Endocr. Relat. Cancer 13, 921.

Giaccia, A., Siim, B.G., Johnson, R.S., 2003. Nat. Rev. Drug Discov. 10, 803.

Goldberg, M.A., Dunning, S.P., Bunn, H.F., 1998. Science 242, 1412.

Grasselli, F., Basini, G., Bussolati, S., Bianco, F., 2005. Reprod. Fertil. Dev. 17, 715.

Hockel, M., Vaupel, P., 2001. J. Natl. Cancer Inst. 93, 266.

Hogenesch, J.B., Chan, W.K., Jackiw, V.H., Brown, R.C., Gu, Y.Z., Pray-Grant, M., Perdew, G.H., Bradfield, C.A., 1997. J. Biol. Chem. 272, 8581.

Hu, J.C., Sataur, A., Wang, L., Chen, H., Simon, M.C., 2007. Mol. Biol. Cell 18, 4528.

Jaakkola, P., Mole, D.R., Tian, Y-M., Wilson, M., Gielbert, J., Gaskell, S.J., Kriegsheim, Av., Hebestreit, H.F., Mukherji, M., Schofield, C.J., Maxwell, P.H., Pugh, C.W., Ratcliffe, P.J., 2001. Science 292, 468.

Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, Av., Hebestreit, H.F., Mukherji, M., Schofield, C.J., Maxwell, P.H., Pugh, C.W., Ratcliffe, P.J., 2001. Science 292, 468.

Jiang, B.H., Rue, E., Wang, G.L., Roe, R., Semenza, G.L., 1996. J. Biol. Chem. 271, 17771.

Lando, D., Peet, D.J., Whelan, D.A., Gorman, J.J., Whitelaw, M.L., 2002. Science 295, 858.

Lee, S.G., Lee, H., Rho, H.M., 2001. FEBS Lett. 507, 259.

Lee, H., Bien, C.M., Hughes, A.L., Espenshade, P.J., Kwon-Chung, K.J., Chang, Y.C., 2007. Mol. Microbiol. 65, 1018.

Malich, G., Markovic, B., Winder, C., 1997. Toxicology 124, 179.

McNeil, L.A., Hewiston, K.S., Gleadle, J.M., 2002. Bioorg. Med. Chem. Lett. 12, 1547.

Powis, G., Kirkpatrick, L., 2004. Mol. Cancer Ther. 3, 647.

Pugh, C.W., O'Rourke, J.F., Nagao, M., Gleadle, J.M., Ratcliffe, P.J., 1997. J. Biol. Chem. 272, 11205–11214.

Roessner, C.A., Santander, P.J., Scott, A.I., 2001. Vitam. Horm. 61, 267.

Ruas, J.L., Poellinger, L., Pereira, T., 2002. J. Biol. Chem. 277, 38723.

Semenza, G.L., 1998. Curr. Opin. Genet. Dev. 8, 588.

Semenza, G.L., 2003. Nat. Rev. Cancer 3, 721.

Sohda, M., Ishikawa, H., Masuda, N., Kato, H., Miyazaki, T., Nakajima, M., Fukuchi, M., Manda, R., Fukai, Y., Sakurai, H., Kuwano, H., 2004. Int. J. Cancer 110, 838.

Theodoropoulos, V.E., Lazaris, A.C., Sofras, F., Gerzelis, I., Tsoukala, V., Ghikonti, I., Manikas, K., Kastriotis, I., 2004. Eur. Urol. 46, 200.

Vengellur, A., Woods, B.G., Ryan, H.E., Johnson, R.S., LaPres, J.J., 2003. Gene Expr. 11, 181.

Walmsley, S.R., McGovern, N.N., Whyte, M.K.B., Chilvers, E.R., 2008. Am. J. Resp. Cell Mol. Biol. 38, 251.

Wang, G.L., Semenza, G.L., 1993. Blood 82, 3610.

Wang, G.L., Jiang, B.H., Rue, E.A., Semenza, G.L., 1995. Proc. Natl. Acad. Sci. USA 92, 5510.

Wenger, R.H., 2002. FASEB J. 16, 1151.

Yuan, Y., Hilliard, G., Ferguson, T., Millhorn, D.E., 2003. J. Biol. Chem. 278, 15911.