



# Effect of hypoxia and re-oxygenation on cell invasion and adhesion in human ovarian carcinoma cells

JUN SHI<sup>1</sup>, YINSHENG WAN<sup>2</sup> and WEN DI<sup>1</sup>

<sup>1</sup>Department of OB and GYN, Renji Hospital of Shanghai Jiaotong University Medical School, Shanghai 200001, P.R. China; <sup>2</sup>Department of Biology, Providence College, Providence, RI 02918-0001, USA

Received March 18, 2008; Accepted June 9, 2008

DOI: 10.3892/or\_00000077

**Abstract.** Hypoxia inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) regulates the transcription of a number of genes under hypoxia and other extracellular or intracellular stimulations. It also promotes angiogenesis, tumor metastasis and invasion. To investigate the effect of hypoxia and reoxygenation on cell proliferation, invasion and adhesion, which are all related to ovarian cancer, we applied chemically-induced hypoxia in the cultured human ovarian carcinoma cell line, HO-8910PM. Semi-quantitative RT-PCR results show that CoCl<sub>2</sub> induces the expression of HIF-1 $\alpha$  in a time- and dose-dependent manner. MTT assay results show that CoCl<sub>2</sub>-induced hypoxia inhibits cell proliferation which is recovered by reoxygenation. The Boyden and cell adhesion test results indicate that CoCl<sub>2</sub>-induced hypoxia inhibits cell invasion and adhesion which are markedly enhanced by reoxygenation in the human ovarian carcinoma cell line, HO-8910PM. Collectively, our data provide insight into understanding molecular mechanisms of the invasiveness of ovarian cancer under the conditions of hypoxia and reoxygenation.

## Introduction

Hypoxia inducible factor (HIF)-1 is composed of O<sub>2</sub>-regulated HIF-1 $\alpha$  and constitutively expressed HIF-1 $\beta$  subunits (1), and functions as a transcriptional activator and a master regulator of O<sub>2</sub> homeostasis. HIF-1 $\alpha$  plays a key role in the cellular response to hypoxia, regulating genes that are associated with energy metabolism, erythropoietin, membrane glucose transporters, vascular epithelial growth factors and glycolytic enzymes. Almost 100 genes have been identified

that are regulated by HIF-1. Although HIF-1 $\alpha$  accommodates the tissue physiological hypoxia response, it has been expressed in various types of human tumor cells (2) with abundant data demonstrating that HIF-1 $\alpha$  is closely related to tumor development and metastasis (3).

Hypoxia is one of the basic features in a solid carcinoma microenvironment. HIF-1 plays a very important role in tumor development and metastasis (4,5), and has been considered as one of the most important drug targets for cancer treatment (6). While the molecular and cellular mechanism of HIF-1 under hypoxia is under investigation *in vivo*, a number of chemicals including CoCl<sub>2</sub> or conditions that induce hypoxia are applied in *in vitro* experiments. Our previous studies have shown that UV radiation induces HIF-1 $\alpha$  expression, which may be associated with UV-induced skin cancer (7).

Ovarian cancer remains the leading cause of mortality of gynecological cancer types. Ovarian carcinoma is expected to develop in 1 of 70 women in their lifetime, and one woman in 100 will ultimately succumb to complications associated with this aggressive disease. Studies have shown that despite good initial responses to chemotherapy, 75% of patients will succumb due to disease recurrence, progression and complications (8).

Ovarian cancer cells behave aggressively and are resistant to various cancer drugs. Our previous studies have shown that resistance to drugs such as taxol is due largely to the transient activation of EGFR and expression of survivin (9-12). HIF-1 $\alpha$  has been shown to be associated with cancer chemoresistance and poor prognosis (13), although cancer drugs such as cisplatin and doxorubicin repress vascular endothelial growth factor expression and differentially down-regulate HIF-1 $\alpha$  activity in human ovarian cancer cells (14).

Given that HIF-1 $\alpha$  is involved in cancer cell invasion and metastasis under hypoxia, we undertook this study to investigate the effect of chemically-induced hypoxia and reoxygenation on cell proliferation, invasion and adhesion in cultured ovarian cancer cells. We found that CoCl<sub>2</sub>, one of the hypoxia inducible agents, induces HIF-1 $\alpha$  expression in cultured HO-8910PM cells. Reoxygenation after hypoxia enhances cell proliferation, invasion and adhesion.

## Materials and methods

**Cell culture.** As previously described (11), human ovarian cancer HO-8910PM cells were cultured in RPMI-1640 with

---

**Correspondence to:** Dr Wen Di, Department of OB and GYN, Renji Hospital of Shanghai Jiaotong University Medical School, Shanghai 200001, P.R. China  
E-mail: diwen163@163.com

Dr Yinsheng Wan, Department of Biology, Providence College, 549 River Ave Providence, RI 02918-0001, USA  
E-mail: yswan@providence.edu

**Key words:** hypoxia, invasion, adhesion, ovarian carcinoma

Table I. Cell proliferation in HO-8910PM cells under different hypoxia/reoxygenation conditions.

CoCl <sub>2</sub> ( $\mu$ M)	Cell proliferation			
	H16 h	H24 h	H16 h RO <sub>2</sub> 8 h	H16 h RO <sub>2</sub> 24 h
0	0.765 $\pm$ 0.067	0.859 $\pm$ 0.062	0.830 $\pm$ 0.045	0.824 $\pm$ 0.090
50	0.422 $\pm$ 0.095	0.339 $\pm$ 0.086	0.567 $\pm$ 0.070	0.466 $\pm$ 0.128
100	0.410 $\pm$ 0.145	0.221 $\pm$ 0.062	0.466 $\pm$ 0.053	0.340 $\pm$ 0.056
150	0.356 $\pm$ 0.073	0.219 $\pm$ 0.070	0.283 $\pm$ 0.033	0.321 $\pm$ 0.030
300	0.337 $\pm$ 0.047	0.202 $\pm$ 0.092	0.311 $\pm$ 0.1000	0.307 $\pm$ 0.032

20% FBS and 1% penicillin-streptomycin. The media were changed every other day. The passage period was ~3 days. Cells were trypsinized with 0.25% trypsin provided by the Shanghai Institute of Cell Biology.

**RT-PCR detection of expression of HIF-1 $\alpha$  after hypoxia.** HO-8910PM cells ( $10 \times 10^5$ ) were seeded in 25 cm<sup>2</sup> culture flasks, and cultured for 24–48 h. When the cells were at a confluence of 80%, CoCl<sub>2</sub> was added into the media. Cells were treated with either 150  $\mu$ M of CoCl<sub>2</sub>, and collected at 4, 8, 16, 24 and 48 h after treatment; or they were treated with CoCl<sub>2</sub> at a concentration of 0, 50, 100 and 150  $\mu$ M, and collected at 16 h after treatment. Total RNAs were isolated using TRIzol (Sigma, St. Louis, MO). RT-PCR was performed using kit (Promega, USA). First-strand cDNA was synthesized with 4  $\mu$ g of total cellular RNA and 0.5  $\mu$ g of Oligo(dT) 18 primer. After synthesis, the reaction mixture was immediately subjected to PCR, which was carried out using the PCR system (Promega). The primers for HIF-1 $\alpha$  are AATTCTACACCCACAGGCCA (sense) and AGGGCCGGACTCGTGATACT (antisense). The primers for  $\beta$ -actin as a control are TCCGATGGAAGCACTAGACA (sense) and TCAAAGCGACAGATAACACG (antisense). The HIF-1 $\alpha$  PCR regimen was: 94°C/5 min, 94°C/30 sec, 58°C/30 sec, 72°C/30 sec for 30 cycles, 72°C for 10 min, and 4°C refrigeration. The  $\beta$ -actin PCR regimen was: 94°C/5 min, 95°C/30 sec, 56°C/30 sec, 72°C/45 sec for 28 cycles, 72°C for 7 min, and 4°C refrigeration. PCR products were electrophoresed on 1.5% agarose gels, and the bands were visualized with ethidium bromide and photographed with a camera. Densitometric analysis was used for band quantification using NIH image software.

**MTT dye assay.** As previously described (11),  $0.2\text{--}0.4 \times 10^5$  HO-8910PM cells in 200  $\mu$ l of complete medium were seeded into a 96-well plate, and the plate was incubated at 37°C in a 5% CO<sub>2</sub>/95% air incubator overnight. Cells were treated at different concentrations of CoCl<sub>2</sub> (0, 50, 100, 150 and 300  $\mu$ M) for 16 and 24 h, and re-oxygenated for 8 and 24 h after being treated with CoCl<sub>2</sub> for 16 h. In the last 4 h of the time period, 20  $\mu$ l of MTT (5 mg/ml) were added into the media. When the time period ended, 150  $\mu$ l of DMSO were added to the wells. After having discarded the medium, the plate was placed on a shaker at low speed for 10 min. The absorption at 492 nm was measured. The data from three wells were used for statistical analysis in each experiment from all the groups.

**Cell invasion assay.** Cells were prepared for the control; hypoxia 24-h; and hypoxia 16-h and re-oxygenated 24-h groups. Twelve-millimetre-diameter polycarbonate filters (8- $\mu$ m pore size, Millipore, USA) at 1:3 dilution in serum-free medium were used. HO-8910PM cells ( $10 \times 10^4$ ) in 400  $\mu$ l of complete medium were seeded into the inner chamber. Media (600  $\mu$ l) were added to the lower chamber, and the plate was incubated at 37°C in a CO<sub>2</sub> incubator for 16 h. Cells on the lower surface of the filter were stained with haematoxylin and counted under microscope.

**Cell adhesion test.** Cells were prepared for the control; hypoxia 16-h; hypoxia 24-h; as well as the hypoxia 16-h and re-oxygenated 24-h groups. Limnin (1  $\mu$ g/cm<sup>2</sup>, Sigma) was fixed in a 24-well plate at 4°C overnight. BSA (5%) was used to block for 60 min at 37°C. Hank's was used to wash the plate. Prepared cells ( $10 \times 10^5$ ) were seeded in the wells of a 24-well plate at 37°C in a CO<sub>2</sub> incubator. Cells were trypsinized with 0.25% trypsin after being cultured for 30, 90, 120 and 180 min, respectively. The cells that adhered to the wells were counted under microscope.

## Results

**Effect of CoCl<sub>2</sub>-induced hypoxia on cell proliferation in cultured HO-8910PM cells.** Existing data have shown that CoCl<sub>2</sub> induces hypoxia and inhibits cell proliferation in other cancer cells. To examine the effect of CoCl<sub>2</sub>-induced hypoxia on cell proliferation in ovarian cancer HO-8910PM cells, the cells were cultured in 96-well plates, and MTT dye assay was performed. The results showed that CoCl<sub>2</sub> treatment inhibits cell proliferation in a time- and dose-dependent manner (Table I). However, reoxygenation after hypoxia markedly enhances cell proliferation.

**Effect of CoCl<sub>2</sub> on HIF-1 $\alpha$  expression in HO-8910PM cells.** To investigate the mechanism of CoCl<sub>2</sub>-induced inhibition of cell proliferation and enhancement by reoxygenation post-hypoxia, we measured the expression of HIF-1 $\alpha$  using RT-PCR. Cells were cultured in 6-well plates and treated with 150  $\mu$ M of CoCl<sub>2</sub>. The cells were collected at different time points (4, 8, 16, 24 and 48 h) and total RNAs were extracted. RT-PCR results showed that CoCl<sub>2</sub> induces HIF-1 $\alpha$  expression in a time-dependent manner. The expression starts at 4 h and peaks at ~24 h and remains elevated at 48 h (Fig. 1A and B). CoCl<sub>2</sub> also induces HIF-1 $\alpha$  expression in a dose-dependent



Treatment	30 min	90 min	120 min	180 min
Control	5.0%	5.5%	9.3%	15.7%
Hypoxia 16 h	4.0%	3.2%	6.8%	11.3%
Hypoxia 24 h	0.3%	0.5%	0.7%	1.0%
Hypoxia 16 h RO <sub>2</sub> 24 h	5.3%	9.0%	9.5%	17.0%

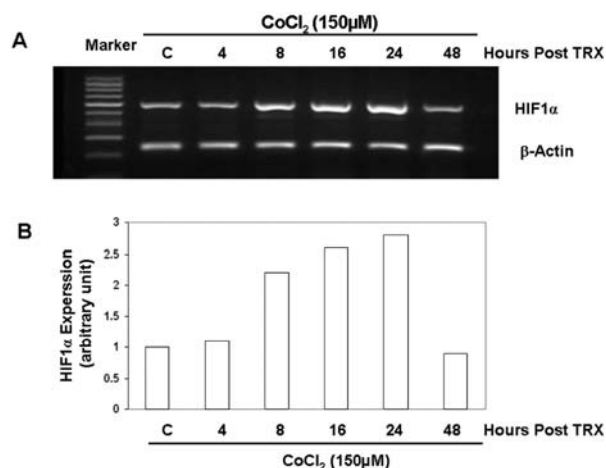


Figure 1. Effect of  $\text{CoCl}_2$  on HIF-1 $\alpha$  expression (time course) in HO-8910PM cells. Cells were cultured and treated with 150  $\mu\text{M}$  of  $\text{CoCl}_2$ . The cells were collected at various time points after treatment (4, 8, 16, 24 and 48 h) for the detection of HIF-1 $\alpha$  expression by RT-PCR (A). The bands were quantified (B).

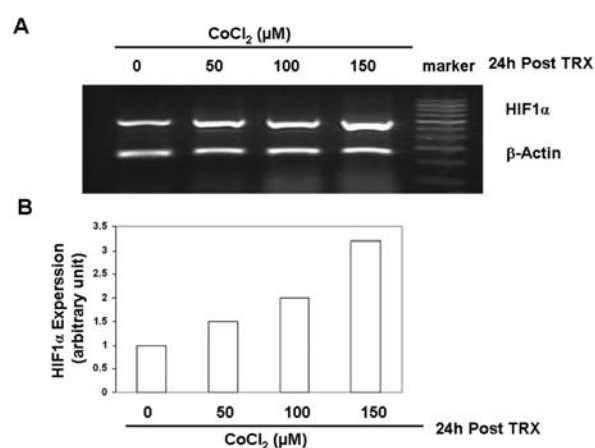


Figure 2. Effect of  $\text{CoCl}_2$  on HIF-1 $\alpha$  expression (dose response) in HO-8910PM cells. Cells were cultured and treated with various concentrations of  $\text{CoCl}_2$  (50, 100 and 150  $\mu\text{M}$ ). The cells were collected at 24 h after treatment for the detection of HIF-1 $\alpha$  expression by RT-PCR (A). The bands were quantified (B).

manner at a concentration of 150  $\mu\text{M}$ , which induces the highest HIF-1 $\alpha$  expression (Fig. 2A and B).

*Effect of  $\text{CoCl}_2$ -induced hypoxia on cell invasion in the HO-8910PM cells.* To study cell invasion under conditions

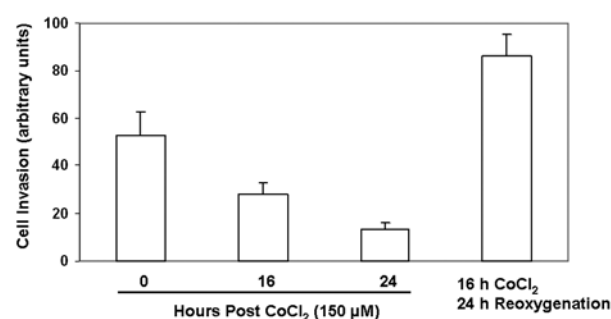


Figure 3. Effect of hypoxia and reoxygenation on cell invasion in HO-8910PM cells. Cells were cultured and prepared in the control;  $\text{CoCl}_2$ -treated 16- and 24-h; and  $\text{CoCl}_2$  16- and 24-h reoxygenation groups. A cell invasion test was performed as described in Materials and methods. Cell invasion was presented as arbitrary units.

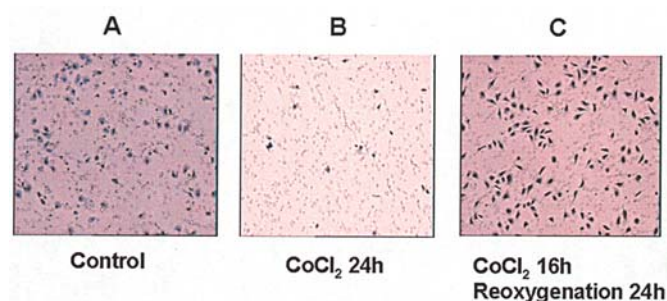


Figure 4. Effect of hypoxia and reoxygenation on cell adhesion in HO-8910PM cells. Cells were prepared for the control; hypoxia 16-h; hypoxia 24-h; hypoxia 16-h and reoxygenated 24-h groups. A cell adhesion test was performed as described in Materials and methods.

of hypoxia and reoxygenation, the Boyden cell invasion test was used. The results showed that after 24-h hypoxia, cell invasion decreases greatly (the number of cells through the artificial basement membrane is  $13.33 \pm 3.06$  and that of the control group is  $52.67 \pm 10.21$ ). However, after being under hypoxia for 16 h and being reoxygenated for 24 h, the cell invasion is enhanced sharply (the number of cells through the artificial basement membrane is  $86.00 \pm 9.17$  and that of the control group is  $52.67 \pm 10.21$ ) (Fig. 3).

*Effect of  $\text{CoCl}_2$ -induced hypoxia on cell adhesion in the HO-8910PM cells.* To study cell adhesion under hypoxia and reoxygenation, the Limnin cell adhesion test was used. The results showed that compared to the control group, hypoxia



induced by  $\text{CoCl}_2$  inhibits cell adhesion in a time-dependent manner. However, reoxygenation after hypoxia enhances cell adhesion (Table II, Fig. 4).

## Discussion

HIF-1 $\alpha$  is induced by hypoxia, and by other factors such as chloride ion, desferrioxamine, and oncogenes.  $\text{CoCl}_2$  is a chemical reagent and remains an important tool for studying the role of HIF-1 $\alpha$  in a number of cells, including skin (7). In this study, we used the ovarian cancer HO-8910PM cell line, which is a high metastasis serous cystadenocarcinoma one. Our study shows that there is HIF-1 $\alpha$  expression in HO-8910PM cells and it is induced by  $\text{CoCl}_2$ . However, the question of whether the expression of HIF-1 $\alpha$  is tightly correlated with a malignant grade of ovarian carcinoma remains to be addressed (15,16).

Our study, using MTT dye assay, shows that  $\text{CoCl}_2$  inhibits cell proliferation in HO-8910PM cells. Choosing an optimal  $\text{CoCl}_2$  concentration and culture time is very important for the establishment of a hypoxia cell model. The results presented above demonstrate that  $\text{CoCl}_2$  induces HIF-1 $\alpha$  expression in HO-8910PM cells in a time- and dose-dependent manner at mRNA level (Figs. 1 and 2). Other studies have shown that the regulation of HIF-1 $\alpha$  occurs at the protein level (17-19). In this study, we used 150  $\mu\text{M}$  as the optimal concentration for the induction of hypoxia and 24 h as the optimal culture time.

Available evidence indicates that tumor cell apoptosis or survival is determined by a degree of hypoxia. Studies have shown that hypoxia/reoxygenation increases cell proliferation, suggesting that HIF-1 $\alpha$  is a key modulator under hypoxia. Our MTT data show that cell growth is inhibited under chemical hypoxia induced by  $\text{CoCl}_2$ . Furthermore, the inhibition is in a time- and dose-dependent manner. As expected, a high concentration of  $\text{CoCl}_2$  induces cell death in cultured HO-8910PM cells.

Metastasis and invasion are very important biological characteristics of malignant tumors (20). Our study shows that cell invasion and adhesion are enhanced under hypoxia/reoxygenation treatment when HIF-1 $\alpha$  is overexpressed by  $\text{CoCl}_2$ . Our data suggest that HIF-1 $\alpha$  overexpression regulates the expression of a number of related genes such as VEGF, which is associated with tumor angiogenesis. The regulatory effect of HIF-1 $\alpha$  may take place at the transcriptional and post-transcriptional level (21).

HIF-1 $\alpha$  also up-regulates  $\text{MMP}_2$  and down-regulates E-cad, which decreases the power of intercellular adhesion and increases the power of extracellular matrix, promoting tumor cell invasion and metastasis (22). Hypoxia is a challenge for promoting tumor cell malignancy. If tumor cells survived under hypoxia, the cell malignant behavior would be more powerful than before hypoxia. Therefore, HIF-1 $\alpha$  is a promoter of tumor cell deterioration and metastasis.

Thus, HIF-1 $\alpha$  has been considered as one of the most important target proteins of neoplasm-targeted therapies. It is envisioned that better understanding of the molecular role of HIF-1 $\alpha$  in promoting dissemination and metastasis in ovarian cancer would eventually improve prognosis and increase

patient survival. Our data provide insight into understanding the molecular mechanism of cell proliferation, invasion and adhesion under hypoxia/reoxygenation in ovarian cancer cells.

## Acknowledgements

This research was supported in part by a grant from the National Natural Science Foundation of China (30471808, W.D.) and a grant from NIH (P20 RR016457 from INBRE Program of the National Center for Research Resources, Y.W.).

## References

1. Wang GL, Jiang BH, Rue EA and Semenza GL: Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O<sub>2</sub> tension. *Proc Natl Acad Sci USA* 92: 5510-5514, 1995.
2. Jubb A and Hillan K: Expression of HIF-1 alpha in human tumours. *J Clin Pathol* 58: 1344, 2005.
3. Yasuda M, Miyazawa M, Fujita M, *et al*: Expression of hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) and glucose transporter-1 (GLUT-1) in ovarian adenocarcinomas: difference in hypoxic status depending on histological character. *Oncol Rep* 19: 111-116, 2008.
4. Zhong H, De Marzo AM, Laughner E, *et al*: Overexpression of hypoxia-inducible factor 1 $\alpha$  in common human cancers and their metastases. *Cancer Res* 59: 5830-5835, 1999.
5. Maxwell PH: The HIF pathway in cancer. *Semin Cell Dev Biol* 16: 523-530, 2005.
6. Park SY, Jeong KJ, Lee J, *et al*: Hypoxia enhances LPA-induced HIF-1 $\alpha$  and VEGF expression: their inhibition by resveratrol. *Cancer Lett* 258: 63-69, 2007.
7. Li Y, Bi Z, Yan B and Wan Y: UVB radiation induces expression of HIF-1 $\alpha$  and VEGF through the EGFR/PI3K/DEC1 pathway. *Int J Mol Med* 18: 713-719, 2006.
8. Chan JK, Pham H, You XJ, *et al*: Suppression of ovarian cancer cell tumorigenicity and evasion of cisplatin resistance using a truncated epidermal growth factor receptor in a rat model. *Cancer Res* 65: 3243-3248, 2005.
9. Qiu L, Di W, Jiang Q, *et al*: Targeted inhibition of transient activation of the EGFR-mediated cell survival pathway enhances paclitaxel-induced ovarian cancer cell death. *Int J Oncol* 27: 1441-1448, 2005.
10. Qiu L, Wang Q, Di W, *et al*: Transient activation of EGFR/AKT cell survival pathway and expression of survivin contribute to reduced sensitivity of human melanoma cells to betulinic acid. *Int J Oncol* 27: 823-830, 2005.
11. Qiu L, Zhou C, Sun Y, *et al*: Crosstalk between EGFR and TrkB enhances ovarian cancer cell migration and proliferation. *Int J Oncol* 29: 1003-1011, 2006.
12. Ji C, Cao C, Lu S, *et al*: Curcumin attenuates EGF-induced AQP3 up-regulation and cell migration in human ovarian cancer cells. *Cancer Chemother Pharmacol* (In press).
13. Fillies T, Werkmeister R, van Diest PJ, Brandt B, Joos U and Buerger H: HIF1-alpha overexpression indicates a good prognosis in early stage squamous cell carcinomas of the oral floor. *BMC Cancer* 5: 84, 2005.
14. Duyndam MC, van Berkel MP, Dorsman JC, Rockx DA, Pinedo HM and Boven E: Cisplatin and doxorubicin repress vascular endothelial growth factor expression and differentially down-regulate hypoxia-inducible factor 1 activity in human ovarian cancer cells. *Biochem Pharmacol* 74: 191-201, 2007.
15. Nakayama K, Kanzaki A, Hata K, *et al*: Hypoxia-inducible factor 1 alpha (HIF-1 alpha) gene expression in human ovarian carcinoma. *Cancer Lett* 176: 215-223, 2002.
16. Birner P, Gatterbauer B, Oberhuber G, *et al*: Expression of hypoxia-inducible factor-1 alpha in oligodendrogliomas: its impact on prognosis and on neoangiogenesis. *Cancer* 92: 165-171, 2001.
17. Semenza GL: HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J Appl Physiol* 88: 1474-1480, 2000.
18. Maxwell PH and Ratcliffe PJ: Oxygen sensors and angiogenesis. *Semin Cell Dev Biol* 13: 29-37, 2002.



SPANDIDOS<sup>®</sup> RH, Kvietikova I, Rolfs A, Gassmann M and Marti HH: Hypoxia-inducible factor-1 alpha is regulated at the post-mRNA level. *Kidney Int* 51: 560-563, 1997.

20. Friedmann Y, Vlodavsky I, Aingorn H, *et al*: Expression of heparanase in normal, dysplastic, and neoplastic human colonic mucosa and stroma. Evidence for its role in colonic tumorigenesis. *Am J Pathol* 157: 1167-1175, 2000.
21. Skinner HD, Zheng JZ, Fang J, Agani F and Jiang BH: Vascular endothelial growth factor transcriptional activation is mediated by hypoxia-inducible factor 1alpha, HDM2, and p70S6K1 in response to phosphatidylinositol 3-kinase/AKT signaling. *J Biol Chem* 279: 45643-45651, 2004.
22. Imai T, Horiuchi A, Wang C, *et al*: Hypoxia attenuates the expression of E-cadherin via up-regulation of SNAIL in ovarian carcinoma cells. *Am J Pathol* 163: 1437-1447, 2003.