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Original article

The Role of Hypoxia-Inducible Factor-1α and -2α in Androgen Insensitive Prostate Cancer Cells☆

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Abstract

Objectives: The aim of this study was to investigate the effects of induction and knocking down of hypoxia-inducible factor (HIF)-1α and/or -2α on tumor biology in androgen insensitive prostate cancer cell lines.

Materials and methods: The induction patterns of HIF-1α and -2α after treatment with $ZnSO_4$ were evaluated in PC3 and DU145 cells. Both cell lines were transfected with siRNA targeted against HIF-1α and/or -2α, and the expression patterns of these 2 HIF isoforms were examined. We next performed cell counting Kit-8 (CCK-8) assays and matrigel invasion assays. Potential additive effects of HIF blockade to chemotherapy (docetaxel) or target agents (sunitinib and sorafenib) were examined. In addition, gene expression changes were determined in $ZnSO_4$-treated DU145 cells using Western blotting.

Results: $ZnSO_4$ affected the expression of HIF in a dose-dependent manner. HIF expression was increased within the first 3 hours but then decreased. Cells in which HIF-1α and/or -2α had been knocked down using siRNA showed decreased cell viability. Invasion abilities were increased by $ZnSO_4$ treatment in both cell lines overexpressing HIF. However, invasion potencies were decreased in response to treatment with HIF siRNAs. Blocking HIF prominently augmented the antitumor effects of target agents. The underlying mechanism could be associated with p21, cMET, IGF-1, and GLUT-1.

Conclusions: Our results demonstrate that HIF-1α and -2α are important for cell proliferation and invasion ability in prostate cancer. Together, our results indicate that combinations of target agents with HIF knockdown may represent a promising strategy for the treatment of prostate cancer.

Keywords: Prostate cancer; Hypoxia-inducible factor; siRNA; Zinc

1. Introduction

In developed countries, prostate cancer (CaP) was the most frequently diagnosed cancer and the third leading cause of death in men in 2008 [1]. Advanced or metastatic CaP initially responds to androgen deprivation therapy [2]; however, the therapeutic efficacy of this treatment does not last, and the disease eventually becomes hormone-refractory CaP [3]. Although systemic chemotherapy is used to treat hormone-refractory CaP, responses are frequently poor [4].

Hypoxia-inducible factor (HIF)-1 is a heterodimer consisting of a HIF-1α subunit and a constitutively expressed HIF-1β subunit. In normoxic conditions, HIF-1α is regulated by ubiquitination and its interaction with the von Hippel-Lindau tumor suppressor protein (pVHL), which results in the rapid degradation of HIF-1α by the 26S proteasome. This process is mediated by prolyl hydroxylase domain-containing (PHD) proteins. Under hypoxic conditions, PHD activity is inhibited and HIF-1α hydroxylation and the association between HIF-1α and pVHL is decreased, thus resulting in increased levels of HIF-1α protein [5]. Due to genetic mutations, expression of HIF-1α is often increased in human cancers even under normoxic condi-

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tions. HIF-1α overexpression is frequently associated with the aggressive features of cancer [6].

HIF-1α overexpression has been observed in primary CaP, in bone metastatic sites, and in high-grade prostate intraepithelial neoplastic lesions [7,8]. One clinical study revealed that overexpression of HIF-1α was independently associated with rapid biochemical recurrence after radical prostatectomy or radiotherapy in a localized CaP cohort [9]. Because androgen stimulates the protein expression and transcriptional activity of HIF-1 in CaP, it has been postulated that the efficacy of androgen deprivation therapy, which has been a mainstay of CaP treatment, could be based in part on its ability to down-regulate HIF-1α [5]. In CaP cells, HIF-1α expression has been shown to be induced by zinc, even under normoxic conditions [10]. $ZnCl_2$ may act by stimulating reactive oxygen species (ROS) production and inhibiting the prolyl hydroxylation of HIF-1α under conditions of oxidative stress. Under normal physiologic conditions, the prostate contains extremely high concentrations of zinc [11]. It is possible that HIF-1α functions as an intrinsic defense molecule that enables prostate cells to survive in a zinc-rich environment, and zinc may be a natural inducer of HIF in CaP cells.

HIF-2α is another HIF protein with structural homology to HIF-1α that is also regulated by oxygen through the PJDs [12]. Like HIF-1α, HIF-2α regulates hypoxia-inducible genes, which are often coexpressed in tumors. The differences in function and tissue distribution between these 2 proteins in human tumor biology are still being defined, and relatively little is known regarding the biology and function of HIF-2α in CaP [12].

In the present study, we investigated the induction patterns of HIF-1α and -2α by $ZnSO_4$ in androgen-insensitive CaP cell lines. We also evaluated changes in tumor biology after manipulating HIF-1α and/or -2α expression using zinc induction or siRNA. Potential synergism between the effects of chemotherapy (docetaxel) or target agents (sunitinib and sorafenib) and HIF-1α and/or -2α blockade were also evaluated. Finally, the molecular mechanisms of these effects were investigated. Our findings may be clinically relevant for the development and clinical application of HIF target therapy or novel combination therapies with chemotherapy or target agents to treat advanced CaP.

2. Materials and methods

2.1. Cell lines and culture

Androgen-insensitive PC3 and DU145 cells were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). The culture medium used throughout this study was DMEM (Gibco BRL; Life Technologies, Carlsbad, CA) containing 5% heat-inactivated fetal bovine serum (FBS; Mediatech, Herndon, VA), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown as monolayer cultures and were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.2. Induction patterns of HIF-1α and -2α by $ZnSO_4$

To evaluate the dose response of HIF induction by $ZnSO_4$, each cell line was treated with $ZnSO_4$ at 0, 50, 100, 150, 200, and 250 μm for 2 and 4 hours. After stimulation, the cells were harvested, and then lysed in 500 μl of cell lysis buffer (1 M Tris-Cl, pH 7.4, 0.5 M NaCl, and 0.5 M EDTA) supplemented with the protease inhibitor, pepstatin (0.7 mg/ml). Cell lysates were centrifuged at 14,000 rpm for 15 minutes at 4°C. Total protein was resolved on 8% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and blocked with 5% skim milk in 1% Tween 20/TBS. The membranes were incubated overnight at 4°C with mouse monoclonal anti-HIF-1α antibody (NB100–123, clone H1α67, 1:500; Novus Biologicals, Littleton, CO) or the anti-HIF-2α antibody (NB100–132, clone ep190b, 1:500; Novus Biologicals). After incubation with secondary antibodies, protein expression was detected with an ECL developing kit (GE Healthcare, Buckinghamshire, UK).

To investigate the relationship between $ZnSO_4$ exposure time and the induction of HIF expression, PC3 and DU145 cells were treated with $ZnSO_4$ at 100 and 200 μm, respectively. Cells were collected at 0, 1, 2, 3, 4, and 6 hours after zinc treatment, and the levels of HIF-1α and -2α were determined by Western blotting as previously described.

2.3. Transfection of HIF-1α and/or -2α target siRNA and its confirmation

siRNAs targeting HIF-1α (GenBank number NM_001530) and -2α (GenBank number NM_001430) were purchased from Invitrogen (Carlsbad, CA). PC3 (2.5×10^5/well) and DU145 (2.0×10^5/well) cells were seeded on 6-well plates. After 24 hours, cells were transfected with either HIF-1α or HIF-2α siRNA, using lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol.

PC3 or DU145 cells were stabilized for 24 hours and then treated with $ZnSO_4$ at a concentration of 100 or 200 μm, respectively. Cells were harvested after 4 and 24 hours of treatment, and the expression levels of HIF-1α and -2α were determined by Western blots.

After confirming effective knockdown of HIF-1α and -2α in both cell lines, HIF protein and RNA expression was evaluated using Western blotting and RT-PCR, respectively. Test groups were as follows: (A) Control (untransfected cells), (B) $ZnSO_4$-treated untransfected cells, (C) $ZnSO_4$-treated, control siRNA (Invitrogen)-transfected cells, (D) $ZnSO_4$-treated, HIF-1α siRNA-transfected cells, (E) $ZnSO_4$-treated, HIF-2α siRNA-transfected cells, and (F) $ZnSO_4$-treated cells transfected with both HIF-1α and -2α siRNA. PC3 and DU145 cells were treated with 100 and 200 μm

$ZnSO_4$, respectively. This test scheme was used through the study, unless otherwise indicated.

For RT-PCR experiments, total RNA was extracted using RNeasy kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Aliquots of 1 μg of total RNA were used for complementary DNA (cDNA) synthesis with a reverse transcription system (Promega, Madison, WI) and oligo(dT) in a total reaction volume of 20 μl. Primer sequences used for the amplification of HIF-1α were 5′-TCACCACAGGACAGTACAGGATGC-3′ (forward) and 5′-CCAGCAAAGTTAAAGCATCAGGTTCC-3′ (reverse). Sequences for HIF-2α were 5′-GATGGCGACATGATCTTTCTGTC-3′ (forward) and 5′-CACTTCATCCTCATGAAGAAGTCCC-3′ (reverse). PCR products were separated by gel electrophoresis on 1% agarose gels and were visualized by ethidium bromide staining and UV irradiation.

2.4. Cell viability measurements

After siRNA transfections, cells were allowed to recover and stabilize for 24 hours. After 24 hours of $ZnSO_4$ treatment, cell viability was assessed using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Gaithersburg, MD) according to the manufacturer's protocol. The experimental groups examined were outlined in the scheme above. Briefly, 10 μl of CCK-8 solution was added to 200 μl of medium in each well, and the absorbance at 450 nm was measured using a Thermomax microplate reader (Molecular Devices, Hercules, CA) after a 4-hour incubation.

2.5. Cell invasion assays

Each well of the upper chamber of a transwell insert (12 μm pores) coated with matrigel (0.7 mg/ml; Collaborative Research, Inc., Boston, MA) was plated with 2×10^4 cells. The lower chamber contained 400 μl of RPMI medium. After 48 hours, non-invading cells were removed using a cotton swab. The cells that had adhered to the bottom surface of the membrane during this period were stained with Hema-3 and evaluated under a microscope. These cells were washed with 10% acetic acid and pooled with the cells that had migrated to the lower chamber. Invasion ability was then quantitatively determined using CCK-8 assays.

2.6. Evaluation of potential additive effects between HIF knockdown and treatment with chemo or target agents

The chemotherapeutic agent, docetaxel, and the multiple tyrosine kinase inhibitors (TKIs), sunitinib and sorafenib, were evaluated. The approximate half-maximal inhibitory concentrations (IC_{50}) of each drug in each cell line had been determined in a preliminary study. The IC_{50} of docetaxel was 500 nM in PC3 cells and 250 nM in DU145 cells. The IC_{50} of sunitinib was 2.5 μm in both cell lines. The IC_{50}s of sorafenib in PC3 and DU145 cells were 15 and 12.5 μm, respectively. After 24 hours of treatment with each drug at a concentration equal to its IC_{50}, CCK-8 assays were performed.

2.7. Investigations of molecular biological mechanism by Western blots

After 4 hours of treatment with 200 μm $ZnSO_4$, total proteins were extracted from DU145 cells, and Western blots were performed using primary antibodies against AKT, p-AKT, cyclin D, p21, cMET, IGF-1, and GLUT-1 (Cell Signaling Technology, Danvers, MA).

2.8. Statistical analysis

Unless otherwise indicated, data sets consist of at least 3 biological replicates, and data are expressed as the mean ± standard error of the mean. Statistical significance was de-

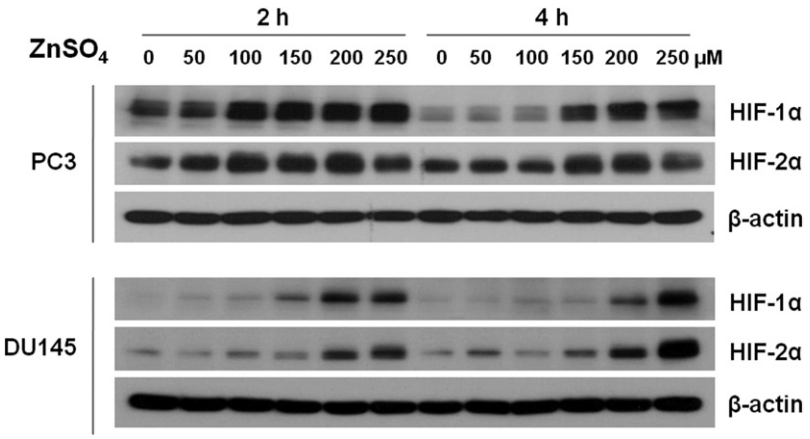

Fig. 1. HIF-1α and -2α induction patterns in response to different $ZnSO_4$ concentrations. HIF-1α and -2α were induced by $ZnSO_4$ in a dose-dependent manner in both cell lines.

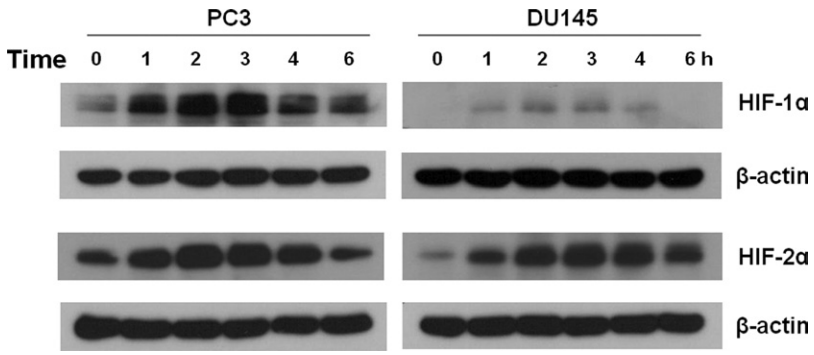

Fig. 2. The induction patterns of HIF-1α and -2α by $ZnSO_4$ exposure over time. HIF-1α and -2α expression was increased over the first 3 hours of $ZnSO_4$ exposure, but expression decreased in both cell lines after 4 hours of exposure.

termined using Student's *t*-tests, and differences with a *P* value of <0.05 were considered statistically significant.

3. Results

3.1. Induction patterns of HIF-1α and -2α by ZnSO4

HIF-1α and -2α were dose-dependently induced by ZnSO4 in both cell lines (Fig. 1). The stabilization of HIF-1α and -2α was increased up to 3 hours and then decreased after 4 hours (Fig. 2). The dose dependency and time course of induction patterns were similar for HIF-1α and -2α in both cell lines.

3.2. siRNA transfections of HIF-1α and/or -2α

It was confirmed that $ZnSO_4$ increased the expression of HIF-1α and -2α proteins, but HIF siRNA blocked this effect in both cell lines. Cotransfection of HIF-1α and -2α siRNA also has been demonstrated to block this effect (Fig. 3). In contrast to the protein levels, the RNA levels of HIF-1α and -2α were not increased by $ZnSO_4$, but they were reduced when the relevant siRNAs were transfected into either PC3 or DU145 cell lines (Fig. 4). The control siRNA did not alter the expression of HIF RNA or protein.

3.3. Cell viability measurements

When siRNAs directed against HIF-1α and -2α were transfected into cells, cell viability decreased significantly compared with $ZnSO_4$-treated controls. For example, cell viability (expressed as the percentage of control) decreased from 99.7 $\pm$ 6.4 to 74.0 $\pm$ 5.8 ($P = 0.041$, PC3) and from 100.2 $\pm$ 1.1 to 57.6 $\pm$ 7.8 ($P = 0.006$, DU145) when cells were transfected with siRNA against HIF-1α. However, the simultaneous knockdown of both HIF-1α and -2α did not lead to an additional decrease in cell proliferation (76.9 $\pm$ 3.2 for PC3 and 71.4 $\pm$ 12.0 for DU145) (Fig. 5).

3.4. Cell invasion assays

Invasion abilities were increased after $ZnSO_4$ treatment in both cell types, and both cell types showed increased expression of HIF-1α and -2α after $ZnSO_4$ treatment (108.7 $\pm$ 14.9; $P = 0.590$ for PC3 and 134.9 $\pm$

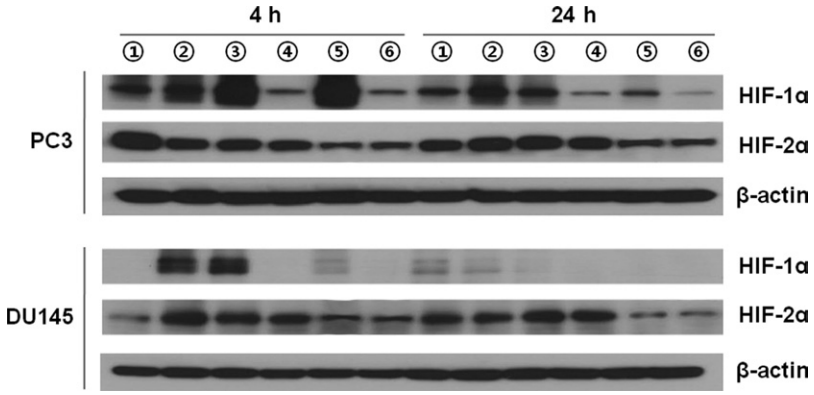

Fig. 3. Western blot confirmation of siRNA-mediated HIF-1α and/or -2α knockdown in prostate cancer cells. HIF-1α and -2α were induced by $ZnSO_4$ (100 μm in PC3 cells and 200 μm in DU145 cells), but this induction was blocked in both cell lines by transfections of the relevant siRNAs. ① Control (untransfected cells), ② $ZnSO_4$-treated untransfected cells, ③ $ZnSO_4$-treated, control siRNA-transfected cells, ④ $ZnSO_4$-treated HIF-1α siRNA-transfected cells, ⑤ $ZnSO_4$-treated HIF-2α siRNA-transfected cells, and ⑥ $ZnSO_4$-treated cells transfected with both HIF-1α and -2α siRNA.

Fig. 4. RT-PCR confirmation of siRNA-mediated HIF-1α and/or -2α knockdown in prostate cancer cells. RNAs of HIF-1α and -2α were not increased by $ZnSO_4$ (100 μm in PC3 cells and 200 μm in DU145 cells), but they were decreased by transfections of relevant siRNAs in both cell lines. ① Control (untransfected cells), ② $ZnSO_4$-treated untransfected cells, ③ $ZnSO_4$-treated, control siRNA-transfected cells, ④ $ZnSO_4$-treated HIF-1α siRNA-transfected cells, ⑤ $ZnSO_4$-treated HIF-2α siRNA-transfected cells, and ⑥ $ZnSO_4$-treated cells transfected with both HIF-1α and -2α siRNA.

4.7; $P = 0.002$ for DU145). However, invasion potencies were decreased more in $ZnSO_4$-treated cells than in control cells after siRNA-mediated knockdown of HIF-1α and/or -2α (Fig. 6). For example, cell invasion ability was decreased to 70.5 $\pm$ 14.6 ($P = 0.141$, PC3) and 83.0% $\pm$ 9.6% of control ($P = 0.008$, DU145) when HIF-1α and -2α target siRNAs were simultaneously transfected. In general, these effects were more remarkable in DU145 cells than in PC3 cells.

3.5. Evaluation of potential additive effects between HIF-1α and/or -2α knockdown with chemo- or target agents

HIF knock-down did not enhance antitumor effect of docetaxel in PC3 cells. In contrast, antitumor effect of docetaxel was enhanced in DU145 cells. When HIF-1α and -2α were knocked down, the antitumor effects of sunitinib and sorafenib were augmented in both cell lines (Table 1).

3.6. Investigations of the molecular mechanism using Western blots

Western blot analysis of proteins isolated from DU145 cells revealed that the levels of p-AKT levels were decreased in cells transfected with HIF-1α and/or -2α siRNA. In contrast, the total levels of AKT were not significantly affected. Cyclin D levels were not changed, but p21 levels were decreased by HIF knockdown. The expression patterns of cMET, IGF-1, and GLUT-1 were similar to the expression pattern of p21 (Fig. 7).

4. Discussion

HIF is a member of the basic helix-loop-helix (bHLH)-PER-ARNT-Sim (PAS) family of transcription factors. The HIF α-subunit heterodimerizes with the β-subunit, and this complex binds to hypoxic responsive elements (HREs) [5]. Binding to HREs activates the transcription of many down-

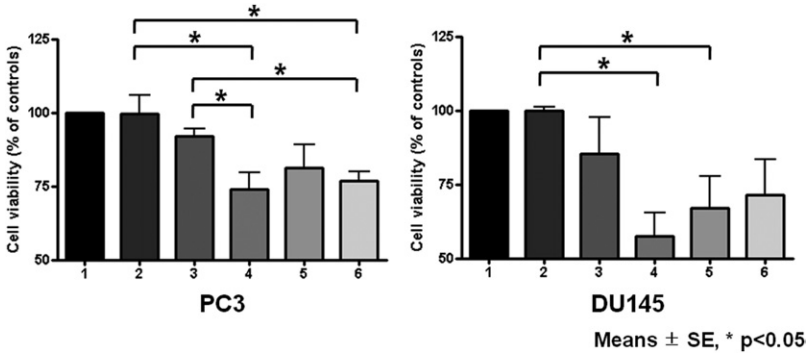

Fig. 5. Cell viability assays after HIF-1α and/or -2α knockdown in prostate cancer cells treated with $ZnSO_4$. Transfections of HIF-1α and -2α target siRNAs led to decreased cell viability compared with $ZnSO_4$-treated untransfected cells. ① Control (untransfected cells), ② $ZnSO_4$-treated untransfected cells, ③ $ZnSO_4$-treated, control siRNA-transfected cells, ④ $ZnSO_4$-treated HIF-1α siRNA-transfected cells, ⑤ $ZnSO_4$-treated HIF-2α siRNA-transfected cells, and ⑥ $ZnSO_4$-treated cells transfected with both HIF-1α and -2α siRNA.

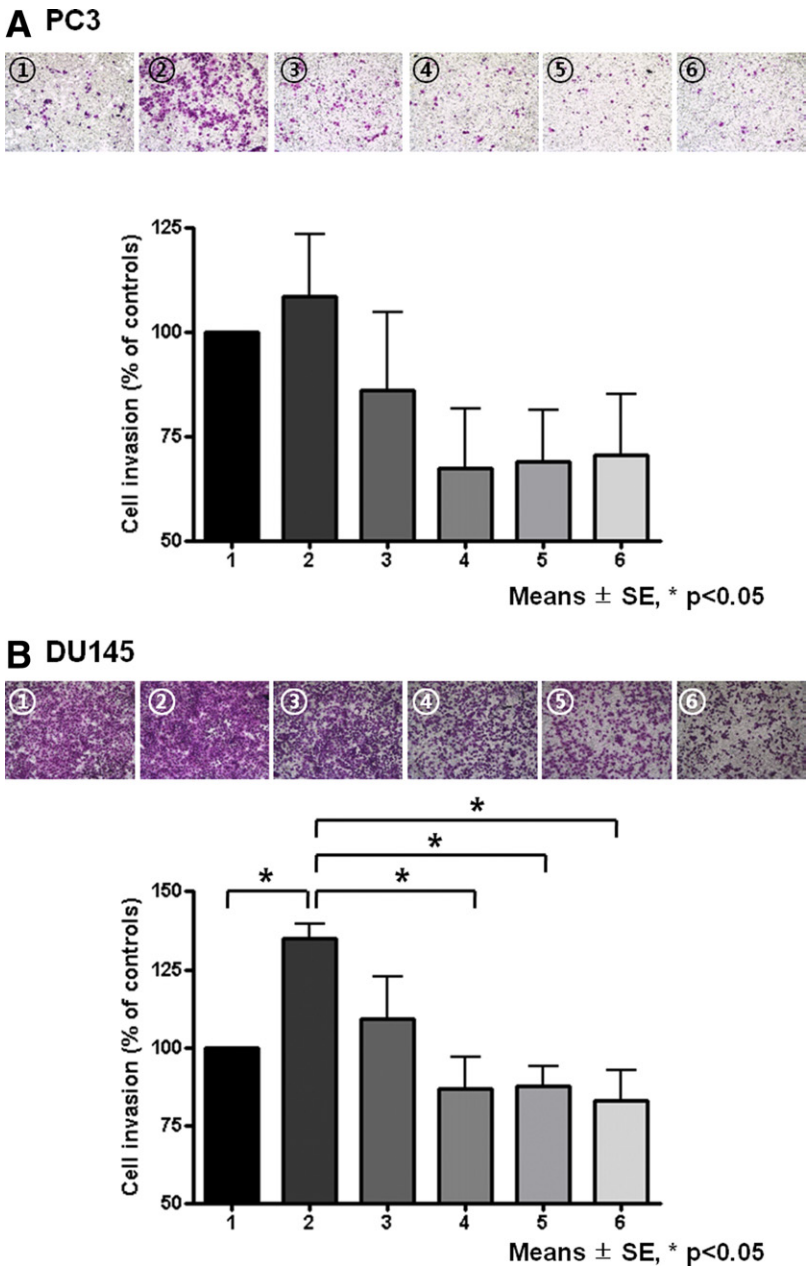

Fig. 6. Matrigel invasion assays. Invasion abilities were increased after $ZnSO_4$ treatment in both cell types. However, invasion potencies were decreased more in siRNA-treated cells than in control cells. ① Control (untransfected cells), ② $ZnSO_4$-treated untransfected cells, ③ $ZnSO_4$-treated, control siRNA-transfected cells, ④ $ZnSO_4$-treated HIF-1α siRNA-transfected cells, ⑤ $ZnSO_4$-treated HIF-2α siRNA-transfected cells, and ⑥ $ZnSO_4$-treated cells transfected with both HIF-1α and -2α siRNA. (Color version of figure is available online.)

stream genes that code for proteins that are involved in angiogenesis (e.g., VEGF), survival/proliferation (e.g., IGF), glucose metabolism (e.g., GLUT-1), and invasion/metastasis (e.g., cMET). As a consequence of this transcriptional activation, cancer cells become more aggressive and display a treatment-resistant phenotype [6]. There are 3 HIF-α genes (HIF-1α, HIF-2α, and HIF-3α) [12]. Although HIF-1α is the most widely described and most well-known isoform, a great deal of evidence indicates that HIF-2α plays as important a role as HIF-1α in cancer [13]. HIF-2α has a structure that is similar to HIF-1α, but recent data suggest that their expression patterns may differ in different types of cancers. For example, HIF-1α is highly expressed in most human breast cancer cell lines, but HIF-2α expression is low or absent in the more aggressive cell line [14]. In VHL-defective renal cell carcinomas, HIF-2α promotes tumor growth while HIF-1α retards it, and the HIF system behaves unusually in a number of ways [15]. The characteristics and role of HIF-3α remain poorly understood, but it has been suggested that an alternative splice form of HIF-3α binds to and inhibits the transcriptional activity of HIF-1α [16].

In contrast to other cell types, it is known that HIF-1α

Table 1
Enhancing effects of HIF-1α and/or -2α siRNA to the docetaxel or target agents (sunitinib or sorafenib) in prostate cancer cells (CCK-8 assay results expressed as the percentage of control after 24 hours of treatment)

Drug	−	−	−	−	−
Si-HIF-1α	−	−	+	−	+
Si-HIF-2α	−	−	−	+	+
Control SiRNA	−	+	−	−	−
PC3	100	86.9	79.4	72.9	74.8
DU145	100	92.7	81.8	81.1	89.3
Drug	+	+	+	+	+
Si-HIF-1α	−	−	+	−	+
Si-HIF-2α	−	−	−	+	+
Control SiRNA	−	+	−	−	−
Docetaxel					
PC3	88.3	87.2	82.5	79.2	82.1
DU145	76.5	63.0	50.2	49.1	50.2
Sunitinib					
PC3	75.3	72.1	48.6	50.0	50.3
DU145	70.8	60.8	45.6	49.0	53.6
Sorafenib					
PC3	39.3	32.3	24.5	27.8	28.2
DU145	34.6	23.2	19.4	18.1	21.9

can be induced by $ZnCl_2$ in CaP cells [10]. In our study, we demonstrated that both HIF-1α and HIF-2α can be induced by $ZnSO_4$ in PC3 and DU145 CaP cells. In addition, the expression patterns of HIF-1α and -2α were fairly similar with respect to their dose response curves and peak time of expression. Excessive zinc inhibits mitochondrial aconitase, which converts citrate to isocitrate in the Krebs cycle and, consequently, causes the depletion of adenosine triphosphate (ATP) and promotes apoptosis. However, prostate tissue requires high zinc levels for its proper function [11]. Prostate tissue and fluid contain 1,000–3,000 μmol/kg and 9,000 μmol/kg of zinc, respectively. Thus, prostate cells have likely developed the ability to stabilize HIFs in response to zinc to overcome zinc toxicity. This mechanism may be related to the expression levels of zinc transporters in the cell membrane [17]. Indeed, HIF-1α and/or -2α knockdown in PC3 and DU145 cells significantly reduced cell viability. However, the simultaneous knockdown of both HIF-1α and -2α did not show an additive effect. Thus, it is likely that HIF-1α and -2α promote cell survival via similar mechanisms in prostate cells.

The acquisition of aggressive phenotypes in cancer cells frequently results from alterations in cell viability. To evaluate this change towards an aggressive phenotype, invasion ability, which is a representative aggressive feature of cancer, was examined. As expected, invasion abilities were significantly increased when HIF-1α and/or -2α were stabilized. In contrast, when HIF-1α and/or -2α were knocked down, invasiveness decreased. These results demonstrate that both HIF-2α and HIF-1α play important roles in regulating cancer invasion ability.

In this study, results seemed more significant in the DU145 than PC3 cells. We do not have definitive evidence to explain this result. However, there is some data to suggest that PC3 cells appear to have stronger antioxidant activity and different metabolic adaptation compared with DU145 [18]. HIF may play a more important role in DU145 because HIF is regulated by ROS and prolyl hydroxylase. This should be further evaluated.

Docetaxel is the widely used chemotherapeutic agent that can improve the survival of CaP patients [19,20]. However, the survival benefit of docetaxel is only approximately 2 months, and the median survival is no longer than 20

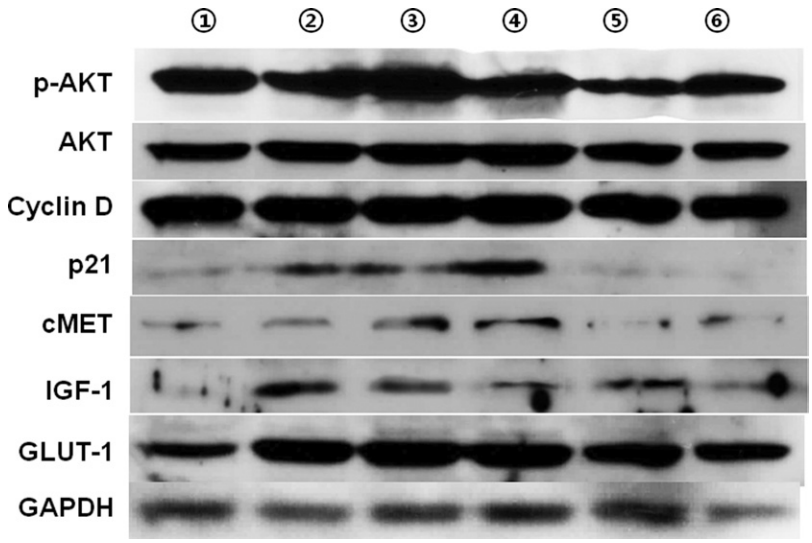

Fig. 7. Investigations of molecular mechanisms by Western blots. Proteins were isolated from DU145 cells 4 hours after $ZnSO_4$ treatment. AKT levels were not affected by HIF siRNA, but p-AKT levels were decreased in cells treated with HIF-1α and/or -2α siRNA. Cyclin D levels were not changed by $ZnSO_4$ treatment or HIF knockdown. In contrast, p21 levels were increased by $ZnSO_4$ treatment and decreased by HIF siRNA. The expression patterns of cMET, IGF-1, and GLUT-1 were similar to p21.

months. Thus, more effective treatment protocols are required. Because HIF is an important transcriptional factors in the development of cancer treatment resistance, it may represent a potential therapeutic target [5,6,21]. In this study, blocking HIF-1α and/or -2α using siRNA enhanced antitumor effect when combined with docetaxel not in PC3 but in DU145 cells. However, augmented antitumor effect was more prominently shown in both cell lines when sunitinib or sorafenib was used. This effect is more prominent in DU145 cells than in PC3 cells. Sunitinib and sorafenib are multiple TKIs that block important signaling pathways, including vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR) signaling [22]. This molecular pathway is closely related with HIF regulation, and there is evidence that multiple TKIs regulate HIF-1α and -2α [23]. This can be an explanation of more enhanced antitumor effect by HIF blockade in target agents rather than in docetaxel. Among the numerous TKIs, these 2 molecules have been the most studied in CaP. Unfortunately, clinical trials examining the use of sunitinib or sorafenib alone in the treatment of CaP exhibited disappointing results. However, strategies to combine multiple TKIs with docetaxel or anti-androgen therapy appear to be promising and await phase III trials [24,25]. Combining multiple TKIs with HIF targeting therapy is another potential treatment option. There are many potential small molecules to directly or indirectly target HIF as the next generation anticancer therapeutics [26]. Further investigations of this combined therapy should be evaluated, including a more complete determination of its molecular mechanisms. In another set of preliminary experiments, we also evaluated the antitumor activity of temsirolimus and everolimus, 2 inhibitors of the mammalian target of rapamycin (mTOR) pathway, in CaP cells. However, both CaP cell lines showed strong resistance to these mTOR inhibitors, and we did not incorporate the mTOR inhibitors in the current study.

We also evaluated changes in protein expression in DU145 cells using Western blots. Although we found total AKT levels to be largely unchanged, p-AKT levels were decreased when HIF isoforms were suppressed. Thus, AKT signaling pathway may be involved in cell viability changes in CaP cells. Cyclin D levels were not changed by either $ZnSO_4$ treatment or HIF knockdown, but p21 levels were increased by $ZnSO_4$ treatment and decreased by HIF knockdown. Thus, it appears likely that anti-apoptotic effects rather than effects on the cell cycle might mediate the altered cell survival observed after HIF blockade. Expression of cMET was similar to that of p21; thus invasion ability might be regulated by cMET downstream of HIF. Interestingly, IGF-1, which is known to affect anti-apoptotic activity and cancer aggressiveness, and GLUT-1, which plays a role in metabolic adaptations of cancer cells, also showed similar expression patterns as p21.

In the future, we will demonstrate our results by in vivo experiments in an animal model. Specifically, we will focus in combination between HIF blockage and target agents in CaP.

In summary, our results showed that HIF-1α and -2α can be stabilized in response to $ZnSO_4$ treatment in PC3, and DU145 cells, even under normoxic conditions. Our results demonstrate that HIF-1α and -2α are important for cell proliferation and invasion ability in CaP. In addition, our results suggest that a combinatorial strategy involving target agents with HIF-blocking therapy may be promising in the treatment of CaP.

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