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Original Article

Hypoxia regulates SOX2 expression to promote prostate cancer cell invasion and sphere formation

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Abstract: SOX2 is an embryonic stem cell marker that in prostate cancer has been associated not only with tumorigenesis but also metastasis. Furthermore hypoxia in primary tumors has been linked to poor prognosis and outcomes in this disease. The goal of the present study was to investigate the impact of hypoxia on SOX2 expression and metastasis-associated functions in prostate cancer cells. A tissue microarray of 80 samples from prostate cancer patients or healthy controls was employed to examine the expression of HIF-1α and its correlation with SOX2. The role of SOX2 and HIF-1/2α in the regulation of cell invasion and sphere formation capacity under hypoxic conditions was investigated *in vitro* using short hairpin RNA (shRNA)-mediated knockdown in three human prostate cancer cell lines. HIF-1α expression was significantly elevated in malignant prostate tissue compared to benign or normal tissue, and in tumor samples its expression was highly correlated with SOX2. In prostate cancer cells, acute and chronic exposures to hypoxia that resulted in elevated expression levels of HIF-1α and HIF-2α, respectively, also induced SOX2. Genetic depletion of SOX2 attenuated hypoxia-induced cell functions. Knockdown of HIF-1α, but not HIF-2α, decreased acute hypoxia-mediated cell invasion and SOX2 up-regulation, whereas only HIF-2α gene silencing reduced sphere formation capacity and chronic hypoxia-mediated SOX2 up-regulation. Enhanced SOX2 expression and HIF-1α or HIF-2α associated phenotypes are dependent on the time duration of exposure to hypoxia. The present results indicate that SOX2 may be a key mediator of hypoxia-induced metastasis-associated functions and hence may serve as a potential target for therapeutic interventions for metastatic prostate cancer.

Keywords: Hypoxia, hypoxia inducible factor, SOX2, invasion, stemness

Introduction

Prostate cancer is a leading cause of death in men in the US. While highly curable if localized, patients with metastatic disease have a 5-year survival rate of only 31% (1). Indeed metastatic prostate cancer remains largely incurable. A better understanding of the mechanisms underlying the spread of prostate cancer cells should aid the development of treatment strategies that improve outcomes for patients with advanced disease.

The embryonic stemness gene SOX2 is a core transcription factor known to sustain tumor initiating cell (TIC) pluripotency and self-renewal [1] and to function as an oncoprotein [2, 3]. Several laboratories, including our own, have established its importance in tumor initiation [3-7] and metastasis-associated functions such as migration and invasion [8-10]. In prostate cancer models we previously reported SOX2 to be significantly involved in tumorigenesis [7] and TIC invasion [8]. Furthermore, in patient tumor samples assessed by tissue microarray analysis, we noted that SOX2 expression (mRNA and protein levels) were associated with Gleason grade [7].

Hypoxia is an essential feature of the microenvironment of many solid tumors including those of the prostate [11, 12]. Two types of hypoxia have been shown to exist in solid tumors [13, 14]. Tumor cells at the limits of oxygen diffusion from blood vessels may experience chronic or "diffusion-limited" hypoxia. Such hypoxic conditions usually last for relatively long periods of hours or days [14]. In contrast, tumor cells exposed to transient hypoxia as a consequence of intermittent blood flow fluctuations are considered to experience acute or "perfusion-limited" hypoxia [14, 15]; typically characterized by

relatively short hypoxic-oxic cycles lasting for minutes to hours [16]. Using a variety of measurements including microelectrodes, hypoxic markers, and hypoxia-associated molecules, hypoxia has been demonstrated to be a common feature of prostate cancer [17-20] associated with a poor prognosis [19-21]. Several reports have correlated hypoxia with biochemical failure in prostate cancer patients undergoing radiotherapy [18, 19, 21], suggesting that hypoxia increases the potential of both resistance and malignancy of prostate cancer cells.

A key mechanism mediating the adaptation of hypoxia is the induction of hypoxia-inducible factor 1α (HIF-1α) [22]. This transcription factor regulates a large number of genes that play critical roles in many aspects of cancer biology including stem cell maintenance, metabolic reprogramming, angiogenesis, invasion, metastasis, and resistance to therapy [23, 24]. HIF-1α expression has therefore become a typical indicator of intratumoral hypoxia and is considered a potential target for therapeutic interventions. In prostate cancer accumulation of HIF-1α has been associated with poor patient prognosis and aggressive tumor phenotypes [25-27].

Hypoxia not only facilitates metastasis-associated functions, but also can contribute to stem cell maintenance [28]. Cancer stem cells or tumor initiating cells (TIC) are a subpopulation of tumor cells that selectively possess tumor initiation and self-renewal capacity and the ability to give rise to bulk populations of non-tumorigenic cancer cell progeny through differentiation [29, 30]. Such cells were initially described in leukemia [31] but subsequently have been identified in a variety of solid tumors including prostate cancer [32]. It further has been suggested that TIC exist in hypoxic regions of tumors [33] and low oxygen tensions drive and maintain the stemness phenotype; majorly mediated by HIF-2α, another hypoxia-regulated transcriptional factor that shares similar sequence and regulatory machinery with HIF-1α [22, 34]. Such observations are supported by the molecular findings that HIF-2α drives several key genes associated with stem cell self-renewal and multi-potency [34, 35]. Although HIF-2α has been noted in prostate cancer [36, 37], its functional role remains unclear.

Recent evidence indicates that hypoxic conditions may also induce the expression of embryonic stem cell markers, including SOX2, in a HIF-dependent manner. Such findings have been reported in colon cancer, cervical cancer and glioblastoma [38, 39] but to our knowledge, the regulation of SOX2 under hypoxia has not been examined in prostate cancer. The focus of the present study was to investigate the impact of acute and chronic oxygen deprivation on SOX2 expression and metastasis-associated functional behaviors of prostate cancer cells.

Materials and methods

Human cell lines, tissues and hypoxia

Human prostate cancer cells (PC-3, DU145, LNCaP) were purchased from American Type Culture Collection. Cells were maintained in appropriate media plus 10% fetal bovine serum (FBS) in humidified 5% CO_2 at 37°C. The human prostate cancer tissue microarrays (TMA) were purchased from Cybrdi. For hypoxic culture conditions, cells were incubated in glass dishes in a modular incubator chamber (Oxygen Sensors, Gladwyne, PA) flushed with a gas mixture containing 1% O_2 (hypoxia) balanced with 5% CO_2 and N_2 at 37°C. For reoxygenation after hypoxic incubation, cells were transferred back to a 5% CO_2 in air environment.

Immunohistochemical analysis of tissue microarrays

TMA slides containing duplicate cores from 40 prostate tissues were purchased from Cybrdi (MD, USA) and stored at 4°C until use. After air drying and equilibrating at room temperature for 2 h, the slides were sequentially deparaffinized in 2 changes of xylene, rehydrated through a series of graded alcohols and blocked for endogenous peroxidase activity for 10 min in 3% hydrogen peroxide diluted in methanol. Optimal staining required 25 min of heat antigen retrieval in 10 mM Citrate buffer pH 6.0 using a microwave oven. Slides were blocked in 2% normal rabbit serum for 30 min, and then with Avidin and Biotin solutions (Vector Labs) for 30 min. Anti-human HIF-1α (BD transduction) was applied to tissue sections at 4 μg/ml and incubated overnight at 4°C. Slides were washed twice for 5 min in TBS buffer and stained using the ABC-Elite kit (Vector Labs) fol-

lowing the manufacturer's instructions. For the negative control, tumor tissue slides were only stained by the secondary antibody [7]. Positive staining was detected with DAB (Vector Labs) using chromogen and hematoxylin 560 (SurgiPath) as the counterstain. Slides were cleared in xylene and mounted by Cytoseal (Richard-Allan Scientific). To assess nuclear staining, an arbitrary system was used by a pathologist blinded to sample identity. Twenty random fields were examined and the overall percentage and intensity of positive nuclear staining was histologically scored. H-scores will be determined by assigning a score of 0 to 3, based on the percentage of cells staining positive in a field with the following metric: 0, no positive cells; 1, 1-33% positive; 2, 33-67%; and 3, 67-100%, and then multiplying this value by the staining intensity score (1-3, where 1 and 3 represent weak and intense staining, respectively) [40].

Western blotting

Western blotting was performed as described [7, 41, 42]. Briefly, whole cell lysates were prepared in a lysis buffer with protease inhibitor cocktail (Pierce) at 4°C, followed by centrifugation at 13,000×g for 10 min. Extracts were separated by SDS/PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in 20 mM Tris-HCl (pH 7.5), 500 mM sodium chloride, and 0.05% Tween 20 for 2 h and then incubated with primary antibodies HIF-1α (BD Bioscience), HIF-2α, OCT3/4, β-actin (Cell Signaling), SOX2 (Santa Cruz), Nanog (BioLegend) in the same buffer with 1% BSA (fraction V). After washing, the blots were incubated with an HRP-conjugated secondary antibody and visualized with an enhanced chemilluminescence detection system (Amersham).

Cell invasion

Matrigel invasion assays were used according to the manufacturer's instructions (BD Biosciences). Briefly, cells were washed, suspended in a serum-free medium, and plated onto Matrigel-coated invasion chambers. Chambers were placed into wells of a 24 well plate containing media supplemented with 10% FBS as a chemoattractant. Chambers were incubated for 24 h at which time any cells remaining inside the inserts were removed using a cotton swab. Cells that successfully invaded through the Matrigel were fixed with 4% paraformaldehyde, stained with crystal violet, the membranes were mounted onto slides, photographed and cells that invaded in 4 randomly-selected fields were counted using ImageJ software (NIH).

Prostate spheroid culture

Prostate spheroid cultures were prepared from DU145 and PC-3 cells as described previously [41]. Briefly, spheroids were generated by growing cells in suspension culture using ultralow attachment plates (Corning). The cells were cultured at a density of 500 cells/ml in serum free DMEM/F12-50/50 supplemented with 20 ng/ml EGF (Biosource), 10 ng/ml bFGF (Invitrogen), 5 μg/ml heparin (Sigma), 2 nM Glutamate, 1% penicillin-streptomycin (50 IU penicillin and 50 μg/ml streptomycin), 0.2% BSA, 1× B27 without Vitamin A and 1× Insulin-Transferrin-Selenium-A (Gibco). The culture medium was changed every 3 to 4 days.

shRNA knockdown

For shRNA knock-down experiments, plasmid vectors encoding SOX2 (TR309173), HIF-1α (TG320380) or HIF-2α (TG315484) were used (Origene). Cancer cells (1.5×10^5/well) were seeded into a 6 well plates in a growth medium without antibiotics on the day before transfection. Cells were then washed with Optimem medium (Invitrogen) and transfected with plasmids containing shRNAs using Lipofectamine (Invitrogen). Stably transfected cells were selected by puromycin, and single-cell colonies were harvested for Western blot detection.

Statistical analysis

The Wilcoxon nonparametric rank sum test or Student's t test was applied to investigate difference between two individual groups. Correlation of H score was analyzed by Spearsman test. All statistical analysis was performed using GraphPad Prism 5.0 software (San Diego, CA). A threshold of $P<0.05$ was defined as statistically significant.

Results

HIF-1α is overexpressed in prostate cancer and correlates with SOX2 expression

We first analyzed HIF-1α levels by immunohistochemistry on a TMA containing prostate tissue samples that were normal, benign (BPH), or

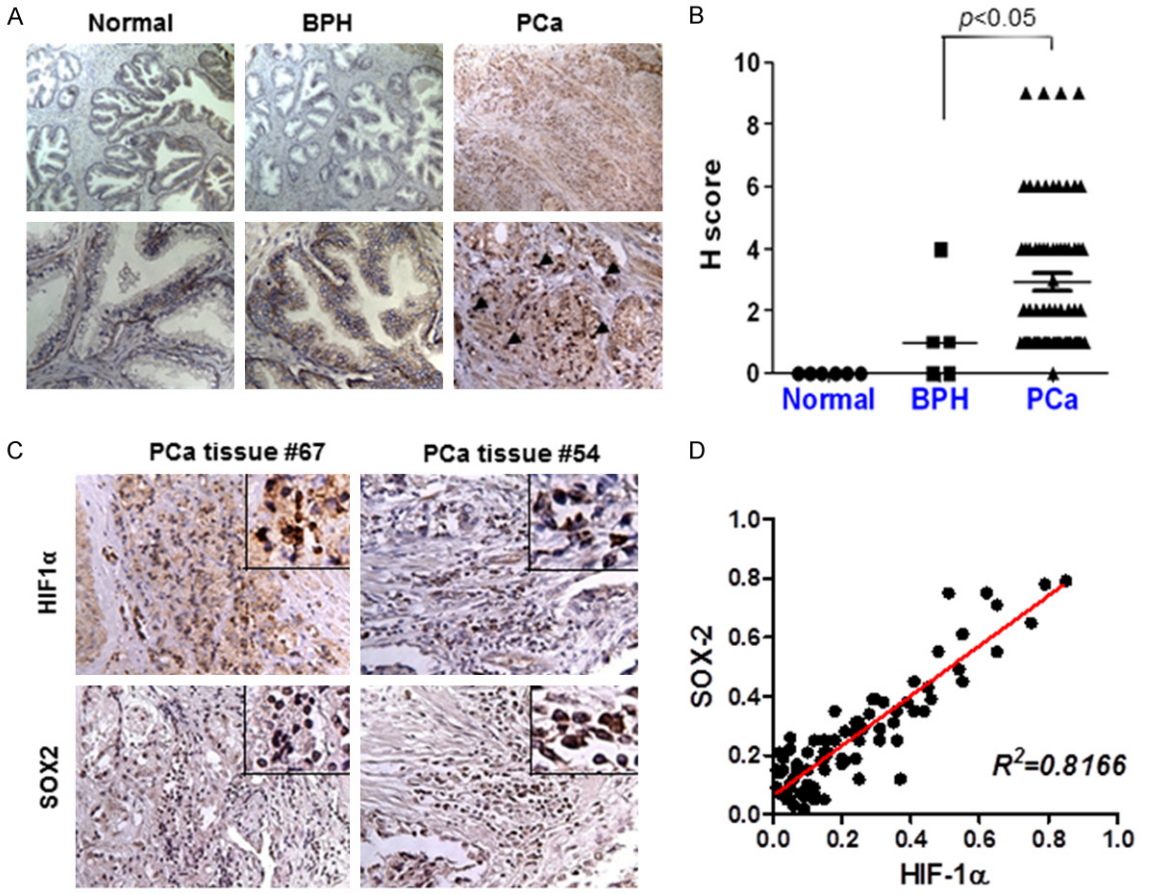

Figure 1. HIF-1α expression and its correlation with SOX2 in prostate tissues. A. Immunostaining for HIF-1α was performed using prostate tissue arrays. Representative images from normal, BPH, and PCa staining were shown. Brown color indicates positive nuclear staining. The magnification of top and bottom lanes is ×5 and ×20, respectively. B. Semiquantitative analysis of IHC staining was performed for all samples that assessed both the percentage of cells stained and the intensity of the staining, and the analysis was reported as the H score of these two parameters. *P* value was calculated by Wilcoxon nonparametric rank sum test. C. Representative images of HIF-1α and SOX2 IHC staining on sample samples of TMA #54 and #67. High magnification images of positive-stained regions are presented in the upper right coner. D. Correlation analysis of HIF-1α and SOX2 by the H score using Spearsman test.

malignant. HIF-1α staining was found to be more intense and widespread in prostate cancer than in normal tissues (**Figure 1A**). Furthermore, the H score, which reflects the percentage of cells stained and the intensity of the staining [40], was significantly higher in prostate cancer tissues than in non-cancerous tissues (**Figure 1B**). Using the same TMA we previously reported that SOX2 positive cells significantly increased in prostate neoplastic tissue compared to BPH or normal prostate (P<0.05) [7]. We now used this TMA to assess HIF-1α and SOX2 expression in prostate cancer tissues. **Figure 1C** shows tumor sections with high co-staining of HIF-1α and SOX2 in two representative samples. When H scores for all the samples were compared, a significant correlation between SOX2 and HIF-1α emerged (P<0.001, Spearsman rank test, **Figure 1D**) suggesting that the expression of human embryonic stem cell maker SOX2 was strongly associated with hypoxia in prostate cancer.

Hypoxic exposures enhance SOX2 expression in prostate cancer cells

When DU145, PC-3 and LNCaP prostate cancer cells were exposed to hypoxia for various periods of time, the expression of SOX2, but not other stem cell markers (Nanog, Oct3/4, c-Myc), was increased significantly in response to both acute (<24 h) and chronic (≥24 h) hypoxia in all 3 lines (**Figure 2A**, **2B**). Increased SOX2 protein expression could be maintained under hypoxia

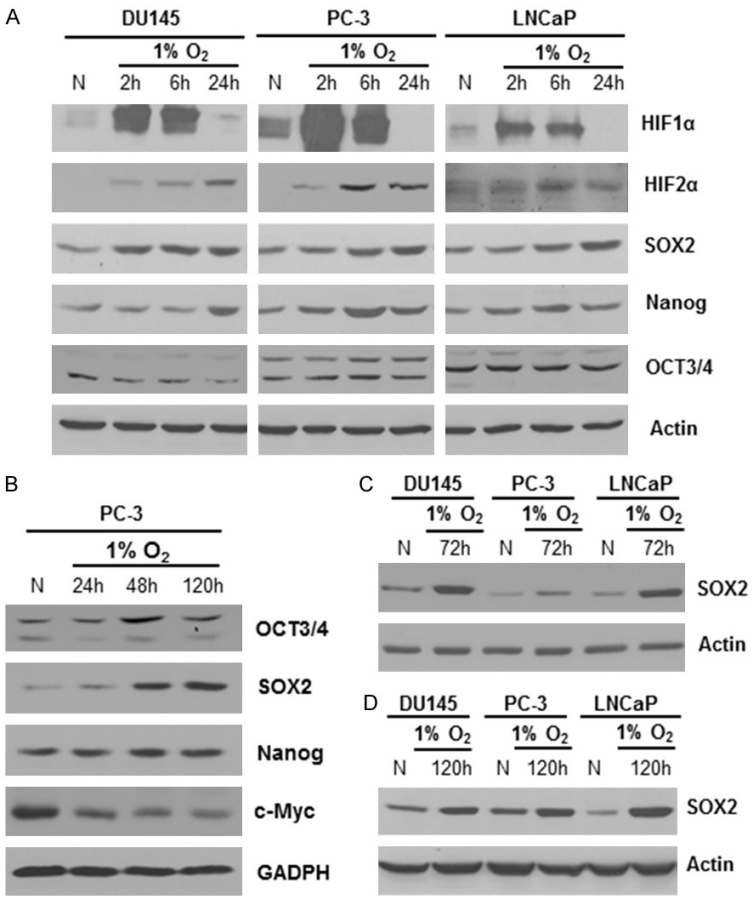

Figure 2. Impact of hypoxia on an embryonic stem cell marker expression. DU145, PC-3 and LNCaP cells were exposed to 1% oxygen for times ranging from 2 to 24 h (A), 24 to 120 h (B) or 72 and 120 h (C and D). Whole cell lysates were analyzed by western blot using actin as a loading control.

lasting for 3 to 5 days (**Figure 2B-D**). Our data showed that HIF-1α levels in prostate cancer cells significantly increased in less than 6 h and decreased after 24 h of hypoxia (**Figure 2A**). In contrast, HIF-2α protein levels were barely detected at 2 h of hypoxia (**Figure 2A**), but then increased as the exposure time increased from 6 to 72 h (**Figures 2A** and **4C**).

HIF-1α and SOX2 regulate acute hypoxia-induced prostate cancer cell invasion

We have constructed SOX shRNA to stably downregulate SOX2 expression in several prostate cancer cell lines. Knockdown of SOX2 significantly inhibits cell invasion [41] in PC-3 (**Figure 3A**, **3B**) and other cell lines (data not shown). Moreover, short-term hypoxic exposure leads to a significant enhancement of cell invasion in PC-3 cells [43] (**Figure 3B**). To explore whether hypoxia-induced invasion is mediated by SOX2, we examined the functional role of SOX2 as well as HIFα proteins in facilitating cell invasion following exposure to short-term hypoxia. The results showed that shRNA transfection of PC-3 cells decreased the SOX2 expression induced by 6 h hypoxia (**Figure 3A**) and significantly impaired the hypoxia enhanced tumor cell invasion (**Figure 3B**).

Transfecting DU145 or PC-3 cells with plasmids encoding shRNA targeting HIF-1α not only resulted in a 90-95% HIF-1α protein knockdown for cells exposed to either normoxia or 6 h hypoxia (**Figure 3C**) but also significantly decreased SOX2 expression (**Figure 3C**). Functionally, HIF-1α shRNA significantly inhibited the short-term hypoxia-induced enhanced invasion of both DU145 and PC-3 cells (**Figure 3D**).

To determine whether the HIF-2α protein was responsible for the hypoxia-induced invasiveness we studied HIF-2α knockdown cells. shRNA effectively inhibited HIF-2α and SOX2 expression in both normoxic and hypoxic PC-3 cells, but had little effect on HIF-1α expression (**Figure 3E**). Furthermore, unlike HIF-1α knockdown which abolished the hypoxia-enhanced tumor cell invasion (**Figure 3D**), HIF-2α knockdown cells still demonstrated a robust increase in invasion when exposed to acute (6 h) hypoxia (**Figure 3F**).

Chronic hypoxia elevates sphere formation capacity of prostate cancer cells

The ability to grow as non-adherent spheroids has been widely used to assess cancer stem cell characteristics. To determine the impact of hypoxia on prostate cancer cell sphere formation, PC-3 and LNCaP cells were exposed to 6

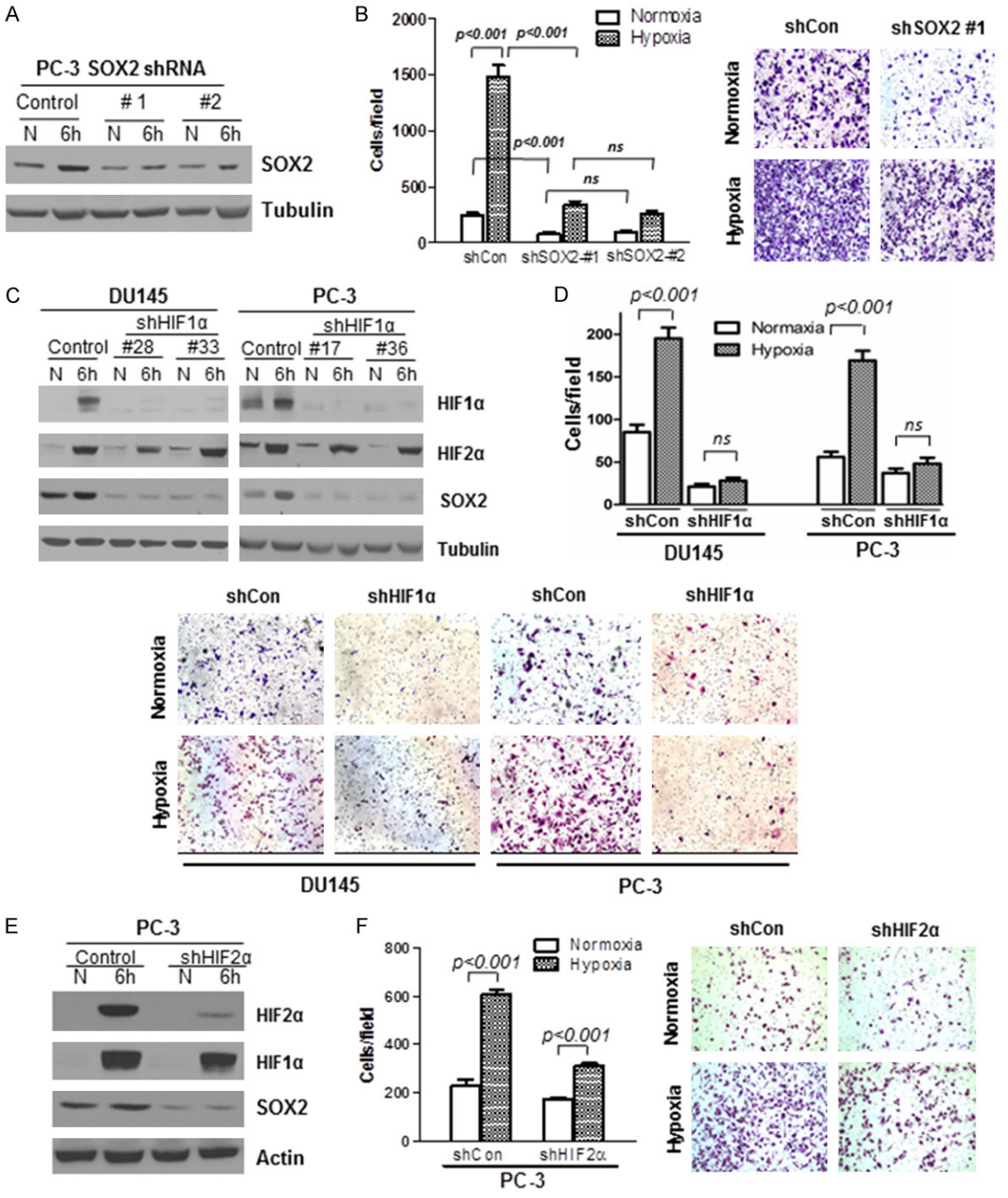

Figure 3. Acute hypoxia-induced, SOX2-mediated cell invasion is dependent on HIF-1α. Prostate cancer cells were stably transfected with shRNA of SOX2 (A, B), HIF-1α (C, D) or HIF-2α (E, F) or scramble shRNA (Control). Transfected cells were exposed to 1% oxygen for 6 h and protein expression was detected by western blot using actin as a loading control (A, C, E). To test cell invasion, transfected cells were seeded into invasion chambers (5×10^4/chamber) and exposed to 1% O_2 for 6 h then re-cultured under aerobic conditions for 18 h. Invaded cells were scored 24 h after cell seeding. Column, mean; bars, SD (n=4). ns = no significance (t-test) (B, D, F). P value was analyzed by t test.

or 24 h of hypoxia prior to being maintained under sphere forming conditions in normoxia for 7 days. The results showed that short term hypoxia (6 h) had no effect on the number and size of spheres formed (**Figure 4A**). In contrast, spheres formed from PC-3 and LNCaP cells pre-

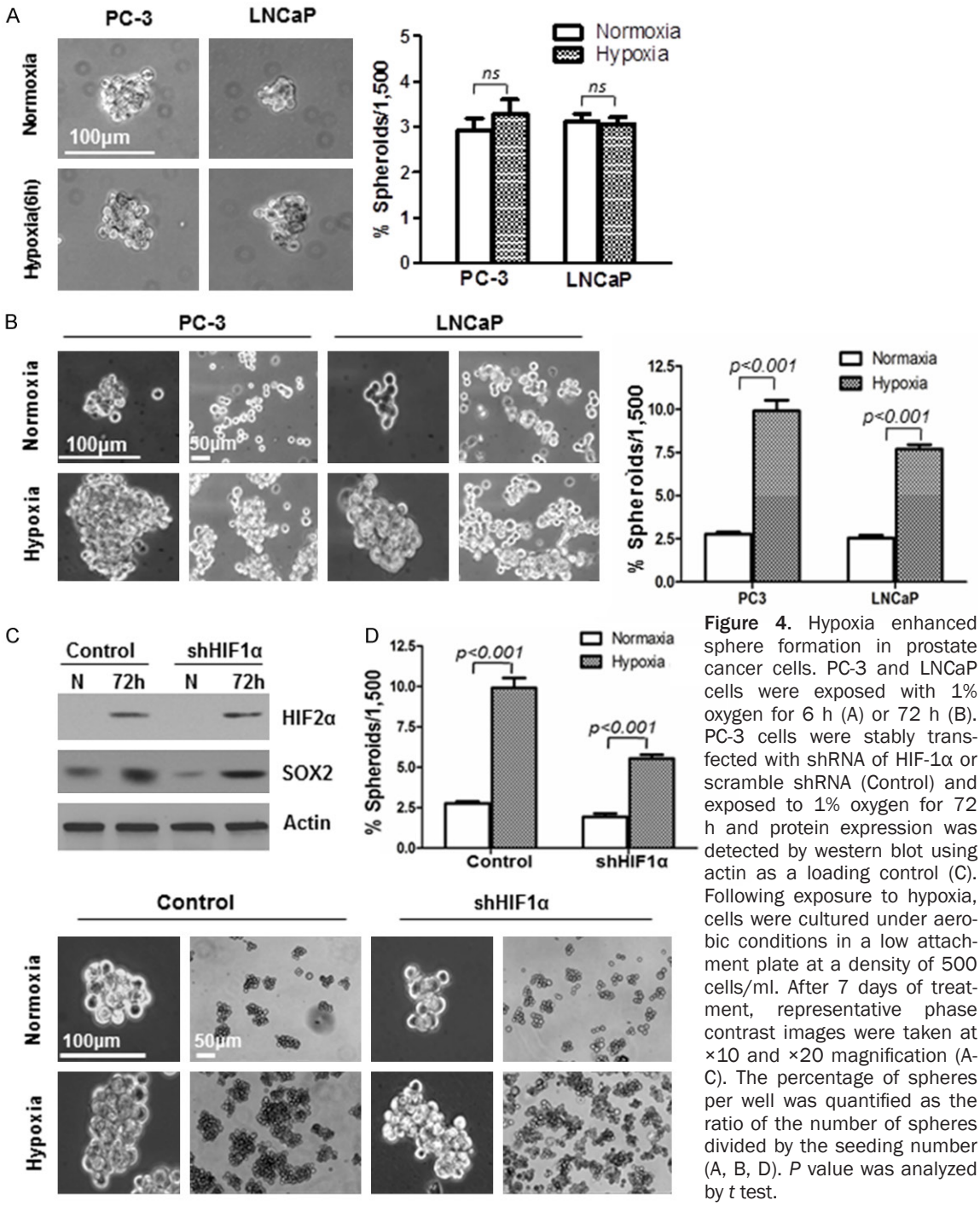

Figure 4. Hypoxia enhanced sphere formation in prostate cancer cells. PC-3 and LNCaP cells were exposed with 1% oxygen for 6 h (A) or 72 h (B). PC-3 cells were stably transfected with shRNA of HIF-1α or scramble shRNA (Control) and exposed to 1% oxygen for 72 h and protein expression was detected by western blot using actin as a loading control (C). Following exposure to hypoxia, cells were cultured under aerobic conditions in a low attachment plate at a density of 500 cells/ml. After 7 days of treatment, representative phase contrast images were taken at ×10 and ×20 magnification (A-C). The percentage of spheres per well was quantified as the ratio of the number of spheres divided by the seeding number (A, B, D). *P* value was analyzed by *t* test.

treated with prolonged hypoxia (72 h) were significantly larger and greater in number than those arising from normoxic prostate tumor cells (**Figure 4B**).

To determine whether HIF-1α contributed to sphere formation under prolonged hypoxia, HIF-1α knockdown or control PC-3 cells were exposed to 1% O_2 for 72 h followed by maintenance under normoxia for 7 days. With HIF-1α knockdown, neither SOX2 nor HIF-2α expression was clearly reduced by hypoxic exposure (**Figure 4C**). Moreover, sphere formation in HIF-1α knockdown cells was markedly enhanced in size and number by prolonged hypoxia (**Figure 4D**) suggesting that HIF-1α knockdown cells

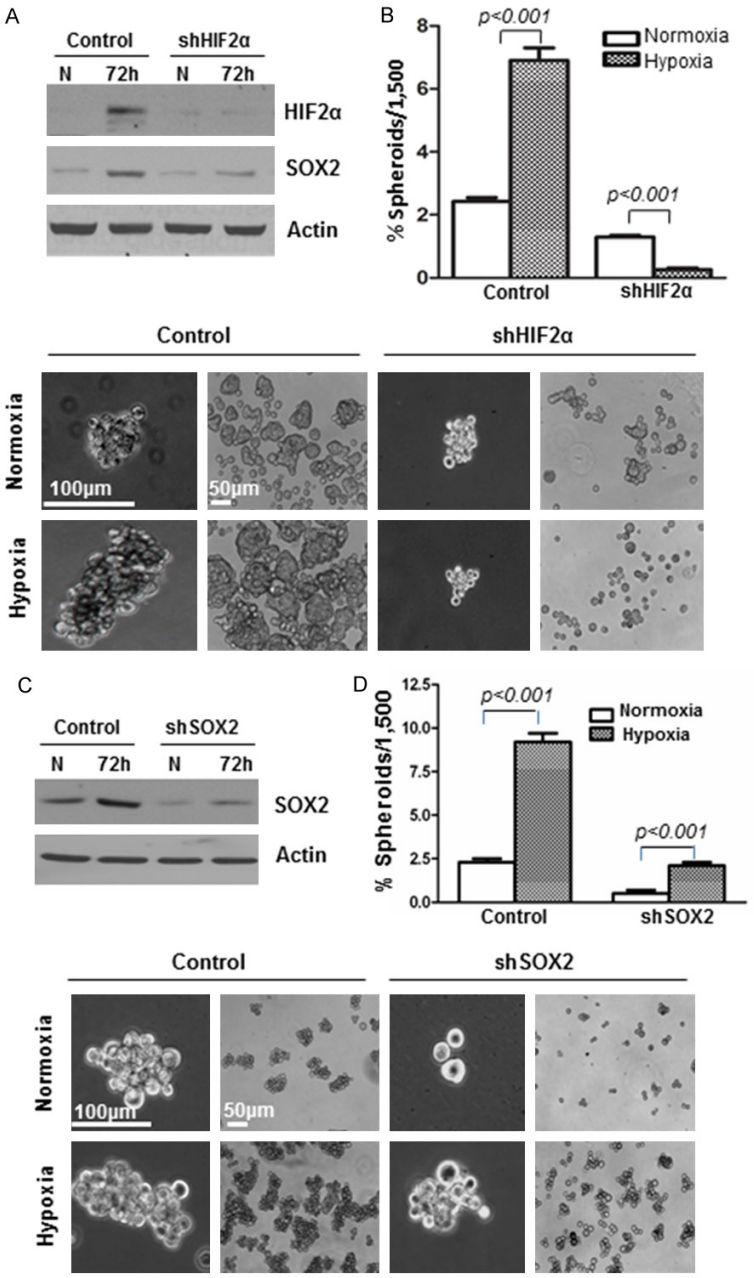

Figure 5. Chronic hypoxia-induced, SOX2-mediated sphere formation is dependent on HIF-2α. Prostate cancer cells were stably transfected with shRNA of HIF-2α (A, B) SOX2 (C, D), or scramble shRNA (Control). Transfected cells were exposed to 1% oxygen for 72 h and protein expression was detected by western blot using actin as a loading control (A, C). To test sphere formation, transfected cells were exposed to 1% oxygen for 72 h and cells were cultured under aerobic conditions in a low attachment plate at a density of 500 cells/ml. After 7 days of treatment, representative phase contrast images were taken at ×10 and ×20 magnification (B, D). The percentage of spheres per well was quantified as the ratio of the number of spheres divided by the seeding number (B, D). *P* value was analyzed by *t* test.

retained hypoxia-induced sphere forming potential.

HIF-2α and SOX2 regulate chronic hypoxia-induced sphere formation capacity

To test whether hypoxia-induced sphere formation was dependent on HIF-2α, HIF-2α knockdown cells and control cells were exposed to 72 h of hypoxia followed by sphere culturing under aerobic conditions. HIF-2α shRNA dramatically suppressed both HIF-2α and SOX2 expression (**Figure 5A**) and sphere formation (**Figure 5B**). To examine the role of SOX2 in hypoxia-induced sphere formation, PC-3 shSOX2 cells were exposed to long-term (72 h) hypoxia. Although only a 30-40% SOX2 knockdown efficiency was achieved (**Figure 5C**), this reduction was sufficient to significantly reduced sphere formation under normoxic conditions compared to control cells (**Figure 4D**). However, chronic hypoxia still induced SOX2 expression in SOX2 shRNA cells (**Figure 5C**) and sphere forming ability was enhanced by hypoxia even in the SOX2 knockdown cells (**Figure 5D**).

Discussion

Hypoxia is a common feature of solid tumors, creating an environment where undifferentiated stem-cell like tumor cells can exist. In prostate cancer, hypoxia is generally associated with disease progression and poor prognosis [19-21]. Furthermore, growing evidence suggests that hypoxia is able to induce stem-cell like characteristics (as indicated by elevated human embryonic stem cell makers including OCT3/4, NANOG, SOX2, KLF4 and c-MYC) in several cancers [23, 28, 38]. Although overlap between HIF-1α,

NANOG, and OCT4 expression has been observed in primary prostate tumors [38], expression of Nanog and Oct3/4 were highly enriched in HIF-1α positive tumor regions. The current study reveals a similar relationship between HIF-1α and SOX2 in prostate tumor tissues. Our findings further suggest SOX2 as a hypoxia-responsive gene that contributes to prostate cancer cell invasion and sphere formation mediated by low oxygen tensions. Exposure of prostate cancer cells to both short-term and prolonged hypoxia increased the expression of SOX2 but not the other stem cell markers evaluated, suggesting that perhaps SOX2 is uniquely augmented by hypoxic exposure in prostate cancer cells.

HIF proteins play a critical role in cellular response to hypoxia [24, 44] and elevations in HIF expressions have been correlated with poor patient survival in a variety of cancers [25-27]. These studies have focused largely on HIF-1α but recent reports also suggest an important role for HIF-2α. For example, it has been reported that HIF-2α may be preferentially expressed in neuronal tumor cells that exhibit cancer stem cell characteristics [45] and that HIF-2α selectively regulates SOX2 in glioblastoma, although the detail mechanism remains unclear [39]. In the present study, we show that hypoxia-induced SOX2 expression can be dependent on both HIF-1α and HIF-2α suggesting a novel role for SOX2 in hypoxia-mediated prostate cancer cell dissemination and stemness. While HIF-1α primarily regulates acute hypoxia-induced cell invasion, HIF-2α controls chronic hypoxia-induced sphere formation. In the presence of low oxygen, the sphere forming ability of prostate cancer cells that lack HIF-2α and SOX2 but not HIF-1α is significantly impaired. Conversely, the invasive capacity of prostate cancer cells that lack HIF-1α and SOX2 is greatly impaired, and not compensated for by HIF-2α expression. Since knockdown of HIF-2α fails to enhance tumor cell invasion even with reduced SOX2 expression, it appears that although SOX2 contributes to hypoxia-induced invasion, other invasion-related proteins induced by HIF-1α may also be involved.

Taken together, the present results support a predominant role of HIF-1α in controlling SOX2-mediated cell invasion induced by short-term hypoxia while SOX2 and HIF-2α interplay during prolonged hypoxia significantly impacts stemness. The alteration of SOX2 expression by hypoxia suggests an adaptive role of SOX2 in tumor cells to the aberrant tumor microenvironments in solid tumors and implies a possible mechanism by which tumor cells may acquire more aggressive behavior and/or stem cell-like characteristics after exposure to low oxygen tensions. Our findings that SOX2 contributes to prostate carcinoma cell invasion and stemness particularly under hypoxic conditions support the notion that this transcription factor may act as a key signaling mediator that modulates tumor cell characteristics and behavior in response to hypoxia. The present data therefore suggest that SOX2 may serve as a potential target for therapeutic interventions for metastatic prostate cancer.

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Disclosure of conflict of interest

None.

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