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Stabilization of Snail by HIF-1α and TNF-α is required for hypoxia-induced invasion in prostate cancer PC3 cells

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Abstract Hypoxia has been involved in the development of tumor by regulating the expression of invasiveness-associated genes. However, the specific function of hypoxia in cancer cell invasion is still unclear. The aim of the present study was to determine the role of hypoxia in invasion of prostate cancer PC3 cells and to investigate the underlying mechanisms. We found that hypoxia significantly increased the invasive activity of PC3 cells, via up-regulation of the expression of hypoxia inducible factor 1α (HIF-1α) and the autocrine production of tumor necrosis factor α (TNF-α). More important, TNF-α cooperated with HIF-1α in promoting stabilization of Snail, a transcriptional repressor of E-cadherin expression, which lead to the up-regulation of invasiveness-associated genes MMP-9, fibronectin and vimentin. Snail silencing by specific siRNA significantly inhibited hypoxia-induced invasion of PC3 cells, indicating an essential role of Snail in conferring the malignant phenotype to cancer cells under hypoxic conditions. In conclusion, our data demonstrate that hypoxia promoted the invasiveness of prostate cancer PC3 cells via HIF-1α- and TNF-α-induced stabilization of Snail, suggesting a signaling mechanism involving HIF-1α/TNF-α/Snail that mediates invasiveness hypoxic tumor cells in the absence of neoangiogenesis.

Keywords Hypoxia · Invasion · Hypoxia inducible factor 1α (HIF-1α) · Tumor necrosis factor (TNF-α) · Prostate cancer

Introduction

It is well known that hypoxia is the most important micro-environmental factor in the development and progression of cancer [1, 2]. In most solid tumors, cellular hypoxia could induce mutagenesis and increase invasiveness of malignant cells [3]. It has been reported that hypoxia inducible factor 1 (HIF-1), composed of α and β sub-units, plays a critical role in the cellular adaption to hypoxia [4]. Thus, HIF-1α activation and/or over-expression have been shown to correlate with the increased invasiveness and metastatic potential of solid tumors, such as breast and bladder cancer. Conversely, HIF-1α loss-of-function has elicited the opposite effects [5, 6]. However, little is known about whether HIF-1α plays a similar oncogenic role in the growth of human prostate cancer cells.

Inflammation has been identified as a tumor promoting factor, which might increase the risk of tumor metastasis [7, 8]. In the absence of neoangiogenesis, hypoxic tumors accumulate different inflammatory cells, which regulate tumorigenic growth and contribute to metastatic potential. The conditions of low oxygen or hypoxia induce the production of various pro-inflammatory cytokines by tumor cells through the activation of key signaling pathways such as NF-κB and PI3K/AKT, leading to the up-regulation of invasiveness-associated genes [9]. Thus, hypoxia is thought to be a potent factor in modulation of inflammatory response

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by the activation of a number of regulatory cytokines such as tumor necrosis factor α (TNF-α), which has been regarded as a very important pro-inflammatory cytokine produced by immune cells. Accumulating evidence also suggests that TNF-α could induce invasion of tumor cells [10, 11]. However, the association of hypoxia and TNF-α expression in tumor metastasis has not been explored. In this study, we, thus, focused on elucidation of the regulatory mechanisms underlying tumor metastasis in hypoxic conditions and on the association between HIF-1α and TNF-α in hypoxia-mediated invasion of prostate cancer cells.

Materials and methods

Cell culture and induction of hypoxia

Human prostate cancer PC3 cells were maintained in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5 % CO_2 at 37 °C. Hypoxia was induced by incubation of PC3 cells in hypoxic-chambers in the atmosphere containing 1 % O_2, 5 % CO_2 and 94 % N_2. In addition, $CoCl_2$ (100 μM) (Sigma, St. Louis, MO, USA) was used as a hypoxia mimetic agent to simulate hypoxic conditions.

Cell viability assay

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Beyotime Institute Biotechnology, China). Briefly, 5×10^3 cells were seeded in 100 μl medium per well in 96-well plates and incubated in normoxic or hypoxic conditions. After the treatment for the indicated times, 20 μl MTT (5 mg/ml) was added to each well and the cells were incubated for additional 4 h. Then discarded cell supernatants and added DMSO to dissolve MTT crystals. The values of absorbance were detected at 570 nm using a microplate reader. The relative levels of cell viability were defined as the absorbance of treated samples versus that of unmanipulated control and the unmanipulated control cells were designated as 100 %.

Conditioned medium (CM)

Briefly, 5×10^6 cells were cultured in 5 ml of RPMI 1640 supplemented with 10 % FBS and incubated under normoxic conditions for 24 h. The medium was removed and replaced by 1 ml of serum-free RPMI 1640 and after incubation for additional 24 h under normoxic or hypoxic conditions, cells were removed by centrifugation and the supernatant (conditioned medium, CM) was used for further experiments.

Table 1 Primer sequences for qRT-PCR

Gene	Primer	Product (bp)
HIF-1α	F5′-GCCGAGGAAGAACTATGAA-3′	100
(NM_001530)	R5′-CACTGAGGTTGGTTACTGT-3′	
Snail	F5′-CTTCCAGCAGCCCTACGA-3′	209
(NM_005985)	R5′-AGCCTTTCCCACTGTCCTC-3′	
MMP9	F5′-CGCTGGGCTTAGATCATTC-3′	126
(NM_004994)	R5′-CAGGGCGAGGACCATAGA-3′	
E-cadherin	F5′-ATCTTCAATCCCACCACG-3′	239
(NM_004360)	R5′-TGTAGAATGTACTGCTGCTT-3′	
Fibronectin	F5′-ATGGAGGAAGCCGAGGTT-3′	186
(NM_212482)	R5′-AGCGGTTTGCGATGGTAC-3′	
Vimentin	F5′-AGGAGGCAGAAGAATGGTA-3′	345
(NM_003380)	R5′-AGGTGGCAATCTCAATGTC-3′	
GAPDH	F5′-TGAAGGTCGGAGTCAACGG-3′	225
(NM_002046)	R5′-CCTGGAAGATGGTGATGGG-3′	

Invasion assay

The invasion assay was performed using Boyden chemotaxis chambers with 8 μm pore Matrigel-coated polycarbonate membrane (Costar, US). Briefly, 5×10^4 cells resuspended in serum-free RPMI 1640 medium were placed in the upper chamber, while the lower chamber was filled with 10 %FCS-containing RPMI 1640 or the CM served as chemoattractants. After incubation at normoxic or hypoxic conditions for 24 h, the cells in the upper chamber were removed and the migrated cells at the bottom of the polycarbonate membrane were fixed with 4 % paraformaldehyde and stained with 0.1 % crystal violet. The number of invading cells was counted in three randomly chosen fields under the microscope.

Real-time PCR

Total RNA was isolated using the Trizol Reagent kit (Invitrogen, US) and cDNA was synthesized using the SuperScript First-Strand Synthesis kit (Invitrogen) according to the manufacturer's protocol. Real-time PCR was performed using cDNA as a template, specific primers (Table 1) and the SYBR master mix (TaKaRa, Japan). The relative mRNA expression for each sample was determined by normalizing the target gene expression to that of the GAPDH.

Western blot analysis

Total proteins extracted from PC3 cells with 1× cell lysis buffer (Pierce, US) were resolved by 10 % SDS-PAGE and transferred to 0.45 μm nitrocellulose membrane (Millipore, Bedford, MA, USA). Primary antibodies against HIF-1α,

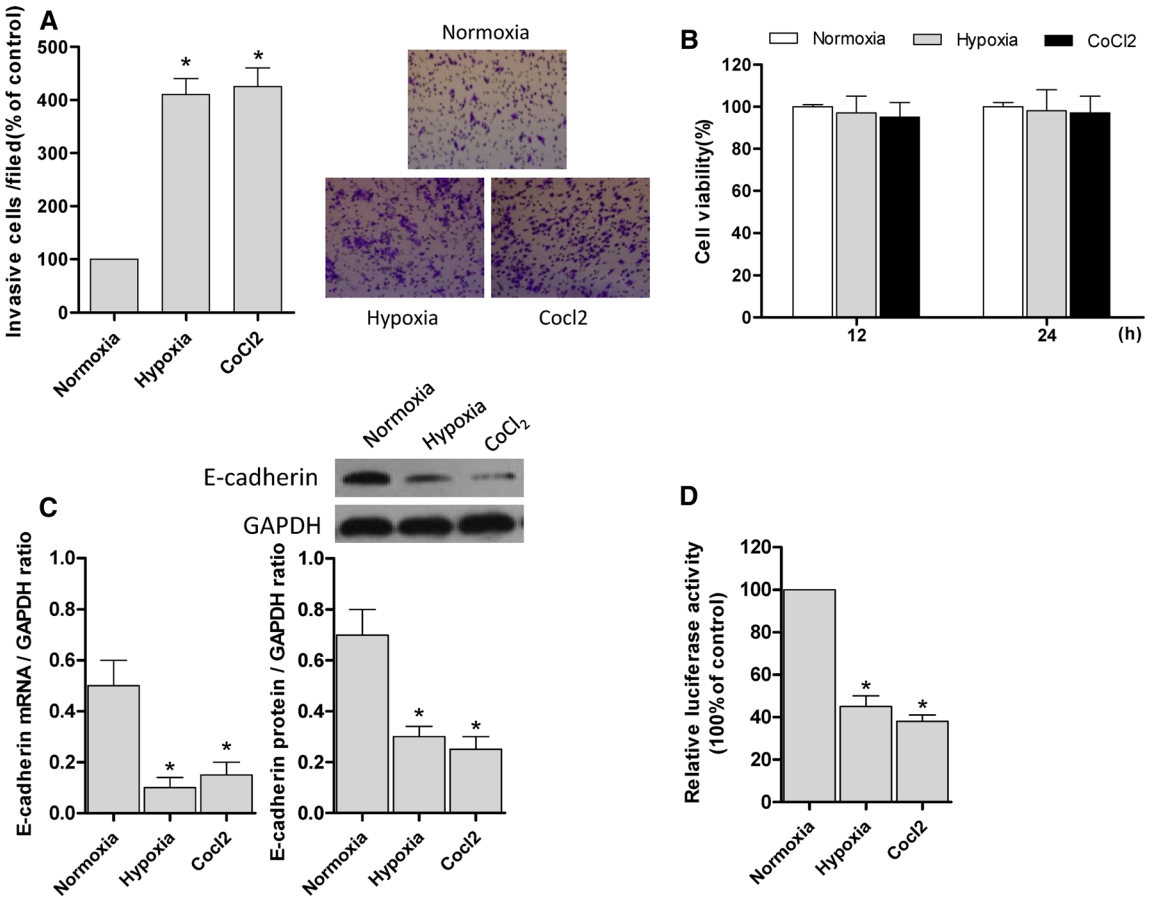

Fig. 1 Hypoxia induces invasion of PC3 cells. **a** Stimulatory effect of hypoxia and its mimetic agent on cell invasion. Images are representative of at least three separate experiments; magnification, ×200. **b** Hypoxia does not affect the viability of PC3 cells. **c** The effect of hypoxia on E-cadherin expression analyzed by qRT-PCR and western blotting. **d** The effect of hypoxia on the activity of E-cadherin promoter. Data are expressed as mean ± SD of three independent experiments. * $P < 0.05$ compared to normoxia

E-cadherin, vimentin, fibronectin, MMP-9, Snail and GAPDH were used respectively to probe the proteins, following by incubation with horseradish peroxidase-conjugated secondary antibodies. Protein bands were detected using a chemiluminescence method (ECL, Pierce), according to the manufacturer's instruction.

Plasmids and siRNAs

The pcDNA3.1/HIF-1α expression vector was constructed by subcloning cDNA the encoding full-length HIF-1α sequence into the pcDNA3.1 plasmid (Invitrogen). For RNA interference, the targeting sequences were designed using RNAi algorithm available online (http://www.ambion.com/techlib/misc/siRNA_finder.html). The specific siRNA targeting HIF-1α (5′-ACGACAAGAAAAAGATAAGTTCT-3′) and Snail (5′-GCCCTCCGACCCCAATCGG-3′) and control siRNA were obtained from Ribobio (Guangzhou, China). PC3 cells were transfected using Lipofectamine^{TM} 2000 (Invitrogen) according to the manufacture's protocol.

Transfection and luciferase reporter assays

The pGL3-E-cadherin-basic plasmid was kindly provided by Jun Li (Chong Qing Cancer Institute, China). For transfection, cells at 1×10^6 per well were plated in 6-well plates and allowed to grow to 70 % confluence. Then cells were transiently co-transfected with pGL3-E-cadherin-basic and the *Renilla* luciferase plasmid pRL-TK (Promega, US) used to normalize for transfection efficiency. Six hour after the transfection, cells were subjected to either under normoxic or hypoxic conditions for 24 h and harvested using Passive Lysis Buffer (Promega). The transcriptional activity of the E-cadherin promoter was determined using dual luciferase system (Promega), according to the manufacturer's instructions.

Analysis of cytokine release by ELISA

1×10^6 cells were seeded into 6-well plates and incubated under normoxic or hypoxic conditions for 24 h. Cell culture supernatants were collected by centrifugation and

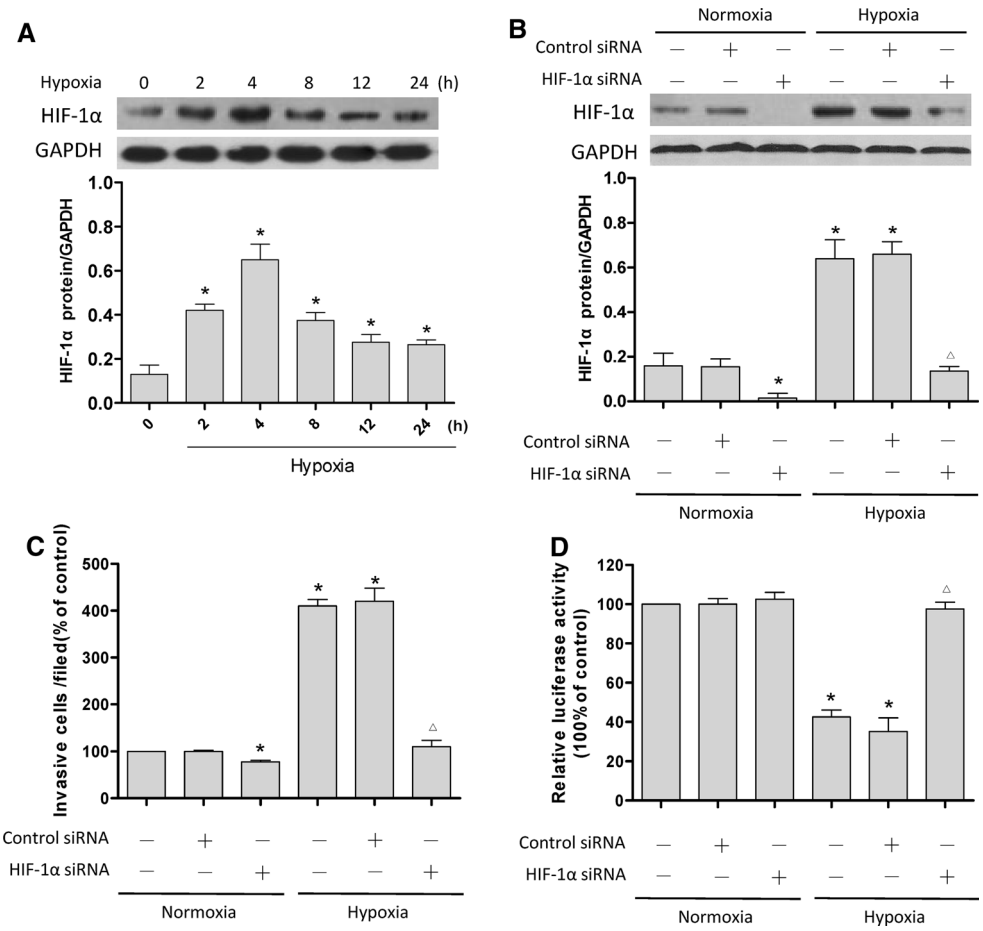

Fig. 2 HIF-1α mediates hypoxia-induced invasion of PC3 cells. **a** Cells were incubated in hypoxic conditions for the indicated times. The HIF-1α protein level was determined by western blotting. **b–d** Cells were transfected with HIF-1α siRNA or control siRNA for 12 h and incubated in normoxic or hypoxic conditions for additional 24 h. HIF-1α expression were determined by western blotting (**b**) and PC3 cell invasive ability was investigated by the invasion assay as described in "Materials and methods" (**c**). **d** Analysis of E-cadherin activity using the luciferase reporter assay. Data are expressed as mean ± SD of three independent experiments. * $P < 0.05$ compared to control (0 h) or normoxia; $^{\Delta}$ $P < 0.05$ compared to hypoxia

analyzed for cytokine content using a quantitative sandwich ELIZA assay (R&D Systems, US) according to the manufacturer's instruction.

Statistical analysis

All statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The data are presented as mean ± SD of at least three independent experiments. Statistical significance ($P < 0.05$) was determined by one-way ANOVA or Student–Newman–Keuls (SNK)-q test.

Results

Hypoxic conditions induced invasiveness of PC3 cells

To evaluate a potential role of hypoxia in PC3 cell invasive activity, the invasion assay was performed. As indicated in Fig. 1, hypoxia markedly increased the invasive activity of PC3 cells. The administration of the hypoxia mimetic agent $CoCl_2$ was also observed to increase the invasive ability of PC3 cells (Fig. 1a). However, hypoxia and $CoCl_2$ had no significant effects on cell viability (Fig. 1b). The analysis of gene expression indicated that both hypoxia and its mimetic agent could induce invasion of PC3 cells through inhibition of the transcriptional activity of the E-cadherin promoter and down-regulation of E-cadherin expression (Fig. 1c, d).

Hypoxia promoted invasion of PC3 cells through upregulation of HIF-1α expression

Hypoxic stimulation of PC3 cells significantly increased the expression of HIF-1α protein, which peaked at 4 h and returned back to the basal level between 12 and 24 h after the treatment (Fig. 2a). To determine whether hypoxia-induced invasion of PC3 cells was mediated by HIF-1α, PC3 cells were transfected with HIF-1α-specific siRNA and subjected to normoxic or hypoxic conditions. HIF-1α siRNA caused dramatic reduction in HIF-1α protein level (Fig. 2b) and significant inhibition of the invasive activity of hypoxia-treated PC3 cells compared to those of control siRNA-transfected cells (Fig. 2c). Moreover, the transcriptional

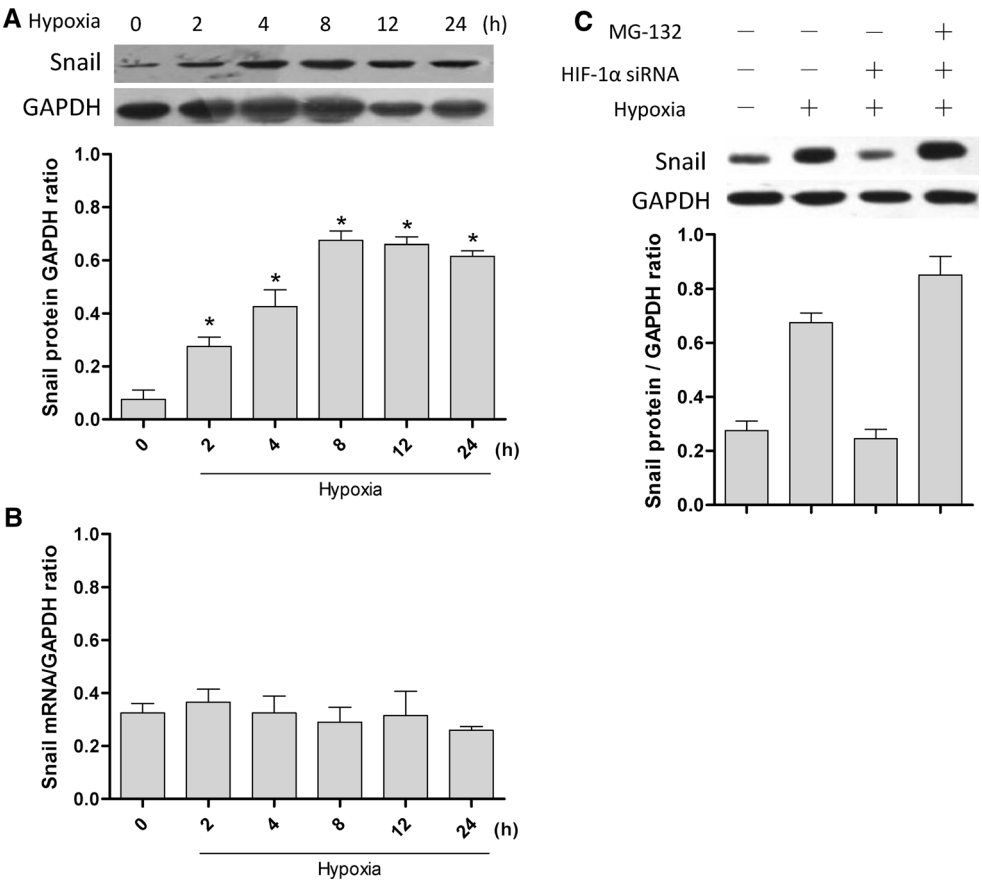

Fig. 3 HIF-1α induced Snail stabilization. PC3 cells were incubated in hypoxic conditions for the indicated times and the expression of Snail protein was determined by western blotting (**a**) and Snail mRNA by qRT-PCR (**b**). PC3 cells were pretreated with the proteasome inhibitor MG-132 or HIF-1α-specific siRNA for 12 h and then subjected to normoxic or hypoxic conditions for additional 24 h; Snail protein expression was determined by western blotting (**c**). Data are expressed as mean ± SD of three independent experiments. * $P < 0.05$ compared to untreated control

activity of the E-cadherin promoter was also significantly increased after HIF-1α knock-down (Fig. 2d).

HIF-1α stabilized Snail protein expression

Previous studies have revealed that Snail, a transcriptional repressor of the adhesion protein E-cadherin, could promote cancer cells invasion [12]. We therefore asked whether the Snail gene was associated with the hypoxia-induced cell invasion of PC3 cells. It was shown that hypoxia remarkably up-regulated Snail expression in a time-dependent manner. The protein expression of Snail increased 2 h after exposure to hypoxic conditions, reaching its maximum at 8 h and remaining at this level for up to 24 h after hypoxic treatment. However, Snail mRNA expression did not change under hypoxic conditions, indicating that hypoxia regulated Snail expression at the post transcriptional level (Fig. 3a, b). Next, we test whether HIF-1α could affect Snail expression. For this, HIF-1α siRNA-transfected PC3 cells were subjected to hypoxia and analyzed for Snail protein expression. Western blotting analysis revealed that the downregulation of HIF-1α resulted in a significantly reduced Snail expression. However, the inhibitory effect of HIF-1α siRNA was attenuated by the treatment with the proteasome inhibitor MG-132(Fig. 3c), indicating that the reduction in HIF-1α level caused the degradation of Snail through ubiquitin–proteasome pathway.

Hypoxia induced autocrine activation of TNF-α

Recent studies have indicated that cell autocrine activation plays a critical role in cancer metastasis [13]. To investigate whether autocrine mechanism was associated with hypoxia-mediated invasion of PC3 cell, they were treated with the CM of normoxic and hypoxic cultures. We surprisingly found that CM of hypoxic cells strongly stimulated the invasive activity of PC3 cells, as compared with the cells cultured in the presence of normoxic CM (Fig. 4a). We next tested the release of cytokines TNF-α, interleukin-1 (IL-1), IL-6, IL-8, interferon-γ (INF-γ), transforming growth factor β (TGF-β) and nerve growth factor (NGF) and a chemokine granulocyte-colony stimulating factor (G-CSF) by PC3 cells subjected to normoxic and hypoxic conditions. Our data revealed that the expression levels of TNF-α and IL-6 were dramatically increased in hypoxia-treated PC3 cells (Fig. 4b). To confirm whether these two inflammatory cytokines were the factors that regulated the invasive ability of PC3 cells

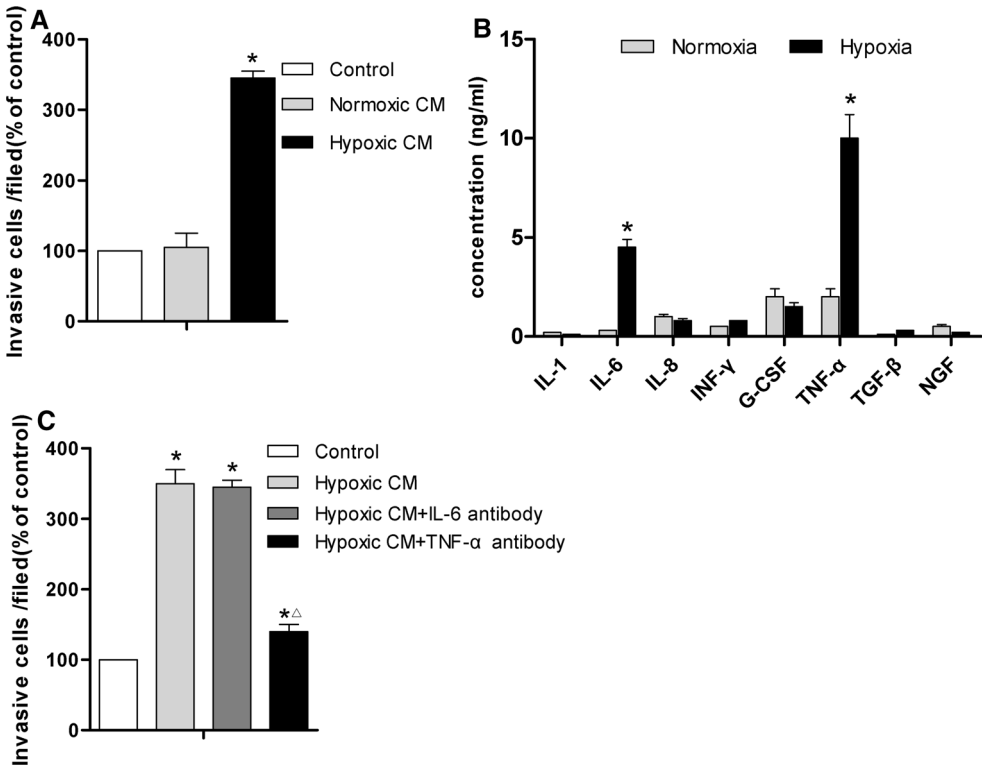

Fig. 4 Autocrine TNF-α activity is required for hypoxia-induced invasion of PC3 cells. **a** Effect of the CM on the invasiveness of PC3 cells. The CM was collected as described in "Materials and methods". Cells were treated with CM under normoxic conditions and cell invasive activity was determined by the invasion assay. **b** Release of cytokines by PC3 cultured under normoxic or hypoxic conditions for 24 h. Cytokine concentrations in culture supernatants were determined by quantitative sandwich ELISA. **c** Effects of TNF-α and IL-6 on PC3 cell invasion. Cells were pre-treated with or without TNF-α or IL-6 antibody (1 μg/ml) for 1 h, incubated in the CM produced by hypoxic PC3 for additional 24 h, and cell invasion was analyzed as described. Data are expressed as mean ± SD of three independent experiments. * $P < 0.05$ compared to control or normoxia; $^{\Delta}$ $P < 0.05$ compared to hypoxic CM

under the hypoxic conditions, cells were pre-treated with anti-TNF-α or anti-IL-6 antibodies and then treated with the CM of hypoxic cultures containing elevated levels of TNF-α and IL-6. While the CM significantly stimulated the invasive activity of normoxic PC3 cells, pre-incubation of PC3 cells with anti-TNF-α antibody abrogated the CM-induced invasion. However, the anti-IL-6 antibody did not affect the induction of PC3 cell invasion by the CM (Fig. 4c). These data further support the notion that the autocrine activation of TNF-α may be responsible for the hypoxia-induced invasion of cancer cells.

TNF-α cooperated with HIF-1α in promoting Snail stabilization

Previous studies have demonstrated that the activation of NF-κB signaling pathway by TNF-α may play an important role in Snail stabilization [14, 15]. Thus, we next examined whether TNF-α could regulate Snail expression. The Snail protein was up-regulated in PC3 cells stimulated with TNF-α. However, the increase in Snail expression in hypoxic conditions was greater than that elicited by TNF-α stimulation (Fig. 5a, b). Therefore, we hypothesized that TNF-α and HIF-α synergistically induced Snail expression and stabilization, leading to the enhanced invasiveness of PC3 cells. To test this, PC3 cells transfected with pcDNA-HIF-1α were treated with TNF-α and examined for Snail expression. The results showed that TNF-α-mediated Snail expression was enhanced in HIF-1α-expressing PC3 cells confirming our hypothesis of TNF-α and HIF-α synergistic activity in inducing Snail expression. Moreover, the increase in Snail expression due to TNF-α and HIF-α synergism was similar to that in the hypoxia-treated PC3 cells (Fig. 5b). Similar results were observed for the regulation of E-cadherin promoter, which was more effectively inhibited in HIF-1α-transfected PC3 cells treated with TNF-α than in cells treated with TNF-α or HIF-1α separately (Fig. 5c).

Hypoxia stabilized Snail, enhanced invasion and modulated the expression invasiveness-associated genes

We next investigated the impact of Snail knockdown on hypoxia-induced invasion of PC3 cells. Our results showed

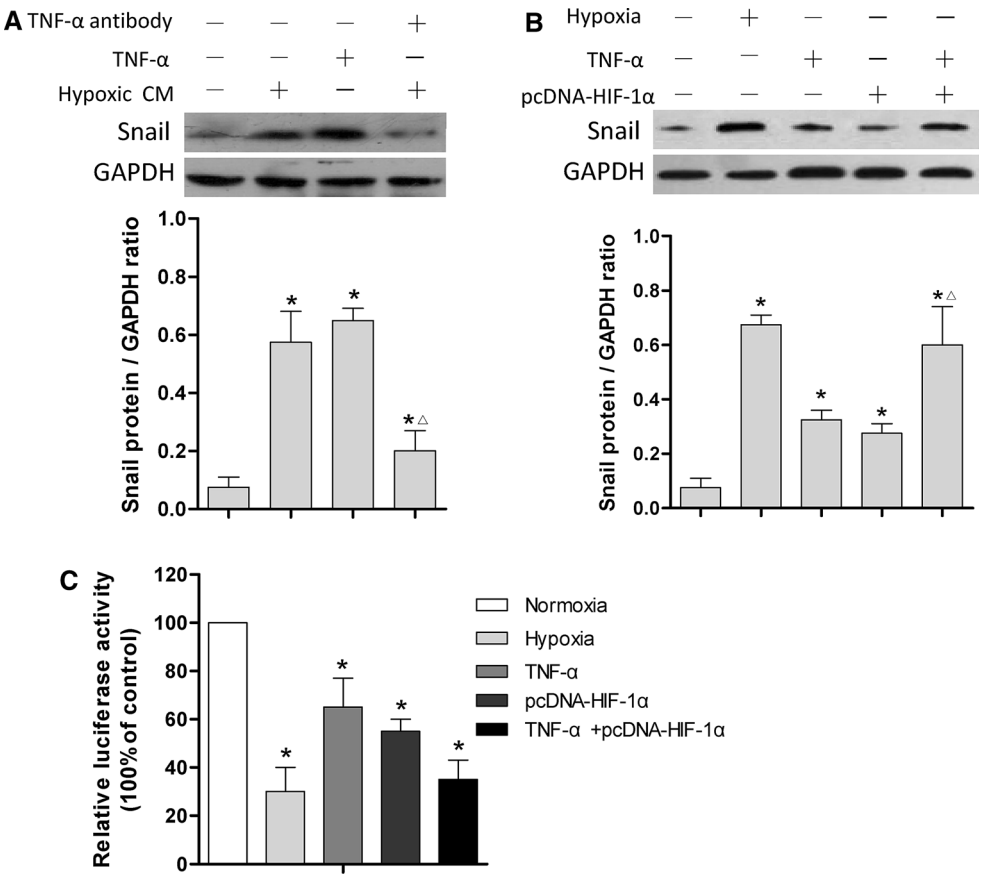

Fig. 5 Synergistic effect of HIF-1α and TNF-α on stabilization of Snail. Cells were pre-treated with or without TNF-α antibody (1 μg/ml) for 1 h and incubated in the hypoxic CM for additional 24 h. Snail protein expression was analyzed by western blotting (**a**). Cells were treated with pcDNA-HIF-1α or/and TNF-α cytokine (10 ng/ml) for 24 h under normoxic conditions; Snail protein expression was determined by western blotting (**b**) and E-cadherin promoter activity was analyzed by the luciferase reporter assay (**c**). Data are expressed as mean ± SD of three independent experiments. * $P < 0.05$ compared to normoxia; $^{\Delta}$ $P < 0.05$ compared to separate treatment with Hypoxic CM, TNF-α, or pcDNA-HIF-1α

that knocking down of Snail expression by siRNA inhibited hypoxia-induced invasion of PC3 cells, a similar effect was observed in HIF-1α- and TNF-α-stimulated cells (Fig. 6a, b). Given that Snail regulated the expression of invasiveness-associated genes, such as matrix metalloproteinase 9 (MMP9), fibronectin and vimentin [16, 17], we next investigated whether hypoxia also affected the expression of these genes. Our results demonstrated that hypoxia-induced mRNA and protein expression of MMP9, fibronectin and vimentin. However, PC3 incubation with Snail-specific siRNA effectively inhibited the hypoxia-induced up-regulation of these invasiveness-associated genes (Fig. 6c, d).

Discussion

Prostate cancer is the most common and second most fatal malignant in males in the Western world [18]. The prognosis of prostate cancer is determined by the presence or absence of metastases caused by the invasion of the constituent tumor cells [19, 20]. Thus, a better understanding of the mechanisms underlying the invasive characteristic of prostate cancer cells is critical for the development of improved treatment strategies to treat prostate cancer. In this study, we showed that the stabilization of Snail plays a critical role in hypoxia-induced invasion of prostate cancer cells and that the stabilization effect is associated with HIF-1α expression and TNF-α autocrine activity.

Hypoxia is a critical factor in tumor microenvironment associated with tumor growth, increased angiogenesis and metastasis [3, 21, 22]. Hypoxic response is mainly mediated by HIF-1α [4]. In the present study, we found that hypoxia could increase the invasive activity of PC3 cells by upregulating the expression of HIF-1α; these results were confirmed by the inhibition of the hypoxia-induced invasion by HIF-1α-specific siRNA. We also found that the hypoxia-induced inhibition of the E-cadherin promoter activity was attenuated by silencing of HIF-1α expression, indicating that E-cadherin may be related to the hypoxia-induced invasion in PC3 cells.

E-cadherin protein is characteristic for epithelial cells. Down-regulation of E-cadherin is one of the biomarkers of epithelial-mesenchymal transition (EMT) implicated in cancer progression and metastasis [23]. Several zinc-finger transcriptional repressors, such as Slug, Snail and Twist, have been shown to directly repress the transcription of E-cadherin. Snail has been identified as the most important E-cadherin transcriptional regulator [24], therefore we investigated whether hypoxia-mediated E-cadherin regulation is associated with Snail. Our data show that hypoxia

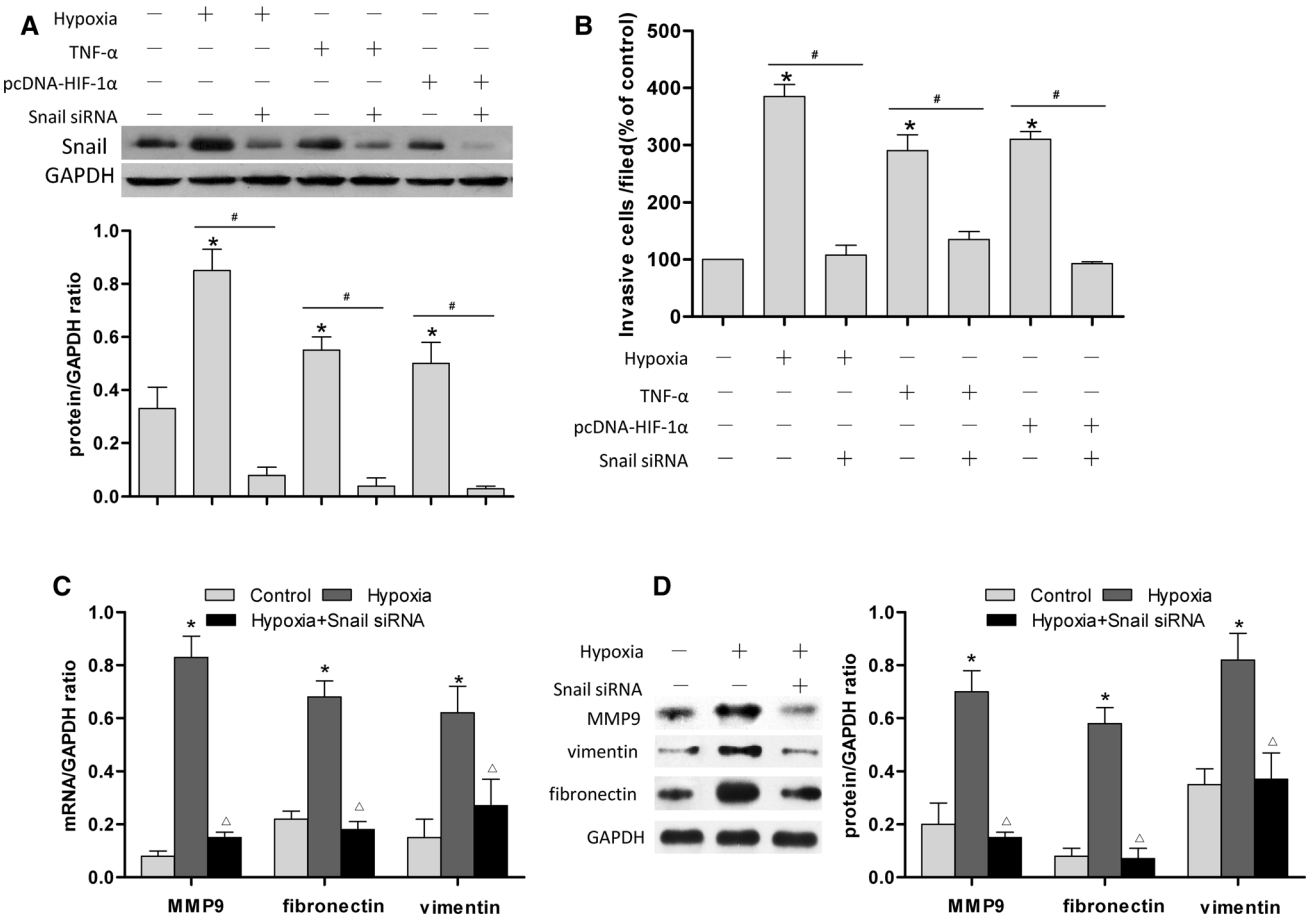

Fig. 6 Regulation of Snail by HIF-1α and TNF-α is required for hypoxia-induced invasion of PC3 cells. PC3 transfected with Snail-specific siRNA for 12 h were subjected to hypoxia, treated with TNF-α, or transfected with pcDNA-HIF-1α. Snail protein expression was determined by western blotting (**a**) and the cell invasive activity was analyzed by the invasion assay (**b**). PC3 cells transfected with Snail siRNA for 12 h were incubated in hypoxic or normoxic conditions for additional 24 h. The mRNA and protein expression levels of MMP-9, vimentin, and fibronectin were determined by qRT-PCR (**c**) and western blotting analysis (**d**), respectively. Data are expressed as mean ± SD of three independent experiments. * $P < 0.05$ compared to normoxia; $^{\#}$ $P < 0.05$; $^{\Delta}$ $P < 0.05$ compared to hypoxia

treatment markedly increased the Snail protein expression but did not affect Snail mRNA, suggesting that hypoxia regulates Snail expression at post-transcriptional level. Interestingly, silencing HIF-1α by siRNA attenuated hypoxia-induced Snail protein up-regulation. Previous studies have demonstrated that Snail half-life, which is less than 30 min, is mainly regulated by ubiquitin–proteasome pathway [25]. In our study, when PC3 cells were pretreated with the proteasome inhibitor MG-132, the concentration of Snail protein was significantly elevated, suggesting that HIF-1α may stabilize Snail by suppressing its degradation in ubiquitin–proteasome pathway.

Recent studies have reported that cancer cells supported their own invasive phenotype in an autocrine fashion by the release of pro-inflammatory cytokines in the hypoxic environment [26]. In the current study, we found that PC3 cells released several pro-inflammatory cytokines, including IL-6, IL-8, IFN-γ, and TNF-α and a chemokine G-CSF. Interestingly, the levels of IL-6 and TNF-α were markedly elevated under hypoxic conditions. IL-6 and TNF-α are important pro-inflammatory cytokines involved in the regulation of various physiological and pathological processes. Both are produced in response to cell stress, including ischemia and hypoxia, and can induce cancer cell proliferation, invasion and angiogenesis [27, 28]. However, it has not been clear whether there is a direct association between these two cytokines and induction of prostate cancer cell invasion under hypoxic conditions. Therefore, further studies are performed to identify the functions of these pro-inflammatory cytokines in PC3 cells by neutralizing antibodies. Our results demonstrated that TNF-α neutralizing antibody partially inhibited the CM-mediated induction of PC3 cell invasiveness, whereas neutralizing antibodies against IL-6 had no inhibitory effect. These results are consistent with the involvement of TNF-α-based autocrine mechanism in the induction of prostate cancer cells invasion suggested in previous studies.

In fact, the expression of Snail is regulated by a complex integrated signaling network that includes PI3K/AKT/GSK-3β and NF-κB pathways [29]. It has been shown that TNF-α can promote tumor cell invasion through the induction of genes encoding NF-κB dependent invasiveness-associated molecules [30]. We hypothesized that hypoxia-induced TNF-α autocrine activation may directly or indirectly up-regulate the expression of Snail, resulting in an increase in cell invasive activity. To address this hypothesis, PC3 cells were cultured in the CM with or without TNF-α antibody and analyzed for Snail protein expression. The results indicated that the CM induced the up-regulation of Snail protein level in PC3 cells. At the same time, the treatment with TNF-α alone produced a similar effect in up-regulation of Snail protein level. However, pretreatment with TNF-α antibody significantly inhibited the CM-mediated induction of Snail protein expression. These results suggest that hypoxia-induced TNF-α autocrine mechanism could stabilize the Snail protein leading to increase in the invasive activity of PC3 cells.

Given that both HIF-1α and TNF-α contributed to stabilization of the Snail protein in PC3 cells under hypoxic conditions, we investigated their synergistic effect on Snail expression. The results showed that simultaneous stimulation by HIF-1α and TNF-α further up-regulated the expression level of Snail protein, confirming HIF-1α and TNF-α synergism induction of Snail expression. Given that Snail is the critical regulator of cell invasion under hypoxic conditions, we investigated the impact of Snail knockdown on hypoxia-induced invasion of PC3 cells and found that it inhibited hypoxia-induced invasion of PC3 cells, similarly to HIF-1α and TNF-α-stimulation. In addition, the expression of Snail-regulated genes MMP-9, vimentin, and fibronectin associated with cell invasion was up-regulated by Snail inhibition.

Collectively, our data suggest that hypoxia-triggered invasion of prostate cancer cells is mediated via HIF-1α- and TNF-α-induced stabilization of Snail. Our study reveals an important mechanism underlying hypoxia-induced invasion of cancer cells, which can have significant implications in the development of treatment strategies for prostate cancer. Further investigation of this novel signaling mechanism in cancer invasion and metastasis is essential for providing a novel therapeutic target to prevent and treat prostate cancer.

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