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Sulforaphane Inhibits Nonmuscle Invasive Bladder Cancer Cells Proliferation through Suppression of HIF-1α-Mediated Glycolysis in Hypoxia

Yong Xia,[†,‡] Taek Won Kang,[‡] Young Do Jung,[*,‡] Chao Zhang,[*,§,⊥] and Sen Lian[*,§,⊥]

[†]Department of Urology, New York University School of Medicine, 423E 23ST, New York, New York 10010, United States

[‡]Research Institute of Medical Sciences, Chonnam National University Medical School, Gwangju, 501-190, Korea

[§]Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Southern Medical University, Guangzhou Guangdong, 510515, China

[⊥]Guangdong Provincial Key Laboratory of Single Cell Technology and Application, Guangzhou, Guangdong 510515, China

ABSTRACT: Bladder cancer is the fourth common cancer among men and more than 70% of the bladder cancer is nonmuscle invasive bladder cancer (NMIBC). Because of its high recurrence rate, NMIBC brings to patients physical agony and high therapy costs to the patients' family and society. It is imperative to seek a natural compound to inhibit bladder cancer cell growth and prevent bladder cancer recurrence. Cell proliferation is one of the main features of solid tumor development, and the rapid tumor cell growth usually leads to hypoxia due to the low oxygen environment. In this study we found that sulforaphane, a natural chemical which was abundant in cruciferous vegetables, could suppress bladder cancer cells proliferation in hypoxia significantly stronger than in normoxia ($p < 0.05$): 20 μM sulforaphane inhibited bladder cancer cell proliferation by 26.1 ± 4.1% in normoxia, while it inhibited cell proliferation by 39.7 ± 5.2% in hypoxia in RT112 cells. Consistently, sulforaphane inhibited cell proliferation by 29.7 ± 4.6% in normoxia, while it inhibited cell proliferation by 48.3 ± 5.2% in hypoxia in RT4 cells. Moreover, we revealed that sulforaphane decreased glycolytic metabolism in a hypoxia microenvironment by downregulating hypoxia-induced HIF-1α and blocking HIF-1α trans-localization to the nucleus in NMIBC cell lines. This study discovered a food sourced compound inhibiting bladder cancer cells proliferation and provided experimental evidence for developing a new bladder cancer preventive and therapeutic strategy.

KEYWORDS: *sulforaphane, hypoxia, HIF-1α, glycolysis, bladder cancer*

INTRODUCTION

Bladder cancer has been known as the fourth most common cancer in men and the ninth most common in women in developed countries.[1] Urologists have classified bladder cancer into two types: nonmuscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC).[2] Nonmuscle invasive bladder cancer, also named superficial bladder cancer, is found on the surface of the inside bladder. It tends to grow into the bladder lumina rather than invade into the bladder mucosa or muscle layers.[3] NMIBC is the most common type of bladder cancer, accounting for 75% of bladder cancer patients.[4] Although the mortality of NMIBC is not high, because of its high recurrence rate, it involves physical agony for patients and high therapy costs.

After surgery for the NMIBC patients, intravesical chemotherapy is usually suggested to prevent cancer from reoccurring.[5] However, because of the side effects of chemotherapy, it is imperative to find a moderate formula to prevent the bladder cancer from returning. Up to now, a variety of food-source natural chemicals' anticancer (or cancer prevention) activities have been discovered; for example, apigenin has been demonstrated to demonstrate cytotoxic activities against colorectal cancer HCT116 cells, breast cancer MCF-7 cells,[6] and play roles in the suppression of bladder cancer T24 cells invasion.[7] Besides, many other food-source natural chemicals also have been studied for anticancer purpose, such as curcumin from turmeric, crocetin from saffron, cyanidins from grapes, epigallocatechin gallate from green tea, genistein from soybean, gingerol from gingers, phenethyl isothiocyanate (PEITC) from cruciferous vegetable and sulforaphane from cruciferous vegetables.[8]

Sulforaphane, 1-isothiocyanato-4-(methylsulfinyl) butane, is a natural compound that includes the isothiocyanate group of organosulfur compounds.[9] Its molecular structural formula is shown in Figure 1A. Sulforaphane is one of the major phytochemicals found in these cruciferous vegetables such as broccoli, cauliflower, brussel sprouts, cabbage, kale, kohlrabi, and so on (Figure 1B). The health benefits of sulforaphane have been known in various aspects: prevention of cardiovascular disease via up-regulation of Nrf2;[10] anti-inflammation function due to suppression of transcription factor NF-κB;[11] prevention from memory dysfunction and improvement of cognitive function.[12] Recently, the anticancer function of sulforaphane has attracted much attention: sulforaphane is able to inhibit breast cancer stem cells via down-regulation of the Wnt/β-catenin self-renewal pathway in the xenograft mice model;[13] sulforaphane shows a chemo-

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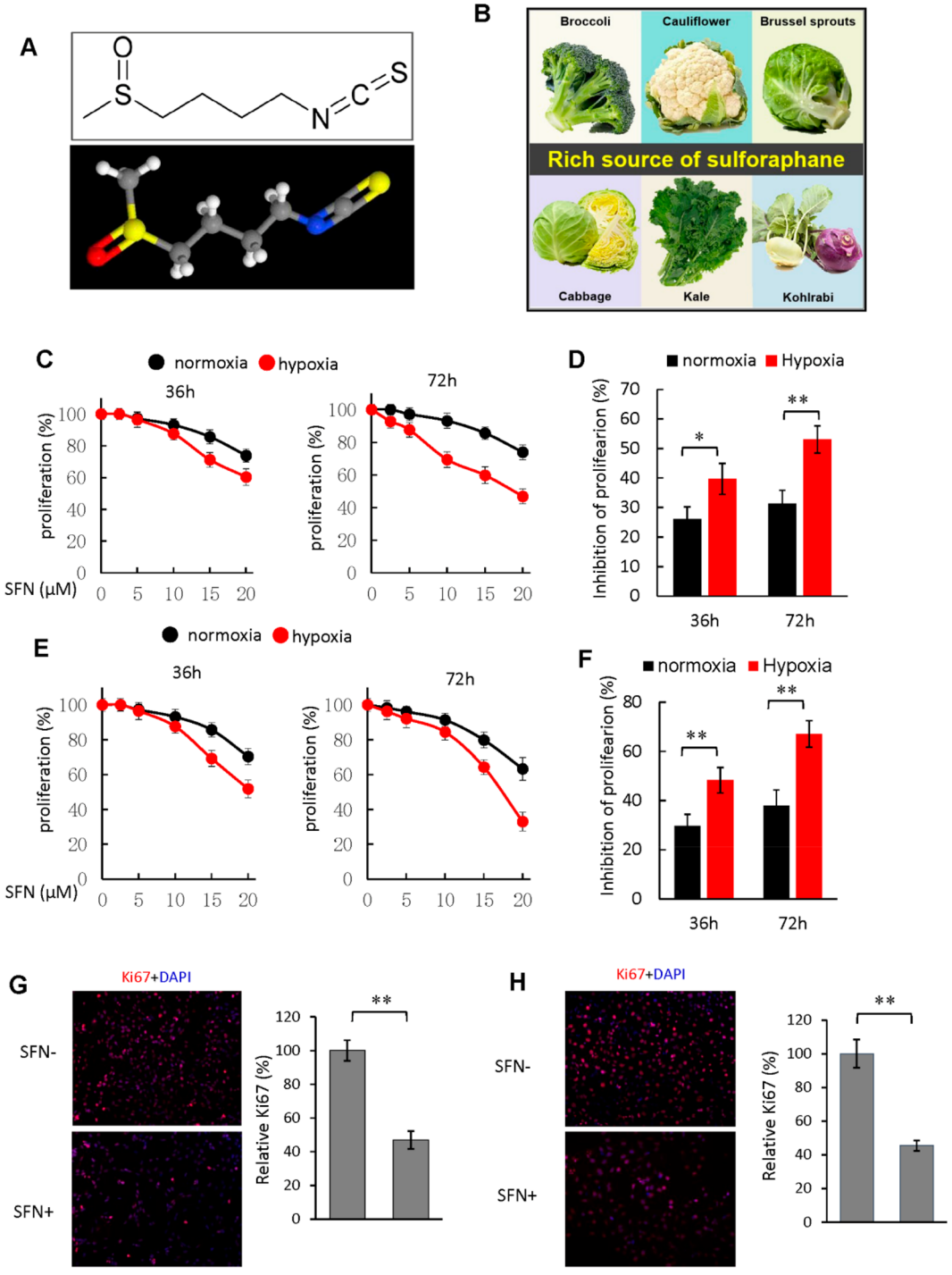

Figure 1. Sulforaphane (SFN) inhibits bladder cancer cells proliferation under hypoxic conditions. (A) SFN formula (top) and structure (bottom); (B) the natural sources of SFN; (C) SFN inhibits RT112 cells proliferation in 36 and 72 h; (D) comparison of inhibition of proliferation between hypoxia and normoxia in RT112 cells, * $p < 0.05$, ** $p < 0.01$; (E) SFN inhibits RT4 cells proliferation; (F) comparison of inhibition of proliferation between hypoxia and normoxia in RT4 cells in 36 and 72 h, ** $p < 0.01$; (G) Ki67 immunofluorescence staining in RT112 cells under hypoxic conditions, ** $p < 0.01$; (H) Ki67 immunofluorescence staining in RT4 cells under hypoxic conditions, ** $p < 0.01$.

preventive effect in prostate cancer cells through up-regulation of Nrf2 expression via mediation of Nrf2's CpGs demethylation.[14] However, as for the anticancer function of sulforaphane against bladder cancer, especially nonmuscle invasive bladder cancer, the investigation is not deep enough. In our present study, we discovered sulforaphane inhibited bladder cancer cells growth via a repression of glycolysis in a hypoxia microenvironment by decreasing hypoxia-induced HIF-1α and blocking HIF-1α localization through a potential mechanism of attenuating hypoxia-induced PHD inactivation in NMIBC cell lines.

MATERIALS AND METHODS

Chemicals. Sulforaphane (SFN, purity ⩾98%, by UPLC) was purchased from Millipore, Burlington, MA, USA. Sulforaphane was dissolved into dimethyl sulfoxide (DMSO). DMSO was obtained

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from Sigma-Aldrich, Saint Louis, MO, USA. Polybrene and DAPI were obtained from Thermo Fisher Scientific, Waltham, MA, USA.

Clinical Data Analysis and Specimen Preparation. The expression level of HIF-1α in normal urothelium (n = 4) and nonmuscle invasive bladder cancer (n = 22) has been collected from Modlich's bladder data set[15] via the Oncomine platform (www.oncomine.org). The unidentified clinical specimens were collected from Chonnam National University Hwasun Hospital under the institutional review board (IRB) approval (CNUH-06-070).

Cells and Culture Conditions. The human bladder cancer cell lines RT112 cells and RT4 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The RT112 and RT4 cells were cultured in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Gibco) at 37 °C with 95% air and 5% CO_2 for the normoxic cell culture, and with 2% O_2, 5% CO_2, and 93% N_2 for the hypoxic cell culture. The embryonic kidney HEK-293T cells were from ATCC, and cultured in DMEM containing 10% FBS at 37 °C with 5% CO_2.

Proliferation Assay. RT112 or RT4 cells (5×10^3) were seeded in a 96-well flat bottom cell culture plate (Corning, Kennebunk, ME, USA) in DMEM medium containing 10% FBS with different concentrations of sulforaphane (Millipore) under normoxic or hypoxic (2% oxygen) conditions. After 36 or 72 h, cell proliferation was tested with WST-1 reagent (Abcam, Cambridge, MA): 10 μL of WST-1 solution was added to each well and incubated at 37 °C for 40 min. Then, the absorbance of 450 nm was read in a 96-well plate reader.

Immunocytofluorescense Staining. Cultured cells were fixed on slides with 4% paraformaldehyde (PFA) and permeated with 0.25% Triton X-100 in PBS for 20 min. The nonspecific antibody-binding sites were blocked with 1% BSA in PBS for 1 h. The primary antibodies rabbit anti-Ki67 (1:250, Abcam) or rabbit anti-HIF-1α (1:200, Abcam) were added to stain the specific site in the fixed cells. After overnight incubation (4 °C), the secondary antibody (1:500, Alexa Fluor 594, Invitrogen, USA) was employed to test the specific primary antibody, and 1 μg/mL DAPI was used to stain the cell nucleus (10 min, dark). After washing three times, the immunofluorescence antibody stained cells were mounted with aqua-mount and were imaged with a fluorescence phase contrast microscope. The quantitative analysis was then performed in the ImageJ software (version 1.50i).

Measurement of HRE Luciferase Activity. The Hypoxia-Response Element (HRE) reporter activity was tested by transient transfection of an HRE-luciferase reporter construct (Qiagen, Germantown, MD, USA) using a HRE Reporter Assay Kit (Qiagen). When the RT112 or RT4 cells grew to 55−60% confluence, the HRE reporter plasmids were transfected into the cells with lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After the cells were transfected for 12 h, the medium was changed with DMEM with 10% FBS. Ten hours later, the cells were cultured in a hypoxia incubator.

Measurement of Glycolysis. Agilent Seahorse technology provides a window into the study of the Warburg effect and other factors which could drive tumorigenesis or tumor growth. We employed the Agilent Seahorse assay system to test the cancer cell glycolysis metabolism. Bladder cancer RT112 and RT4 cells were cultured onto XF24 cell culture microplates (Seahorse Bioscience, Santa Clara, CA, USA) at 3×10^4 cells/well. When the confluence reached 60%, the cells were cocultured with SFN or same volume of DMSO in normoxia or hypoxia for 12 h. Before testing, the culture media were replaced with 500 μL of XF Base DMEM medium (Agilent, Santa Clara, CA, USA) containing 5 mM HEPES (Agilent), 1 mM sodium pyruvate (Sigma), and 2 mM Glutamine (Sigma). The extracellular acidification rate (ECAR) assays were initiated by successively adding glucose, a mixture of rotenone and antimycin (Rot/AA), and 2-deoxy-glucose (2-DG) in XFe24 an extra-cellular flux analyzer (Agilent) following the manufacturer's instructions. The working concentration of glucose, Rot/AA, and 2-DG is 10 mM, 0.5 μM, and 50 mM, respectively. The ECAR data were automatically recorded via wave software (Agilent). After the ECAR test, the cell number of each well was recorded and the ECAR values were normalized by the control group.

Western Blotting. Cells harvested from culture plates were lysed by mammalian whole cell lysis reagent (Thermo Scientific, Rockford, IL, USA) containing 1× protease and phosphatase inhibitors (Thermo Scientific). Then a Pierce BCA Protein Assay Kit (Thermo Scientific) was employed to determine the protein concentration. Thirty micrograms of proteins for each sample was loaded into each lane of SDS-PAGE gel. After running the gel, proteins were electrically transferred from the gel onto a PVDF membrane. BSA in TBST (5%) was used to block the nonspecific antibody binding sites. Then the membrane was incubated with the following: First, antibodies at 4 °C overnight were added, which were rabbit anti-HIF-1α (1:1000, Abcam), rabbit anti-MEK1/2 (1:1000, Cell Signaling Technology), rabbit anti-Hydroxy-HIF-1α (Pro564) (1:1000, Cell Signaling Technology), rabbit anti-Histone H3 (1:2000, Cell Signaling Technology) and mouse anti-β-Actin (1:5000, Sigma). Then, secondary antibodies, which were goat antirabbit IgG antibody (1:5000, Cell Signaling Technology) or horse antimouse IgG antibody (1:5000, Cell Signaling Technology) were used to incubate the membrane. The final development reaction was initiated by ECL solution (Thermo Fisher Scientific) and the images were recorded with an image forming system.

HIF-1α Lentivirus shRNA Infection. The lentivirus expressing 29-mer against HIF-1α or scramble 29-mer was packaged by cotransfection of plasmid lenti-shHIF-1α (Origene, Rockville, MD, USA), plasmid psPAX2 and the pMD2.G plasmid containing VSV-G into HEK-293T cells using lipofectamine 2000 (Invitrogen) according to the manufacture's instruction. After infection of 12 h, the media was changed using DMEM containing 10% FBS, and the cell culture supernatant was collected after 48 h. When RT112 and RT4 cells grew to 55−60% confluence, cells were infected by coincubation with a cell culture supernatant containing packaged lentivirus and Polybrene at a concentration of 8 μg/mL. The stable transfected cells were selected with 0.5 μg/mL puromycin (Gibco).

Reverse Transcriptional PCR and qPCR. The total RNA was extracted from RT112 and RT4 cells using TRIzol reagent (Invitrogen). The complementary DNA (cDNA) was synthesized by M-MLV transcriptase (Promega) with random primers. Then, the real-time PCR was performed using HIF-1α and GAPDH specific primers in a Fast SYBR Green master mix solution (Applied Biosystems, Foster City, CA, USA). Sequences of primers were as follows: HIF-1α forward primer 5′-GGA TGA TGA CTT CCA GTT ACG-3′ and reverse primer 5′-CTG TGC TTT GAG GAC TTG C-3′; GAPDH forward primer 5′-AAC TTT GGT ATC GTG GAA GGA C-3′ and reverse primer 5′-GGG ATG ATG TTC TGG AGA GC-3′.

Cell Fractionation. To investigate HIF-1α in cytoplasm and nucleus separately, cell fractionation was performed with a cell fractionation kit (Cell Signaling Technology) according to the manufacture's instruction. The fraction of cytoplasm and nucleus was boiled with 3× SDS-loading buffer, and then Western blotting was employed to examine the HIF-1α protein in cytoplasm and nucleus, respectively.

Statistics. The column data which are shown as ±SD, represent the mean of at least three experiments. Differences between every two data sets were determined by t tests, and differences among more than two data sets were determined by the one-way ANOVA test with SPSS 17.0 software (IBM, USA). Differences were considered as significant when $p < 0.05$.

RESULTS AND DISCUSSION

Sulforaphane Inhibits Bladder Cancer Cells Proliferation in Hypoxic Condition. As shown in Figure 1C, sulforaphane inhibited the NMIBC cell line RT112 cells proliferation in a dose-dependent manner in both 36 h treatment and 72 h treatment. It was worth noting that sulforaphane showed more inhibitory effect on RT112 cells in hypoxia than in normoxia (Figure1D). Interestingly, sulfor-

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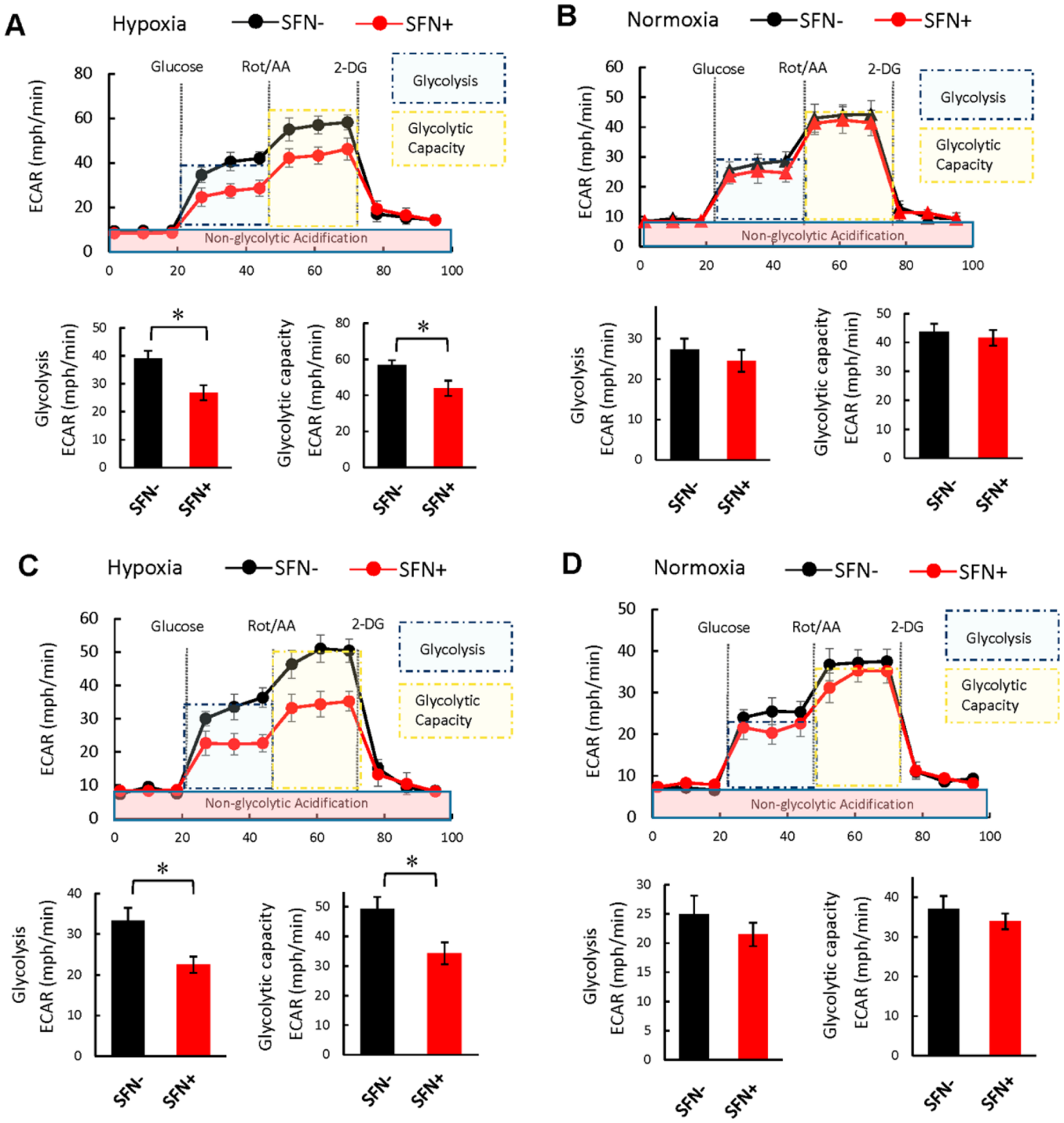

Figure 2. SFN inhibits glycolysis more effectively in hypoxia than normaxia. (A) Glycolysis assay under hypoxia conditions with (red) or without (black) SFN in RT112 cells, ** $p < 0.01$. (B) Glycolysis assay under normoxia conditions with (red) or without (black) SFN in RT112 cells. (C) Glycolysis assay under hypoxia conditions with (red) or without (black) SFN in RT4 cells, ** $p < 0.01$. (D) Glycolysis assay under normoxia conditions with (red) or without (black) SFN in RT4 cells.

aphane also dose-dependently inhibited cell proliferation in another NMIBC cell line RT4 cells (Figure1E) and showed a stronger inhibitory effect on RT4 cells in hypoxia than in normoxia (Figure1.F). To verify sulforaphane's antiproliferation function in hypoxia, immuno-fluorescence staining was performed. As shown in Figure 1G, the cell number and fluorescence intensity of Ki67 positively stained cells with sulforaphane treatment sample were lower than the sample without sulforaphane treatment, which indicated that sulforaphane remarkably decreased the proliferation in RT112 cells under hypoxia conditions. Consistently, in RT4 cells, sulforaphane also suppressed cell proliferation by reduction of the Ki67 level under hypoxia conditions (Figure 1H). Although other scientists discovered that sulforaphane inhibited the growth of human bladder cancer cells via arresting the cell cycle in the G2/M phase,[16] in this study, we are the first to compare the inhibitory effect of sulforaphane against cell proliferation between hypoxia and normoxia. To

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Table 1. Inhibition (Percentage) of the Proliferation of Bladder Cancer Cells by SFN

cell line	RT112				RT4			
treatment time	36 h		72 h		36 h		72 h	
[SFN] (μM)	N	H	N	H	N	H	N	H
0	0 ± 3.4	0 ± 2.5	0 ± 2.9	0 ± 3.1	0 ± 3.7	0 ± 2.5	0 ± 3.7	0 ± 3.5
2.5	0.8 ± 2.6	1.3 ± 3.6	1.1 ± 3.3	7.2 ± 4.2	0.3 ± 2.6	0.6 ± 3.6	1.7 ± 4.1	3.8 ± 4.7
5	1.7 ± 3.9	3.1 ± 4.9	3.8 ± 4.0	12.4 ± 4.5	2.8 ± 3.2	3.5 ± 3.9	3.9 ± 3.9	7.9 ± 5.1
10	6.7 ± 3.8	11.5 ± 3.9	9.9 ± 4.6	30.6 ± 4.8	6.9 ± 4.3	12.4 ± 3.9	8.7 ± 3.8	15.6 ± 4.5
15	14.3 ± 4.5	28.6 ± 4.6	19.6 ± 3.8	40.2 ± 5.1	15.4 ± 4.0	30.8 ± 4.6	20.2 ± 4.5	35.8 ± 4.3
20	26.1 ± 4.1	39.7 ± 5.2	31.3 ± 4.5	53.1 ± 4.6	29.7 ± 4.6	48.3 ± 5.2	36.8 ± 6.5	67.1 ± 5.4

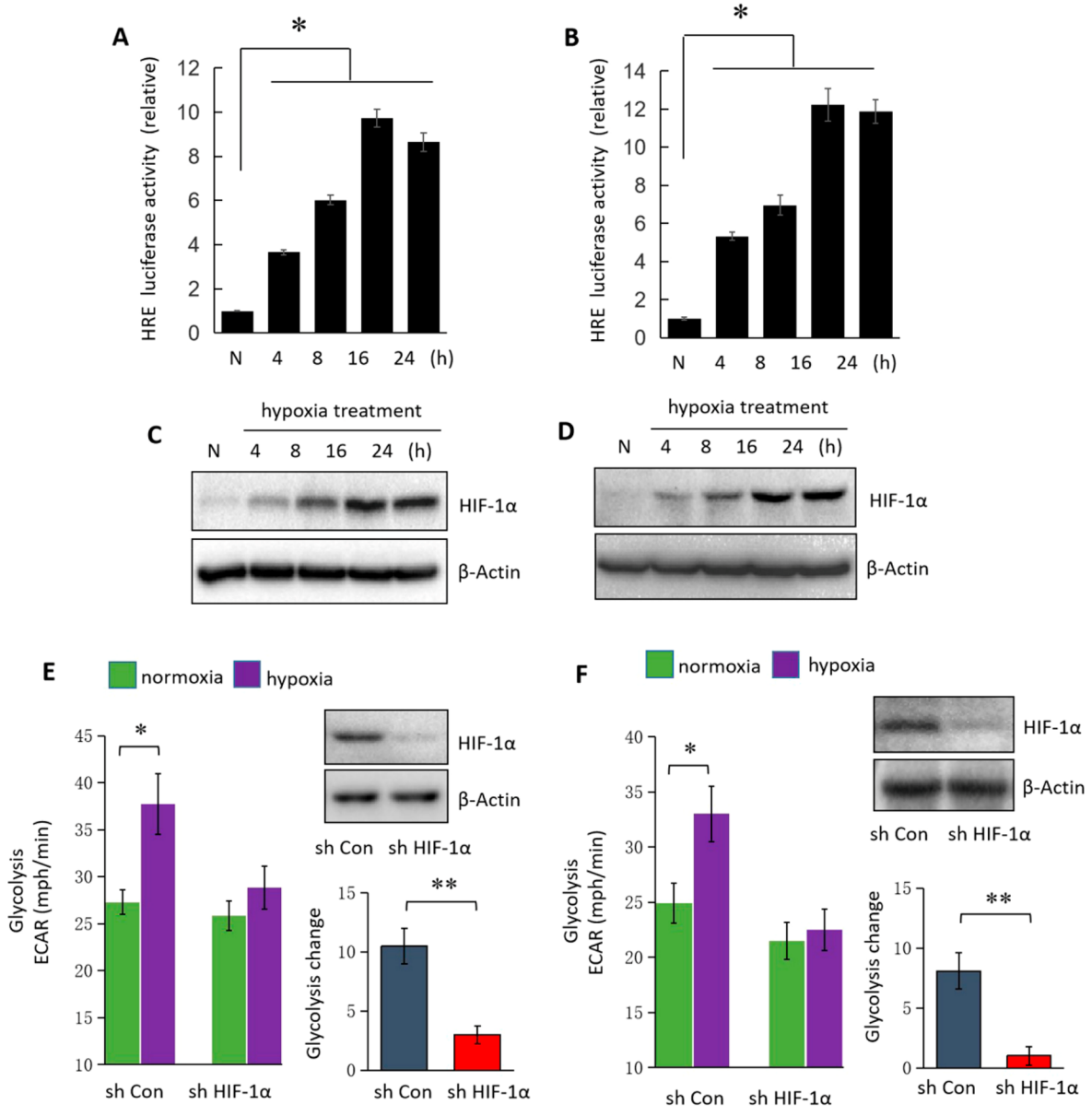

Figure 3. Hypoxia up-regulated glycolysis via stimulation of HIF-1α expression. (A) HRE-luciferase under hypoxia during 4−24 h in RT112, * $p <$ 0.05. (B) HRE-luciferase under hypoxia during 4−24 h in RT4, * $p < 0.05$. (C) HIF-1α expression under hypoxia during 4−24 h in RT112. (D) HIF-1α expression under hypoxia during 4−24 h in RT4. (E) Change of glycolysis between normoxia and hypoxia in wild type RT112 cells and HIF-1α knockdown RT112 cells, * $p < 0.05$, ** $p < 0.01$. (F) Change of glycolysis between normoxia and hypoxia in wild type RT4 cells and HIF-1α knockdown RT4 cells, * $p < 0.05$, ** $p < 0.01$.

reveal the mechanism of how sulforaphane inhibited the proliferation of bladder cancer cells, we investigated the effects of sulforaphane on the metabolism of bladder cancer cells.

Sulforaphane Inhibits Bladder Cancer Cells Glycolysis under Hypoxic Conditions. Warburg[17] discovered that many tumors tended to produce excess lactic acid even in the

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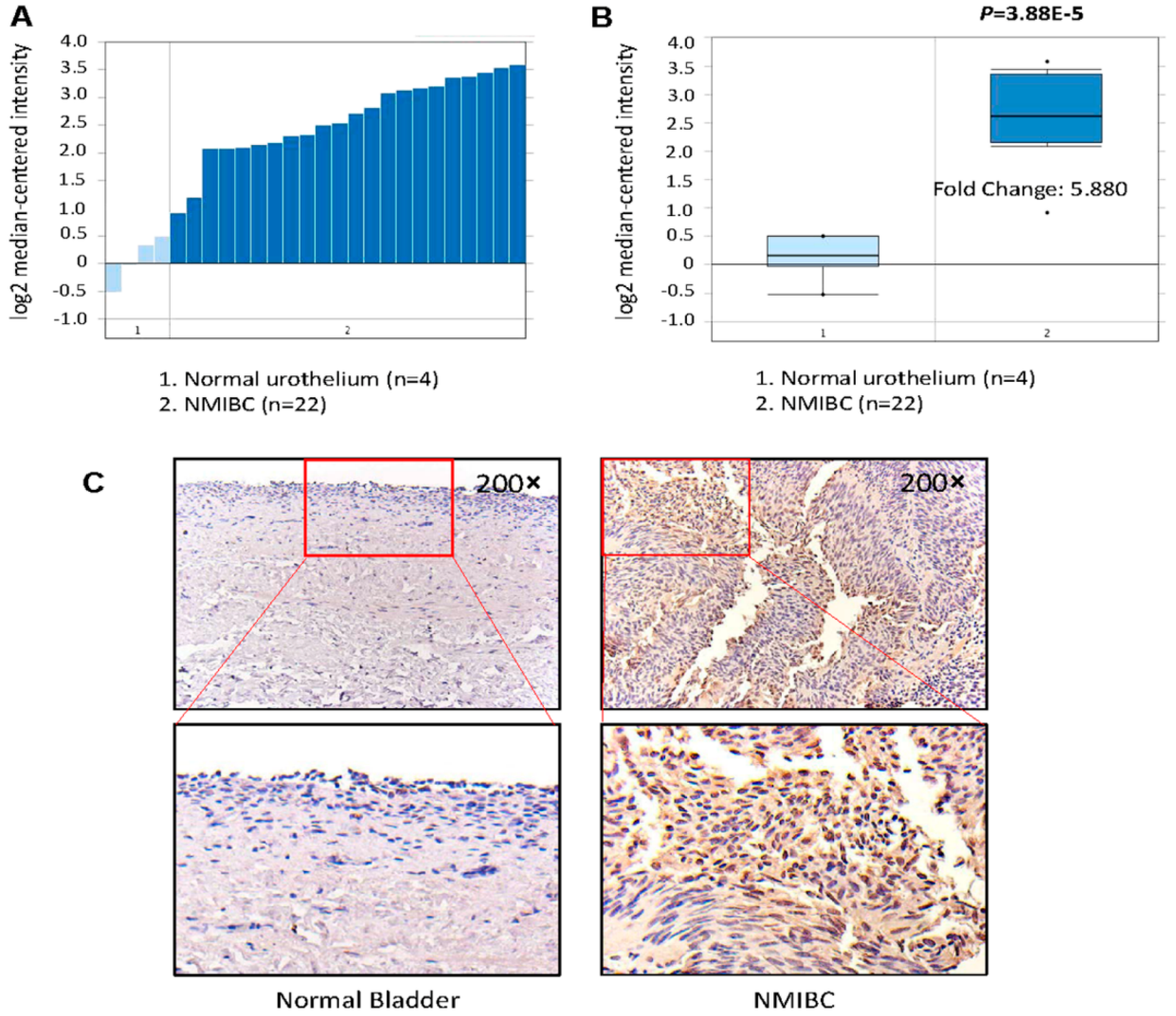

Figure 4. HIF-1α is highly expressed in nonmuscle invasive bladder cancer: (A) expression level of HIF-1α from clinical sample database; (B) box figure (data are from panel A); (C) immunohistochemistry staining for HIF-1α in normal bladder (left) and nonmuscle invasive bladder cancer (right) specimen.

presence of oxygen and established a correlation between the growth of cancer cells and glycolysis for the first time.[18] Raghunand et al. stated that the high glycolysis phenotype in cancer cells might be selected in periods of cyclic hypoxia in the stage of cancer cells early growth.[19] Moreover, other studies also found that hypoxia could elevate glycolysis,[20,21] so we raised the question could sulforaphane suppress hypoxia-induced glycolysis in bladder cancer cells. As shown in Figure 2, the rectangle with light green represented glycolysis, and the rectangle with light yellow represented glycolytic capacity (glycolytic capacity represents the maximum ability to produce ATP when all the other energy supply pathways are blocked). The ECAR curves shown in Figure 2A represented the RT112 cells treated with sulforaphane (red spots and curve) and without sulforaphane (black spots and curve) under hypoxic conditions. The inserted figures below Figure 2A, showed that sulforaphane decreased both glycolysis and glycolytic capacity of RT112 cells under hypoxia conditions. However, Figure 2B showed that there is no significant difference of glycolysis and glycolytic capacities in RT112 between the sulforaphane treatment group and the control group under normoxic conditions. In RT4 cells, we also found sulforaphane suppressed glycolysis and glycolytic capacity in hypoxia, but not in normoxia (Figure 2C,D). The above data indicated that sulforaphane might suppress hypoxia-elevated glycolysis rather than intrinsical glycolysis. Consistent with our findings, it has been reported that sulforaphane is able to decrease glucose metabolism in turn-delayed senescence of fibroblasts,[22] and our present study is the first to report sulforaphane can reduce hypoxia-induced glycolysis in nonmuscle invasive bladder cancer cells (Table 1). The next interesting question is how sulforaphane suppresses hypoxia up-regulated glycolysis. Can sulforaphane inhibit hypoxia-induced molecules which play an essential role in hypoxia-induced glycolysis?

Hypoxia Induces HIF-1α Expression in RT112 and RT4. Hypoxia response elements (HRE) are DNA regions containing gene promoter sequences which are able to bind specific transcription factors and trigger or enhance transcription of genes under hypoxic conditions. As shown in Figure 3A and 3B, hypoxia-induced HRE luciferase activity in a time-dependent manner in 4−24 h in both RT112 and RT4 cells. The Western blotting results showed that hypoxia increased the HIF-1α protein level in a time-dependent manners in RT112 and RT4 cells (Figure 3C,D). To

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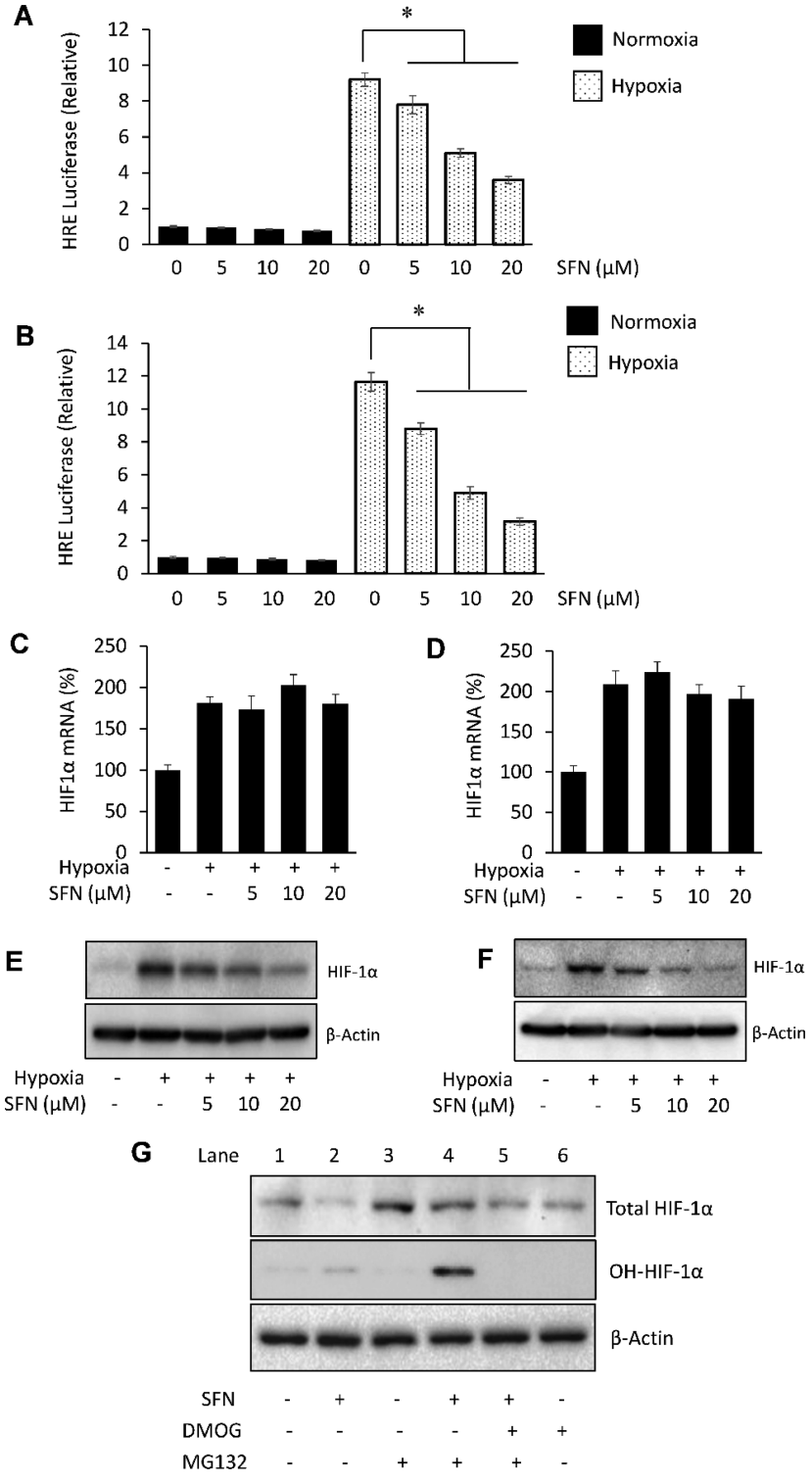

Figure 5. SFN inhibits hypoxia-induced HRE luciferase via decreasing HIF-1α protein level. (A) HRE luciferase under nomoxic and hypoxic conditions with or without SFN treatment in RT112 cells, * $p < 0.05$. (B) HRE luciferase under nomoxic and hypoxic conditions with or without SFN treatment in RT4 cells, * $p < 0.05$. (C) HIF1α mRNA under nomoxic and hypoxic conditions with or without SFN treatment in RT112 cells. (D) HIF-1α mRNA under nomoxic and hypoxic conditions with or without SFN treatment in RT4 cells. (E) HIF-1α protein under nomoxic and hypoxic conditions with or without SFN treatment in RT112 cells. (F) HIF-1α protein under nomoxic and hypoxic conditions with or without SFN treatment in RT4 cells. (G) Total HIF-1α and hydroxylated HIF-1α protein in hypoxia with or without SFN, DMOG, and MG132 treatments in RT4 cells.

investigate the role of HIF-1α under hypoxia-induced glycolysis, we knocked down the *HIF1A*, and compared the glycolysis between *HIF1A* knockdown cells and the control in normoxia and hypoxia. As shown in Figure 3E,F, after *HIF1A* was knocked down, less glycolysis was induced by hypoxia, which indicated HIF-1α might be indispensable for hypoxia-elevated glycolysis. One reason why HIF-1α plays an important role in the regulation of glycolysis might be that

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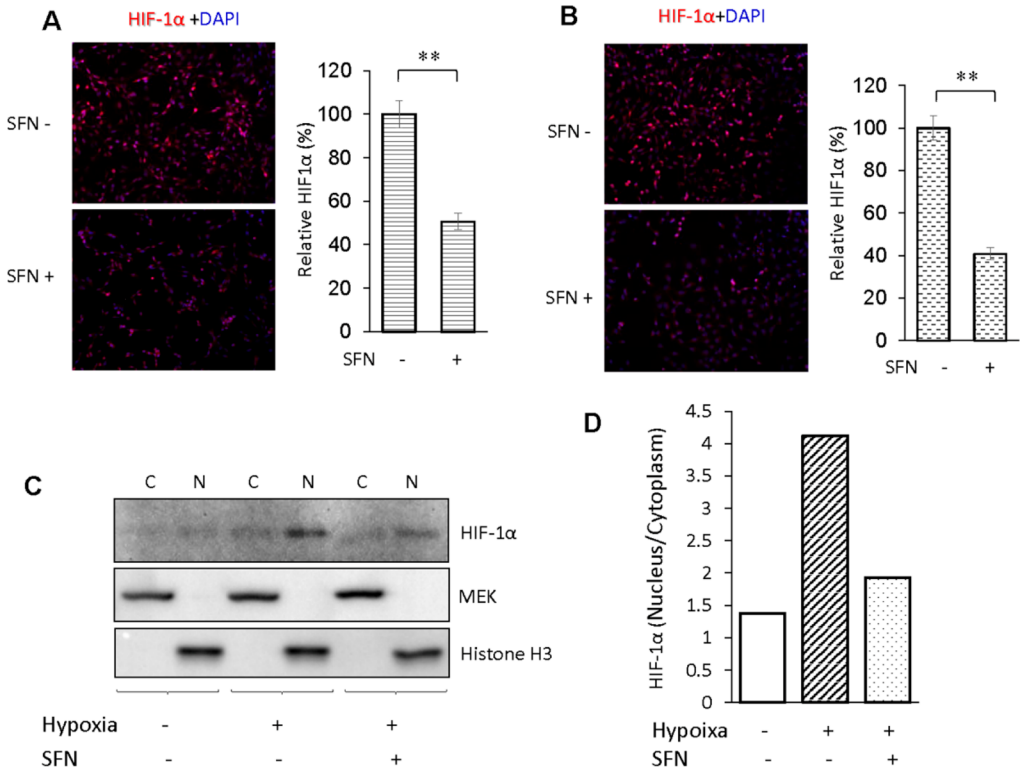

Figure 6. SFN inhibits hypoxia-induced HIF-1α translocation to nucleus: (A) HIF-1α immunofluorescence staining in RT112 cells under hypoxic conditions with or without SFN treatment, ** $p < 0.01$; (B) HIF-1α immunofluorescence staining in RT4 cells under hypoxic conditions with or without SFN treatment, ** $p < 0.01$; (C) HIF-1α in cytoplasm and nucleus fraction in hypoxia with or without SFN treatment in RT4 cells; (D) ratio between HIF-1α in nucleus and HIF-1α in cytoplasm (data are from panel C).

HIF-1α can drive the higher expression of many glycolytic enzymes, such as enolase, pyruvate kinase, and so on.[23,24] Consistent to our discovery, other scientists also found the similar phenomenon that HIF-1α elevated macrophage glycolysis in immunologic responses.[25]

HIF-1α Is Highly Expressed in Nonmuscle Invasive Bladder Cancer. Tumor hypoxia is the microenvironment in which tumor cells are in relatively low oxygen. When a solid tumor proliferates quickly, it promptly depletes the blood supply, leading to a shortage of oxygen in some tumor regions.[26,27] It is discovered that, in order to sustain the continuous high speed proliferation in such challenging hypoxic environments, tumor cells actively change their metabolism.[28] HIF-1, hypoxia-inducible factor 1, is one of the important molecules that are up-regulated by cells to adapt the hypoxic environment.[29] HIF-1α, the alpha subunit of HIF-1, is known to induce more than 60 genes transcription assisting in promoting and increasing oxygen delivery to hypoxic regions. It also up-regulates genes involved in cell survival and proliferation as well as glucose metabolism.[30] Using the oncomine platform, we analyzed the HIF-1α expression level in normal bladder urothelium and nonmuscle invasive bladder cancer. Through RNA-seq data,[15] we found the HIF-1α in nonmuscle invasive bladder cancer is significantly higher than normal bladder urothelium (Figure 4A,B). Moreover though in-house immunohistochemical staining, we also found that the expression of HIF-1α in nonmuscle invasive bladder cancer was higher than that in normal bladder (Figure 4C). All the results in Figure 3 and Figure 4 indicate that HIF-1α is the key molecule induced by hypoxia, playing a crucial role in helping cells adapt to the hypoxic environment and promoting cell survival and growth. Next, we examined whether sulforaphane was able to inhibit hypoxia-elevated HIF-1α.

Sulforaphane Inhibits Hypoxia Activated HRE via Decreasing HIF-1α Protein. The effects of sulforaphane on hypoxia-induced HRE were studied by monitoring the HRE luciferase activity. As shown in Figure 5A,B, sulforaphane obviously suppressed hypoxia-induced HRE activation in a dose-dependent manner in RT112 and RT4 cells. To further determine the mechanism of how sulforaphane attenuated the hypoxia-stimulated HRE activity, mRNA of HIF-1α was detected. As the RT-qPCR results in Figure 5C,D showed, sulforaphane did not affect hypoxia-induced HIF-1α mRNA upregulation. However, the Western blotting results in Figure 5E,F showed that sulforaphane was able to reduce HIF-1α at the protein level in a dose-dependent manner. The above results indicated that sulforaphane decreased the HIF-1α protein level without affecting its transcription in hypoxia.

Generally, HIF-1α protein levels are low in normoxia, due to the oxygen-sensitive prolyl hydroxylases (PHDs) triggered HIF-1α hydroxylation and ubiquitination-dependent degradation via the Von Hippel–Landau (VHL) protein.[31,32] In hypoxia, the PHDs cannot hydroxylate HIF-1α efficiently so that the HIF-1α protein level increases in hypoxia because VHL is not able to degrade the HIF-1α without hydroxylation.[30] In other words, hypoxia leads to an accumulation of HIF-1α via inactivation of PHDs.[33] To investigate the mechanism of how sulforaphane decreased the protein level of HIF-1α, we employed DMOG (dimethyloxalylglycine) with the concentration of 1 mM to inhibit HIF-1α hydroxylation and used MG132 with the concentration of 10 μM to suppress

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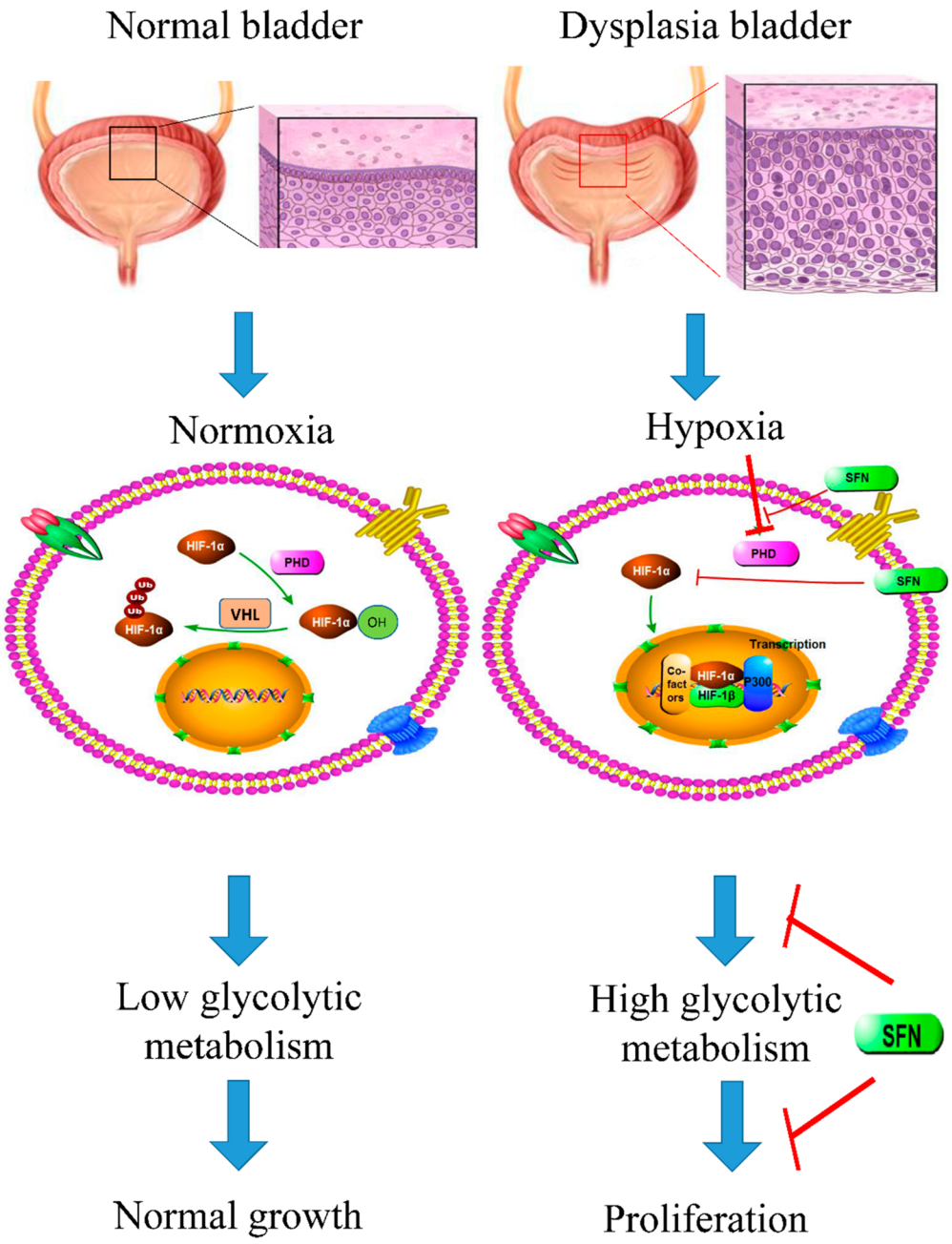

Figure 7. Mechanism diagram of SFN inhibiting cell proliferation under hypoxic conditions.

ubiquitination-dependent degradation of HIF-1α. As shown in Figure 5G (lane 2 vs lane 1), in hypoxia, without DMGO nor MG132, sulforaphane decreased the total HIF-1α protein; whereas when the cells were treated with MG132, the hydroxylated HIF-1α was higher in the sulforaphane treatment group (lane 4 vs lane 3), which indicated that sulforaphane might be able to attenuate hypoxia-induced PHD inactivation. Generally, in normoxia HIF-1α is at a low level and mostly maintained in the cytoplasm, but hypoxia can rapidly increase the amounts of HIF-1α and promote HIF-1α to enter into nucleus and bind to the core DNA with the sequence of 5′-TACGTG-3′ within the hypoxia response element (HRE) and in turn promote target gene expression.[19,34] In a further step, we examined whether sulforaphane affected HIF-1α subcellular translocation in hypoxia.

Sulforaphane Inhibits Hypoxia-Induced HIF-1α Translocation. The immunocytofluorescense staining results in Figure 6A,B showed that sulforaphane was able to reduce HIF-1α protein in hypoxia in RT112 and RT4 cells, which was consistent with the result shown in Figure 5. To investigate the effect of sulforaphane on HIF-1α nuclear translocation, cell fractionation and Western blotting was performed in RT4 cells. As shown in Figure 6C, under normoxic conditions, HIF-1α protein was in low levels in both cytoplasm and nucleus. However, under hypoxic conditions, the HIF-1α protein level greatly increased, especially in the nucleus. In contrast, with 10 μM sulforaphane treatment, the HIF-1α protein level in the nucleus and cytoplasm is much lower than that without sulforaphane treatment in hypoxia. Interestingly, sulforaphane notably decreased the ratio of nucleus HIF-1α/cytoplasm HIF-1α which was obviously elevated by hypoxia (Figure 6D). The

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above results indicated that hypoxia-induced HIF-1α translocation was inhibited by sulforaphane in nonmuscle invasive bladder cancer cells.

This study is of great significance because (1) the bladder is a special organ in which the chemical infused fluids are easy to access. Also, (2) animal preclinical tests have shown that sulforaphane can be absorbed by various tissues and organs including the bladder, implying the potential benefits of sulforaphane for bladder treatment.[16,35] Finally, (3) sulforaphane can be metabolized via the mercapturic acid pathway to form *N*-acetylcysteine- cysteine- and cysteinylglycine- conjugates which are excreted into urine.[36] In conclusion, it is reported for the first time that sulforaphane is able to inhibit the proliferation of nonmuscle invasive bladder cancer cells through suppression of glycolysis via reduction of the HIF-1α protein level and decrease of HIF-1α nuclear translocation (Figure 7). This study furnishes a novel insight into how sulforaphane inhibits bladder cancer cells proliferation, and provides experimental evidence for developing a new bladder cancer preventive and therapeutic strategy.

AUTHOR INFORMATION

Corresponding Authors

*Tel.: (+86) 20- 62789385. Fax: (+86) 20- 62789385. E-mail: senlian@i.smu.edu.cn.

*Tel.: (+86) 20- 62789385. Fax: (+86) 20- 62789385. E-mail: zhangchao1558@126.com.

*Tel.: (+82)-61-3792772. Fax: (+82)-61-3792781. E-mail: ydjung@chonnam.ac.kr.

ORCID

Sen Lian: 0000-0002-7295-9216

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Notes

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