KLF5 promotes hypoxia-induced survival and inhibits apoptosis in non-small cell lung cancer cells via HIF-1α

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Abstract. Transcription factor Krüppel-like factors 5 (KLF5) is overexpressed in a wide range of tumor tissues and acts as a prognostic factor in cancer. However, the role of KLF5 in non-small cell lung cancer is not clear. Hypoxia plays a vital part in the development of cancer via hypoxia-inducible factor 1 (HIF-1). Our study showed that hypoxia (1% O₂) increased cell viability, clonality and proliferation and inhibited cell apoptosis in A549 cells. The expression of HIF-1α and KLF5 was increased time-dependently in hypoxia. Using small interfering RNA (siRNA) targeting KLF5 or HIF-1α, we demonstrated that KLF5 or HIF-1α knockdown inhibited hypoxia-induced cell survival and promoted cell apoptosis by actively downregulating cyclin B1, survivin and upregulating caspase-3. Given the similar effect of KLF5 and HIF-1α on cell survival, an attempt was made to investigate the putative interaction of them in hypoxia. KLF5 was revealed to co-immunoprecipitate with HIF-1α and hypoxia increased the amount of KLF5 and HIF-1α complex. Moreover, silencing of KLF5 decreased HIF-1α expression while KLF5 was not affected by HIF-1α inhibition in hypoxia, confirming the effect of KLF5 on upregulation of HIF-1α. In conclusion, this study identified hypoxia as a tumor promoter by triggering KLF5 → HIF-1α → cyclin B1/survivin/caspase-3 in lung cancer cells.

Introduction

Lung cancer is the most common cancer and responsible for 19.4% of cancer death with 87% mortality rate worldwide. A third of patients with lung cancer are Chinese and the fatality is as high as 91.3% in China (1). Deregulated cell proliferation and failure of cell apoptosis are critical events to propel neoplastic progression (2). Besides the internal straightforward linear accumulation of oncogenic mutations, potential proliferation and apoptosis-inhibitory signals responding to external pressure account for evolution of tumor. Hypoxia is a characteristic driver of solid carcinoma. During the process of tumor formation and rapid growth, oxygen diffusion limitation and poor blood perfusion lead to hypoxia (3). To adapt to low oxygen level, anomalous proliferation and reduced apoptosis which result in lower supply of oxygen have been developed in hypoxic tumors (4). The vicious circle of tumor progression has long been a major focus for cancer treatment. However, previous studies showed an inconsistent role of hypoxia in A549 cells (5,6).

Hypoxia-inducible factor 1 (HIF-1) α plays a central role in this process as a master regulator of oxygen homeostasis. Clinical data indicate that HIF-1α expression is either positively associated with tumor stage in brain tumor (7) or correlates with longer median survival time among patients with non-small cell lung cancer (8). Consistently, in vitro studies present evidence that HIF-1α has a contradictory effect on cell proliferation and apoptosis in lung cancer A549 cells (9,10). It is proposed that HIF-1α level is associated with tissue- and cell-special response to hypoxia (11). Von Hippel-Lindau tumor suppressor protein (pVHL), which is often defective or inactive in cancer, mediated the ubiquitination and degradation of HIF-1α in normoxia (12). In hypoxia, P53 is revealed to have directly negative effect on the stability of HIF-1α or indirectly negative impact via pVHL (13). A recent study showed that P53 regulates LPA-stimulated HIF-1α expression through binding to the promoter of HIF-1α competed with Krüppel-like factors 5 (KLF5) in colon cancer cells (14). Since hypoxia exerts synergistic effect with LPA on HIF-1α expression and cell survival (15), KLF5 may be potentially involved in the regulation of HIF-1α following hypoxia. KLF5 as a zinc finger transcription factor, is known to be regulated by hypoxia in tumors (16). The overexpression of KLF5 is reported to be associated with better patient survival in non-small lung cancer (17) or shorter overall survival in breast cancer (18). In view of this fact, it is likely that the conflicting response to hypoxia of HIF-1α and KLF5 may rely on the types and stages of tumors.

In this study, we assessed the effect of HIF-1α and KLF5 on cell survival in A549 cells exposed to hypoxia, and a
hypothesis was made that there may be interaction between HIF-1α and KLF5 involved in the adaptation to hypoxia. We further explored the function regulation of HIF-1α by KLF5 to advance knowledge on cancer remission.

Materials and methods

Cell lines and culture. Human non-small cell lung cancer cell line A549 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in RPMI-1640 supplemented with 10% (vol/vol) FBS (Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin. Cells were maintained at 37°C under normoxic condition in a 5% CO₂, 95% ambient air incubator (Hera cell 150, Heraeus, Langenese, Germany) or hypoxic condition in a modular incubator (Galaxy R, RS Bitotech, Alloa, UK) flushed with a gas mixture consisting of 1% O₂, 5% CO₂, and 94% N₂.

Cell viability assay. Approximately 2,000 cells were seeded in each well of 96-well plate (Corning, Acton, MA, USA). After 72-h treatment, 10 µl Cell Counting Kit-8 (CCK-8) solution for each well of 96-well plate (Corning, Acton, MA, USA). After 45 min, the optical density (OD) values (measuring wavelength at 450 nm, reference wavelength at 630 nm) were obtained using an ELx800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Cell colony formation. Cells (100) were allocated in each well of 6-well culture cluster (Corning). After attachment to plates, A549 cells with indicated treatment were exposed to 20% O₂ or 1% O₂ for 14 days. Colonies were washed three times with cold PBS, fixed with 4% (vol/vol) paraformaldehyde for 20 min and then stained with 0.2% (w/v) crystal violet for 10 min. All colonies visible with the naked eye (>50 cells) were counted.

Analysis of cell proliferation by CFSE dilution. Cells for labeling were diluted to 1·5x10⁵ per ml in FACS buffer (PBS/0.1% BSA) and stained with an equal volume of carboxyfluorescein diacetate succinimidyl ester (CFSE) at a final working concentration of 10 µM for 10 min at 37°C. The staining was quenched by the addition of 5-fold volumes of complete RPMI-1640 medium and incubating for 5 min at 37°C. Cells were pelleted and re-suspended in PRMI medium three times. Then cells were plated in 6-well plates at 1·x10⁶ cells per well under normoxic or hypoxic condition for 72 h. Harvested cells were measured using flow cytometer (BD Biosciences, San Jose, CA, USA) with 488 nm excitation and analyzed with BD FACSDiva 6.1.

Analysis of cell apoptosis by Annexin V/PI assay. Cells were incubated in dark for 15 min at room temperature (RT) in the presence of 5 µl Annexin V-FITC and 5 µl of PI. The stained cells were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA) to identify the early apoptotic (Annexin V+/PI-) and late apoptotic (Annexin V+/PI+) cells.

RNA isolation and real-time PCR. Total RNA was extracted using TRIzol (TakaRa, Dalian, China) from A549 cells. RNA (0.5 µg) was converted into cDNA in a final volume of 10 µl using the cDNA RT–PCR kit (TakaRa, Dalian, China). Then cDNA was used as a template for quantitating gene expression using SYBR Green real-time PCR kit (TakaRa) in an ABI PRISM Fast 7500 system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The amplification conditions were preheat at 95°C for 30 sec, 40 cycles of 95°C for 5 sec, 60°C for 34 sec followed by 95°C for 15 sec and 60°C for 1 min. Primers were as follows: β-actin F-5’-agccagacatcccccaagtt-3’, R-5’-gggcaagaggtctactct-3’; HIF-1α F-5’-ctcctcactctccaccaac-3’, R-5’-tttttctgctctttgtttgtggtt-3’; KLF5 F-5’-ccagtgaatgctctccacac-3’, R-5’-ggtttctcaaatggtggtact-3’; caspase-3 F-5’-atacagctcttcactaatcaga-3’, R-5’-cagcagctgctcgtttt-3’; BIRC F-5’-ccagcgcctctacattcaaga-3’, R-5’-cataagctgcttctagctgct-3’; caspase-3 F-5’-atacagctcttcactaatcaga-3’, R-5’-cagcagctgctcgtttt-3’; caspase-3 F-5’-atacagctcttcactaatcaga-3’, R-5’-cagcagctgctcgtttt-3’.

Transfection of siRNA. Small interfering RNA (siRNA) constructed with HIF-1α or KLF5 and the non-targeting negative control siRNA were synthesized by Ruibo Biotechnology Co. (Guangzhou, China). The target sequences were the following: HIF-1α sense 5’-ugcucuuugugggugauua-3’, antisense 5’-uagucuuugugggugauua-3’; KLF5 sense 5’-aagcaccgagccagca-3’, antisense 5’-ugucacccgagccagca-3’. A549 cells (2x10⁵ cells/well) were transfected in the 6-well plates and were transfected with siRNA at a final concentration of 50 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Cells were harvested after 24-h incubation for RNA isolation and protein were extracted at 72 h after transfection.

Immunoblotting and co-immunoprecipitation. Whole cells in 6-well plates were washed with cold PBS and isolated by RIPA lysis buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS) for 30 min on ice. The lysates were centrifuged at 12,000 rpm for 10 min, boiled with an addition of 5X SDS sample buffer and stored at -20°C. Protein samples (50 µg) were resolved in 10% SDS-PAGE at 100 V for 2 h and transferred to a 0.22-µm pore size polyvinylidene fluoride (PVDF) membrane (Immobilon, Millipore) at 200 mA for 2 h using Trans-Blot Electrophoretic TransferCell (Bio-Rad Laboratories, Richmond, CA, USA). The membrane was blocked and incubated with the appropriate primary antibody: anti-HIF antibody (Novus Biologicals, NB100-134, 1:1,000), anti-KLF5 antibody (Santa Cruz, sc-22979, 1:200), anti-GAPDH antibody (Proteintech, 10494-1-AP, 1:3,000), anti-cyclin B1 (Proteintech, 55004-1-AP, 1:1,000), anti-survivin (Proteintech, 10508-1-AP, 1:1,000), anti-caspase-3 (Proteintech, 19677-1-AP, 1:1,000). After washing by Tris-buffered saline (TBS) containing 0.1% Tween-20, blots were incubated with secondary antibody conjugated to horseradish peroxidase (HRP) (Proteintech, SA00001-1, SA00001-2,
1:4,000) for 2 h at RT. The signals were detected by enhanced chemiluminescent ECL kit (Pierce, Rockford, IL, USA) for 2 min and captured by Kodak X-ray film. For co-immunoprecipitation (co-IP) analyses, cells were lysed in NP-40 lysis buffer (1% NP-40). Protein samples (800 µg) were incubated with the appropriate primary antibody (1-2 µg) and agarose beads (A/G plus, Santa Cruz Biotechnologies) overnight at 4°C, followed by immunoblotting (as described above).
Statistical analysis. Statistical analyses were carried out using GraphPad Prism (version 5.0). The Student's t-test was used to make a statistical comparison between groups, two paired. For multi-group analysis of variances, one-way ANOVA with Dunn's multiple comparison test and two-way ANOVA with Bonferroni post-test were performed. All experiments were repeated at least four times and the results are expressed as the mean ± SEM. P<0.05 was considered to be statistically significant.

Results

Hypoxia promotes proliferation of A549 cells and inhibits apoptosis. Uncontrollable survival is a pivotal characteristic in the process of tumor development. In order to identify the effect of hypoxia on lung cancer cells, we performed CCK-8 test to measure cell viability, colony forming assay for cell clonality, CFSE dilution assay for cell proliferation and Annexin V/PI assay for cell apoptosis in A549 cells exposed to 20% or 1% O2. According to CCK-8 test results, hypoxic cell viability evaluated by mitochondrial activity was significantly higher than normoxic cells (Fig. 1A). In colony forming assay, colony numbers of cells under hypoxic condition for 14 days increased 3-fold compared with cells in normoxia (Fig. 1B and C). CFSE dilution assay showed that hypoxia significantly increased cell proliferation after a 3-day treatment (Fig. 1D and E). Percentages of apoptotic cells were detected by Annexin V and PI staining and hypoxia reduced both the early stage and the late stage apoptotic cells (Fig. 1F and G).

Hypoxia upregulates the expression of HIF-1α and KLF5. To investigate the role of HIF-1α and KLF5 in lung cancer, we tested the expression of HIF-1α and KLF5 in A549 cells exposed to hypoxia for different times by real-time PCR and immunoblotting. HIF-1α mRNA levels were increased in response to hypoxia within 0.5 h, peaked at 1 h and returned to basal levels after 8 h of stimulation. Similarly, the mRNA levels of KLF5 were elevated after 0.5 h and peaked at 2 h, then returned to basal levels 8 h later (Fig. 2A). As expected, HIF-1α and KLF5 protein levels were rapidly elevated exposed to 1% O2 after 0.5 h and continued to rise steadily up to 4 h (Fig. 2B and C).

Effect of KLF5 and HIF-1α on cell proliferation and apoptosis. To better characterize the role of HIF-1α and KLF5 in
Figure 3. KLF5 and HIF-1α mediate proliferation and apoptosis of hypoxic A549 cells. (A) CCK-8 test was conducted in A549 cells transfected with negative control siRNA, HIF-1α-siRNA or KLF5-siRNA in normoxia or hypoxia for 3 days. (B) Representative colonies are shown for indicated cells exposed to different O₂ concentrations. (C) The number of colonies is shown. (D) Analysis of indicated cells labeled CFSE following exposure to 20% O₂ or 1% O₂ for 3 days. (E) Proliferation indices were calculated for each treatment group. (F) Annexin V/PI assay was done to determine apoptosis of transfected cells exposed to 20% O₂ or 1% O₂ for 3 days. (G) Percentage of cell death containing early apoptosis, and later stage apoptosis in transfected cells under different O₂ supply condition. Data (A, C, E and G) are shown as mean ± SEM; n=4, *P<0.05, **P<0.01, ***P<0.001 (two-way ANOVA with Bonferroni post-test). si-NC, negative control siRNA; si-HIF, HIF-1α siRNA; si-KLF5, KLF5 siRNA; N, normoxia; H, hypoxia.
cell survival following hypoxia, we measured cell viability, clonality, proliferation and apoptosis in cells treated with siRNA targeting HIF-1α or KLF5. Cell viability with a significant increase in hypoxia were decreased to normoxic level by transient silencing of HIF-1α or KLF5. However, downregulation of HIF-1α and KLF5 had no impact on cell viability in normoxia (Fig. 3A). Consistently, HIF-1α and KLF5 inhibition resisted cell survival and accelerated cell apoptosis in hypoxia and did not affect normoxic cells (Fig. 3B-G).

**Regulation of downstream target genes and proteins.** We assessed whether KLF5 or HIF-1α knockout affected the expression of relevant target genes and proteins. The results showed that KLF5 and HIF-1α inhibition significantly decreased the expression of cyclin B1, survivin and increased caspase-3 expression in mRNA and protein levels in hypoxia and did not affect normoxic cells (Fig. 3B-G).

**HIF-1α interacted with KLF5.** Since HIF-1α and KLF5 both are hypoxia-regulated transcriptional activators and involved in tumor progression, co-IP assay was performed in A549 cells exposed to 20% or 1% O2 to confirm the direct correlation of HIF-1α and KLF5. KLF5 was found to co-immunoprecipitate with HIF-1α and vice versa. The amount of co-immunoprecipitated KLF5 was increased in hypoxia compared with normoxia (Fig. 4A). Immunoblots described the same amounts of input and hypoxia-induced increase in HIF-1α and KLF5 (Fig. 4B).

**Hypoxia-induced HIF-1α overexpression is KLF5-dependent.** To evaluate the functional interaction of HIF-1α with KLF5 in hypoxia, HIF-1α and KLF5 expression was measured in A549 cells exposed to 20% or 1% O2 with the presence of KLF5 or HIF-1α siRNA. Suppressing KLF5 by siRNA significantly reduced HIF-1α expression compared with negative controls under hypoxic condition while HIF-1α expression did not change in normoxia. Likewise, treating cells with HIF-1α siRNA did not affect the expression of KLF5 either in hypoxia or normoxia (Fig. 6).

**Discussion**

Hypoxia is described to be an important stimulating factor to sustain an aggressive growth phenotype in cancer cells. Multiple biological processes have been developed in hypoxic tumors, including activation of glycolysis and angiogenesis, pro-survival and invasiveness of cancer cells, metastasis and resistance to chemotherapy and radiation, which lead to poorer prognosis (21-23). The role of hypoxia in alveolar epithelial cells remains ambiguous as a pro-apoptotic factor or proliferation promoter. Therefore, we assessed the effect of hypoxia on A549 cells in several ways and found that prolonged hypoxia...
HIF-1α plays an important role in the adaptation to hypoxia, which is underscored by the association with prognosis. Plenty of HIF-1α inhibitors, including chemical inhibitors, protein and nucleic acid agents, have been studied for targeted cancer therapy. KLF5 is predominantly present in epithelial tissues characterized by active proliferation and exerts pleiotropic effects on the regulation of tumor progression, cell proliferation and apoptosis by binding to similar GC-rich promoter of target genes (27). In our experiments, expression of HIF-1α and KLF5 was upregulated time-dependently in A549 cells exposed to 1% O₂. Interestingly, peak time of HIF-1α protein expression was identical with KLF5. Hypoxia-induced increase of cell viability, clonality, proliferation and decrease of cell apoptosis were abrogated by siRNA targeting HIF-1α or KLF5, implying their contribution to hypoxic microenvironment.

Cell proliferation is dependent on the response to mitogenic signals to enter the cell cycle. There is evidence that KLF5 mediates the activation of cyclin B1/Cdc2 complex to accelerate mitosis (28). Apoptosis is controlled by caspase family (29) and caspase-3 plays a key role in both the extrinsic and the intrinsic apoptosis pathways (30). Survivin, as a direct inhibitor of caspase-3, is implicated in promoting tumorigenesis and resisting chemotherapy and radiation-induced apoptosis in non-small cell lung cancer (31). Recent studies show that KLF5 is involved in the upregulation of survivin via direct interaction with p53 (32), and knockout of KLF5 induces caspase-3 cleavage through pERK/MKP-1 signaling. HIF-1α is revealed to induce survivin expression at the transcriptional level in prostate cells (33) and bind to the promoter of caspase-3 (34). As expected, cyclin B1 and survivin were downregulated and caspase-3 was increased by HIF-1α or KLF5 ablation. Therefore, our experiments clearly demonstrated that KLF5 and HIF-1α played a vital role in the regulation of cell survival and cell apoptosis via cyclin B1, survivin and caspase-3 in hypoxic lung cancer cells.

The expression and activity of HIF-1α are regulated in tumor development by multiple signaling pathways and proteins, including PKC/P13K/AKT/mTOR (35-37), Ras/MER/ERK (38), MAPK (39,40), EGFR and IGF1R autocrine signaling (41), PTEN (37,42,43). Under hypoxic condition, stabilized HIF-1α protein dimerizes with HIF-1β in the nucleus and the complex binds to a core DNA hypoxia response element (HRE) (44), activating the expression of numerous tumor promoter genes, including GLUT1 (10), VEGF (45), NOS (46), IGFl-2 (47) and ID2 (48). The increase of HIF-1α mRNA and protein levels in our study supported the view that hypoxia-induced HIF-1α accumulation originate from de novo gene transcription and the protein stabilization (49). An array of proteins participate in the regulation of HIF-1α degradation via binding to HIF-1α, including ERRα (50), STAT3 (51), Hsp90 (52) and VHL (53). In our study, KLF5 formed a complex with HIF-1α and the interaction between them was oxygen-dependent. Furthermore, silencing of KLF5 markedly inhibited HIF-1α production while HIF-1α inhibition did not affect KLF5 expression, suggesting a possible role of KLF5 in preventing the degradation of HIF-1α following hypoxia. HIF-1α is recently proposed by Mori et al to regulate KLF5 expression by modulating its interaction with KLF5 in pancreatic cancer cell line (54). Mori et al showed that HIF-1α co-immunoprecipitates with KLF5 and vice versa, independent of hypoxia. The expression of HIF-1α is not affected by the downregulation of KLF5. Because of the particularly overexpression of HIF-1α in pancreatic cancer cells, the functional regulation of KLF5 and HIF-1α are performed in normoxia. Therefore, the differences between Mori et al and our study may be accounted for by the types of cancer and the duration of hypoxia. The new finding substantiated the direct interaction of KLF5 with HIF-1α and the upregulation of HIF-1α by KLF5 in the adaptation to hypoxia supported tumor growth, providing a promising therapeutic target for cancer therapy.

KLF5 was strongly overexpressed in hypoxic A549 cells and mediated hypoxia-induced upregulation of HIF-1α. Considering the multiple regulations of cell viability, clonality, proliferation and apoptosis in hypoxia, KLF5 may be an attractive target for therapy of lung cancer. Thus, treatment protocols using KLF5 inhibitors require further investigation.

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References


