HAF drives the switch of HIF-1α to HIF-2α by activating the NF-κB pathway, leading to malignant behavior of T24 bladder cancer cells

ZHENFENG GUAN1, CHEN DING1, YIQING DU1, KAI ZHANG1, JIAN NING ZHU1, TINGTING ZHANG1, DALIN HE2, SHAN XU1, XINYANG WANG1 and JINHAI FAN2

1Oncology Research Lab, Key Laboratory of Environment and Genes Related to Diseases, Ministry of Education; 2Department of Urology, The First Affiliated Hospital of Medical College of Xi'an Jiaotong University, Xi'an, P.R. China

Received October 7, 2013; Accepted November 19, 2013

DOI: 10.3892/ijo.2013.2210

Abstract. Hypoxia is a characteristic feature of solid tumors, leading to malignant behavior. During this process, HIF family members (HIFs) and the NF-κB pathway are activated. In addition, the hypoxia-associated factor (HAF) is reported to participate in the regulation of HIFs. However, the precise relationship among HIFs, HAF and the NF-κB pathway in bladder cancer (BC) remains unknown. In the current investigation, T24 BC cells were exposed to hypoxia, or by plasmid transfection to overexpress HAF or RelA (P65) to demonstrate their roles. The results indicate that hypoxia leads to the elevation of HAF plus activation of the NF-κB pathway, accompanied by the switch of HIF-1α to HIF-2α, resulting in the enhanced ability of malignancy in T24 cells. In order to further demonstrate the significance of this switch, HIF-1α and HIF-2α were co-transfected into T24 cells with HIF-β, respectively. The following results indicate that the T24HIF-2α/β cells show enhanced ability of malignancy, accompanied by the maintenance of stem-cell markers, but the T24αβ/β cells show higher expression of metabolism-related genes. Boyden assays and wound-healing assays indicate the enhanced ability of malignancy, accompanied by the switch of HIF-1α to HIF-2α, resulting in the enhanced ability of malignancy in T24 cells. In order to further demonstrate the significance of this switch, HIF-1α and HIF-2α were co-transfected into T24 cells with HIF-β, respectively. The following results indicate that the T24HIF-2α/β cells show enhanced ability of malignancy, accompanied by the maintenance of stem-cell markers, but the T24αβ/β cells show higher expression of metabolism-related genes. Boyden assays and wound-healing assays indicate the enhanced ability of malignancy for T24 bladder cancer.

Introduction

Regional hypoxia is common feature of solid tumors, leading to the induction of the transcriptional factor hypoxia inducible factor (HIF) (1). Prolonged-term exposure to hypoxia, which mimics the tumor microenvironment, drives a perpetual epithelial to mesenchymal transition (EMT), whereas short-term exposure to hypoxia induces a reversible EMT (2). The discrepancy of cell behavior change driven by differing terms of hypoxia can be regarded as the manifestation of different activated target genes induced by different HIF family members (HIFs).

HIFs are composed of two heterodimeric proteins belonging to the basic helix-loop-helix (bHLH)/Per-ARNT-Sim (PAS) domain family of transcription factors (3,4), the former contains three subunits: HIF-1α, HIF-2α and HIF-3α (5,6), in particular, HIF-1α and HIF-2α (also known as EPAS1) are the two best studied members of the HIF-α family (7), HIF-3α is a new one (8-11). The latter is also known as the aryl hydrocarbon receptor nuclear translocator (ARNT) or simply HIF-β, including HIF-1β, HIF-2β and HIF-3β (8). Although HIF-1α and HIF-2α share several common targets such as VEGF (12,13), both isoforms may regulate distinct transcriptional targets (14). HIF-1α is responsible for the regulation of genes encoding enzymes involved in the glycolytic pathway but HIF-2α targets to the genes involved in invasion such as the matrix metalloproteinase (4,14,15) or the stem-cell related genes such as Oct-4 (16), both isoforms may regulate distinct transcriptional targets (14). HIF-1α capable of dimerizing with HIF-β binding to the hypoxia-responsive DNA element, resulting in the activation of hypoxia-responsive genes (7). Besides the above, HIF-α can be regulated by other factors, such as hypoxia-associated factor (HAF) (8) and the nuclear factor-κB (NF-κB) pathway.

HAF, also known as SARTI (16), is reported to be able to bind to and induce the degradation of HIF-1α in PHD-pVHL-
independent way (16). But this binding of HAF to HIF-2α leads to its elevation instead degradation (6,16). In addition, HAF is elevated in many solid tumors, such as breast cancer, and brain tumors, etc.

The NF-κB family is composed of structurally homologous transcription factors, including NF-κB1, NF-κB2, RelA, RelB and c-Rel, which bind to IκB enhancers as homo- or heterodimers (19,22,23). van Uden et al (23) reported that the HIFs could be upregulated by NF-κB, and they found the binding site of NF-κB in the promoter region of the HIFs. Degradation of IκB leads to the activation of the pathway, resulting in the nuclear translocation of the NF-κB complexes, predominantly RelA/P50 (P65/P50) and P50/c-Rel dimers (24). This activation occurs in inflammation as well as in the progression of cancer and hypoxia.

Herein, we report that in the bladder cancer T24 cells, prolonged exposure to hypoxia induces the elevation of HAF, resulting in the switch of HIF-1α to HIF-2α, the process of which is mediated by NF-κB pathway. This leads to more malignant behavior and maintenance the stem-cell markers of T24 cells, giving us a further clue to understand the mechanism for the progression of bladder cancer.

Materials and methods

Western blotting. Cells were harvested at 80% confluence, and washed with cold PBS three times. Total cellular protein lysates were prepared with RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP40 and 0.5% sodium deoxycholate] containing proteinase inhibitors [1% cocktail and 1 mM PMSF, both from Sigma (St. Louis, MO, USA)]. Nuclear protein was prepared using the kits (lot no. BSP001) obtained from Sangon Biotech Co., Ltd. (Shanghai, China) strictly according to its protocol. Total of 30 µg of protein was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked at room temperature for 1 h with 5% skim milk in Tris-buffered saline without Tween-20 (pH 7.6, TBS). Polyclonal antibodies were applied at different dilutions (Table I) by 5% skim milk in TBS at 4˚C overnight, followed by washing with TBST (with Tween-20, pH 7.6). Membranes were incubated with secondary antibodies (supplied by Licor, Rockford, IL, USA) coupled to the first antibody at room temperature in the dark for 1 h, followed by washing as above in the dark, drying with neutral absorbent paper and scanning by Odyssey detection system (Licor). MG-132 (Sigma) was used to inhibit the proteasome-dependent degradation if necessary (10 µM, 4 h before the protein harvest). GAPDH (for total cell fraction) and Hist1H1a (for nuclear fraction) were used as the loading controls.

Real-time PCR. Total RNA of the related groups of the cell was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and quantitated by absorbance at 260 nm. RNA (2 µg) was reverse transcribed using Revert Aid™ First Strand cDNA Synthesis kit (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturer’s protocol. For real-time PCR, we used the SYBRR Premix Ex Taq™ II system (Takara Biotechnology Co., Ltd., Dalian, China) and the Bio-Rad CFX96™ Real-time system (Bio-Rad, CA, USA). SYBRR Premix Ex Taq II (12.5 µl), 1 µl primer (10 µM, primers see the Table II), 200 ng cDNA and 9.5 µl DD water mixed together, following the second stage, pre-degeneration for 95°C, 30 sec, one repeat, and PCR reaction, 95°C 5 sec followed by 60°C, 30 sec, 35 repeats, and the third

Table I. The antibodies used.

| Gene ID      | Antibody   | Dilutions | Species | Supplied by     |
|--------------|------------|-----------|---------|-----------------|
| NM_004360.3  | E-cadherin | 1:600     | Homo    | Santa Cruz     |
| NM_001792.3  | N-cadherin | 1:300     | Homo    | Santa Cruz     |
| NM_003380.3  | Vimentin   | 1:300     | Homo    | Santa Cruz     |
| NM_003068.4  | Slug/Snail2| 1:400     | Homo    | Santa Cruz     |
| NM_001174096.1| Zeβ1 | 1:300     | Homo    | Santa Cruz     |
| NM_001530.3  | HIF-1α     | 1:500     | Homo    | Santa Cruz     |
| NM_001430.4  | HIF-2α     | 1:300     | Homo    | Santa Cruz     |
| NM_001668.3  | HIF-β      | 1:300     | Homo    | Santa Cruz     |
| NM_005146.4  | HAF        | 1:400     | Homo    | Santa Cruz     |
| NM_001145138.1| P65 | 1:300     | Homo    | Santa Cruz     |
| NM_004530.4  | MMP2       | 1:400     | Homo    | Santa Cruz     |
| NM_004994.2  | MMP9       | 1:400     | Homo    | Santa Cruz     |
| NM_002046.4  | GAPDH      | 1:15,000  | Homo    | Santa Cruz     |
| NM_024865.2  | Nanog      | 1:400     | Homo    | Millipore      |
| NM_001173531.1| Oct-4 | 1:300     | Homo    | Millipore      |
| NM_020529.2  | IκB        | 1:400     | Homo    | Santa Cruz     |
| NM_020529.2  | p-IκB      | 1:300     | Homo    | Santa Cruz     |
| NM_005325.3  | Hist1H1a   | 1:300     | Homo    | Santa Cruz     |
stage of dissociation, 95°C, 15 sec followed by 60°C, 30 sec, and another 95°C, 15 sec, then the data were collected. GAPDH was used as the internal control.

Cell culture. Human bladder cancer cell line T24 and J82 were obtained from ATCC (American Type Culture Collection, ATCC, USA) and cultured in the DMEM (Invitrogen) supplemented by 10% FBS (Invitrogen). For normal condition, the cell was cultured in the atmosphere with 5% CO\textsubscript{2} at 37°C. For mimicking the hypoxic conditions, the cell was cultured in an atmosphere with 1% O\textsubscript{2} and 99% CO\textsubscript{2} at 37°C (Incubator: Thermo Scientific, Germany).

Boyden chamber assay and wound healing assay. Migration and invasion were tested by Boyden chamber assay, the chambers were obtained from Millipore (Millipore, Switzerland). For migration assay, 0.2 ml FBS-free DMEM suspension with 10,000 cells was added to the upper chamber in a 24-well plate, and 0.8 ml FBS-free DMEM was added to the lower chamber. After 12 h of incubation, the chambers were washed with PBS (pH 7.4) three times to remove the cells in the upper chamber and were fixed with 4% formalin for 5 min, then stained with crystal violet (0.01% in ethanol) for 5 min followed by washing three times. The cells were counted in an inverted microscope, and five visions were randomly taken at x200, the average number of the cells were analyzed; for the invasion assay, the suspension of the upper chamber contained 0.2 ml mixture (FBS-free DMEM/Matrigel=8/1, Matrigel, Sigma) and 10,000 cells, the incubating times was 36 h, other steps were the same as the migration test.

Wound healing assay was carried out by scratching a 6-well dish with a 10-μl pipette tip when the dish was at 80% confluence (including T24\textsuperscript{p65/β}, T24\textsuperscript{HIF-2α/β} and T24\textsuperscript{vec/vec}). The width of the scratches was compared at 0, 12 and 24 h after scratching.

Construction of a stable clone cell line. In order to understand the role of HAF and NF-κB in T24 cell, the pFRT/TO/HIS/FLAG/HA-SART1 and gfp-rel a (Addgene plasmid 38087 and

| Gene ID          | Gene        | Primers                              |
|------------------|-------------|--------------------------------------|
| NM_001145138.1  | p65 Forward | ACGAATGACAGAGCGGTGTATAAGG            |
|                  | Reverse     | CAGAGCTGCTTGGCGGATTAG                |
| NM_001197325.1  | HIF-β Forward | CTCCTGTTGACAGTGGTTCTGTA            |
|                  | Reverse     | CAGGCTTGTATGTGGACGTCTGTC             |
| NM_001530.3     | HIF-1α Forward | TTAGCTCATGTTGGGAGCTTCC              |
|                  | Reverse     | AGCAATTCATCCTGTCATTTCC              |
| NM_001430.4     | HIF-2α Forward | CATGCAGCTAGCTCCGAGAAACA            |
|                  | Reverse     | GCTTTGCGAGCTACCCGTA                 |
| NM_005146.4     | HAF Forward | AAGTACAGCGGAGGGAGGAATAC             |
|                  | Reverse     | TTCTATCTGCTGAGCCT                                                                 |
| NM_002046.4     | GAPDH Forward | AACAGGACACCCACCCATCT                |
|                  | Reverse     | CATACCAGAACGAAATGCTTC              |
| NM_020529.2     | IκBα Forward | GATCGGCCATTGGAAGGG                   |
|                  | Reverse     | GCAATTTCCTGCTGAGTG                 |
| NM_024865.2     | Nanog Forward | CTAAGAGTTGGGAGAAAAAC                 |
|                  | Reverse     | CTTGTTAGAGAGAGATGTGTTTT            |
| NM_00117351.1   | Oct-4 Forward | TTAGGCTAGAGGAAGATGTGTT              |
|                  | Reverse     | GGAAGAAGGGACTGAGTAGGTGTG            |
| NM_005165.2     | ALDOC Forward | CTTGCCAAACCATCACAGGAT              |
|                  | Reverse     | CACCACCCCTTGGTCAACCTT               |
| NM_000291.3     | PGK1 Forward | CTGTGGTACTGAGACGACGCAAGGA            |
|                  | Reverse     | CAGGACCATCCTCAAATCTCTG              |
| NM_001135239.1  | LDHA Forward | TGCTACGAGGTTGTAGTCAAGCT              |
|                  | Reverse     | ATGCACCAGCCGTAGAGTTCT               |
| NM_001145138.1  | p65-siRNA Antisense | AAGAGCATCATGAAGAGAGATGCTTC          |
|                  | Sense       | AAACCTCTCTTCATGATGTCCCTCGTCTC       |

Table II. Primers for real-time PCR and siRNA sequence.
23255, http://www.addgene.org), two plasmids were transfected into the T24 cells, respectively. Lipofectamine™ 2000 (Life Technologies, USA) was used for transfection strictly according to its protocol, and selected by Blasticidin and G418 (8 and 600 µg/ml) respectively.

For understanding the roles of HIFs, pcDNA3.1-hif-1α and pcDNA3.1-hif-2α (Addgene plasmid 18949 and 18950, http://www.addgene.org) were cotransfected with pCMV-hif-β (pCMV-HIF-β-hygro, HG13010-M, Sino Biological Inc. China) into the T24 cell, respectively. Both the subclones containing hif-1α/β and hif-2α/β were selected by G418 plus hygromycin (600 and 80 µg/ml, respectively).

Inhibition of NF-κB pathway. Pyrrolidine dithiocarbamate (PDTC) as an inhibitor of NF-κB pathway was obtained from Sigma (Sigma-Aldrich, USA) and the final concentration was 10 µM in the medium for the last 24 h before the protein/total RNA was extracted or Boyden chamber assay. In addition to PDTC, siRNA for knocking down the expression of p65 was used in parallel experiments. siRNA was transfected into T24 haf cells with Lipofectamine 2000 according to its protocol, and the scrambled sequence was used as a control.

Immunofluorescence staining for nuclear translocation of NF-κB. After designated treatment, the cells were washed three times with cold PBS (pH 7.4) followed by fixing with 4% paraformaldehyde for 15 min, permeabilized in 0.5% Triton X-100 for 10 min, and incubated in 1% BSA blocking solution for 1 h. Fixed cells were incubated overnight in 4°C with rabbit anti-human-P65 in 1% BSA. Cells were washed and incubated with mouse anti-rabbit TRITC (Red) IgG antibody (Santa Cruz, USA) diluted 1:100 in blocking buffer for 1 h. Nuclei were stained with DAPI for 5 min. Cells were examined with a fluorescent microscope equipped with narrow band-pass excitation filters to individually select for red, and blue fluorescence. Cells were observed through the Image Pro Plus system mounted on a fluorescent microscope (Olympus, Japan), the experiment was repeated thrice.

Statistical analysis. ANOVA test was used for analyzing the discrepancy of three or more than three groups. The Student’s t-test was used to detect any statistically significant difference between two groups. P<0.05 was considered statistically significant.

Results

Hypoxia contributes to EMT and the enhanced ability of migration/invasion, accompanied by the elevation of HAF. Tumor regional hypoxia is a common feature in solid tumors, leading to behavior change of the tumor cells in order to fit the microenvironment. Based on this, we mimicked the hypoxic environment under the condition of 1% O2 supplemented with 99% CO2 in order to observe the behavior change of our T24 cells. As indicated in Fig. 1A, the oxygen starvation for 48 h indicates EMT of T24 cells, accompanied by enhanced ability of migration/invasion (Fig. 1B and C). Previously it was reported that prolonged-term hypoxia induced the expression HAF in other tumors, our results proved this point in bladder cancer T24 cell (Fig. 1D and E).

Prolonged hypoxia results in the elevation of HIF-2α and HIF-β but decreases HIF-1α in protein, however, increasing all the HIFs in mRNA. As reported, the prolonged-term of
Figure 2. Hypoxia induces the nuclear translocation of NF-κB and the degradation of IκB/p-IκB, which also occurred in the T24haf cells, resulting in the EMT and elevation of NF-κB. (A) Western blotting showing HAF induces the upregulation of N-cadherin, vimentin, Slug, Zeb1, MMP2, MMP9, and T-P65, but downregulation of E-cadherin and HIF-1α, accompanied by the elevation of HIF-2α, HIF-β. (B) Immunofluorescence of the nuclear translocation of P65 induced by prolonged hypoxia. (C) Western blotting indicating the elevated p-IκB in T24-HAF cells in the presence of PDTC. (D) Western blotting showing the HAF-overexpression contributing to the elevation of p-IκB but without visible change of IκB. (E) Western blotting indicating the prolonged term hypoxia induces the elevation of p-IκB but without visible change of IκB. (F) Western blotting of the HAF-overexpression induces the accumulation of P65 in the nuclear.

Figure 3. HAF overexpression-induced malignancy, NF-κB nuclear translocation, EMT and alternation of HIFs in T24 cell can be inhibited by PDTC. (A) Representative images of Boyden chamber assay for the attenuated ability of migration/invasion in T24-HAF cells in the presence of PDTC. (B) Western blotting showing the phenomenon induced by PDTC, manifested as the decreased N-cadherin, vimentin, Zeb1, Slug, MMP2, MMP9, and increased E-cadherin. (C) Real-time PCR indicating that haf contributes to the elevation of hif-1α, hif-2α and hif-β but has no visible effect on p65 and iκB. (D) Immunofluorescence suggesting the nuclear translocation of P65 induced by HAF-overexpression, and its inhibition by PDTC. (E) Western blotting of the elevation of HIF-2α and HIF-β was inhibited by PDTC. (F) Real-time PCR of the HAF-induced elevation of hif-1α, hif-2α and hif-β was inhibited by PDTC, which still has no visible effect on p65 and iκB.
hypoxia led to differing expression profiles of the HIFs (11,16). Based on this, we detected all the HIFs in the different hypoxic time-points in T24 cells. As indicated by Fig. 1D, HIF-2α and HIF-β were increased from 0 to 48 h, but HIF-1α was decreased after 18 h. However, the mRNA of all the HIFs were increased (Fig. 1E).

HAF contributes to EMT and the enhanced ability of migration/invasion in T24 cells. In order to understand the role of HAF in hypoxia-induced phenotype, the haf plasmid was transfected into T24 cells (Fig. 2A), western blotting indicated the extremely increased expression of EMT-related genes and the enhanced ability of migration/invasion (Fig. 3A).

HAF mimicks the phenomenon occurring to HIFs induced by hypoxia. In order to explore the roles of HAF on HIF-1α, HIFs were detected in the lysate of T24 cells (Fig. 2A), western blotting indicated the extremely increased expression of EMT-related genes and the enhanced ability of migration/invasion (Fig. 3A).

HAF, consistent with hypoxia, contributes to the degradation of IκB, leading to the activation of NF-κB pathway. As previously reported, in the NF-κB pathway, the degradation of IκB led to the liberation of NF-κB and resulted in the activation of this pathway. As shown in Fig. 2E, hypoxia resulted in decreased IκB and nuclear accumulation of NF-κB (Fig. 1F). In addition, immunofluorescence also indicated the nuclear translocation of NF-κB by hypoxia exposure (Fig. 2B). Based on the phenomenon above, we postulated that HAF may play a role in the degradation of IκB, which was decreased in the T24haf cells in an unknown way (Fig. 2D). Both western blotting (Fig. 2F) and immunofluorescence (Fig. 3D) suggested that overexpression of HAF in T24 cell induced the nuclear accumulation of NF-κB, leading to the activation of this pathway.

The role of HAF on EMT and HIFs is inhibited by PDTC or p65 knockdown. Large amount of investigations have pointed out that the NF-κB pathway plays an important role during the process of EMT in the progression of bladder cancer (25). Degradation of IκB led to the activation of this pathway and resulted in the upregulation of EMT-related genes (24,26-30) and HIFs (23). Based on this, we used PDTC, an inhibitor of the NF-κB pathway (31,32), to attenuate the transcription of the target genes of this pathway. In addition, siRNA for knocking down p65 was used in parallel with PDTC (data not shown). As expected, the nuclear translocation of NF-κB was inhibited (Fig. 3D) and HIFs were all decreased (Fig. 3E), leading
to the reversal of EMT induced by HAF-overexpression in T24 cells (Fig. 3B), accompanied by the attenuated ability of migration/invasion (Fig. 3A). However, this inhibitor had no significant effect on HAF (Fig. 3E).

NF-κB contributes to EMT and the enhanced ability of migration/invasion in T24 cells. The dimer of p65/p50 was the critical member among all the combinations of NF-κB family, p65 was the activator subunit for activating the transcription of the target genes (22,33). In order to explore its roles in T24 cells, the gfp-p65 was transfected into T24 cells (Fig. 4B-D), resulted in the increasing of HIFs, which gave us evidence that NF-κB contributed to the elevation of HIFs. Thus, at least, NF-κB contributed to EMT and the elevation of HIFs in T24 cells, leading to the enhanced ability of migration/invasion (Fig. 4E and F).

The switch of HIF-1α to HIF-2α initiates to the different target genes, accompanied by the enhanced ability of migration/invasion in T24 cells. As has been reported (6), although HIF-1α and HIF-2α share several common targets, both isoforms may induce distinct transcriptional target genes (16). In order to observe this discrepancy in T24 cells, by cotransfection of the two subunits into the T24 cells (Fig. 5C and D), we proved the discrepancy of target genes (Fig. 5A, B, E and F) and the ability of migration/invasion (Fig. 6A, C and D). Herein we chose the cotransfection of the β subunit with α subunit because α subunit must couple with β subunit, which is greatly lower in T24 cells (Fig. 1D), to activate their target genes.

Discussion

Hypoxia, a characteristic feature of solid tumors, has emerged as a pivotal factor of the tumor since it can activate the related genes of the tumor cells in order to adapt to the microenvironment to promote tumor progression and resistance to therapy (34). Among large numbers of genes, the key candidate is widely accepted to be the HIF family, which can be regulated by an oxygen- or pVHL-dependent mechanism. Recently, HAF is reported to regulate the HIFs both in an oxygen- and pVHL-independent way. In the present study, we
provide evidence that HAF contributes to the degradation of HIF-1α directly and promotes the transcriptional activation of all HIFs by activating the NF-κB pathway, leading to the elevation of HIFs in mRNA indirectly. The combination of two aspects results in the switch of HIF-1α/β to HIF-2α/β followed by the activation of different target genes, leading to the malignant behavior of tumor cells. To our knowledge, this is the first study involved in the NF-κB pathway during the process of HAF induced switch of HIF-1α/β to HIF-2α/β in bladder cancer cells.

Guan et al: HAF DRIVES THE SWITCH OF HIF-1α TO HIF-2α BY ACTIVATING THE NF-κB PATHWAY

Figure 6. The migration/invasive ability in vitro. (A and C) Boyden chamber assay indicating the enhanced ability of migration/invasion in T24hif-2α/β. (B) Real-time PCR showing the efficiency of cotransfection of hif-1α/2α/β in the T24 cell. (D) Wound healing assay indicating the enhanced ability of malignancy in T24hif-2α/β.

Figure 7. Schematic diagram as summary of the present investigation. Short-term hypoxia leads to the stabilization of HIF-1α, targeting the metabolism-related genes, such as PGK1, ALDOC and LDHA, to adapt to the hypoxic environment. The prolonged hypoxia is more sophisticated. Firstly, this hypoxia induces HAF. On the one hand, the induced HAF contributes to the degradation of IκB in an unknown way, leading to the activation of NF-κB pathway, which can be inhibited by PDTC resulting in the increase of target genes, such as HIF-1α, HIF-2α and HIF-β. On the other hand, HAF leads to the degradation of HIF-1α directly. The combination of the two aspects result in the switch of HIF-1α to HIF-2α, leading to the activation of migration/invasion and stem-cell related genes to maintain invasive and self-renewal capacity of tumor cells.
The HIF-α/β heterodimer binds to the hypoxic response elements (HREs) of target genes on hypoxia (35) to promote their expression, leading to the tumor behavior change to adapt to the hypoxic environment. We noted that on the different time-point of hypoxia, the expressions of the HIFs are different (Fig. 1D and E). It is reported that HIF-1α but not HIF-2α is more sensitive to the oxygen, and can be degraded within 5 min (8,21) in normal atmosphere, but this protein can keep the stable status in hypoxia. However, compared to the reported results (4,36,37), our data indicate that this stable status on hypoxia can be destroyed in protein, but not mRNA by the prolonging time of hypoxic exposure. Compared with HIF-1α, hypoxia induces the elevation of HIF-2α and HIF-β instead of reduction.

Hypoxia induces the expression of HAF, accompanied by the different changes of HIFs in T24 and J82 cells (J82 cell data not shown), the process of which could be mimicked by HAF overexpression. Our results are consistent with the investigation (16) that HAF contributes to the switch of HIF-1α/β to HIF-2α/β. The importance of this switch is the different target genes of the two dimers. It is reported that HIF-1α/β preferentially induces glycolytic enzyme genes (8,38,39), but HIF-2α/β induces genes involved in invasion and are stem cell related (8,16). Our data in T24 cells provide evidence on this point that HIF-1α/β promotes the expression of metabolism-related genes (Fig. 5E and F), but HIF-2α/β affect the induction of EMT and migration/invasion-related genes, also including the stem cell-related genes (Fig. 5B, E and F). This discrepancy indicates the initiation of the differing ability of migration/invasion (Fig. 6A, C and D). In order to explore whether HIF-1α affects the HIF-2α/β induced malignant behavior of T24 cells, we transfected hif-1α into T24hif-2α/β cells (Fig. 6). To our surprise, there is almost no discrepancy of malignancy between T24hif-1α/β and T24hif-2α/β cells, which indicates that there must be a more complex mechanism during this process. Besides the above, the enhanced ability of migration/invasion can be induced also by the NF-κB pathway directly.

In bladder cancer, Levidou et al (40) demonstrated that NF-κB nuclear expression emerged as an independent prognosticator of adverse significance. While, this nuclear expression was regarded also as a marker of activation of NF-κB pathway, in the process where the degradation of IκB is the key step (30). We noted that either in hypoxia or in the T24hif-1α cells, IκB is decreased, accompanied by the nuclear translocation of NF-κB (Figs. 1E and F, 2B, D, E and F and 3D), which means, at least, HAF is one of the factors contributing to the degradation of IκB in an unknown mechanism and leads to the activation of the NF-κB pathway.

It was reported (39,41) that HIF-1α is a target gene for NF-κB, and the promoter of HIF-1α contained a NF-κB binding site (42,43). Furthermore, NF-κB also controlled HIF-β directly and HIF-2α indirectly, making NF-κB a key regulator of the HIF family (1,23,44). Our activation or inhibition results also illustrate this point. The activation or inhibition of the NF-κB pathway is significantly accompanied by the inverse EMT related markers and the ability of migration/invasion in our T24 cells.

In conclusion, our present study summary is shown in Fig. 7. There are still several problems to be explored in our following work. For instance, HAF is reported as an E3 ubiquitin ligase that binds and ubiquitinates HIF-1α by an oxygen and pVHL-independent mechanism, but our results indicate that this protein also plays roles in the phosphorylation of IκB (Fig. 2D). Phosphorylation of IκB, which also occurs in hypoxia (Fig. 2E), is the first and one of the prerequisite steps for its degradation. It seems that HAF can induce the phosphorylation followed by ubiquitination of IκB in some way, resulting in the activation of the NF-κB pathway in T24 cells. In addition, we note that total P65 was elevated either by hypoxia exposure (Fig. 1A) or by HAF-overexpression (Fig. 2A), but this elevation seems not to be inhibited by PDTC (Fig. 3B), while, the N-P65 can be inhibited by PDTC. This gives us a clue there must be some sophisticated mechanisms involved in this process. We found that HAF and NF-κB pathway play key roles in the switch of HIF-1α/β to HIF-2α/β in hypoxia, thus providing a blueprint for future investigation of this signal pathway.

Acknowledgements

This study was supported by National Natural Science Foundation of China grants (no. 81072105).

References

1. Nathke I and Rocha S: Antagonistic crosstalk between APC and HIF-lalpha. Cell Cycle 10: 1545-1547, 2011.
2. Yoo YG, Christensen J, Gu J and Huang LE: HIF-1alpha mediates tumor hypoxia to confer a perpetual mesenchymal phenotype for malignant progression. Sci Signal 4: p4, 2011.
3. Haase VH: Hypoxia-inducible factors in the kidney. Am J Physiol Renal Physiol 291: F271-F281, 2006.
4. Henze AT and Acker T: Feedback regulators of hypoxia-inducible factors and their role in cancer biology. Cell Cycle 9:  2749-2763, 2010.
5. Berr A, et al: The hypoxia-inducible factor hydroxylases bring fresh air into hypoxia signalling. EMBO Rep 7:  41-45, 2006.
6. Koh MY and Powis G: Passing the baton: the HIF switch. Trends Biochem Sci 37:  364-372, 2012.
7. Yoo YG, Christensen J and Huang LE: HIF-1alpha confers aggressive malignant traits on human tumor cells independent of its canonical transcriptional function. Cancer Res 71: 1244-1252, 2011.
8. Loboda A, Jozkowicz A and Dulak J: HIF-1 and HIF-2 transcription factors - similar but not identical. Mol Cells 29: 435-442, 2010.
9. Majmundar AJ, Wong WJ and Simon MC: Hypoxia-inducible factors and the response to hypoxic stress. Mol Cell 40: 294-309, 2010.
10. Rohwer N and Cramer T: Hypoxia-mediated drug resistance: novel insights on the functional interaction of HIFs and cell death pathways. Drug Resist Updat 14: 191-201, 2011.
11. Heddleston JM, Li Z, Lathia JD, Bao S, Hjelmeland AB and Rich JN: Hypoxia inducible factors in cancer stem cells. Br J Cancer 102: 789-795, 2010.
12. Tafani M, Schito L, Pellegrini L, et al: Hypoxia-increased RAGE and P2X7R expression regulates tumor cell invasion through phosphorylation of Erk1/2 and Akt and nuclear translocation of NF-κB. Carcinogenesis 32: 1167-1175, 2011.
13. Yamakuchi M, Yagi S, Ito T and Lowenstein CJ: MicroRNA-22 regulates hypoxia signaling in colon cancer cells. PLoS One 6: e20291, 2011.
14. Zhdanov AV, Dmitriev RI, Golubeva AV, Gavrilova SA and et al: Novel insights on the functional interaction of HIFs and cell death pathways. Drug Resist Updat 14: 191-201, 2011.
15. Rich JN: Hypoxia inducible factors and their role in cancer biology. Cell Cycle 9: 2749-2763, 2010.
16. Tafani M, Schito L, Pellegrini L, et al: Hypoxia-increased RAGE and P2X7R expression regulates tumor cell invasion through phosphorylation of Erk1/2 and Akt and nuclear translocation of NF-κB. Carcinogenesis 32: 1167-1175, 2011.
16. Koh MY, Lemos RJ, Liu X and Powis G: The hypoxia-associated factor switches cells from HIF-1alpha- to HIF-2alpha-dependent signaling promoting stem cell characteristics, aggressive tumor growth and invasion. Cancer Res 71: 4015-4027, 2011.

17. Li Z and Rich JN: Hypoxia and hypoxia inducible factors in cancer stem cell maintenance. Curr Top Microbiol Immunol 345: 21-30, 2010.

18. Yeung TM, Gandhi SC and Bodmer WF: Hypoxia and lineage specification of cell line-derived colorectal cancer stem cells. Proc Natl Acad Sci USA 108: 4382-4387, 2011.

19. Yeramian A, Santacana M, Sorolla A, et al: Nuclear factor-kappaB2/p100 promotes endometrial carcinoma cell survival under hypoxia in a HIF-1alpha independent manner. Lab Invest 91: 859-871, 2011.

20. Xue Y, Li NL, Yang JY, Chen Y, Yang LL and Liu WC: Phosphatidylinositol 3'-kinase signaling pathway is essential for Rac1-induced hypoxia-inducible factor-1alpha and vascular endothelial growth factor expression. Am J Physiol Heart Circ Physiol 300: H2169-H2176, 2011.

21. Nys K, Maes H, Dudek AM and Agostinis P: Uncovering the role of hypoxia inducible factor-1alpha in skin carcinogenesis. Biochim Biophys Acta 1816: 1-12, 2011.

22. Ghosh G, Wang VY, Huang DB and Fusco A: NF-kappaB regulation: lessons from structures. Immunol Rev 246: 36-58, 2012.

23. van Uden P, Kenneth NS, Webster R, Muller HA, Mudie S and Rocha S: Evolutionary conserved regulation of HIF-1beta by NF-kappaB. PLoS Genet 7: e1001285, 2011.

24. Sun SC: The noncanonical NF-kappaB pathway. Immunol Rev 246: 125-140, 2012.

25. Paliwal P, Arora D and Mishra AK: Epithelial mesenchymal transition in urothelial carcinoma: twist in the tale. Indian J Pathol Microbiol 55: 443-449, 2012.

26. Jing Y, Han Z, Zhang S, Liu Y and Wei L: Epithelial-mesenchymal transition in tumor microenvironment. Cell Biosci 1: 29, 2011.

27. Wu ST, Sun GH, Hsu CY, et al: Tumor necrosis factor-alpha induces epithelial-mesenchymal transition of renal cell carcinoma cells via a nuclear factor kappa B-independent mechanism. Exp Biol Med 236: 1022-1029, 2011.

28. Harhaj EW and Dixit VM: Regulation of NF-kappaB by deubiquitinases. Immunol Rev 246: 107-124, 2012.

29. Gilmore TD and Wolenski FS: NF-kappaB: where did it come from and why? Immunol Rev 246: 14-35, 2012.

30. Kanarek N and Ben-Neriah Y: Regulation of NF-kappaB by ubiquitination and degradation of the IkappaBAs. Immunol Rev 246: 77-94, 2012.

31. Brennan P and O’Neill LA: 2-mercaptoethanol restores the ability of nuclear factor kappa B (NF kappa B) to bind DNA in nuclear extracts from interleukin 1-treated cells incubated with pyrrolidine dithiocarbamate (PDTC). Evidence for oxidation of glutathione in the mechanism of inhibition of NF kappa B by PDTC. Biochem J 320: 975-981, 1996.

32. Hayakawa M, Miyashita H, Sakamoto I, et al: Evidence that reactive oxygen species do not mediate NF-kappaB activation. EMBO J 22: 3356-3366, 2003.

33. Julien S, Puig I, Caretti E, et al: Activation of NF-kappaB by Akt upregulates Snail expression and induces epithelial mesenchyme transition. Oncogene 26: 7445-7456, 2007.

34. Vaupel P and Mayer A: Hypoxia in cancer: significance and impact on clinical outcome. Cancer Metastasis Rev 26: 225-239, 2007.

35. Weidemann A and Johnson RS: Biology of HIF-1alpha. Cell Death Differ 15: 621-627, 2008.

36. Uchida T, Rossignol F, Matthay MA, et al: Prolonged hypoxia differentially regulates hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression in lung epithelial cells: implication of natural antisense HIF-1alpha. J Biol Chem 279: 14871-14878, 2004.

37. Myllyharju J and Schipani E: Extracellular matrix genes as hypoxia-inducible targets. Cell Tissue Res 339: 19-29, 2010.

38. Jiang H, Zhu Y, Xu H, Sun Y and Li Q: Activation of hypoxia-inducible factor-1alpha via nuclear factor-kappaB in rats with chronic obstructive pulmonary disease. Acta Biochim Biophys Sin 42: 483-488, 2010.

39. G-Antigiotopoulou P, et al: Clinical significance of nuclear factor (NF)-kappaB level in urothelial carcinoma of the urinary bladder. Virchows Arch 452: 295-304, 2008.

40. Gorlach A and Bonello S: The cross-talk between NF-kappaB and HIF-1: further evidence for a significant liaison. Biochem J 412: e17-e19, 2008.

41. Taylor CT and Cummins EP: The role of NF-kappaB in hypoxia-induced gene expression. Ann NY Acad Sci 1177: 178-184, 2009.

42. van Uden P, Kenneth NS and Rocha S: Regulation of hypoxia-inducible factor-1alpha by NF-kappaB. Biochem J 412: 477-484, 2008.

43. Nam SY, Ko YS, Jung J, et al: A hypoxia-dependent upregulation of hypoxia-inducible factor-1 by nuclear factor-kappaB promotes gastric tumour growth and angiogenesis. Br J Cancer 104: 166-174, 2011.