

FoxO6 inhibits cell proliferation in lung carcinoma through up-regulation of USP7

HONG-JUN HU, LI-GUO ZHANG, ZHEN-HUA WANG and XI-XI GUO

Department of Thoracic Tumor Surgery 2, Xinxiang Central Hospital, Xinxiang, Henan 453000, P.R. China

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Abstract. Emerging evidence has suggested that misregulation of oncogenes and/or tumor suppressors has a crucial role in the development of lung carcinoma. The present study demonstrated that the expression levels of forkhead box O6 (FOXO6) were downregulated in lung cancer tissue samples, as compared with those in adjacent normal tissue. Overexpression of FOXO6 inhibited the proliferation of A549 human lung cancer cells, whereas knockdown of endogenous FOXO6 expression enhanced cell proliferation. Furthermore, ectopic FOXO6 expression induced the expression of ubiquitin-specific-processing protease 7 (USP7). As a result of this regulation, FOXO6 overexpression led to an elevation of p53 protein expression levels in A549 cells. In conclusion, the results of the present study indicated that the FOXO6/USP7 molecular network has an important role in the regulation of lung cancer development.

Introduction

Lung cancer comprises two types: Small cell lung cancer and non-small cell lung cancer, and is one of the most commonly diagnosed malignancies worldwide (1,2). Previous studies have demonstrated that the development of lung cancer arises from a dysregulation of numerous oncogenes and tumor suppressors, including p53, phosphatase and tensin homolog, as well as β -catenin (3,4). However, the underlying regulatory mechanisms remain poorly understood.

Roles of the Forkhead transcription factor family (FOXO) have previously been reported in cell proliferation, differentiation, apoptosis and metabolic pathways (5). There are four members of the FOXO family in humans: FOXO1, -3, -4 and -6. The role of FOXO1 in tumor cell proliferation has been well documented (6). FOXO1 overexpression has been shown

to result in cell-cycle arrest through upregulation of p27 and p21 and downregulation of cyclin D1 (5). Furthermore, FOXO3 is also considered a tumor suppressor in numerous types of cancer, including neuroblastoma (7), as well as colon (8) and thyroid cancer (9). Previous studies have demonstrated that FOXO6 may regulate memory consolidation and synaptic function (10). Additionally, FOXO6 have been identified to promote gluconeogenesis and integrate insulin signaling with microsomal triglyceride transfer protein for the regulation of very low-density lipoprotein production in the liver (11,12). However, the functions of FOXO6 in tumorigenesis remain unclear. In the present study, the expression levels of FOXO6 in lung cancer tissue were determined by quantitative PCR and western blot analysis. Additionally, USP7, a ubiquitin-specific protease, was shown to promote p53 protein stabilization (13). Therefore, the role and mechanism of FOXO6 in the regulation of USP7 expression was further investigated.

Materials and methods

Tissue samples. A total of 30 paired primary lung cancer tissue and adjacent normal tissue samples were obtained from patients at the Department of Thoracic Tumor Surgery 2 (Xinxiang Central Hospital, Xinxiang, China). All patients provided informed consent. The present study was approved by the Institutional Review Board of Xinxiang Central Hospital. The study was approved by the ethics committee of Xinxiang Central Hospital (Xinxiang, China). Written informed consent was obtained from the patients or their families.

Cell culture and reagents. The A549 human lung cancer cells were provided by The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Invitrogen Life Technologies, Shanghai, China), 100 IU/ml penicillin (Invitrogen Life Technologies) and 100 mg/ml streptomycin (Invitrogen Life Technologies). The cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Tumor necrosis factor (TNF α (5 ng/ml), interleukin (IL)-1 β (2 ng/ml) and IL-6 (10 ng/ml) were obtained from Sigma-Aldrich. Cells were seeded into 6-well plates and treated with these cytokines or the vehicle control (PBS). Following either 24 or 36-h incubation, the cells were harvested for RNA or protein extraction.

Correspondence to: Dr Hong-Jun Hu, Department of Thoracic Tumor Surgery 2, Xinxiang Central Hospital, 56 Jinsui Avenue, Xinxiang, Henan 453000, P.R. China
E-mail: hong_junhu@126.com

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Promoter construction and luciferase assays. The 5'-regulatory sequence of the human ubiquitin-specific-processing protease 7 (USP7) promoter was cloned by polymerase chain reaction (PCR) and inserted into a pGL3 vector (Promega Corp., Madison, WI, USA) using Taq enzymes (Invitrogen Life Technologies). Site-directed mutagenesis was conducted using a PCR kit (Toyobo Co., Ltd., Osaka, Japan) with the following primer (mutation sites in bold): 5'-CCATGCTTTGGGGAACGACTACA-3'. MDM2 and YY1 expression levels were measured using quantitative PCR. All the transient transfection experiments were performed on cells seeded into 24-well plates at 70-80% confluence, using Lipofectamine[®] 2000 (Invitrogen Life Technologies), according to the manufacturer's instructions. Luciferase activities were normalized against pRL-TK activity (Promega Corporation) using the Dual Luciferase Reporter Assay system (Promega Corp.).

Small interfering (si)RNA, RNA extraction and quantitative PCR (qPCR). Non-targeting siRNA (5'-UCUAUACGGUCUACUACGG-3') and FOXO6 siRNA (5'-CAUGACUUAGCAUACGAAGUAC-3') oligonucleotides were obtained from Genepharma Co., Ltd. (Shanghai, China). Tissues were homogenized by liquid nitrogen grinding and total RNA was extracted using TRIzol[®] reagent (Invitrogen Life Technologies). First-strand complementary DNA (cDNA) synthesis was performed for each RNA sample using the Promega Reverse Transcription system (Promega Corp.). Random primers were used to prime cDNA synthesis. qPCR was performed using SYBR Green Premix Ex Taq (Roche Diagnostics, Basel, Switzerland) on a Light Cycler 480 (Roche Diagnostics). The relative quantification for each target gene was corrected to GAPDH mRNA values. The following primers were used: FOXO6 forward, 5'-GGCGGCGCTCGTATACC-3' and reverse, 5'-TACACAGGGCCGGCCG-3'; USP7 forward, 5'-GGCTCCTGGCATTAGGTCA-3' and reverse, 5'-CTGGCTAATTGTGCTGATGT-3'; p53 forward, 5'-TGGAGCCCGTGAATTAGAGC-3' and reverse, 5'-TCTGGTTCATCTTAGAAACCAC-3'; p27 forward, 5'-ACCTTAACCAAGTGTCTCCTA-3' and reverse, 5'-CCCAGCCTTGAAACAATCC-3'; p21 forward, 5'-ACTCTTCGTGCAAGGGCG-3' and reverse, 5'-GCTCAAGAAAGTGCTGATCCC-3'; and GAPDH forward, 5'-CATGTACGCCATATCCAGGC-3' and reverse, 5'-CTCCATTGATGTCACGCGGAT-3' (Biosune Co., Shanghai, China). PCR conditions included an initial holding period at 95°C for 5 min, followed by a two-step PCR program comprising 94°C for 5 sec and 60°C for 30 sec for 40 cycles. Relative quantification analysis of the gene expression results were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis. Extracts from the cells or tissues were prepared using lysis buffer containing 50 mM Tris-HCl (pH 6.8), 100 mM 2-ME, 2% w/v SDS and 10% glycerol. Proteins in the supernatants were quantified using a Bicinchoninic Acid Protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Equal amounts of protein (50 μ g) were separated by 10% SDS-PAGE. Proteins electrophoretically separated on denaturing gels were transferred to polyvinylidene difluoride membranes (Millipore,

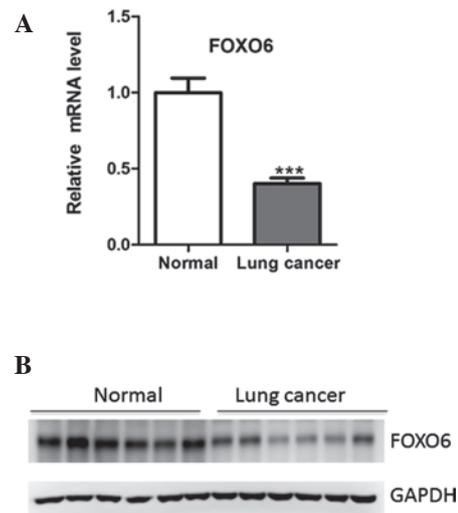


Figure 1. Downregulation of FOXO6 in lung cancer tissues. (A) mRNA and (B) protein expression levels of FOXO6 were analyzed in lung cancer and adjacent normal tissue samples by quantitative polymerase chain reaction and western blotting, respectively. FOXO6, forkhead box 6.

Bedford, MA, USA). Then membranes were blocked using 10% non-fat milk and washed by PBST solution (Beyotime Company, Nantong, China). Anti-GAPDH antibody was purchased from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA). Rabbit polyclonal anti-FOXO6 (ab48730), rabbit monoclonal anti-p53 (EPR17343), rabbit polyclonal anti-p21 (ab7960), rabbit monoclonal anti-p27 (Y236), rabbit polyclonal anti-growth arrest and DNA damage-inducible 45 (GADD45; ab105060), rabbit monoclonal anti-signal transducer and activator of transcription 3 (Stat3; EPR787Y), rabbit polyclonal anti-FOXO1 (ab70382), mouse monoclonal anti-E2F transcription factor 1 (E2F1; 8G9), rabbit monoclonal anti- β -catenin (E247) and rabbit polyclonal anti-retinoblastoma (Rb; ab6075) antibodies were all purchased from Abcam (Cambridge, MA, USA). All the antibodies were used at 1:2,000 dilution and incubated overnight at 4°C. The proteins were visualized using an enhanced chemiluminescence detection kit (GE Healthcare Life Sciences, Little Chalfont, UK).

Bromodeoxyuridine (BrdU) assay. A cell proliferation enzyme-linked immunosorbent assay (BrdU kit; Beyotime Company) was used to measure the incorporation of BrdU during DNA synthesis, according to the manufacturer's instructions. Following transfection for 24 h, BrdU was added and the cells were incubated for an additional 4 h at 37°C.

Chromatin immunoprecipitation (ChIP) assays. The A549 cells were fixed with 1% formaldehyde, followed by numerous intervals of sonication. The immunoprecipitated DNA fragments were quantified by PCR. The following primer sequences used were: Regions -600 to -400 forward, 5'-CAGCTTCGACGTCAC-3' and reverse, 5'-GACTGCAACATTGCACT-3'; and regions -3,000 to -2,800 forward, 5'-CATTAGCATCAGATCCATTA-3' and reverse, 5'-CAGCATCAGGACTTACGCA-3'.

Transcription element search system (TESS) analysis. TESS software <http://www.cbil.upenn.edu/cgi-bin/tess> was used to automatically identify potential FOXO6 motifs.

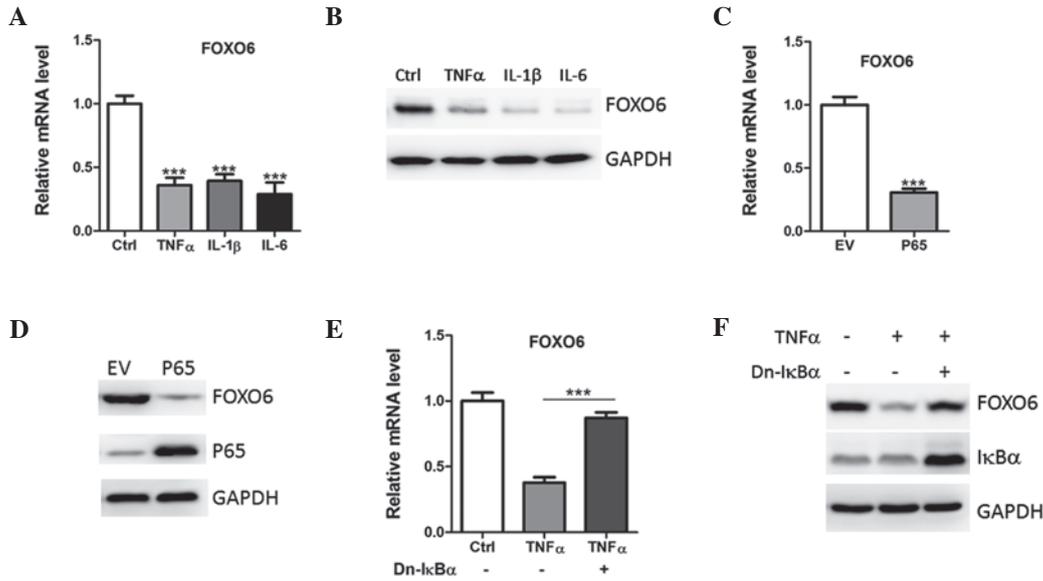


Figure 2. NF-κB activation downregulates FOXO6 expression. (A) mRNA and (B) protein expression levels of FOXO6 were analyzed in A549 human lung cancer cells treated with Ctrl or TNFα (5 ng/ml), IL-1β (2 ng/ml) or IL-6 (10 ng/ml) by qPCR and western blotting, respectively. (C) mRNA and (D) protein expression levels of FOXO6 were analyzed in A549 cells transfected with EV or p65 by qPCR and western blotting, respectively. (E) mRNA and (F) protein expression levels of FOXO6 in A549 cells transfected with dominant-negative (Dn) IκBα, in the absence or presence of TNFα (5 ng/ml). FOXO6, forkhead box O6; TNFα, tumor necrosis factor-α; IL, interleukin; EV, empty vector; Ctrl, vehicle control; qPCR, quantitative polymerase chain reaction.

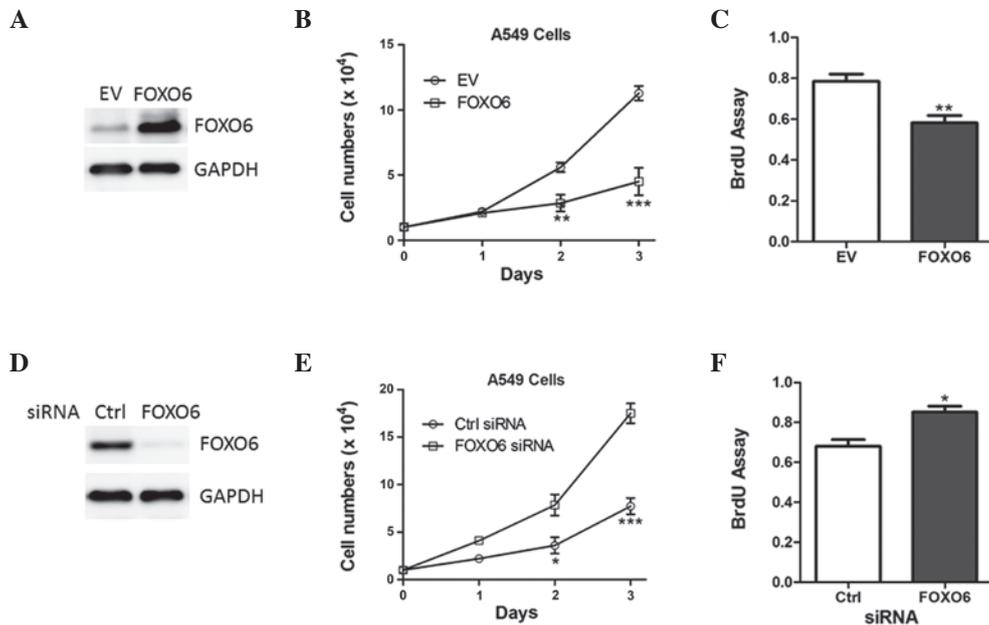


Figure 3. Effects of FOXO6 on cell proliferation. (A) FOXO6 expression was determined by western blotting in A549 human lung cancer cells transfected with EV or FOXO6. (B) Growth curve of A549 cells transfected with EV or FOXO6. (C) Cell proliferative potential was determined in A549 cells transfected with EV or FOXO6, using a BrdU assay. (D) FOXO6 expression was determined by western blotting in A549 cells transfected with siRNA oligonucleotides targeting FOXO6 or Ctrl. (E) Growth curve of A549 cells transfected with siRNA oligonucleotides targeting FOXO6 or Ctrl. (F) Cell proliferative potential was determined in A549 cells transfected with FOXO6 siRNA or Ctrl, using a BrdU assay. FOXO6, forkhead box O6; siRNA, small interfering RNA; BrdU, bromodeoxyuridine; Ctrl, scramble siRNA; EV, empty vector; qPCR, quantitative polymerase chain reaction.

Statistical analysis. Values are expressed as the mean ± standard error of the mean. Statistical analyses were performed using GraphPad software version 5.0 (La Jolla, CA, USA). Statistical differences were determined by Student's t test. P<0.05 was considered to indicate a statistically significant difference.

Results

Reduced expression of FOXO6 in lung cancer tissue. FOXO6 mRNA expression levels were initially measured by qPCR in 30 paired lung cancer and adjacent normal tissue samples. FOXO6 mRNA expression levels were significantly

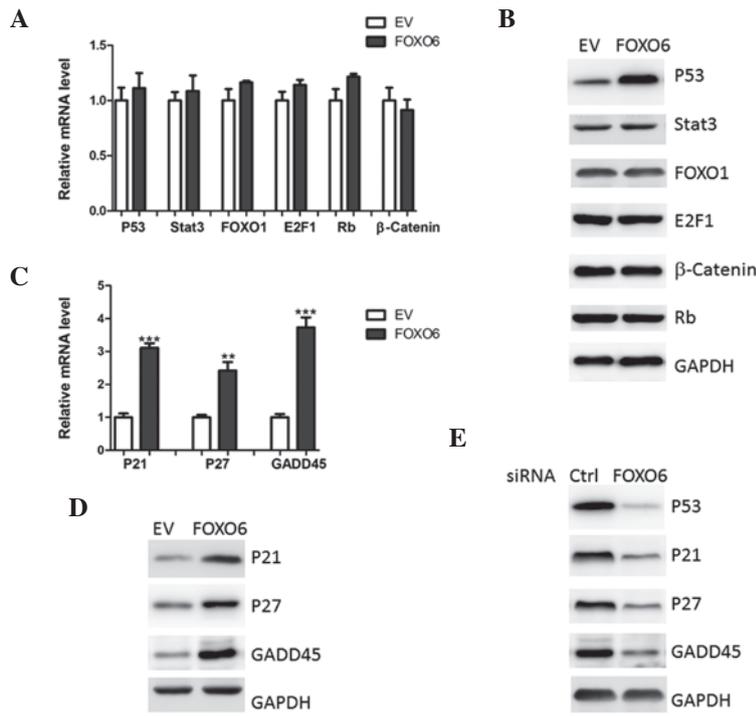


Figure 4. FOXO6 upregulates p53 protein expression levels. (A) mRNA and (B) protein expression levels of p53, Stat3, FOXO1, E2F1, Rb and β -catenin were analyzed in A549 human lung cancer cells transfected with EV or FOXO6 by qPCR and western blotting, respectively. (C) mRNA and (D) protein expression levels of p21, p27 and GADD45 were analyzed in A549 cells transfected with EV or FOXO6, by qPCR and western blotting, respectively. (E) Protein expression levels of p53, p21, p27 and GADD45 were analyzed by western blotting in A549 cells transfected with FOXO6 or Ctrl. FOXO1/O6, forkhead box O1/O6; Stat3, signal transducer and activator of transcription 3; E2F1, E2F transcription factor 1; Rb, retinoblastoma; GADD45, growth arrest and DNA damage-inducible 45; siRNA, small interfering RNA; qPCR, quantitative polymerase chain reaction; EV, empty vector; Ctrl, scramble siRNA.

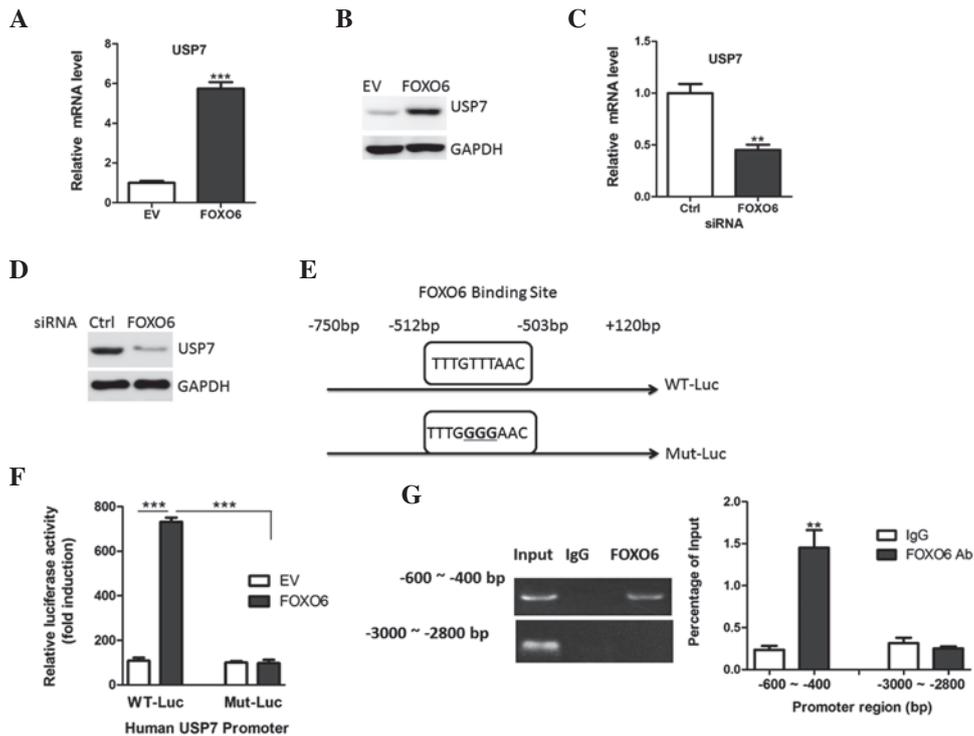


Figure 5. FOXO6 transcriptionally regulates USP7 expression. (A) mRNA and (B) protein expression levels of USP7 were analyzed in A549 cells transfected with EV or FOXO6, by qPCR and western blotting, respectively. (C) mRNA and (D) protein expression levels of USP7 were analyzed in A549 cells transfected with FOXO6 or Ctrl by qPCR and western blotting, respectively. (E) Human USP7 promoter constructs containing a potential FOXO6 motif (-512 bp to -503 bp). Point mutations were induced in the FOXO6 motif (TTTGTTTAAC to TTTGTTTAAAC). The transcription start site was set as +1 bp. (F) Promoter region from -750 to +120 bp (Wt-Luc and Mut-Luc) was cloned and co-transfected with FOXO6 expression plasmids in A549 cells. Cells were lysed and luciferase activity was determined 36 hours after transfection. (G) Chromatin immunoprecipitation assays showing the recruitment of FOXO6 onto the USP7 promoter. The promoter region from -3,000 to -2,800 bp was set as a negative control. PCR was performed to quantify the binding. FOXO6, forkhead box O6; USP7, ubiquitin-specific-processing protease 7; siRNA, small interfering RNA; qPCR, quantitative polymerase chain reaction; EV, empty vector; Ctrl, scramble siRNA; WT, wild-typ; Mut, mutated.

downregulated in the lung cancer samples, as compared with those in the adjacent normal tissue (Fig. 1A). This finding was further confirmed by western blot analysis (Fig. 1B).

Identification of FOXO6 as a downstream target of inflammation. The present study aimed to determine potential mechanisms underlying the downregulation of FOXO6. Previous studies have shown that inflammatory response-mediated nuclear factor (NF)- κ B/p65 activation is critical for the development of lung carcinoma (14). To investigate whether NF- κ B/p65 was able to regulate FOXO6, A549 cells were treated with various pro-inflammatory cytokines. Treatment with TNF α , IL-1 β and IL-6 suppressed FOXO6 mRNA and protein expression levels (Fig. 2A and B). Furthermore, FOXO6 expression was also inhibited by p65 overexpression in A549 cells (Fig. 2C and D).

To further analyze whether downregulation of FOXO6 was NF- κ B/p65-dependent, the A549 cells were transfected with dominant negative I κ B α , which inhibits the translocation of p65 from the cytoplasm to the nucleus (15). As predicted, overexpression of I κ B α reversed the inhibition of FOXO6 by TNF α (Fig. 2E and F). Therefore, it is hypothesized that the downregulation of FOXO6 may partly be due to the hyperinflammatory status of the lung cancer tissue.

FOXO6 inhibits lung cancer cell proliferation. To determine the functions of FOXO6, A549 cells were transfected with empty vector or FOXO6 lentiviruses (Fig. 3A). Overexpression of FOXO6 reduced the cell proliferation of A549 cells (Fig. 3B), which was further confirmed by a bromodeoxyuridine analysis (Fig. 3C).

In addition, endogenous FOXO6 expression in A549 cells was deleted by siRNA targeting FOXO6 (Fig. 3D). Ablation of FOXO6 increased the number of A549 cells and enhanced the cell proliferative abilities (Fig. 3E and F).

FOXO6 overexpression induces p53 protein accumulation in lung cancer cells. To investigate the molecular mechanisms of FOXO6 on cell proliferation, the expression levels of numerous oncogenes and tumor suppressors were determined. None of the transcription factors were altered at the transcriptional level in response to FOXO6 overexpression (Fig. 4A). However, western blot analysis demonstrated that p53 protein expression levels were increased in the A549 cells overexpressing FOXO6 (Fig. 4B). In addition, downstream targets of p53, including p21, p27 and GADD45 (16), were also upregulated by FOXO6 overexpression (Fig. 4C and D). Furthermore, FOXO6 deficiency resulted in reduced levels of p53, p21, p27 and GADD45 (Fig. 4E). These results indicated that FOXO6 may modulate p53 protein expression at the post-transcriptional or translational level.

FOXO6 modulates p53 protein expression through upregulation of USP7. It has been well-established that p53 protein abundance is regulated by various proteins, including USP7, mouse double minute 2 homolog (MDM2) and Yin Yang 1 (YY1) (17-19). In the present study, USP7 expression levels were markedly increased in the A549 cells with FOXO6 overexpression (Fig. 5A and B), whereas MDM2 and YY1 expression remained unaltered (data not shown). Conversely,

knockdown of FOXO6 expression inhibited USP7 expression (Fig. 5C and D).

The present study aimed to investigate the regulatory pathway involving FOXO6 and USP7. A potential FOXO6 motif (TTTGTTTAAC) was identified at -512 bp to -503 bp using TESS software (<http://www.cbil.upenn.edu/cgi-bin/tess>) (Fig. 5E). A luciferase reporter was generated containing the binding site from position -750 bp to +120 bp. USP7 promoter activity was markedly elevated by FOXO6 overexpression (Fig. 5F). However, mutation of the potential binding site completely abrogated the function of FOXO6 (Fig. 5F). ChIP assays also confirmed that FOXO6 protein could bind with the proximal promoter region in A549 cells, but not with the distal region (Fig. 5G). These results suggested that FOXO6 regulated USP7 expression at the transcriptional level.

Discussion

The present study established FOXO6 as a novel tumor suppressor in lung cancer. The results of the present study demonstrated that FOXO6 was downregulated, at least partly, by inflammatory response-mediated NF- κ B/p65 activation in lung cancer tissue. Since cytokines and growth factors secreted by tumor cells are viewed as causative factors in constitutive NF- κ B/p65 activation, these results provide an alternative role for NF- κ B/P65 in tumor cells; however, the regulatory mechanisms still require further study.

The present study showed that FOXO6 was able to upregulate USP7 expression through binding to the proximal region of its promoter. USP7 is a member of the ubiquitin-specific protease family, and was initially considered a binding partner of the herpes simplex virus protein Vmw110/ICP0 (20). Subsequent studies have indicated that USP7 is able to stabilize p53 protein, through a complex mechanism targeting both p53 and its E3 ubiquitin ligase (13). Ubiquitination-mediated modification of p53 protein may regulate its degradation, transcriptional activity and subcellular localization (21). MDM2 is an oncogenic E3 ligase for p53, which promotes p53 degradation and prevents its activation in the presence of genotoxic stress (22). Furthermore, upregulation of MDM2 has previously been observed in numerous types of human cancer, including lung and breast cancer, as well as osteosarcoma (23,24). Therefore, USP7-mediated regulation of p53 protein stability may be a potential therapeutic target for the treatment of human cancer.

In conclusion, the results of the present study indicated an important role for FOXO6 in controlling lung cancer development. Of note, Qinyu *et al* (25) demonstrated that FOXO6 promoted gastric cancer progression through trans-activation of C-myc expression. These results suggested that the roles of FOXO6 may be cell or tissue-specific. Therefore, the precise roles of FOXO6 in cancer biology require further study, using other models, such as tissue-specific FOXO6 knockout mice.

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