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USP22 acts as an oncogene by regulating the stability of cyclooxygenase-2 in non-small cell lung cancer

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ABSTRACT

The histone ubiquitin hydrolase ubiquitin-specific protease 22 (USP22) is an epigenetic modifier and an oncogene that is upregulated in many types of cancer. In non-small cell lung cancer (NSCLC), aberrant expression of USP22 is a predictor of poor survival, as is high expression of cyclooxygenase-2 (COX-2). Despite its oncogenic role, few substrates of USP22 have been identified and its mechanism of action in cancer remains unclear. Here, we identified COX-2 as a direct substrate of USP22 and showed that its levels are modulated by USP22 mediated deubiquitination. Silencing of USP22 downregulated COX-2, decreased its half-life, and inhibited lung carcinoma cell proliferation by directly interacting with and modulating the stability and activity of COX-2 through the regulation of its ubiquitination status. The findings of the present study suggest a potential mechanism underlying the oncogenic role of USP22 mediated by the modulation of the stability and activity of COX-2.

1. Introduction

Lung cancer is the leading cause of cancer-related mortality in the world, accounting for more than one million deaths each year [1]. Approximately 80%–90% of all lung cancers are non-small cell lung cancer (NSCLC) [2]. Current treatments for lung cancer include surgical resection, platinum-based chemotherapy, and radiation therapy alone or in combination. However, the disease is rarely curable and prognosis is poor, with an overall 5-year survival rate of approximately 15% [3]. Despite significant progress in the diagnosis and treatment of lung cancer, its incidence and mortality are still increasing worldwide [1].

Ubiquitin specific protease 22 (USP22) is a ubiquitin hydrolase that catalyzes the deubiquitination of histones H2A and H2B and plays an important role in the regulation of transcription [4]. USP22 is required for the transcription of Myc target genes, and its depletion leads to cell cycle arrest in G1 phase [5]. USP22 expression is upregulated in several malignancies in correlation to metastasis and poor survival [6], and is a poor prognostic factor in patients with NSCLC, bladder cancer, cervical cancer, breast cancer, salivary duct carcinoma, and papillary thyroid carcinoma [4,7]. USP22 plays an important role in tumor progression and oncogenesis. Its expression is associated with metastasis, therapy resistance, and cell cycle progression, and it is therefore considered a biomarker and therapeutic target in cancer [8].

Cyclooxygenase (COX) catalyzes the production of prostaglandins from arachidonic acid and exists in two isoforms, COX-1 and COX-2 [9]. Prostaglandin E2 (PGE2) is the predominant prostaglandin and it exerts its effect via G protein coupled receptors; it has been associated with tumor growth, immunosuppression and angiogenesis [10,11]. Overexpression of COX-2 has been detected in several cancers and is an independent predictor of poor survival in patients with NSCLC [12,13]. The levels of COX-2 are modulated by the ubiquitin/proteasome pathway, which targets misfolded or damaged proteins for degradation by the 26S proteasome through the covalent attachment of ubiquitin chains [14–16]. However, the exact mechanism underlying the ubiquitination-mediated modulation of COX-2 expression remains unclear.

In the present study, we identified COX-2 as a substrate of USP22 and showed that USP22 interacts with and regulates the stability and activity of COX-2 by modulating its ubiquitination status. Our findings suggest a potential mechanism underlying the oncogenic

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role of USP22 in lung cancer via the modulation of COX-2 expression.

2. Materials and methods

2.1. Plasmids and proteins

The cDNA for USP22 in pCMV6-XL5 was obtained from Origene Technologies and used as a template to generate pcDNA3-USP22. pcDNA3-USP22-R was generated to be resistant to USP22 SiRNA 1 using the QuikChange site-directed mutagenesis kit by changing the third base in every codon of the SiRNA sequence (Stratagene). The USP22-C185A mutation was generated using the QuikChange mutagenesis kit. C-terminal Myc-tagged COX-2, COX-1 and USP1 and N-terminal His-tagged ubiquitin were cloned into the pcDNA3 vector.

2.2. Cell culture

The human NSCLC cell lines, A549 *and NCI-H460* were maintained in DMEM media supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin and cultured at 37 °C in a 5% CO_2 atmosphere.

2.3. Screening of a USP siRNA library

The USP siRNA library for USPs (four ON-TARGET-plus siRNAs for each USP) was obtained from Dharmacon. For cell-based USP siRNA screening, the four ON-TARGET-plus siRNAs for each USP were pooled at the same concentration of 25 μM. Luciferase siRNA was used as a control (sequence: AACGUACGCGGAAUACUUCGA) and obtained from Dharmacon. Transfection of A549s cells was performed at a final concentration of 25 nM siRNA for 3 days.

2.4. siRNA transfection

For transfection of A549 *and NCI-H460* cells, 3×10^5 cells were plated in a 10-cm dish on day 0. On day 1, 50 μl of Lipofectamine 2000 (Invitrogen) was added to 1.5 ml of Opti-MEM (Invitrogen) and incubated at room temperature for 5 min (solution A). Then, 6 μl of the siRNAs were added to 1.5 ml of Opti-MEM (solution B). Solution A and solution B were mixed and incubated at room temperature for 20 min, and then added to the cells in 1 ml medium and incubated for 4 h at 37 °C and 5% CO_2. SiRNA specific for COX-2 was obtained from Origene.

2.5. In vitro translation of USP22 and USP1

Recombinant USP22 and USP1 proteins were generated using the TNT-coupled transcription/translation system according to the vendor's instructions (Promega). Briefly, a reaction mixture containing 280 μl of rabbit reticulocyte lysate, 8 μl of amino acid mixture, 16 μl of TNT reaction buffer, 8 μl of T7 polymerase, and 4 μg of pcDNA3-USP22 or pcDNA3-USP1 in a volume of 400 μl was incubated at 30 °C for 2 h. The resulting solution was used directly in the immunoprecipitation assay.

2.6. In vitro ubiquitination of COX-2

The ubiquitination of recombinant His-tagged COX-2 was performed using an ubiquitination kit (Boston Biochem). Briefly, 1 μg of His-tagged COX-2 was incubated at 37 °C for 4 h with a ubiquitin conjugation system consisting of 200 μg of HeLa S-100, 5 μM MG-132, 4 μM ubiquitin aldehyde, 600 μM ubiquitin, and 5 μl of energy regeneration solution in a total volume of 50 μl (Boston Biochem). The ubiquitinated COX-2 was purified with Ni-NTA-resin according to the manufacturer's instructions.

2.7. Western blotting

Cultured cells were collected and lysed with a lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP40, 1 mM EDTA, and a complete protease inhibitor cocktail tablet (Roche Applied Science) for 40 min on ice. The extracts were sonicated three times each for 5 s at 20 kHz. The sonicated cell extracts were centrifuged at 20,630 × g for 30 min at 4 °C, and supernatants were collected. Protein concentrations were determined by Pierce™ BCA Protein Assay Kit (Pierce Biotechnology). Equal amounts of protein (30 μg) were separated by 8% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Pierce Biotechnology) with a tank transfer system (Bio-Rad). The membranes were blocked in 5% skim milk at room temperature for 30 min and then incubated with the following primary antibodies: COX-2 (Novus Biologicals) 1:500, USP22 (Abcam, ab4812, a cross-reactive antibody to USP22-C185A) 1:500, far upstream element-binding protein 1 (FBP1, Abcam) 1:500, COX-1 (Abcam) 1:500, USP1 (Proteintech) 1:500, β-Actin (Abcam) 1:1000, at room temperature for 90 min. The bound primary antibody was detected by a secondary horseradish peroxidase-linked appropriate species antibody preparation. Signal detection was performed using an enhanced chemiluminescence system (Santa Cruz Biotechnology).

2.8. Immunoprecipitation

Anti-Myc (20 μg), anti-USP22 (30 μl), or control (30 μl) antibody was incubated with a 30-μl bed volume of protein A/G-agarose beads (Santa Cruz Biotechnology) in 200 μl of phosphate buffered saline (PBS) at room temperature for 60 min. The beads were washed three times with PBS and incubated with 0.5 mg of cell lysate at 4 °C for 4 h in radioimmune precipitation assay (RIPA) buffer (Cell Signaling Technology). The beads were next washed with RIPA buffer three times, resuspended in loading buffer, and analyzed by immunoblotting.

2.9. Enzyme immunoassay for PGE2

A549 cells plated on 12-well plates (1.5×10^5/well) were transfected with 1.5 nM control RNA plus 80 ng of pcDNA3, 1.5 nM USP22 SiRNA 1 plus 80 ng of pcDNA3, or 1.5 nM USP22 SiRNA 1 plus 80 ng of pcDNA3-COX-2 on day 1 in the presence of 20 ng of pcDNA3-EGFP. The cells were transfected again on day 2 and left for another day, then incubated at 37 °C for 15 min with 1 μM arachidonic acid (Sigma) in DMEM. PGE2 concentrations in the medium were determined using a PGE2 Competitive ELISA (Pierce), according to the manufacturer's instructions.

2.10. Cell viability assay

Cell viability was evaluated by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. COX-2, USP22 and control transfectants (1×10^3 cells/well) were seeded into 96-well plates in 100 μl culture medium for 72 h before the addition of 100 μl of MTT reagent (0.5 g/l) to each well and incubation for 90 min at 37 °C. Precipitates were dissolved in DMSO and absorbance was read at 560 nm with reference of 690 nm using a Thermo iEMS Microplate Reader (Thermo Scientific).

2.11. Statistical analysis

All data were shown as means ± SD. All the data were analyzed by Student's unpaired *t*-test. $p < 0.05$ was considered significant. *The half-life of COX-2 was calculated and analyzed using Origin 8.0.*

3. Results

3.1. USP22 modulates the expression of COX-2

Because COX-2 expression is a predictor of survival in NSCLC and its levels are regulated by the ubiquitin proteasome system, we examined the possible regulation of COX-2 stability by USP22. For this purpose, a USP siRNA library was transfected into A549 *and NCI-H460* cells and the PGE2 concentrations were examined at 72 h after transfection. Among the USPs identified in the screen as capable of downregulating COX-2, we focused on USP22 because of its known role as an oncogene and predictor of poor survival in patients with NSCLC. To verify the effect of the siRNAs, the four ON-TARGET-plus siRNAs against USP22 were transfected into A549 *and NCI-H460* cells together with pcDNA3-USP22, and cell lysates were analyzed by western blotting. All four siRNAs silenced USP22 expression compared to control siRNA (Fig. 1A and D). We then examined the effect of USP22 silencing on COX-2 expression and the levels of FBP1, a regulator of the expression of key oncogenes and cell cycle associated molecules such as c-Myc and p21 [17,18], and a substrate of USP22 whose levels are controlled by ubiquitination [19]. Our results showed that USP22 silencing downregulated COX-2 and FBP1 in A549 *and NCI-H460* cells compared to control siRNA (Fig. 1B and E). To examine the specificity of USP22 against COX-2, A549 cells were co-transfected with siRNA against USP22 and a plasmid encoding siRNA resistant USP22 (USP22R). The results of western blot analysis showed that USP22R restored the levels of USP22 and COX-2 downregulated by USP22 siRNA (Fig. 1C and B). The effect of USP22 knockdown on the stability of COX-2 was examined by transfection of A549 cells with control siRNA or USP22 siRNA for 72 h and treatment with cycloheximide for different times. Western blot analysis showed that *USP22 siRNA significantly reduced the half-life of COX-2 in A549 cells from 2.5 ± 0.57 h to 1.18 ± 0.34 h* (Fig. 1G and H, $p < 0.05$). Ectopic expression of USP22 upregulated the levels of COX-2, whereas expression of the catalytically inactive C185A USP22 mutant had no effect, confirming that USP22 regulates the stability of COX-2 in A549 cells (Fig. 1I).

3.2. COX-2 is a substrate of USP22

To examine the potential interaction between USP22 and COX-2, A549 *and NCI-H460* cells were transfected with vectors expressing USP22 and Myc-tagged COX-2 and cell lysates were immunoprecipitated against anti-Myc or control IgG antibodies. The results showed that USP22 co-immunoprecipitated with COX-2 (Fig. 2A and D). Similar results were obtained when lysates were immunoprecipitated with anti-USP22 antibodies (Fig. 2B and E). To verify the direct interaction between USP22 and COX-2, recombinant USP22 was incubated with His-tagged COX-2 or COX-1 and proteins were isolated by affinity chromatography. As shown in Fig. 2C, USP22 was pulled down together with COX-2 but not with COX-1, confirming the specificity of the interaction between USP22 and COX-2. To examine whether COX-2 is a substrate of USP22, full

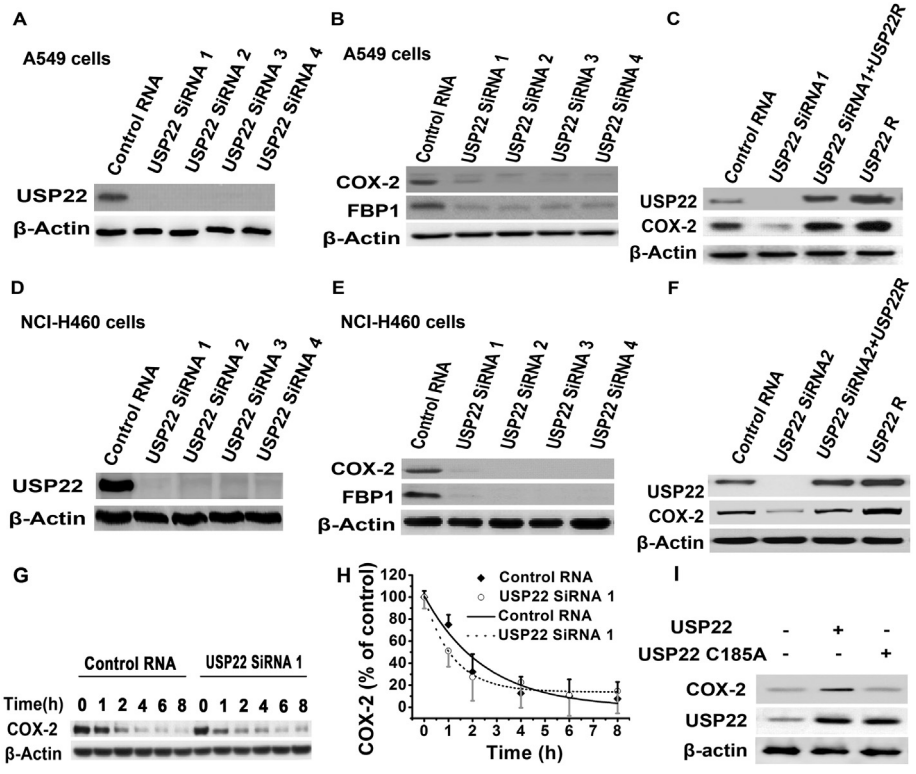

Fig. 1. Effect of USP22 silencing on COX-2 expression. A549 *and NCI-H460* cells were transfected with pcDNA3-USP22 and control SiRNA or ON-TARGET-plus USP22 SiRNA for 24 h. The levels of USP22 (A and D), COX-2 and FBP1 (B and E) were analyzed by western blotting with β-actin as the loading control. (C and F) A549 cells were transfected with control RNA or *two different USP22 SiRNAs* in the absence or presence of pcDNA3-USP22-R for 72 h, and the expression of COX-2 and USP22 was analyzed. (*G*), A549 cells were transfected with USP22 SiRNA for 72 h and then treated with cycloheximide for the indicated times. COX-2 levels were analyzed by western blotting. (H) Quantified to determine the half-life of COX-2 in D. n = 3. (I) A549 cells were transfected with pc-DNA3.1 (−) Myc-COX-2 and pcDNA3-USP22 or pcDNA3-USP22-C185A for 24 h. COX-2 and USP22 levels were analyzed by western blotting.

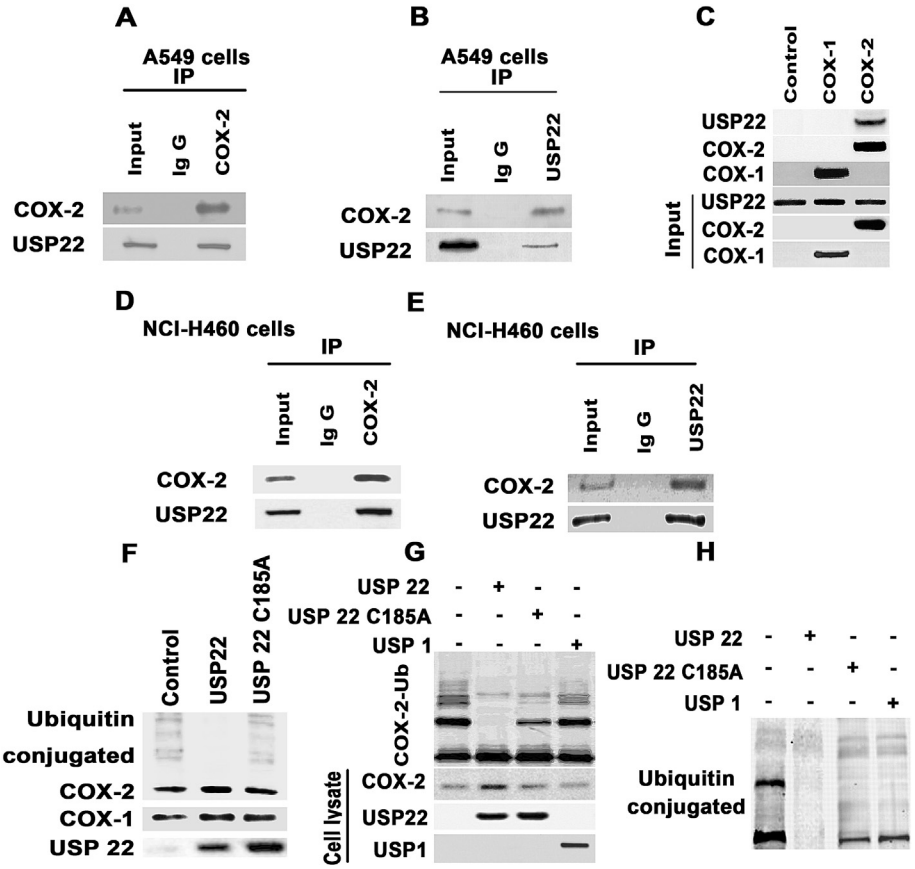

Fig. 2. USP22 interacts with COX-2 in lung carcinoma cells. A549 (A and B) and *NCI-H460* (D and E) cells were transfected with pcDNA3-USP22 and pcDNA3.1 (−) Myc-His-COX-2 for 24 h, and then treated with 20 μM MG-132 for 4 h and subjected to immunoprecipitation against rabbit IgG or anti-Myc or anti-USP22 antibody and immunoblotting against USP22 and Myc. (C) Recombinant USP22 was generated using the TNT-coupled transcription/translation system, incubated with buffer alone or His-tagged COX-2 or COX-1 and subjected to Ni-NTA affinity chromatography followed by immunoblotting against COX-2, COX-1, and USP22. (F) USP22 activity assays were performed using purified ubiquitinated His-tagged COX-2 and either USP22 or USP22-C185A followed by immunoblotting against COX-2 and USP22. (G and H) A549 cells were transfected with pcDNA3-COX-2 and pcDNA3-His8-ubiquitin alone or in the presence of pcDNA3, pcDNA3-USP22, pcDNA3-USP22-C185A, or pcDNA3-Myc-USP1. Cells were treated with 30 μM MG-132 for 4 h and ubiquitinated proteins were purified using Ni-NTA beads and immunoblotted against COX-2, USP22, USP1(G). Cell lysates prepared in E were also blotted with anti-ubiquitin antibody (F).

length His-tagged COX-2 was ubiquitinated and incubated with USP22 or the catalytically inactive mutant USP22 C185A. Western blot analysis showed that incubation with USP22, but not with USP22 C185A, resulted in the removal of ubiquitin conjugated to COX-2 (Fig. 2F). To confirm the specificity of USP22 for the deubiquitination of COX-2, the effect of another deubiquitinating enzyme, USP1 was determined using ubiquitination assays. As shown in Fig. 2G, the levels of ubiquitinated COX-2 were reduced from control levels in the presence of US22, whereas ubiquitinated COX-2 was increased in the presence of USP1 or USP22 C185A. Cell lysates were also probed for COX-2, USP22 and USP1 (Fig. 2G, lower panel) and against an anti-ubiquitin antibody, which showed similar levels of ubiquitinated proteins in these lysates (Fig. 2H). Taken together, these results indicate that COX-2 is a substrate for USP22 and its stability is regulated by USP22 mediated deubiquitination.

3.3. USP22 modulates COX-2 stability and activity and their effect on cell proliferation

To further examine the role of USP22 in tumorigenesis via its regulation of COX-2, we assessed the viability of A549 cells in which the expression of USP22 and COX-2 was knocked down via siRNA. Ectopic expression of COX-2 restored COX-2 levels in cells treated with USP22 siRNA (Fig. 3A). Cell viability assessed with the MTT assay showed that knock-down of USP22 and COX-2 significantly reduced cell viability to approximately 63% and 47%, respectively, of control cells, whereas overexpression of COX-2 restored cell viability to above control levels when cells were co-treated with USP22 siRNA and a plasmid overexpressing COX-2 ($P < 0.01$, Fig. 3B), indicating that USP22 affects cell viability via the modulation of COX-2 levels. To determine the effect of USP22 on the function of COX-2, we analyzed the levels of the proinflammatory prostaglandin PGE2. Silencing of USP22 or COX-2 significantly decreased the production of PGE2, whereas overexpression of COX-2 restored PGE2 levels downregulated by USP22, confirming that USP22 modulates COX-2 expression and activity (Fig. 3C).

4. Discussion

In eukaryotic cells, protein levels need to be tightly regulated to maintain homeostasis. The ubiquitin proteasome system is one of the mechanisms responsible for protein clearance, which among other functions, serves to prevent the activation of molecular signaling pathways such as those leading to cancer development [20]. Deubiquitinating enzymes (DUBs) catalyze the removal of ubiquitin from proteins and are subdivided into cysteine proteases and metalloproteases. USPs, which belong to the first group, are the largest family, with more than 60 members identified to date [21]. USPs contain short well-conserved cysteine and histidine boxes

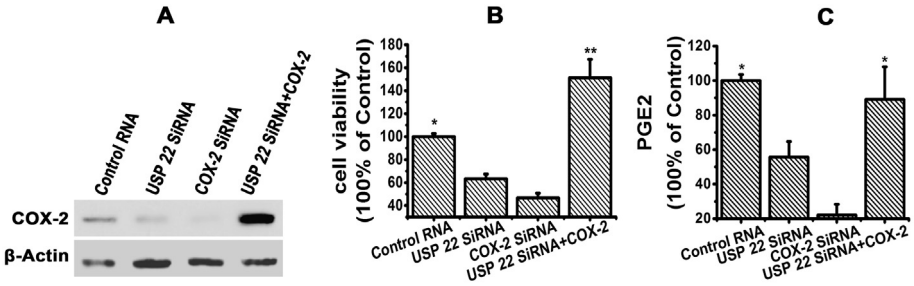

Fig. 3. USP22 silencing-induced inhibition of cell proliferation and prostaglandin E2 production are rescued by COX-2. A549 cells were transfected with siRNAs against USP22 and COX-2 in the presence or absence of a plasmid expressing COX-2. Cell lysates were analyzed by immunoblotting against COX-2 with β-actin as the loading control (A). Cell viability was determined by the MTT assay (B) and PGE2 levels were analyzed by ELISA (C). *, $p < 0.05$, **, $p < 0.01$. Versus the USP22 SiRNA groups.

essential for catalytic activity separated by variable sequences whose amino acid divergence may contribute to their broad substrate specificity. DUBs are involved in many biological processes and their role in human malignancies is just beginning to be understood [22]. Several DUB inhibitors have been developed and are being tested as potential anti-cancer agents. However, the development of USP inhibitors is hindered by their lack of specificity, and the identification of specific substrates for different USPs is therefore essential for the development of effective drugs targeting these important enzymes [21]. USP22 has been implicated in the development and progression of various malignancies including NSCLC; however, its mechanism of action in cancer remains unclear. Here, we identified COX-2 as a substrate of USP22 and investigated the role of USP22 in tumorigenesis via the modulation of COX-2 stability and activity.

In the present study, we showed that silencing of USP22 downregulated COX-2 and decreased its half-life in A549 lung carcinoma cells. USP22 was shown to interact with COX-2 and regulate its stability and activity through the modulation of its ubiquitination status. The modulation of COX-2 expression has been investigated as a potential strategy for the treatment of cancer based on studies showing that prolonged use of nonsteroidal anti-inflammatory drugs, which target COX enzymes, reduces the incidence of lung cancer [12]. The selective COX-2 inhibitor celecoxib has been approved for the treatment of patients with familial adenomatous polyposis. Celecoxib has been suggested as a treatment for lung cancer and as an adjuvant to classical chemotherapeutics in NSCLC [23]. However, the effects of COX-2 upregulation and increased PGE2 production are not clear, as COX-2 upregulation stimulates cell growth but also causes cell cycle arrest, and PGE2 inhibits cell growth in several cancers but also increases cell motility and has growth promoting activity via transactivation of the EGF receptor [24]. COX-2 is a membrane-bound protein that resides in the endoplasmic reticulum (ER) and its degradation by the UPS is preceded by its translocation from the ER to the cytoplasm via the ER-associated degradation system [25]. The stability of COX-2 was shown to be regulated by the COP9 signalosome, a multi-subunit complex that regulates the stability of proteins by affecting their ubiquitination [26]. The modulation of COX-2 levels by ubiquitination and the importance of COX-2/PGE2 signaling in lung cancer have been demonstrated; therefore, our findings indicating the modulation of COX-2 levels by USP22 are relevant, although additional experiments are necessary to further examine the relationship between USP22 upregulation and COX-2 in NSCLC.

The identification of USP22 substrates is important to understand its mechanism of action in relation to tumorigenesis. USP22 removes ubiquitin moieties from histones H2A and H2B, and regulates the expression of c-Myc target genes [5,27]. The modulation of histone H2B ubiquitination by USP22 was shown to underlie the processing of JAK-STAT-inducible genes, suggesting a potential mechanism of USP22 in cancer [28]. FBP1 was identified as a substrate of USP22 and the USP22 mediated deubiquitination of FBP1 modulates its recruitment to target loci and the regulation of gene expression, suggesting a potential indirect mechanism underlying the oncogenic action of USP22 via the modulation of transcription [19]. USP22 interacts with MDMX and leading to the inhibition of p53-dependent apoptosis [29]. Despite the well documented oncogenic role of USP22 and its upregulation in several malignancies, the identification of USP22 substrates has been limited and its mechanism of action remains to be elucidated. The present findings showing the modulation of COX-2 and PGE2 expression by USP22 mediated deubiquitination of COX-2 and its effect on cell viability in lung carcinoma cells suggests a potential mechanism underlying the oncogenic role of USP22. Future studies should be aimed at further exploring the effects of USP22 silencing on cell proliferation, invasion and metastasis in vitro and in vivo, as well as confirming the involvement of the COX-2/PGE2 axis in the effects of USP22 on tumorigenesis.

Conflict of interest

None declared.

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