p38 mitogen-activated protein kinase inhibits *USP22* transcription in HeLa cells

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Abstract. Elevated expression of ubiquitin-specific processing enzyme 22 (*USP22*) was identified in multiple types of human cancers, and was correlated with tumorigenesis and progression. Despite an increase in the numbers of studies in the physiological function of *USP22*, little is known regarding the regulation of its expression. The 5' flanking sequence of the *USP22* gene was recently characterized. In the present study, *USP22* transcription was regulated by p38 mitogen-activated protein kinase (MAPK). Treatment of human cervical carcinoma (HeLa) cells with SB203580, an inhibitor of p38 MAPK, enhanced basal *USP22* promoter activity and mRNA abundance. Transfection of MAPK kinase 6 (MKK6), an upstream activator of p38 MAPK, resulted in a 40% decrease in *USP22* mRNA, while the dominant negative MKK6 increased the transcription level of the *USP22*, similar to SB203580. Dual luciferase report assays showed that mutations of the Sp1 binding site ahead of the transcription start site abolished the promoting effect of the *USP22* promoter by SB203580. Cisplatin, the activator of p38 MAPK, also suppressed *USP22* expression. This suppression was blocked by SB203580. In conclusion, p38 MAPK acts as an upstream negative regulator of *USP22* transcription in HeLa cells.

Introduction

Ubiquitin-specific processing enzyme 22 (*USP22*) is a novel deubiquitinating enzyme that can cleave ubiquitin (Ub) from Ub-conjugated protein substrates (1). As the subunit of the human SAGA coactivator complex, *USP22* is linked to the regulation of gene transcription by deubiquitinating histones H2A and H2B (2,3). In addition, *USP22* deubiquitinates intracellular protein, including the shelterin protein telomeric repeat binding factor 1 (4), the histone deacetylase sirtuin 1 (5) and the

far upstream element-binding protein 1 fructose-1,6-bisphosphatase 1 (4), and therefore performs an extensive physiological function. A murine study showed that *USP22* also regulates embryonic stem cell differentiation (6). In humans, the *USP22* gene is located on chromosome 17, consists of 14 exons, and is transcribed and produced broadly across various tissues (7). Of note, elevated levels of *USP22* have been identified in numerous types of human cancer, including colorectal (8) lung (9) and breast cancer (10). *USP22* has been indicated in tumorigenesis. Deletion of *USP22* leads to the accumulation of cells in the G_1 phase of the cell cycle (2). For these reasons, *USP22* is a putative cancer stem cell marker. Reducing the rate of *USP22* expression may be a suitable target for cancer therapy (11). However, the mechanisms that lead to *USP22* transcriptional activation, particularly in the human tumor cells, remain unknown.

Previously, *USP22* transcription was activated by mitogen stimulation or viral infection in normal T and B lymphocytes (12), suggesting the regulation of *USP22* gene expression occurs mainly at the transcriptional level. However, the mechanism in which signal transduction pathways regulate *USP22* transcription is unclear. It is well-known that activation of the mitogen-activated protein kinase (MAPK) pathways is the main downstream event in response to mitogen stimulation. Three activated subgroups of MAPKs: Extracellular signal-regulated kinases (ERKs), p38 MAPK and c-Jun N-terminal kinases (JNKs), regulate diverse cellular responses, including cell proliferation, differentiation, survival, the inflammatory response and even cell death (13).

In the present study, p38 MAPK was involved in the regulation of *USP22* transcription, but ERKs and JNKs were not. The chemotherapeutic agent cisplatin suppressed the *USP22* gene partly through p38 MAPK. These results provide novel insights on the molecular mechanisms underlying *USP22* expression.

Materials and methods

Cell cultures. Human cervical carcinoma (HeLa) cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and were cultured in Dulbecco's modified Eagle's medium from Invitrogen (Carlsbad, CA, USA) supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. Cisplatin and MAPK inhibitors U0126, SB203580 and SP600125 were from Sigma-Aldrich (St. Louis, MO, USA).

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Plasmid constructs. The *USP22* promoter fragments inserted into pGL-3 were constructed as described previously (14). Site-directed mutagenesis was carried out within the *USP22* basic promoter p-210/+52 according to the manufacturer's instructions for the MutanBEST kit (Takara Bio Inc., Otsu, Shiga, Japan). Mutagenic primer pairs used for the polymerase chain reaction (PCR) amplification included 5'-GTAG CGTAATCTCCGTCCGC-3' for the CREB/ATF-binding site mutagenesis, 5'-CCTGTAGGCTCTGGGTAGAC-3' for the MYB-binding site mutagenesis, 5'-GGATCGGTG CCTGCCTTGCA-3' for the Sp1-binding site (-7/-12) mutagenesis (complementary reverse primers are not shown, and mutated nucleotides are underlined). All the mutations were confirmed by DNA sequencing. The MAPK kinase 6 (MKK6) expression plasmid and dominant negative MKK6 (DN MKK6) plasmid were provided by Professor Jiahuai Han of Xiamen University (Xiamen, Fujian, China).

Transfections and dual luciferase reporter assay. Cells ($1x10^4$) were plated in 24-well plates 12 h before transfection with 0.5 μg of various *USP22* promoter constructs and 0.1 μg pRL-TK (Promega Corp., Madison, WI, USA) using Lipofectamine 2000 (Invitrogen) in each well. Twenty-four hours after transfection, cells were washed in phosphate-buffered saline and lysed for 30 min at room temperature using the passive lysis buffer (Promega Corp.). Luciferase activity was determined using the dual luciferase reporter assay system (Promega Corp.). The normalized luciferase activity was expressed as the ratio of firefly luciferase activity to Renilla luciferase for each sample. All the transfection experiments were repeated four times.

Total RNA isolation and quantitative PCR (qPCR). Total RNA from cells treated with agents was prepared using TRIzol according to the manufacturer's instructions (Invitrogen). RNA was reverse-transcribed with oligo-dT primers using an RNA PCR kit (AMV) ver. 3.0 (Takara Bio Inc.), and the cDNA fragments were analyzed by qPCR using the SYBR-Green PCR Master mix (Toyobo Co., Ltd., Osaka, Japan) on an ABI 7500 real-time PCR System (Applied Biosystems, Foster City, CA, USA). *USP22* primer pairs were: Forward, 5'-ACC ACCACGCTCACGGACTG-3'; and reverse, 5'-TTGGCTGAG TGTTCAAATCG-3'; P21 primer pairs were: Forward, 5'-GCA GATCCACAGCGATATCC-3'; and reverse, 5'-CAACTGCTC ACTGTCCACGG-3'. GAPDH primers were: Forward, 5'-AGAAGGCTGGGGCTCATTTG-3'; and reverse, 5'-AGG GGCCATCCACAGTCTTC-3'.

Western blot analysis. Cells were lysed with 1X SDS sample buffer. Protein was separated by 10% SDS-PAGE and electroblotted onto nitrocellulose membrane. The membrane was subsequently incubated with anti-USP22 antibody or anti-GAPDH antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and developed using the electrochemiluminescence system (Pierce Biotechnology, Inc., Rockford, IL, USA), according to the manufacturer's instructions.

Flow cytometry. Cells ($1x10^5$) were seeded in 6-well plates overnight and subsequently SB203580 or dimethyl sulfoxide (DMSO) was added. After 12 h, the medium was changed for medium containing cisplatin for a further 12 or 24 h incubation. The cells were harvested by trypsinization, washed with 1X PBS and resuspended in 50 μl of 1X PBS. Cell death was assessed using flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) following staining with fluorescein isothiocyanate-Annexin V and propidium iodide.

Statistical analysis. Numerical data are presented as the mean ± standard error of the mean. Statistical differences between sample means were determined using the unpaired, two-tailed Student's t-test. The significance level was set at α<0.05, and therefore, $P<0.05$ was considered to indicate a statistically significant difference.

Results

p38 MAPK regulates USP22 promoter activity. Pharmacological reagents that inhibit MAPK pathways with different specificity were used to determine whether MAPK signaling is involved in the regulation of *USP22* expression: U0126, SB203580 and SP600125. These selectively inhibit ERK1/2, p38 MAPK and JNK, respectively. HeLa cells were transfected with the reporter construct, pGL-210/+52, which contains the *USP22* basic promoter region from -210 to +52 (14). The transfected cells were cultured for a further 12 h in the presence of 2 μM U0126, 2 μM SB203580, 10 μM SP600125 and 0.1% DMSO as blank controls, and luciferase activity was measured. As shown in Fig. 1A, incubation of 2 μM SB203580 resulted in an ~150% induction of luciferase activity relative to DMSO ($P<0.05$). However, treatment of SP600125 and U0126 did not affect the luciferase activity in the tested cells ($P>0.05$).

To further confirm the induction effects and localize the region in the promoter responsible for this enhancing effect by SB203580, reporter vectors containing a series of 5' terminal deletion constructs of the *USP22* promoter were transfected into HeLa cells and treated with 2 μM SB203580 for 12 h. Luciferase activity showed that SB203580 enhancing *USP22* promoter activity was observed with constructs pGL-2828/+52, pGL-886/+52 and pGL-210/+52, but not with construct pGL-7/+52 (Fig. 1B), suggesting a certain degree of promotion by SB203580 may be mediated through response elements located within the -210/-7 domain. Subsequently, pGL-210/+52 was transfected and incubated with SB203580 for different periods. As shown in Fig. 1C, SB203580 induced *USP22* promoter activity but this was not an early event. Significant increases in promoter activity (~150%) occurred after 12 h incubation.

MKK6 phosphorylates and activates p38 MAPK (15). To confirm whether MKK6 regulates *USP22* expression, DN MKK6 and pGL-210/+52 were transiently co-transfected into HeLa cells. A luciferase assay showed that expression of DN MKK6 increased transcription of *USP22* by 150%, which was similar to the action of SB203580. By contrast, forced expression of a constitutively active MKK6 had a significant inhibitory effect on *USP22* promoter activity (Fig. 1D). All these results demonstrated that p38 MAPK is involved in the regulation of *USP22* promoter activity.

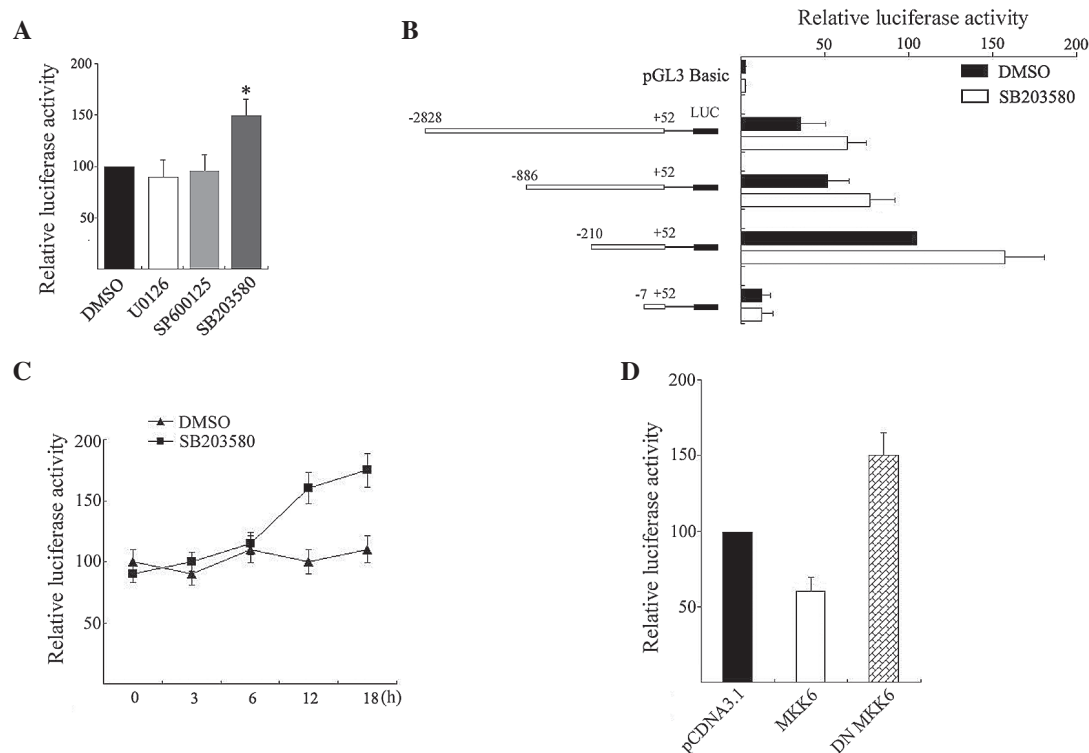*p38 MAPK regulates endogenous USP22 expression*. As the p38 MAPK pathway is involved in regulating *USP22*

Figure 1. p38 MAPK regulated *USP22* promoter activity. (A) Treatment with 2 μM U0126, 2 μM SB203580, 10 μM SP600125 or vehicle DMSO (-) was added to HeLa cells transfected with 0.5 μg *USP22* promoter construct pGL-210/+52 and 0.1 μg pRL-TK. The cells were harvested and assayed for luciferase. The normalized relative luciferase activity for pGL-210/+52 treated with DMSO was set at 100. The p38 inhibitor SB203580 markedly enhanced (*P<0.05 compared to DMSO treated) pGL3-210/+52 activity. (B) HeLa cells were transiently transfected with reporter vectors containing series of 5' terminal deletion constructs of the *USP22* promoter and treated with 2 μM SB203580. After 12 h, cells were harvested and assayed for luciferase. The normalized relative luciferase activity for pGL-210/+52 treated with DMSO was set at 100. (C) HeLa cells were transfected with pGL-210/+52 and incubated with SB203580 for different periods. Cells were harvested and assayed for luciferase. The normalized relative luciferase activity for pGL-210/+52 treated with DMSO for 0 h was set at 100. (D) Effects of MKK6 and DN MKK6 on pGL-210/+52 reporter construct. Cells were transiently transfected with 0.5 μg of reporter construct plus 1.0 μg of pCDNA3.1 (empty vector), or MKK6 or DN MKK6. After 24 h, the transfected cells were analysed for luciferase activity. The normalized relative luciferase activity obtained in cells transfected with pGL-210/+52 and pCDNA3.1 was set at 100. MAPK, mitogen-activated protein kinase; *USP22*, ubiquitin-specific processing enzyme 22; HeLa, human cervical carcinoma; MKK6, MAPK kinase 6; DN MKK6, dominant negative MKK6.

promoter activity, the effects of p38 MAPK on endogenous *USP22* expression were assessed. As shown in Fig. 2A, treatment of HeLa cells with 2 μM SB203580 for 12 h significantly increased *USP22* mRNA expression by ~160% as examined using qPCR. Consistent with the PCR results, USP22 protein levels were also increased by SB203580 incubation (Fig. 2B).

MKK6 and DN MKK6 were transfected into HeLa cells and *USP22* mRNA expression was quantitated. As shown in Fig. 2C, forced expression of MKK6 decreased endogenous *USP22* mRNA and DN MKK6 increased the expression.

p21, a cyclin-dependent kinase inhibitor, is repressed by *USP22* (4). For this reason, levels of *p21* mRNA were measured in response to SB203580. As shown in Fig. 2D, SB203580 treatment for 12 h decreased *p21* mRNA expression in HeLa cells.

To further determine whether p38 MAPK influences *USP22* mRNA stability in HeLa cells, actinomycin D was used to block *de novo* mRNA transcription. The level of mRNA was determined at different points in time using qPCR. When cells were treated with actinomycin D, the half-life of *USP22* mRNA was between 4 and 5 h. When SB203580 was added 12 h before actinomycin D treatment, the half-life of *USP22* mRNA did not change significantly and was similar to that in the cells without SB203580 treatment (Fig. 2E),

excluding the possibility that p38 MAPK influences *USP22* mRNA stability.

p38 MAPK regulates USP22 via the Sp1 binding site. As mentioned above, the region responsible for enhancing *USP22* promoter activity using SB203580 is located within the -210/-7 domain. TFSEARCH analysis showed there to be several potential transcription factor binding sites within this region, including an MYB, a CREB/ATF and a Sp1 binding site. To characterize the involvement of particular *cis* elements in response to SB203580, wild-type and mutant reporter gene constructs of the *USP22* promoters were generated. After plasmids were transfected into HeLa cells, SB203580 was incubated for another 12 h. As shown in Fig. 3, mutation of the MYB and CREB/ATF binding sites did not disrupt the enhancing effect of *USP22* promoters by SB203580. However, mutation of the Sp1 binding site (-7/-13) resulted in increased basal *USP22* promoter activity and abrogated the enhancing promoter activity treated by SB203580 (Fig. 3), suggesting SB203580 elicits promotion of *USP22* transcription through the Sp1 binding site.

Cisplatin represses USP22 expression through p38 MAPK. A previous study has shown that p38 MAPK can be activated by several anticancer reagents, such as cisplatin (16). Whether
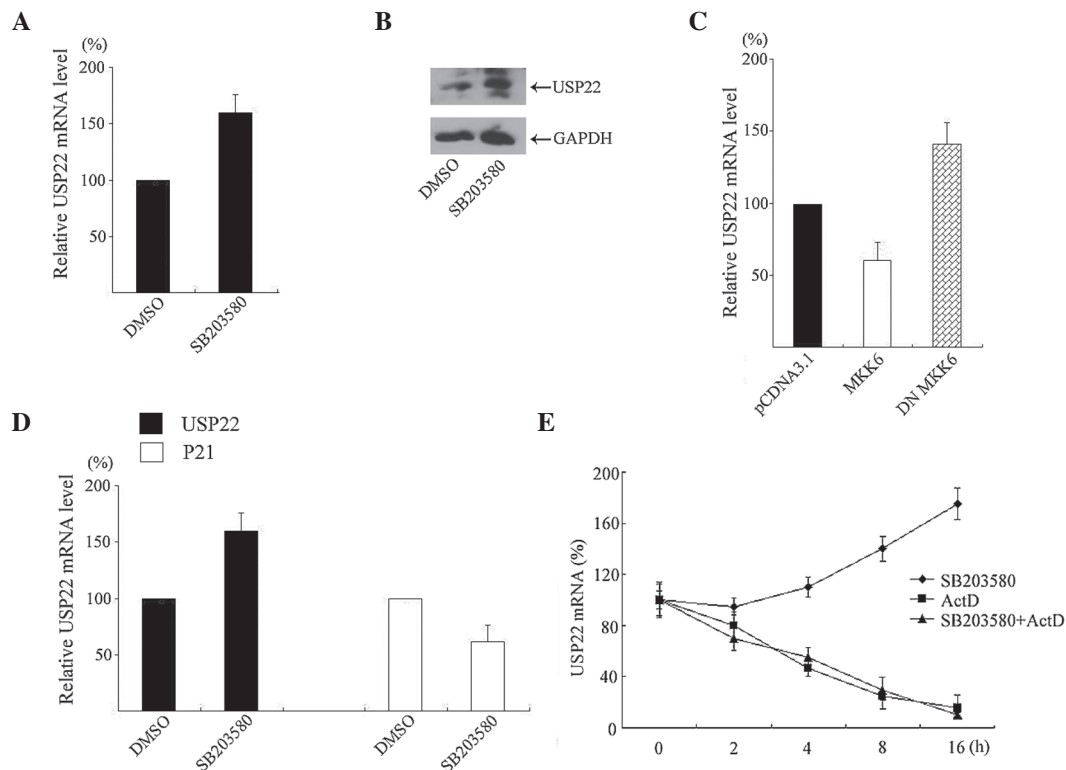
Figure 2. p38 MAPK regulated endogenous *USP22* expression. (A) SB203580 and *USP22* expression. *USP22* transcriptional levels in response to 2 μM SB203580 as examined via RT-qPCR. Data are normalized to *GAPDH* and are representative of independent triplicate experiments. (B) USP22 protein expression levels in response to SB203580 examined via western blot analysis. (C) Dominant negative MKK6 or MKK6 on *USP22* mRNA expression. (D) *p21* transcriptional levels in response to 2 μM SB203580 examined via RT-qPCR. Data are normalized to GAPDH and are representative of independent triplicate experiments. (E) Induction of *USP22* by SB203580 at the transcriptional level. HeLa cells were treated with SB203580 (2 μM), actinomycin D (10 μg/ml) or SB203580 plus ActD for different time periods. *USP22* mRNA expression was determined by RT-qPCR and normalized to *GAPDH* expression. *USP22*, ubiquitin-specific processing enzyme 22; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; HeLa, human cervical carcinoma; MKK6, MAPK kinase 6; DN MKK6, dominant negative MKK6.
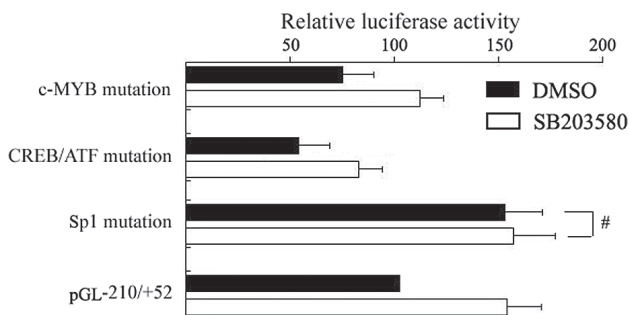

Figure. 3 Effect of mutated binding sites on the reporter assay. HeLa cells were transiently transfected with the c-MYB mutation, CREB/ATF mutation, Sp1 mutation derived from pGL-210/+52 and wild-type pGL-210/+52 for 12 h. The cells were subsequently treated with 2 μM SB203580. After 12 h incubation, cells were analyzed for luciferase activity. The normalized relative luciferase activity for pGL-210/+52 treated with DMSO was set at 100. SB203580 (2 μM) did not enhance promoter activity of Sp1 mutation (#P>0.05). HeLa, human cervical carcinoma; DMSO, dimethyl sulfoxide.

cisplatin can suppress *USP22* expression was explored. HeLa cells were treated with 30 nM cisplatin for 0, 6, 12 and 24 h and *USP22* expression at the mRNA level was analyzed using qPCR. As shown in Fig. 4A, cisplatin can induce an extremely significant decrease in *USP22* mRNA expression in HeLa cells. Significant changes can be observed after 6 h of treatment (52% inhibition).

Subsequently, HeLa cells were co-treated with cisplatin and SB203580 for 12 h. As shown in Fig. 4B, cisplatin decreased *USP22* promoter activity and this suppression was mostly restored in HeLa cells by treatment with 2 μM SB203580. Similarly, SB203580 partially restored the cisplatin-induced decrease in *USP22* mRNA (Fig. 4C). These results indicate that cisplatin can suppress *USP22* expression at the transcriptional level and this suppression is possibly mediated by p38 MAPK.

SB203580 did not protect HeLa cells from cisplatin-induced apoptosis. As overexpression of *USP22* may be a factor for therapeutic resistance, the role of p38 MAPK/*USP22* signaling was determined in the survival of cancer cells exposed to cisplatin. HeLa cells were treated with cisplatin and SB203580 in two ways and were subsequently analyzed by flow cytometry. One group of cells was pretreated with SB203580 for 12 h and subsequently cisplatin was added and allowed to incubate for another 12 or 24 h. The other was co-treated with SB203580 and cisplatin for 12 or 24 h. As shown in Fig. 5A, pretreatment or co-treatment of HeLa cells with SB203580 did not result in less cisplatin-induced apoptosis as determined by Annexin V staining. These results suggest that SB203580-induced promotion of *USP22* expression did not protect HeLa cells against cisplatin-induced apoptosis.
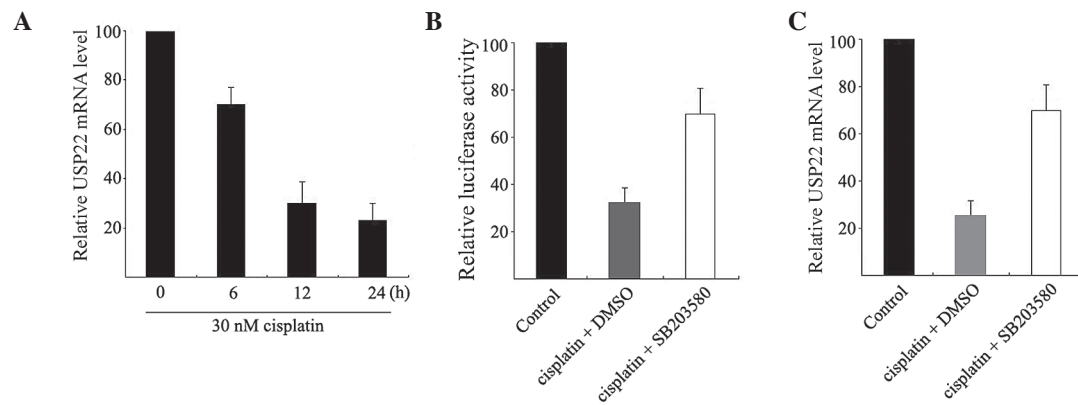
Figure 4. Effect of cisplatin on *USP22* expression. (A) *USP22* mRNA levels were measured by RT-qPCR in HeLa cells incubated for the indicated time with 30 nM cisplatin. The normalized *USP22* mRNA without cisplatin treatment was set at 100. (B) *USP22* promoter activity in response to cisplatin and SB203580 examined via the luciferase assay. (C) *USP22* transcriptional levels in response to cisplatin and SB203580 examined via RT-qPCR. USP22, ubiquitin-specific processing enzyme 22; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.
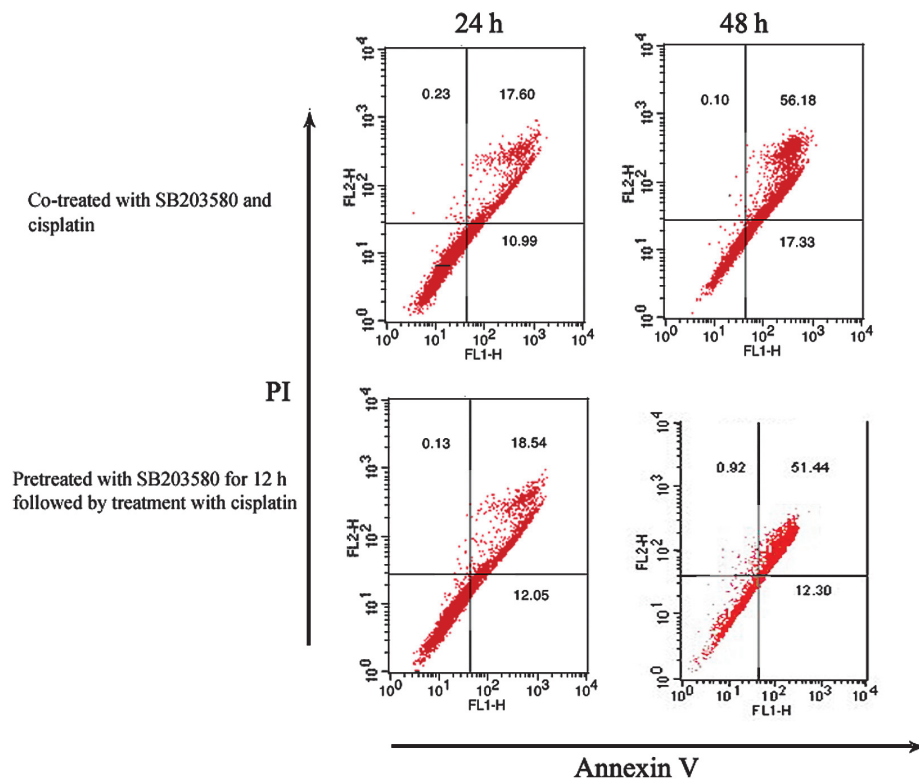

Figure 5. Effect of blocking p38 MAPK on cisplatin-induced apoptosis. HeLa cells were co-treated with SB203580 and cisplatin for 24 or 48 h or pretreated with SB203580 for 12 h and were subsequently treated with cisplatin for 24 or 48 h, and stained with PI and Annexin V. The relative numbers of apoptotic cells are indicated. MAPK, mitogen-activated protein kinase; HeLa, human cervical carcinoma; PI, propidium iodide.

Discussion

Considering the crucial role of *USP22* in carcinogenesis and tumor progression, p38 MAPK regulation of *USP22* may have important implications. Studying signaling pathways that regulate *USP22* expression may lead to identification of new therapeutic targets for cancer therapy. The present study reported that p38 MAPK acts upstream of *USP22* and plays a negative role in *USP22* transcription. p38 MAPK was found to regulate *USP22* transcription via a Sp1 binding site. In addition, cisplatin suppressed *USP22* expression in part through p38 MAPK.

One of the findings of the present study is that *USP22* transcription is controlled by p38 MAPK. Pharmacological inhibitors were used and results showed that the inhibitor of p38 MAPK enhanced *USP22* promoter activity under culture conditions but ERKs and JNKs did not. The further multiple experimental results leading to this conclusion are as follows: i) SB203580 promoted endogenous *USP22* mRNA expression, ii) transfection of the p38 activator MKK6 repressed *USP22* promoter activity and endogenous mRNA expression, and iii) transfection of the p38 inhibitor DN MKK6 enhanced *USP22* promoter activity and endogenous mRNA expression. All these results indicated that p38 MAPK acts as the

upstream regulator in regulation of *USP22* transcription. The p38 MAPK pathway is an important regulator of numerous cellular responses and the target genes regulated by activated p38 MAPK are complex. Initially, cytokine genes, such as tumor necrosis factor-α and interleukin-10 (IL-10), have been described as target genes promoted by p38 MAPK in T cells and monocytes following stimulation (17,18). Recently, p38 MAPK has received increasing attention as a tumor suppressor and its activation suppresses oncogenesis in cancer cells with different patterns (19). For example, *BMI-1*, one of 11 death signature genes as *USP22*, is reported to be downregulated by p38 MAPK by posttranscription modification (7). Cyclin D1, the oncogene, is also negatively regulated by p38 MAPK at the transcription level (20). In the present study, the possibility that p38 MAPK affects the stability of *USP22* mRNA was excluded and p38 MAPK was confirmed to regulate *USP22* at the transcriptional level. Results showed that in response to SB203580 treatment, expression of *USP22* and the key regulator of cell cycle, p21, exhibited the inverse trend. A previous study has demonstrated that p38 MAPK participates in *p21* upregulation, subsequently inhibiting cell growth (21). It is not fully understood how activated p38 MAPK can upregulate *p21* expression. One study showed that p38 MAPK stabilizes *p21* mRNA (22). Another study reported that p38 MAPK can enhance *p21* expression by promoting the transcriptional elongation (23). *USP22* acts upstream of *p21* and represses *p21* expression (4) and the present results indicate that expression of the p38 MAPK promoter *p21* may take place partially through *USP22* downregulation.

p38 MAPK regulation of gene expression is mediated by the activation of a wide range of protein kinases, transcription factors and other proteins. The present study showed that p38 MAPK-induced repression of *USP22* is mediated by a Sp1 binding site. Bioinformatic analysis showed that the 5' flank region of *USP22* is a typical feature of the TATA-less promoter and several DNA motifs are within the basic *USP22* promoter, including an Sp1, a CREB/ATF and a c-MYB binding site. Mutation of these sites has been confirmed to lead to *USP22* promoter activity changes (data not shown). The present study showed only the Sp1 binding site (GGGCGG) ahead of the transcription start site (-7 to -13) to be responsible for SB203580 treatment, as mutations at this site enhance *USP22* promoter activity and abolish the effects of SB203580 on *USP22* promoter activity. Previous studies have shown that the GGGCGG sequence is involved in regulation of *USP22* expression and is specifically bound by Sp1. Despite its history, transcription factor Sp1 is activated by phosphorylation in response to p38 MAPK. For example, it has been reported that p38 MAPK regulation of IL-10 promoters activity is via Sp1. The mechanisms by which p38 MAPK activates Sp1 to regulate *USP22* transcription will be discussed in a future study.

p38 MAPK activation is also necessary for the suppression of cancer cell growth as initiated by a variety of anticancer agents, including cisplatin (24). In the present study, cisplatin was used to activate p38 MAPK in HeLa cells. Treatment of these cells with cisplatin suppressed production of *USP22* mRNA, which was mostly accounted for by decreased promoter activity. Cisplatin exerts its cytotoxic properties by DNA strand-cross links, therefore activating downstream signaling cascades, which ultimately induce apoptosis. In the present study, cisplatin was found to repress *USP22* expression via the p38 MAPK pathway; SB203580 treatment antagonized cisplatin to repress *USP22* expression, and the decrease of the *USP22* promoter by cisplatin was also abolished by disruption of the Sp1 binding site. It has been proved that p38 MAPK is the universal sensor for cisplatin presence and the activation of p38 MAPK is required for apoptosis. Considering the p38 MAPK downregulation of *USP22*, it is not difficult to understand that cisplatin treatment suppresses *USP22* expression.

USP22 is considered as the putative cancer stem cell marker and its overexpression has been associated with therapy resistance. Therefore, it appears that enhanced *USP22* expression by SB203580 may exert anti-apoptotic effects in theory. However, previous studies showed that the role of SB203580 on cisplatin-induced apoptosis in tumor cells remains controversial. Numerous studies showed that the p38 MAPK inhibitor, SB203580, protected cells from cisplatin-induced apoptosis. However, several studies showed that SB203580 did not reverse cell apoptosis but sensitized cells to apoptosis. In the present study, pretreatment or co-treatment of SB203580 was not observed to protect HeLa cells from cisplatin-induced apoptosis, suggesting that although SB203580 enhances *USP22* expression, this is not sufficient to antagonize the cisplatin-induced apoptosis.

In conclusion, the present study provides evidence that p38 MAPK regulates *USP22* promoter activity and its expression in a human cancer cell line, and cisplatin represses *USP22* expression partly through the p38 MAPK pathway. These findings provided new insights on the molecular mechanisms underlying *USP22* expression and may have indications for developing novel therapeutic strategies.

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