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ORIGINAL ARTICLE

Upregulation of MTA1 expression by human papillomavirus infection promotes CDDP resistance in cervical cancer cells via modulation of NF-κB/APOBEC3B cascade

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Abstract

Objective Compelling evidence establishes the etiological role of viral proteins E6 and E7 of high-risk human papillomaviruses (HPV) in cervical carcinogenesis, but their contribution in chemoresistance that leads to advanced metastatic lesions remains poorly defined. Since metastasis-associated protein 1 (MTA1) upregulation and augmentation of APOBEC3B expression are both strongly associated with cervical cancer (CCa) development, and both molecules have been shown to be functionally associated with NF-κB pathway, we therefore sought to investigate the potential mechanistic link between MTA1, APOBEC3B and NF-κB during the pathogenesis of cisplatin (CDDP) resistance in HPV-positive CCa cells.

Methods MTA1 expression was assessed in HPV-transfected CCa cells using quantitative RT-PCR and immunoblotting. Effects of MTA1 deregulation on CDDP chemosensitivity in CCa cells were determined by measuring cell viability, apoptosis and in vivo oncogenic capacity. Finally, we studied the transcriptional regulation of the antiviral DNA cytosine deaminase APOBEC3B by MTA1 using multiple approaches including DNA deaminase activity assay, luciferase reporter assay, chromatin immunoprecipitation, co-immunoprecipitation and transient/stable transfection, at the molecular and functional levels.

Results Expression levels of MTA1 were significantly induced in HPV-positive CCa cells. Transduction experiments showed that the E6 oncoprotein alone was sufficient to cause MTA1 upregulation. Moreover, MTA1 knockdown potentiated CDDP sensitivity in highly metastatic CCa cells. Mechanistically, MTA1 acted as an indirect upstream modulator of APOBEC3B transcription during the pathogenesis of CDDP chemoresistance. HPV-mediated stimulation of APOBEC3B expression was accompanied by the enhanced recruitment of Iκκ α/β and p65 to the NF-κB consensus sites in the *APOBEC3B* promoter, and this recruitment was substantially abrogated by MTA1 siRNA treatment.

Conclusions These findings reveal an obligatory coregulatory role of MTA1 in the indirect regulation of APOBEC3B expression via classical NF-κB pathway, and also suggest that inhibition of MTA1/NF-κB/APOBEC3B cascade may be repositioned to suppress cancer mutagenesis, dampen tumor evolution, and decrease the probability of adverse outcomes from CDDP resistance in CCa.

Keywords MTA1 · Cervical cancer · Cisplatin · NF-κB · APOBEC3B

Introduction

Worldwide, cervical cancer (CCa) is the second most common cancer among women. Chemotherapy is currently the standard treatment for these patients. The chemotherapeutic agent cisplatin (CDDP) is so far the most effective chemotherapeutic regimen to treat recurrent or metastatic cervical cancer, but the overall chemotherapeutic effect is low due to the inevitable occurrence of drug resistance [1]. The precise mechanisms underlying this phenomenon have not been clearly elucidated up to date, but multiple mechanisms are certain to coexist to confer CDDP chemoresistance.

Qiu-ping Jia and Chang-you Yan contributed equally to this work.

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HPV is an ~ 8-kb double-stranded DNA virus. Certain types of HPV have been considered to be necessary risk factors for the development of cervical cancer. Among HPVs, HPV-16 and -18 are found in 70% of all cervical cancer cases [2]. Both HPV-16 and -18 encode viral oncoproteins E6 and E7. E7 causes the stabilization of p53 through inhibiting the ubiquitin ligase activity of Hdm-2 towards p53, whereas E6 protein binds to p53 and to the cellular ubiquitin ligase E6AP which leads to the proteasomal degradation of p53 in HPV-infected cells. Thus, p53 can no longer regulate the G1/S and G2/M checkpoints of the cell cycle. Interestingly, a recent study shows that p53 knockdown does not affect CDDP sensitivity in HPV-positive CCa cells [3]. Since almost all cervical cancers contain wild-type p53 that is abrogated by degradation through the oncoprotein E6, it is possible that other p53-independent pathways may be involved in the development of CDDP resistance.

Metastasis-associated protein 1 (MTA1), a component of the Mi-2/nucleosome remodeling and deacetylase complex, plays a crucial role in the transcriptional regulation of key molecules [4]. MTA1 is one of the most upregulated genes in human cancer. Of particular interest, MTA1 level is tightly associated with tumor progression in CCa [5]. Consistent with these clinical data, silencing MTA1 in vitro reverses the adhesion, migration and invasiveness in CCa cells [6]. These results collectively are indicative of a potent role of MTA1 in CCa. It has been recently shown that MTA1 plays a critical homeostatic role in the regulation of inflammatory responses by serving both as a target and as a component of the NF-kB signaling during the pathogenesis of lipopolysaccharide-induced inflammation [7] and acute lung injury [8]. Nevertheless, the potential role of MTA1 in other inflammatory diseases such as HPV infection is yet to be defined.

The enzymatic activity of APOBEC3B has been implicated as a major source of mutagenesis in multiple human cancers. Interestingly, CCa is among the tumor types displaying the highest APOBEC3B expression levels and activities [2]. HPV infection can induce APOBEC3B expression via regulation of the non-canonical NF-κB pathway in breast and ovary cancer [9], while other groups report that the classical NF-κB pathway is responsible for activation of *APOBEC3B* mRNA expression in breast and CCa cells [10]. These conflicting data could be related to differences in the experimental design and to the use of long-term cultures of cell lines, like HeLa cells. Alternatively, the modulation of *APOBEC3B* expression by different stimuli may be based on the cellular context.

Because MTA1 plays a pivotal homeostatic role in the regulation of inflammatory responses, and because persistent HPV infection is a high-risk factor for the development of CCa, a major unresolved question is whether these two factors regulate the CCa progression in a coordinated manner. A mechanistic study comprising of multiple approaches was therefore designed to elucidate the potential links between this chromatin modifier and the aggressive nature of the chemoresistant CCa cells. Moreover, since MTA1 upregulation and augmentation of APOBEC3B expression are both strongly associated with CCa pathogenesis, and both signaling pathways have been shown to be functionally associated with NF-κB signaling, we then evaluated the potential mechanistic link between MTA1 and APOBEC3B.

Materials and methods

Cell treatment

The CCa cells, including C-33 A (HPV negative), Ca Ski and SiHa (HPV-16 positive), and HeLa and C4-I (HPV-18 positive), were obtained from ATCC (Manassas, VA, USA). Cells were cultuerd in E-medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin under 5% CO_2. C-33 A cells were transfected with the HPV genome using full-length HPV genomes from wild-type HPV-16 (p1322 HPV-16, Addgene, Cambridge, MA, USA) and HPV-18 (p2092 HPV-18, Addgene) and from HPV-18 containing a specific reading frame for E6 (MSCV-C 18E6, Addgene) or E7 (MSCV-C 18E7, Addgene), as described elsewhere [2]. 2×10^5 C-33 A were plated in low-Ca^{2+} incomplete E-medium one day prior to transfection. Cells were then transfected with 3 µg of religated HPV and 1.2 µg of a plasmid conferring neomycin resistance (pEGFP-N1) using Lipofectamine 2000 (Invitrogen, Shanghai, China). HPV-negative controls were transfected with 1.2 µg of pEGFP-N1 alone. On the following 4 days, cells were selected in the presence of G418 (125 µg/ml for 2 days followed by 250 µg/ml for 2 days). Two weeks after transfection, colonies were pooled and expanded. Cells were passaged on a weekly basis and were grown until approximately 90% confluent prior to harvesting of total RNA. Specific knockdown of MTA1 was achieved using specific siRNA as described previously [11]. Specific knockdown of APOBEC3B was achieved by transfecting cells with corresponding shRNA against *APOBEC3B* (sc-72,515) or with a control shRNA (sc-108,060) (Santa Cruz Biotechnology, CA, USA). 48 h after transfection, cells were collected and subjected to other experiments. HeLa/pCMV6-XL5-MTA1/APOBEC3B$^{-/-}$ cell line was generated by sequential transfection of pCMV6-XL5-MTA1 and APOBEC3B shRNA followed by selection with 50 ng/ml puromycin (Invitrogen). The CCa cells stably deprived of endogenous MTA1 were established by transfection of pCMV-shRNA-MTA1 or empty vector, followed by selection with 0.5 µg/ml G418 (Invitrogen).

Measurement of cell survival and apoptosis

24 h after seeding, the culture medium was removed and CDDP were diluted in culture medium at the concentrations as indicated. After a 24-h drug exposure, cell viability was evaluated using the WST-1 viability assay (Roche Applied Science, Mannheim, Germany), according to the manufacturer's protocol. The optical density was measured at 450 nm, with 650 nm as the reference wavelength, on a microplate reader (xMark™ Microplate, Bio-Rad, Hercules, CA, USA). Based on the CDDP dose–response curves, we determined the lethal dose values of LD50 for CDDP in different cells.

Apoptotic cell death in CDDP-treated cells was assessed by spectrophotometry using ApoStrand™ ELISA Apoptosis Detection Kit (ENZO, Farmingdale, NY, USA) at 405 nm, according to the manufacturer's instructions.

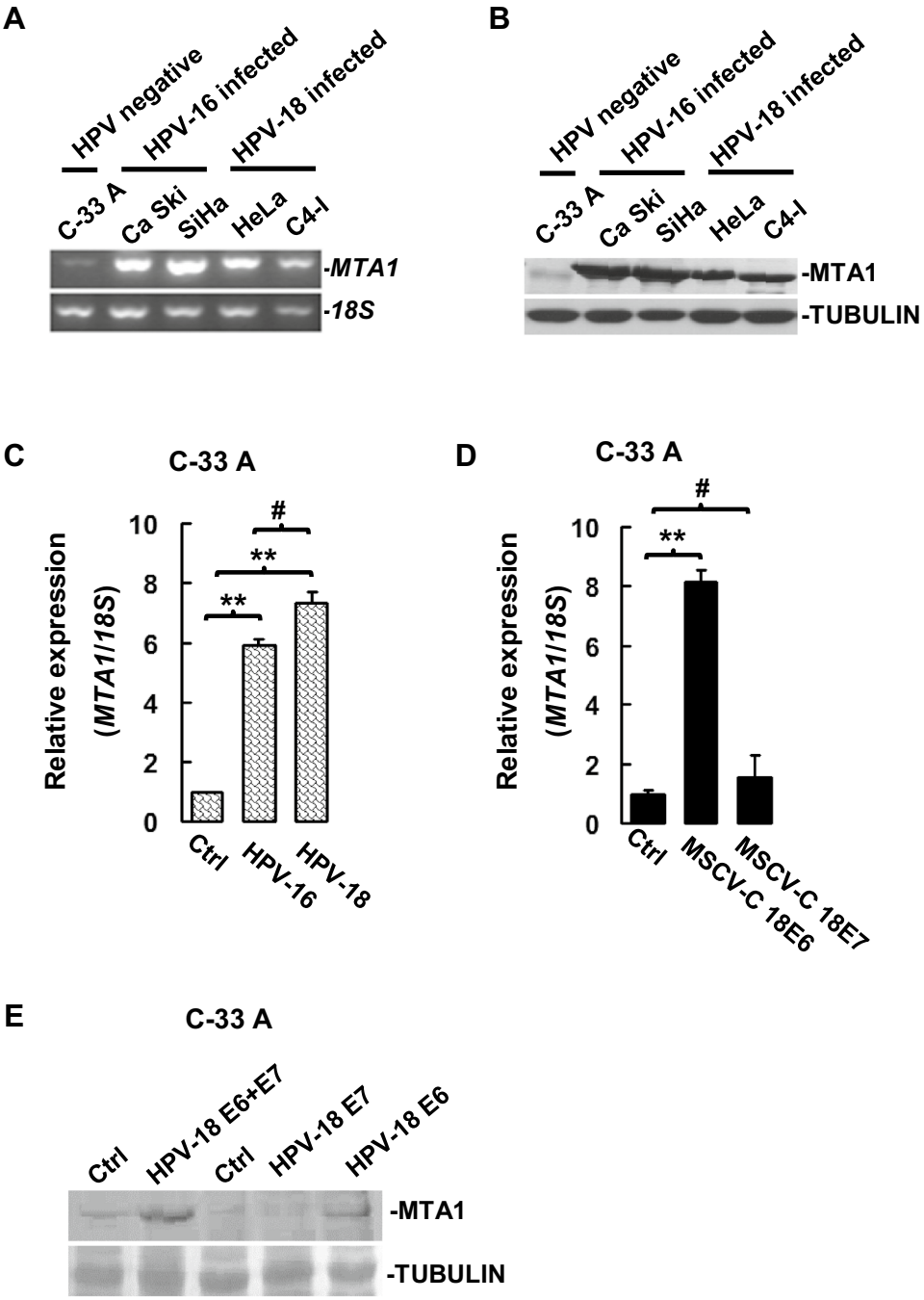
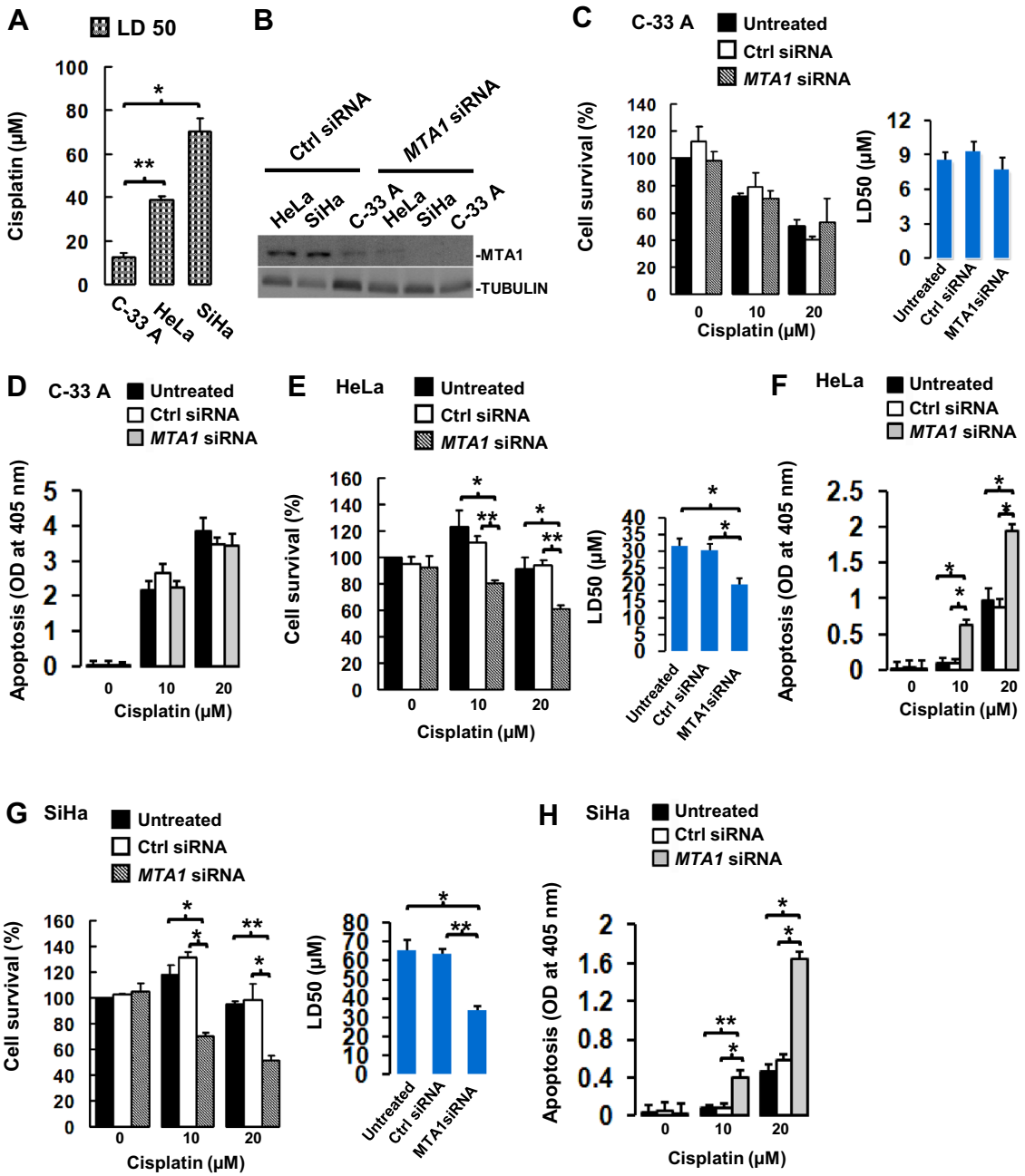


Fig. 1 Induction of MTA1 expression by infection with distinct HPV genomes. **a** Relative expression levels of *MTA1* mRNA in different cervical cancer (CCa) cells were evaluated using RT-PCR as described in the "Materials and methods" section. Parallel amplification of *18S* mRNA served as internal control. **b** Immunoblotting analysis of MTA1 protein levels in different CCa cells. TUBULIN served as a loading control. **c** Relative expression levels of *MTA1* mRNA in C-33 A cells transfected with full-length HPV-16 or HPV-18 were determined using RT-qPCR. Quantitative values are the mean ± SEM of at least three independent determinations (**$P < 0.01$; #$P > 0.05$). **d** *MTA1* mRNA levels in the C-33 A cells transfected with HPV-18 containing a specific reading frame for E6 or E7 were evaluated by RT-qPCR (**$P < 0.01$; #$P > 0.05$). **e** MTA1 protein levels in the C-33 A cells transfected with full-length HPV-18 or HPV-18 containing a specific reading frame for E6 or E7 were assayed using Western blotting. TUBULIN served as a loading control



In vivo chemosensitivity

Cells with different transfections (1×10^6 cells/10 μl PBS) were injected subcutaneously into each flank of the 8-week-old female BALB/c nude mice. When tumor volume reached ~ 150–200 mm^3 (about 2.5 weeks), mice received the intraperitoneal (i.p.) injection of either CDDP (2 mg/kg/2 day) or saline (control) for another 25 consecutive days. Five mice of each experimental group were sacrificed at the time points as indicated after CDDP treatment, and tumor volume was calculated using the formula: V (mm^3) = width^2 (mm^2) × length (mm)/2.

Quantitative RT-PCR (RT-qPCR)

Total RNA was isolated using the NucleoSpin RNA kit (Clontech, CA, USA) according to the manufacturer's protocol. The primers used for detection *MTA1, APOBEC3B* and *18S* were chosen according to previous reports [2, 11]. Amplification of *18S* served as internal control. The relative abundance of each target transcript was quantified using the comparative △△Ct method.

◂**Fig. 2** Endogenous MTA1 protects HPV-positive CCa cells from CDDP-induced cell death. a CCa cells were treated for 24 h with different concentrations of CDDP. Cell viability was determined by WST-1 assay. The lethal dose values LD50 were then determined and plotted in a histogram (*$P<0.05$; **$P<0.01$). **b** 48 h after transfection with MTA1 siRNA or Ctrl siRNA, MTA1 protein levels were determined using Western blotting. **c** Different concentrations of CDDP were added to the C-33 A cells 48 h post-transfection, and C-33 A cells were then cultured for another 24 h. Cell viability was then measured by WST-1 assay. All obtained values were normalized to untreated cells, which were set to the 100% cell viability (*$P<0.05$; **$P<0.01$). Right panel, the lethal dose values LD50 were determined accordingly and plotted in a histogram. **d** C-33 A cells with different transfections were treated for 24 h with various doses of CDDP as indicated, followed by measurement of cell apoptosis using spectrophotometry at 405 nm. **e** Different concentrations of CDDP were added to the HeLa cells 48 h post-transfection, and HeLa cells were then cultured for another 24 h. Cell viability was then measured by WST-1 assay. All obtained values were normalized to untreated cells, which were set to the 100% cell viability (*$P<0.05$; **$P<0.01$). Right panel, the lethal dose values LD50 were determined accordingly and plotted in a histogram (*$P<0.05$; **$P<0.01$). **f** HeLa cells with different transfections were treated for 24 h with various doses of CDDP as indicated, followed by measurement of cell apoptosis using spectrophotometry at 405 nm (*$P<0.05$; **$P<0.01$). **g** Different concentrations of CDDP were added to the SiHa cells 48 h post-transfection, and SiHa cells were then cultured for another 24 h. Cell viability was then measured by WST-1 assay. All obtained values were normalized to untreated cells, which were set to the 100% cell viability (*$P<0.05$; **$P<0.01$). Right panel, the lethal dose values LD50 were determined accordingly and plotted in a histogram (*$P<0.05$; **$P<0.01$). **h** SiHa cells with different transfections were treated for 24 h with various doses of CDDP as indicated, followed by measurement of cell apoptosis using spectrophotometry at 405 nm (*$P<0.05$; **$P<0.01$)

Western blotting

Western blotting was carried out according to previous reports [12]. Membranes were incubated with different primary antibodies including goat anti-MTA1, rabbit anti-TUBULIN (Santa Cruz Biotechnology, Shanghai, China) and rabbit anti-APOBEC3B (Abcam, Hongkong, China).

DNA deaminase activity assay

Deamination reactions were carried out as reported [2]. 4 pmol 5′-ATTATTATTATTCAAATGGATTTATTTATTTATTTATTTATTT-fluorescein was treated with cell extracts (16.5 µl) in the presence of 0.025U uracil DNA glycosylase (UDG), 2 µl 10×UDG buffer (NEB), and 1.75 U RNase A, at 37 °C for 2 h. Abasic sites were cleaved by treatment with 100 mM NaOH at 95 °C for 10 min. Substrates were then separated from product using 15% TBE-urea gel electrophoresis. Gels were finally analyzed using Fuji Film Image Reader FLA-7000.

Luciferase reporter assay

HeLa cells were either stably transfected with pCMV6-XL5-MTA1 or control vectors, or transiently transfected with MTA1 siRNA or control siRNA, as described above [11]. Two days later, cells were transfected using DharmaFECT DUO (Dharmacon) with a human *APOBEC3B* promoter reporter construct system (SwitchGear Genomics, Menlo Park, CA, USA) (pLightSwitch-APOBEC3B; Restriction pair: Mlu1 and Bgl2). Luciferase activity measurements were performed in LightSwitch Assay Reagents as instructed in the manufacturer's protocol.

NF-κB activity

Signosis' NF-κB filter assay was performed to evaluate the activity of NF-κB pathway as instructed by the manufacturer. The bound NF-κB probe was finally measured with luminescence using GloMax™ 20/20 Luminometer (Promega).

Chromatin immunoprecipitation (ChIP)

Two weeks after MSCV-C 18E6 transfection, HeLa cells were transiently transfected with MTA1 siRNA or control siRNA, as described above. 48 h later, cells were harvested and ChIP assay was performed as described elsewhere [13]. The chromatin fragments were amplified by PCR using primers flanking the NF-κB binding sites as reported previously [9].

Co-immunoprecipitation (Co-IP)

C-33 A/MTA1 cells were treated with 20 µM of CDDP for 24 h. Cells were then lysed with the aid of Pierce Co-Immunoprecipitation Kit (Thermo Scientific, Shanghai, China), sonicated and centrifuged at 15,000×*g* for 20 min to obtain the clear supernatant. The lysates (~ 150 µg) were incubated at 4 °C with anti-MTA1 or control IgG antibodies overnight. On the next day, lysates were incubated with protein A-Sepharose (Thermo Scientific) at 4 °C for another 4 h, followed by a thorough elution using Laemmli sample buffer (Thermo Scientific). The eluted protein samples were finally subjected to SDS–PAGE and Western blotting analysis.

Statistical analysis

Results are presented as mean ± SEM from at least three independent experiments. Data normality was determined using normal probability plots and compared using Student's *t* test or one-way analysis of variance (ANOVA) as appropriate, with $P<0.05$ being considered as statistically significant.

Results

HPV infection causes MTA1 upregulation

We first examined the expression profiles of MTA1 in different CCa cells. RT-PCR analysis revealed that *MTA1* mRNA expression was weak in C-33 A cells (HPV negative), moderate in HeLa and C4-I (HPV-18 positive), and relatively high in Ca Ski and SiHa (HPV-16 positive) cells (Fig. 1a). This observation was validated by Western blot at the protein level. As shown in Fig. 1b, Ca Ski and SiHa cells exhibited the highest expression of MTA1 protein compared to the moderate expression in HeLa and C4-I cells. Negligible expression of MTA1 protein was observed in C-33A cells. Among high-risk HPV types, HPV-16 and -18 are mostly detected in the majority of CCa cases [14]. When C-33A cells were transfected with HPV genomes, *MTA1* mRNA expression was induced significantly by transfection of either HPV-16 or HPV-18 genomes, with no difference being detected between these two groups (Fig. 1c). The viral oncoproteins E6 and E7 are invariably expressed in HPV-positive cases [3]. To ask whether HPV-induced *MTA1* upregulation is oncoprotein-specific, we transfected the C-33A cells with HPV-18 containing a specific reading frame for E6 (MSCV-C 18E6) or E7 (MSCV-C 18E7). Apparently, only E6 could induce the MTA1 expression, which was verified at both the transcriptional (Fig. 1d) and translational levels (Fig. 1e). Together, transfection with distinct HPV subtypes could induce MTA1 upregulation, and HPV E6 alone is sufficient to induce MTA1 expression in CCa cells.

MTA1 knockdown potentiates CDDP sensitivity in highly metastatic CCa cells

We next treated C-33 A cells, as well as HeLa and SiHa cells, with different concentrations of CDDP to define the lethal dose values LD50 representing 50% cell death. HPV-positive HeLa and SiHa cells exhibited more resistance to CDDP when compared to HPV-negative C-33 A cells (Fig. 2a), indicating that MTA1 induction may be associated with HPV infection-induced CDDP resistance. To validate this, we transiently knocked down the MTA1 expression in CCa cells using siRNA treatment (Fig. 2b), and the cells were then subjected to a 24-h exposure to CDDP. Ablation of MTA1 expression caused no significant changes in the cell survival rate among untreated, Ctrl siRNA-transfected and MTA1 siRNA-transfected C-33 A cells, which died after CDDP treatment in a dose-dependent manner (Fig. 2c). Consistently, no significant changes in cell apoptosis were observed among untreated, Ctrl siRNA-transfected and MTA1 siRNA-transfected C-33 A cells after CDDP treatment (Fig. 2d). By contrast, MTA1 inhibition rendered the HPV-positive HeLa and SiHa cells more sensitivity to CDDP exposure, at both drug concentrations tested. To be specific, MTA1 siRNA-transfected HeLa cells demonstrated significant decrease in cell survival rate at concentrations of CDDP 10 and 20 μM, with the LD50 value dramatically decreased by ~ 34.8% compared to untreated and Ctrl siRNA-transfected HeLa cells (Fig. 2e). In accordance with these results, CDDP-elicited apoptosis increased in MTA1 siRNA-transfected HeLa cells by ~ 6.4- and ~ 2.2-fold at concentrations of CDDP 10 and 20 μM, respectively (Fig. 2f). Likewise, MTA1 siRNA-transfected SiHa cells demonstrated significant decrease in cell survival rate at concentrations of CDDP 10 and 20 μM, with the LD50 value dramatically decreased by ~ 41.7% compared to untreated and Ctrl siRNA-transfected HeLa cells (Fig. 2g), whereas CDDP-elicited apoptosis increased in MTA1 siRNA-transfected SiHa cells by ~ 5.1- and ~ 2.8-fold at concentrations of CDDP 10 and 20 μM, respectively (Fig. 2h).

To further assess whether enhanced MTA1 expression is a cause of or a result of HPV-induced CDDP resistance, we co-transfected C-33 A cells with MSCV-C 18E6 and MTA1 siRNA. As expected, in the MTA1 siRNA-transfected C-33 A cells, MSCV-C 18E6 was unable to induce MTA1 expression (Fig. 3a). Intriguingly, overexpression of HPV E6 in C-33 A cells was sufficient to induce high resistance to CDDP at both drug concentrations tested, and this resistance could be effectively reversed by MTA1 siRNA treatment (Fig. 3b, c). Consistently, MTA1 inhibition significantly restored the sensitivity to CDDP-elicited apoptosis in C-33 A cells (Fig. 3d). Collectively, these results suggest that endogenous MTA1 is involved in the development of CDDP resistance in HPV-infected CCa cells.

Stable ablation of endogenous MTA1 expression promotes in vivo sensitivity to CDDP treatment

To better evaluate the biologic function of MTA1 in vivo, we employed a xenograft model using HeLa and SiHa cells stably deprived of endogenous MTA1 by shRNA (Fig. 4a, b). When tumor volume reached ~ 150–200 mm^3 (about 2.5 weeks), CDDP (2 mg/kg) or saline (control) was intraperitoneally injected into female nude mice every 2 days for 25 consecutive days. Intriguingly, the tumor volume of the tumors expressing the MTA1 shRNA was notably smaller than that of tumors expressing Ctrl shRNA during the late phase of treatment (Fig. 4c, d).

Augmentation of MTA1 expression by HPV infection stimulates APOBEC3B expression and activity in CCa cells

HPV infection triggers upregulation of the antiviral and cancer genomic DNA deaminase APOBEC3B [9].

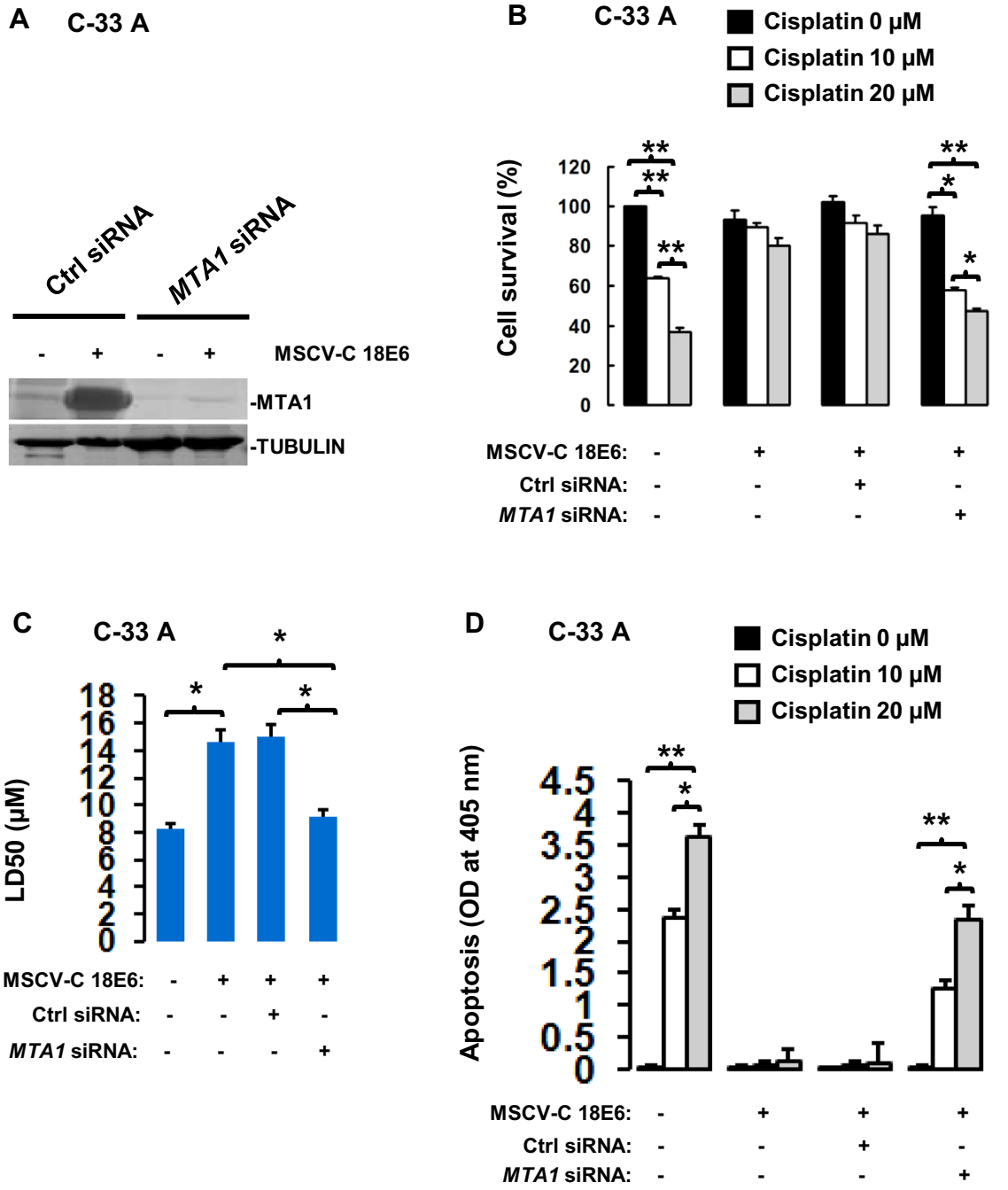


Fig. 3 MTA1 inhibition counteracts the HPV infection-induced CDDP resistance. **a** C-33 A cells transfected with HPV-18 containing a specific reading frame for E6 or empty vector were subjected to siRNA treatment. 48 h later, cells were harvested and MTA1 expression was determined using Western blotting. **b** The above-mentioned C-33 A cells were incubated with different concentrations of CDDP for 24 h, followed by WST-1 viability assay (*$P<0.05$; **$P<0.01$). **c** The lethal dose values LD50 in Panel B were determined accordingly and plotted in a histogram (*$P<0.05$; **$P<0.01$). **d** The C-33 A cells with different transfections were treated with different concentrations of CDDP for 24 h, followed by measurement of cell apoptosis using spectrophotometry at 405 nm (*$P<0.05$; **$P<0.01$)

Consistently, in the current study, transfection of HPV-negative C-33A cells with MSCV-C 18E6 significantly induced APOBEC3B expression at both transcriptional and translational levels. This stimulatory effect could be effectively abolished by MTA1 knockdown (Fig. 5a, b). To further quantify the APOBEC3B functional activity, we performed the single-stranded DNA deaminase assay using protein extracts from the same cells as used for mRNA

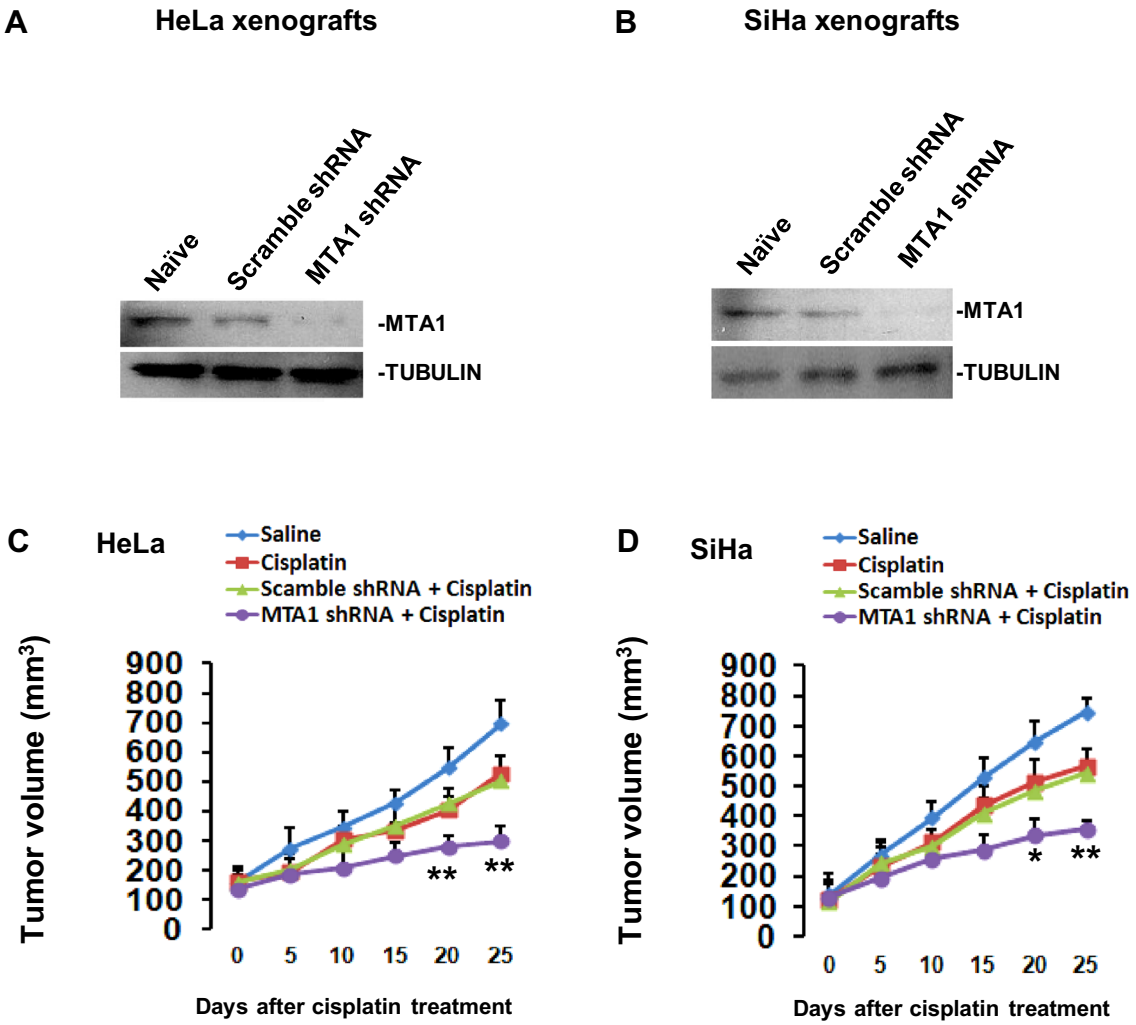


Fig. 4 Stable ablation of endogenous MTA1 expression potentiates in vivo sensitivity to CDDP. **a** Verification of MTA1 deletion in HeLa xenografts was achieved using Western blotting at the end of a 25-day CDDP treatment. **b** Verification of MTA1 deletion in SiHa xenografts was achieved using Western blotting at the end of a 25-day CDDP treatment. **c** Effects of MTA1 repression in HeLa cells on in vivo CDDP sensitivity were evaluated using xenografts model, as described in "Materials and methods" (*$P<0.05$ and **$P<0.01$ when comparing MTA1 shRNA + cisplatin to scramble shRNA + cisplatin). **d** Effects of MTA1 repression in SiHa cells on in vivo CDDP sensitivity were evaluated using xenograft models, as described in Materials and methods (*$P<0.05$ and **$P<0.01$ when comparing MTA1 shRNA + cisplatin to scramble shRNA + cisplatin)

quantification in Fig. 3a. As expected, protein extracts from the cells transfected with MSCV-C 18E6 had deaminase activity more than three times higher than that of the transfection control. This stimulatory effect of 18E6 could be substantially compromised by MTA1 siRNA treatment (Fig. 5c). Therefore, transfection of HPV18 genome could cause an increase in DNA deaminase activity, which is proportional to the APOBEC3B upregulation and requires the participance of MTA1. In another experimental setting, stable transfection with pCMV6-XL5-MTA1 elicited a significant induction in the expression levels of both MTA1 and APOBEC3B, whereas co-transfection with pCMV6-XL5-MTA1 and APOBEC3B shRNA in C-33A cells only suppressed APOBEC3B expression but failed to affect MTA1 expression (Fig. 5d), further confirming that MTA1 may act as an upstream modulator of APOBEC3B signaling. From a functional standpoint, MTA1 overexpression resulted in a noticeable resistance to CDDP in HPV-negative C-33A cells, a similar phenotype observed in HPV-infected cells (Fig. 2f). In contrast, stable deletion of APOBEC3B by shRNA could effectively abolish MTA1 overexpression-induced chemoresistance in C-33A cells (Fig. 5e). Taken together, APOBEC3B may function as a downstream effector to reconstitute MTA1 function in HPV-infected CCa cells.

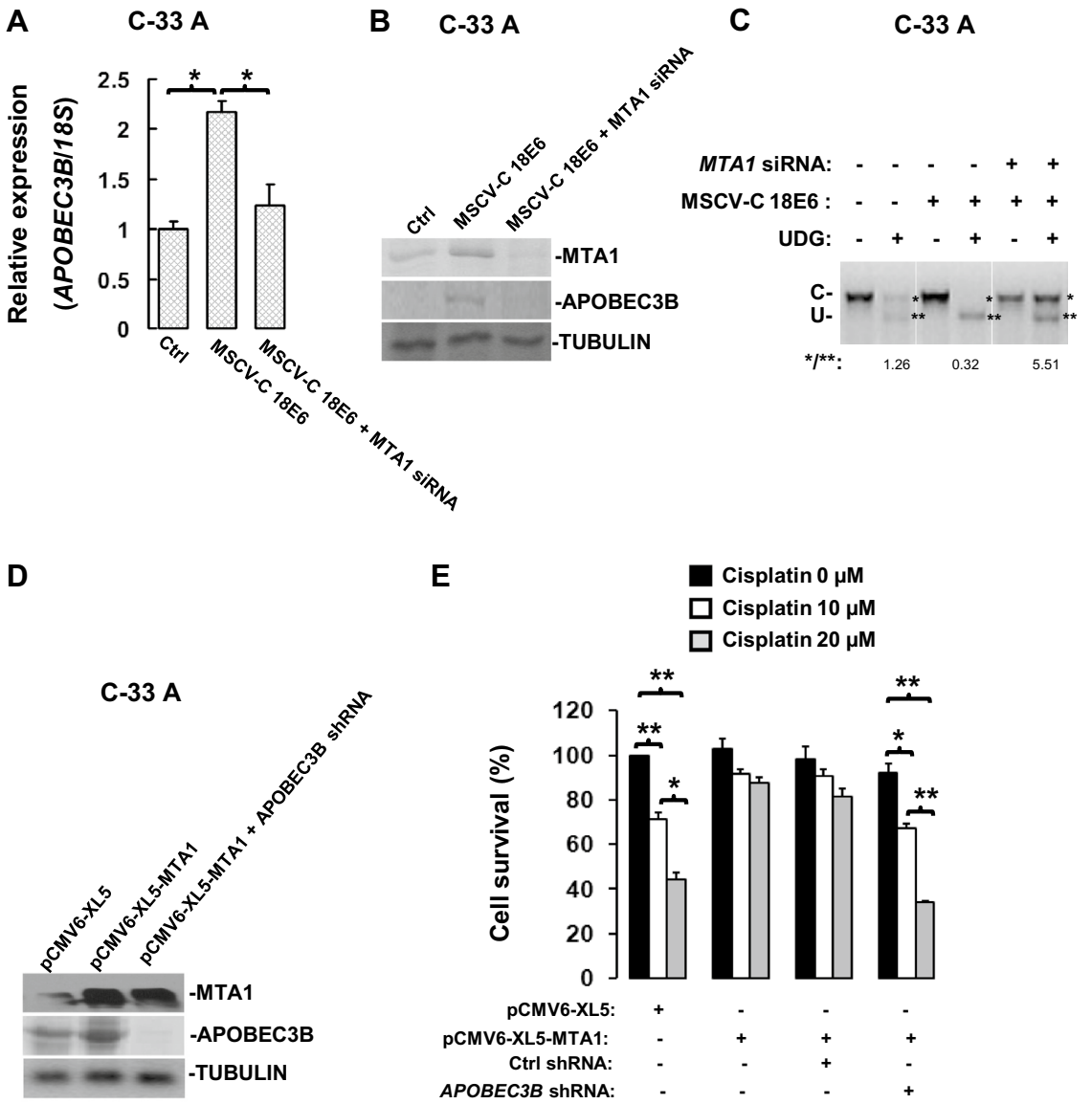
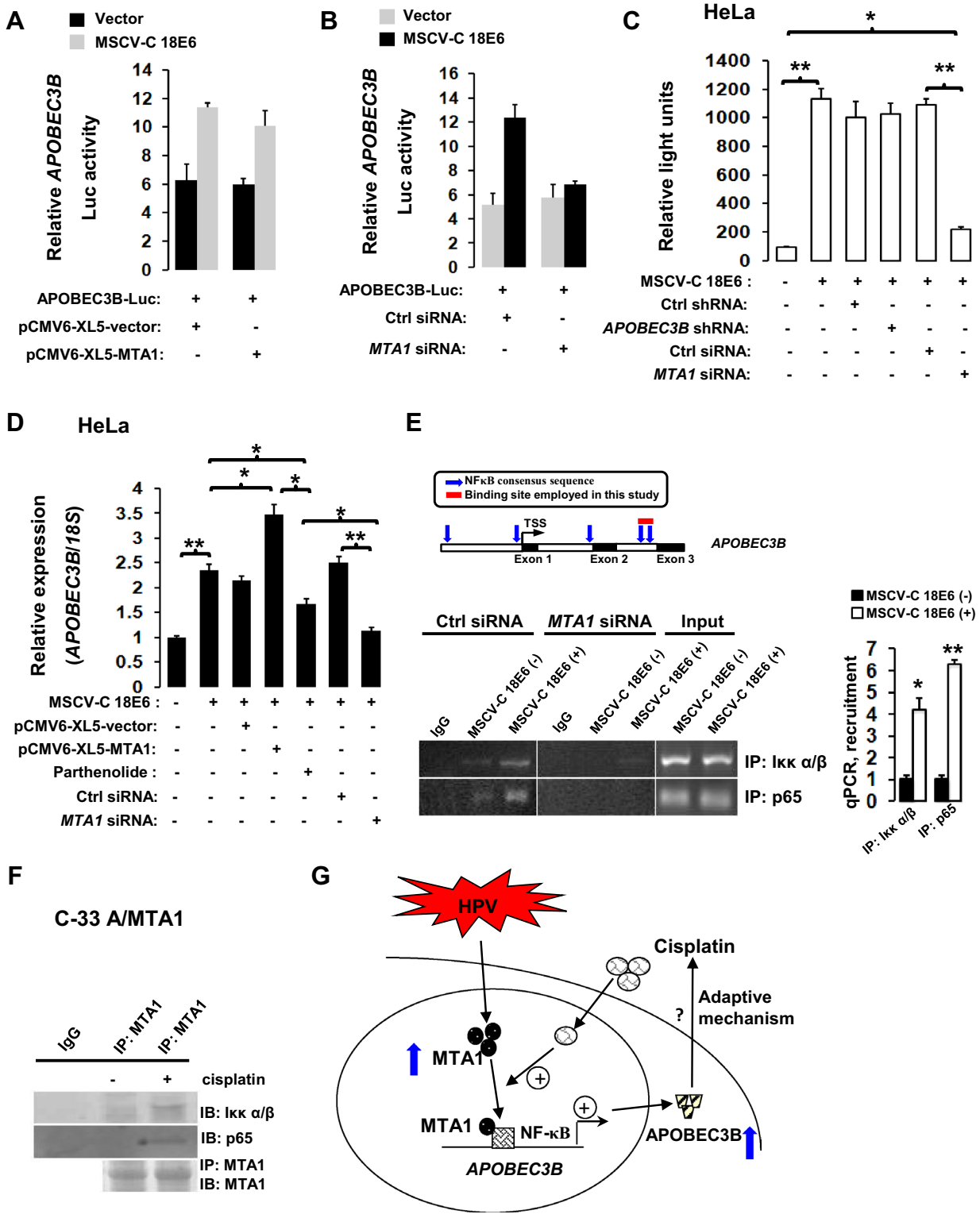


Fig. 5 Augmentation of MTA1 expression by HPV infection causes APOBEC3B upregulation. **a** Effects of E6 overexpression, as well as MTA1 knockdown, on the expression of *APOBEC3B* mRNA were assayed in C-33A cells using RT-qPCR (*$P<0.05$). **b** Effects of E6 overexpression, as well as MTA1 knockdown, on the expression of APOBEC3B protein were assayed using Western blotting. **c** Representative DNA cytosine deaminase assay performed with cell extracts from the same cells as in **a. d** Effects of MTA1 overexpression, as well as APOBEC3B knockdown on the expression levels of MTA1 and APOBEC3B were determined using Western blotting. **d** Effects of MTA1 overexpression, as well as APOBEC3B knockdown on the cell survival rate after a 24-h CDDP exposure were evaluated by WST-1 viability assay (*$P<0.05$; **$P<0.01$)

NF-κB is required for APOBEC3B induction by MTA1 in HPV-infected CCa cells

MTA1 regulates divergent cellular pathways by acting as a crucial transcriptional regulator. We therefore asked whether MTA1 directly regulates APOBEC3B expression at the transcriptional level. Overexpression of the exogenous MTA1 could not stimulate HPV-induced *APOBEC3B* promoter activity. On the contrary, in the presence of MTA1 inhibition, the HPV-induced *APOBEC3B* promoter activity was substantially impaired (Fig. 6a, b), indicative of an indirect modulation of *APOBEC3B* transcription by MTA1. HPV-18 transfection dramatically stimulated NF-κB promoter activity, which could be efficiently blocked by MTA1 siRNA treatment but not by APOBEC3B knockdown (Fig. 6c). Consistently, 18E6-induced *APOBEC3B* upregulation could be reversed by co-treatment with the NF-κB pathway pharmacologic inhibitor parthenolide or by MTA1



siRNA treatment (Fig. 6d). In contrast, transient transfection with APOBEC3B overexpressing vector did not induce any changes on MTA1 transcription in either HeLa or SiHa cells (Supplementary Fig. 1). Therefore, MTA1 acts upstream of APOBEC3B signaling, and the regulation of APOBEC3B expression by MTA1 is dependent on the NF-κB pathway in CCa cells. To provide the direct evidence for the modulation of APOBEC3B by NF-κB signaling, we performed ChIP experiments. Both Ikκ α/β and p65 were recruited to the NF-κB consensus binding site near Exon 3 in response to

◂**Fig. 6** MTA1 indirectly regulates APOBEC3B expression via classical NF-κB signaling. **a, b** Effects of manipulation of MTA1 expression on HPV-induced *APOBEC3B* promoter activity were determined using a luciferase reporter assay. **c** HPV-induced NF-κB activity after MTA1/APOBEC3B knockdown was monitored by NF-κB filter assay (*$P<0.05$; **$P<0.01$). **d** Effects of manipulation of MTA1 expression on HPV-induced *APOBEC3B* mRNA level were assessed using RT-qPCR in HeLa cells, in the presence or absence of co-treatment with the pharmacologic NF-κB inhibitor parthenolide (5 μM) for 6 h (*$P<0.05$; **$P<0.01$). **e** ChIP analysis showing recruitment of the components of NF-κB pathway onto a specific region of the *APOBEC3B* promoter. Right panel, from the same elutes of ChIP analysis, qPCR analysis was also performed to quantify the recruitment (*$P<0.05$; **$P<0.01$ when compared to the values in the cells without MSCV-C 18E6 transfection). **f** C-33A/MTA1 cells were treated with 20 μM of CDDP for 24 h, followed by evaluation of the association between MTA1 and NF-κB components using co-immunoprecipitation assay. **g** Summary diagram of the possible mechanisms related to MTA1-NF-κB function conferring cisplatin resistance through enhancement of *APOBEC3B* transcription in CCa cells

18E6 transfection. This recruitment was effectively attenuated in the presence of MTA1 siRNA (Fig. 6e). To determine whether the observed enhancement of *APOBEC3B* transcription by MTA1 was associated with recruitment of NF-κB components in vivo, we examined the association between MTA1 and the components of NF-κB pathway by co-immunoprecipitation. Apparently, only in the presence of CDDP stimulation, MTA1 was found to be structurally interacting with NF-κB components in CCa cells (Fig. 6f). The available data thus indicate that NF-κB signaling may act as an indispensible modulator of the MTA1-regulated APOBEC3B expression in HPV-infected CCa cells.

Discussion

This is the first study to demonstrate a mechanistic link between HPV infection and upregulation of the master transcriptional regulator MTA1 in CCa cells. We show that MTA1 is highly expressed in HPV-infected CCa cells but weakly expressed in HPV-negative CCa cells. Moreover, transfection of the HPV genome triggers MTA1 upregulation at both mRNA and protein levels, and high-risk E6 alone is sufficient to induce the MTA1 upregulation. HPV infection usually invokes a cellular immune response with regulatory T cells involved in local immune suppression in HPV-associated malignancies. Active immunity may be further suppressed by host factors, such as macrophages and inhibitory cytokines [15]. Interestingly, recent advances have shown that MTA1 plays a critical homeostatic role in the regulation of inflammatory responses [7]. MTA1 can function both as a target and as a component of the NF-κB signaling by regulating a subset of LPS-induced proinflammatory cytokines such as IL-1β, MIP2, and TNF-α. In this context, it is a logical observation that HPV infection causes immunological deregulation, which should lead to the deregulated expression of MTA1 in CCa cells.

The chemotherapeutic agent CDDP appears to most effectively treat advanced/recurrent CCa. However, resistance to CDDP may develop, thus substantially compromising the therapeutic effect [1]. The molecular mechanism underlying CDDP resistance is associated with multiple intertwined signaling pathways: increase in DNA damage repair, inactivation of apoptosis, augmentation of epithelial–mesenchymal transition (EMT) and disruption of microRNA profile [1]. Accumulated evidence establishes MTA1 to be a valid DNA damage-responsive protein with a significant role in maintaining the optimum DNA-repair activity in mammalian cells upon genotoxic stress [11]. Furthermore, dysfunction of apoptosis-related signaling such as Bcl-2 family proteins, p53 and NF-κB contributes to the pathogenesis of cisplatin resistance in CCa [1]. Surprisingly, MTA1 could regulate all these apoptotic pathways as a transcriptional coregulator, by modifying DNA accessibility for cofactors. Additionally, MTA1 is functionally associated with EMT. It has been reported that the Wnt1/β-catenin signaling and E-cadherin, two key components of EMT molecules regulating the motility, invasion, and metastatic potential of cancer cells, are both tightly regulated by MTA1 [16]. Therefore, MTA1 may exert its regulatory effects on chemoresistance at different levels, and the coordinated existence of MTA1 inside various cascade loops may help to integrate the complicated regulatory network underlying CDDP resistance. Of note, MTA1 overexpression could also potentiate docetaxel resistance in human prostate cancer cells [11]. So, the pro-survival effect of MTA1 in response to chemotherapy may exist on a broader range.

Derepression of *APOBEC3B* transcription lead to a mutator phenotype that explains the observed cytosine mutation biases in HPV-positive CCa. Recently, it has been shown that HPV-E6 alone could trigger *APOBEC3B* upregulation [2], but a major unresolved question is how HPV regulates *APOBEC3B* transcription in transformed cells. Our findings provide novel clues by identifying MTA1 as an upstream regulator of APOBEC3B expression. We found that NF-κB is required for MTA1-dependent stimulation of APOBEC3B signaling as pharmacological inhibition of NF-κB activity leads to a substantial reduction in the HPV-induced APOBEC3B expression. Thus, MTA1 may regulate APOBEC3B expression in an indirect manner. A recent publication demonstrates that activation of protein kinase C (PKC) results in specific and dose-responsive increase in APOBEC3B expression and activity, and mechanistically, PKC activation caused the recruitment of RELB, but not RELA, to the *APOBEC3B* promoter implicating the involvement of non-canonical NF-κB signaling [9]. In our study, Iκκ α/β and p65 were both recruited to the NF-κB consensus binding site near Exon 3 in response to HPV infection, which

was substantially inhibited after MTA1 knockdown. So both canonical and non-canonical NF-κB signaling can regulate *APOBEC3B* transcription, and by which way APOBEC3B expression is regulated may largely be decided by the cellular context and upstream signaling.

In summary, the results presented here have identified a critical role of MTA1, both as a target of HPV infection and as a modifier of the NF-κB signaling, in conferring a deregulated inflammatory response against HPV infection. MTA1 upregulation enhances the recruitment of Iκκ α/β and p65 onto *APOBEC3B* promoter, stimulates APOBEC3B expression and thus results in somatic mutagenesis through the cancerous development. To be noted, we believe that MTA1 indirectly binds to the *APOBEC3B* promoter region via first binding to NF-κB components (Fig. 6g). By revealing how APOBEC3B is modulated by MTA1/NF-κB cascade, our findings suggest that MTA1 siRNA and NF-κB inhibitors may be repositioned to suppress cancer mutagenesis, dampen tumor evolution, and decrease the probability of adverse outcomes by HPV infection (such as CDDP resistance) in CCa.

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Compliance with ethical standards

Conflict of interest Qiu-ping Jia declares that she has no conflict of interest. Chang-you Yan declares that he has no conflict of interest. Xue-rong Zheng declares that she has no conflict of interest. Xia Pan declares that she has no conflict of interest. Xin Cao declares that she has no conflict of interest. Lei Cao declares that she has no conflict of interest.

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