



Original Research

Targeting matrix metalloproteinase MMP3 greatly enhances oncolytic virus mediated tumor therapy

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ABSTRACT

In cancer, the extracellular matrix is extensively remodeled during chronic inflammation, thus affecting cell transcription, differentiation, migration and cell-cell interactions. Matrix metalloproteinases can degrade the extracellular matrix of tumor tissues and take important roles in disease progression. Numerous efforts to develop cancer treatments targeting matrix metalloproteinases have failed in clinical trials owing to the ineffectiveness and toxicity of the applied inhibitors. In this study, we investigated the potential of targeting matrix metalloproteinases and oncolytic virus combination in cancer therapy. We found that MMP3 expression was up-regulated in various cancers and MMP3 expression in the tumor cells, but not in other tissues, was important for tumor growth and metastasis. Single treatment of colon cancer with multiple MMP3 inhibitors was not effective in mice. Nevertheless, the therapeutic effect of MMP3 was greatly improved by combination with an oncolytic virus. A potential mechanism of MMP3 in regulating tumor cell proliferation and invasion was mediated via Erk1/2 and NF- κ B signaling. This study reveals that MMP3 is a promising target and the combined treatment with oncolytic virus is a potential strategy for cancer therapy.

Introduction

Matrix metalloproteinases (MMPs) consist of at least 24 calcium-dependent, zinc-containing endopeptidases, most of which are of mammalian origin and classified in four major subgroups based on their domain-structure [1]. The pattern of MMPs expression in tumors varies depending on the function of the MMPs and the type of cancer. For example, in colorectal carcinoma, MMP7 is selectively expressed by malignant epithelial cells, whereas stromal cells express stromelysin-1 and -3 as well as gelatinase A [2,3]. In breast cancer, stromelysin-3 and MMP13 are predominantly expressed by stromal cells [4], and MMP2 is generally found ubiquitously expressed by both malignant and non-malignant cells [5]. In brain tumors, MMP9 is abundantly expressed by malignant cells [6]. Recently, a study based on the Cancer Genome Atlas (TCGA) data analyzed the expression difference of 24 MMPs in 15 different cancer types, and the results showed that MMP3 was significantly upregulated in seven cancer types, suggesting the strong association of MMP3 with tumor progression [7]. A positive correlation between the pattern of MMP3 expression and the tumor invasive and metastatic potential has also been shown in breast, colon, cervix, lung,

and bone tumors [8–11]. Indeed, it was shown that ectopic expression of MMP3 results in ECM reorganization and is sufficient to induce malignant transformation of the epithelium, suggesting that epithelial cells lacking normative mesenchymal cues are more likely to adopt a malignant fate [12]. In addition, overexpression of MMP3 in the mammary epithelium will cause the cleavage of E-cadherin and lead to the transformation between epithelium and mesenchyme [13,14].

Clinical trials of synthetic MMP inhibitors (MMPis) for cancer therapy were performed during the late 1990s and early 2000s [15–17]. However, these studies failed owing to the lack of efficacy and severe side effects. For instance, batimastat was one of the first drugs designed to mimic MMP substrate collagen with broad-spectrum inhibition of MMP family members. Although several phase I studies showed efficacy with direct injection of the drug into the pleural or peritoneal space of patients with malignant effusions or ascites, significant toxicities including pain, pyrexia, transaminitis, dyspnea, cough, and nausea were observed [18,19]. Later, marimastat was developed as a next-generation drug with a similar mechanism to that of batimastat; however, batimastat and more selective inhibitors, including tanomastat and rebimastat, uniformly failed to show a survival benefit. Moreover, many

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patients experienced negative effects on their quality of life, which ultimately led to the stop of further MMPI trials in the mid-2000s [20].

Oncolytic viruses are defined as genetically engineered or naturally occurring viruses that selectively replicate and kill cancer cells without damaging healthy tissues [21]. Since T-vec, a type I Herpes Simplex Virus (HSV) from Amgen, was first approved by the U.S. Food and Drug Administration for the treatment of patients with local melanoma in 2015 [22], many oncolytic viruses have been rapidly developed into clinical trials. We and others have shown the efficacy of virotherapy combined with PD-1/PD-L1 antibodies in mice with Lewis lung carcinoma [23] and patients with advanced melanoma [24]. The effects of MMPs in oncolytic virus therapy is controversial. Previous study showed that overexpression of MMP1 and MMP8 can enhance HSV convective interstitial transport in tumor, thus improve HSV oncolytic efficacy [25]. Another report showed that vaccinia virus carrying MMP9 leads to a degradation of collagen IV, facilitating intra-tumoral viral dissemination, and resulting in accelerated prostate cancer regression in mice [26]. However, a conditionally replicating adenoviruses expressing the TIMP3 transgene, which is an endogenous inhibitor of MMPs, revealed enhanced killing of primary and clonal human glioma cells *in vitro*, and these properties did not result in significantly greater antitumor activity when combined with adenoviral oncolysis *in vivo* models for malignant glioma [27]. In the present study, we showed that MMP3 was upregulated in colon cancer cells. Targeting MMP3 by MMP inhibitor NNGH, lentiviral- or siRNA-mediated knock down together with vesicular stomatitis virus (VSV) virotherapy achieved superior therapeutic efficacy compared with that of monotherapy in the mouse colon cancer, indicating promising clinical application in the future.

Materials and methods

siRNAs, antibodies and chemical inhibitors

Two MMP3 small interfering RNAs (simmp3-1# and simmp3-2#) were purchased from RiboBio Co., Ltd. (siG131219155315, siG131219155335; Guangzhou, China). The siR-Ribo™ negative control was used as control scramble siRNA. The siRNAs were transfected at a concentration of 100 nM using Lipofectamine 2000 reagent (Invitrogen, CA, USA) according to the manufacturer's instructions in the cell assay. For *in vivo* injection, the simmp3-2# was synthesized with cholesterol modification for the efficiency to get into cell. Rabbit anti-MMP3 (ab53015) and GAPDH (G9545) antibodies were obtained from Abcam and Sigma-Aldrich, respectively. The secondary goat anti-rabbit IgG-horse radish peroxidase (HRP) (SQ19844) antibody was purchased from Southern Biotech (4030-05; Birmingham, AL, USA). NF-κB inhibitor Pyrrolidinedithiocarbamate ammonium and Erk1/2 inhibitor (BVD-523) were purchased from Selleck (S3633, S7854) and used in the concentration of 10 μM/mL, 20 μM/mL, respectively. The MMP broad spectrum inhibitor NNGH was purchased from Abcam (ab141259).

Cells and viruses

The human cervical cancer cell (Hela), endometrial carcinoma cell (Hec-1-B), pancreatic cancer cell (Panc-1), colon cancer cell (HT29, Caco-2), Prostate cancer cell (22RV1), breast cancer cell (T47D), lung cancer cell (NCI-1650, A549), stomach adenocarcinoma cell (SGC-7901), glioma cell (U251) and monocytic leukemia cell (THP1) were kindly provided by Prof. Yunsen Li at Soochow University. The human embryonic kidney cell line (HEK293T), Mouse colon cancer cell line (MC-38), African green monkey kidney cell line (Vero), Hela, Hec-1-B, Panc-1, HT29, Caco-2, T47D, NCI-1650 and SGC-7901 were cultured in Dulbecco's Modified Eagle Medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone) and 100 units/mL penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) in a 5% CO₂ incubator at 37 °C. The 22RV1, A549, U251 and THP1 were cultured in RPM1640 Medium (Hyclone) supplemented with 10% fetal bovine

serum and 100 units/mL penicillin/streptomycin in a 5% CO₂ incubator at 37 °C.

Vesicular Stomatitis Virus (VSV) VSV^{M51R} which carries M51R mutation in M gene for virus attenuation was maintained by passage in HEK293T cells as described [28]. Virus titers were determined using a 50% tissue culture infectious dose (TCID₅₀) assay on Vero cell monolayers and calculated by the Reed–Muench method [29].

Generation of MMP3 stable down-regulation cell line

The shRNA sequence (GGAGGUUGAUGAGAAGAA) of mouse MMP3 was designed and cloned into Lentivirus Expression Vector pLL3.7-eGFP to generate the down-regulation plasmid pLL3.7-eGFP-shmmp3. The plasmid was further verified by restriction endonuclease digestion and sanger sequencing.

5 × 10⁶ HEK293T cells in 10 cm dish were co-transfected with pLL3.7-eGFP-shmmp3 (10 μg), Δ8.9 (5 μg) and VSVG (2.5 μg) (Added gene, Cambridge, MA, USA) plasmids for the generation of MMP3 knock down lentivirus. The virus containing supernatant was collected 48 and 72 h post transfection, and was filtered with 0.22 μm filter. MC-38 cells were infected with the viral supernatants supplemented with polybrene (10 μg/mL) for 90 min. The GFP expressing cells were selected by BD FACS AriaTMIII sorter to obtain MC-38-Shmmp3 cells 3 days post infection. After two rounds sorting, more than 97% of cells were GFP positive. The control cell was MC-38-Vector, which was transduced by the pLL3.7-eGFP empty vector packaged lentivirus.

Experiment design

Six to 8 weeks old C57BL/6 female mice were purchased from the Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China) and the C57BL/6 J MMP3 knock out (KO) mice were kindly provided by Prof. Dai at Institutes of Biology and Medical Sciences, Soochow University. The MMP3 KO was based on CRISPR/Cas9 technique targeting MMP3 exon 2–4. All mice were bred in the Department of Laboratory Animal Science, Soochow University in a standard specific pathogen free environment. Animal experimental protocols used in this study followed the Guidelines for the Care and Use of Laboratory Animals (Ministry of Health, China, 1998) and were approved by the Ethics Committee of Soochow University.

MC-38 cells (7 × 10⁵ in 0.1 mL PBS) were subcutaneously inoculated into the right forelimb underarm of each mouse, including MMP3 KO mice. The tumor sizes (width: W and length: L) were measured by a vernier caliper every two days. The volume (V) was calculated by the formula: $V = (L)(W^2)/2$ [23]. Treatment with virotherapy was initiated when tumor volume reached around 50 mm³. Each mouse in the single NNGH treatment group was intratumorally injected with 50 Ki of NNGH (1Ki = 130 nM), and each mouse in the viral monotherapy group was intratumorally injected with 3 × 10⁷ PFU of virus. The combination therapy group was injected with 50 Ki of NNGH and 3 × 10⁷ PFU of virus each time. The virus and inhibitor were injected every two days. The total times for virus injection and inhibitor was 3 and 8, respectively. Animals were euthanized when tumor volume reaches 2000 mm³ or at the end point of observations [30]. Each mouse in the single MMP3 siRNA treatment group was intratumorally injected with 0.5 nM siRNA, and each mouse in the viral monotherapy group was intratumorally injected with 3 × 10⁷ PFU of virus. The combination therapy group was injected with 0.5 nM siRNA and 3 × 10⁷ PFU of virus each time. The detailed treatments were illustrated in the result figures.

For the colon cancer lung metastasis model, mice were injected with 3 × 10⁶ MC-38-Vector and MC-38-Shmmp3 cells through the tail vein. After 20 days, the mice were sacrificed to observe and count the lung tumor formation.

Western blot

MC-38 Cells transfected with 100 nM siRNA were harvested 48 h post-transfection and lysed with $5 \times$ sodium dodecyl sulfate (SDS) lysis buffer. Cellular proteins were separated with 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. After blocking with phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-T) and 5% fat-free milk, membranes were incubated with the primary antibodies anti-mmp3 (1:1000), or anti-GAPDH (1:2000). After washing with PBS-T three times, membranes were incubated with HRP-conjugated secondary goat anti-rabbit IgG (1:5000). Detection was performed with enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The gray intensity of bands was measured using Image J software. The relative gray intensity of interested bands was calculated with the comparison of the loading control GAPDH. Numbers underneath the blot represent the fold changes of relative gray intensity compared with that of the standard (setting=1.00).

Real-time PCR

RNA from experimental cells was extracted using TRIzol reagent (Invitrogen), and transcribed into cDNA with oligo-dT primers (Takara, Dalian, China). Real-time PCR was performed using $5 \times$ PowerUp SYBR Green Master Mix (ABI) and QuantStudio Q6 Flex to measure the mRNA levels of MMP3 and GAPDH. The real-time quantitative PCR was performed using the following primer pairs: GAPDH Forward 5'- GAGC-CAAACGGGTCATCATCT -3'; Reverse 5'- GAGGGGCCATCCACAGTCTT -3'; MMP3 Forward 5'- ACATGGAGA CTTTGTCCCT TTTG -3'; Reverse 5'- TTGGCTGAGTGGTAGAGTCCC -3'. The relative 3 expression level normalized to GAPDH was determined by the $2^{-\Delta\Delta CT}$ method.

5-ethynyl-2'-deoxyuridine (EDU) assay for detection of cell proliferation

EDU is a thymine nucleoside analogue and can be inserted into the replicating DNA molecule during cell proliferation. Based on the conjugate reaction between EDU and dye, it can be used in the efficient and rapid cell proliferation detection [31]. Cell-Light EdU Apollo488 *in Vitro* Kit was purchased from RiboBio Co., Ltd. (C10310-3; Guangzhou, China). MC-38-vector and MC-38-Shmmp3 cells were seeded in a 12-well plate at a density of 1.5×10^5 /well. Twelve hours after inoculation, the cell culture medium was replaced with 1 mL of fresh medium containing 50 μ M EdU, and incubated for another 2 h. The cells were collected and fixed with 1 mL of 4% polymethylmethacrylate for 20 min. After permeating the nuclear membrane with 1 mL 0.5% Triton X-100, 200 μ l $1 \times$ Apollo488 staining reaction solution was added to each group. The cells were kept in the dark for 10 min and then washed 3 times with 0.5% TritonX-100 plus once with PBS. Finally, the cells were resuspended in 500 μ l PBS and detected by flow cytometry.

Matrigel invasion assay

Matrigel was obtained from BD (356,234, Bedford, MA, USA) and thawed at 4 °C overnight and diluted in a concentration of 5 mg/ml with pre-chilled cell culture medium. 25 μ g of Matrigel was placed in the upper chamber of a 24-well Transwell and preincubated at 37 °C for one hour. Then 5.0×10^4 cells premixed with NNGH inhibitor at a final concentration of 10Ki were added to each well. The transwell was incubated at 37 °C for 48 h and the cells on the top of upper chamber were scraped, and the cells on the lower chamber were fixed with 70% alcohol for 5 min, washed with $1 \times$ PBS, and finally stained with 0.5% Crystal violet solution and the number was counted under a light microscope.

Histopathological research

Lungs from the colon cancer lung metastasis model mice were collected 20 days post tail vein injection of tumor cells. The lungs were immersed in 4% paraformaldehyde solution for fixation, then processed for dehydration by 75–100% ethanol. The samples were finally embedded in paraffin and cut into 5 μ m thick slices by Leica RM2235 Rotary Slicer. The slices were then stained with hematoxylin and eosin, and examined under Nikon microscopy.

The detection of MC-38 infection by flow cytometry

MC-38 cells were infected with VSV^{M51R} at MOI=1. The infected cells were harvested 24 h post infection and stained with a rabbit VSV G protein primary antibody (Abcam) for 30 min. The cells were then washed three times with PBS and then stained with Goat-anti-rabbit-633 secondary antibody for 30 min (Novus). After washing, the percentage of VSV G positive cells was measured on a FACS Canto II cytometer (BD Biosciences), and data were analyzed using FlowJo software (BD Biosciences).

Statistical analysis

Graph Pad Prism 5.0 software was used for the experimental data analysis. The experimental data were expressed by Mean + SD. Unpaired *t*-test was used for the comparison of mean values between two groups. * *P* < 0.05 was considered to have significant differences.

Results

High level of MMP3 expression in colon cancer cells

Tumor cells typically express a high level of MMPs. To compare the expression level of MMP3 in different tumor cells, we firstly measured MMP3 expression in 12 human tumor cells by quantitative PCR. The results revealed that HT-29 and Caco-2 human colon cancer cells expressed the highest level of MMP3. To establish a tumor model in immunocompetent mice, we also measured MMP3 expression in MC-38 mouse colon cancer cells. Indeed, MC-38 cells had a high expression level of MMP3, similar to HT-29 cells (Fig. 1A). Furthermore, upregulation of MMP3 was also observed in colon cancer patients. Analysis of the colon cancer cohort from The Cancer Genome Atlas (TCGA) database indicated that MMP3 in colon cancer samples increased 34.54 folds compared with that of normal samples (Fig. 1B). The ability of oncolytic virus to infect tumor cells is a prerequisite for its oncolytic effect. To determine whether the oncolytic virus VSV is prone to infect MC-38 cells, we infected MC-38 cells with VSV^{M51R} [23], an attenuated VSV at MOI=1. The infected cells were stained 24 h post-infection and examined by flow cytometry. The majority of the cells (72.0%) were determined as viral G protein positive (Fig. 1C), suggesting that MC-38 cells are susceptible to VSV infection.

Tumor cell-associated MMP3 expression affects tumor growth

To study the effect of MMP3 on tumor growth, we generated MC-38 cells with stable downregulation of MMP3 via lentiviral-mediated shRNA knockdown. The downregulation of MMP3 in MC-38 cells was confirmed by quantitative PCR and western blotting (Fig. 2A). Although the two designed MMP3 shRNA sequences both were effectively measured by real-time PCR, the Shmmp3-2 sequence exhibited the more inhibition in western blot. Therefore, the knock down cells generated by Shmmp3-2 sequence was used in the following experiment. Next, the effect of MMP3 was investigated in a mouse tumor model using the MMP3-knockdown cells and MMP3 KO mice. As shown in Fig. 2B. The experiment utilized four groups: wild-type (WT) mice and MMP3 KO mice, each inoculated with MMP3-knockdown cells MC-38-Shmmp3 or

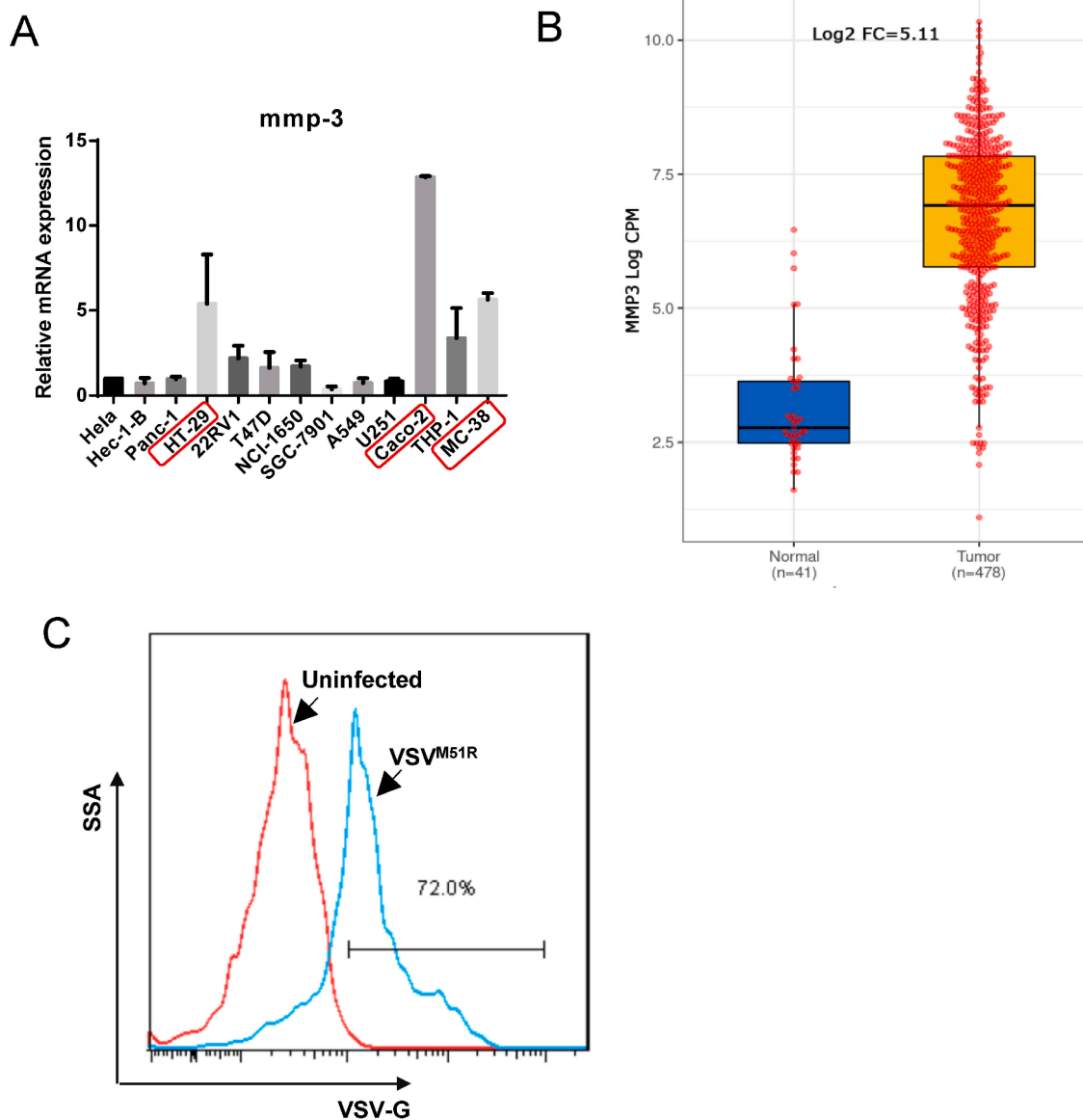


Fig. 1. MC-38 cells had high expression level of MMP3 and were easily succumbed to VSV infection. (A) The relative MMP3 expression in different tumor cells. (B) Boxplot showing normalized and log-transformed MMP3 gene expression levels of tumor and normal samples from TCGA colon cancer cohort. Differential expression analysis indicates significant upregulation of MMP3 in tumor samples Log₂ FC (Fold Change) = 5.11, FDR = 2.98e-30. (C) The MC-38 cells were infected by VSV^{M51R} at MOI=1 for 12 h. The percentage of VSV-G positive cells was measured by flow cytometry as infection efficiency.

control cells MC-38-Vector on 0 day. The tumor size was measured 6 days post-inoculation. Interestingly, differences in tumor size between mice inoculated with knockdown cells and Vector cells were observed at 16 days post-inoculation, and the differences became more obvious over time (Fig. 2C). However, there was no difference in tumor size between MMP3 WT mice or KO mice inoculated with the same cells. There was a significant difference in tumor size between the two groups of mice inoculated with MMP3 knockdown cells compared with those inoculated with control cells, regardless of the type of mice, on day 26 post-inoculation (Fig. 2D). Although all mice in these four groups died, those that received the MMP3-knockdown cells survived longer than the other receiving control cells (Fig. 2E). Taken together, our results suggest that the endogenous MMP3 in tumor cells, but not MMP3 in other tissues, is crucial for tumor growth in mouse models.

Improved effect of the MMP3 inhibitor NNGH on tumor growth when combined with oncolytic virus

Previous studies have shown that the inhibitor NNGH can inhibit the function of MMP3, thus affecting the neuronal apoptosis signal transduction process downstream of caspase-12 [32]. To address whether the therapeutic effects of NNGH can be improved with the combination of oncolytic virus therapy, we firstly verified the effect of NNGH *in vitro*. Transwell assay showed that the number of MC-38 cells on the membrane in the presence of NNGH was significantly reduced 48 h after culture, suggesting that the cells treated with NNGH had impaired migration ability (Fig. 3A). Next, colon cancer tumor models were established in mice (Fig. 3B). The tumor volumes in different groups were measured 7 days post-inoculation (Fig. 3C). At 25 days post-inoculation, tumor size was obviously reduced in the oncolytic virus therapy VSV^{M51R} group compared with that in the PBS group, which was in line with our previous finding [23]. However, the inhibitory role of NNGH on tumor growth was weak *in vivo*, similar to the

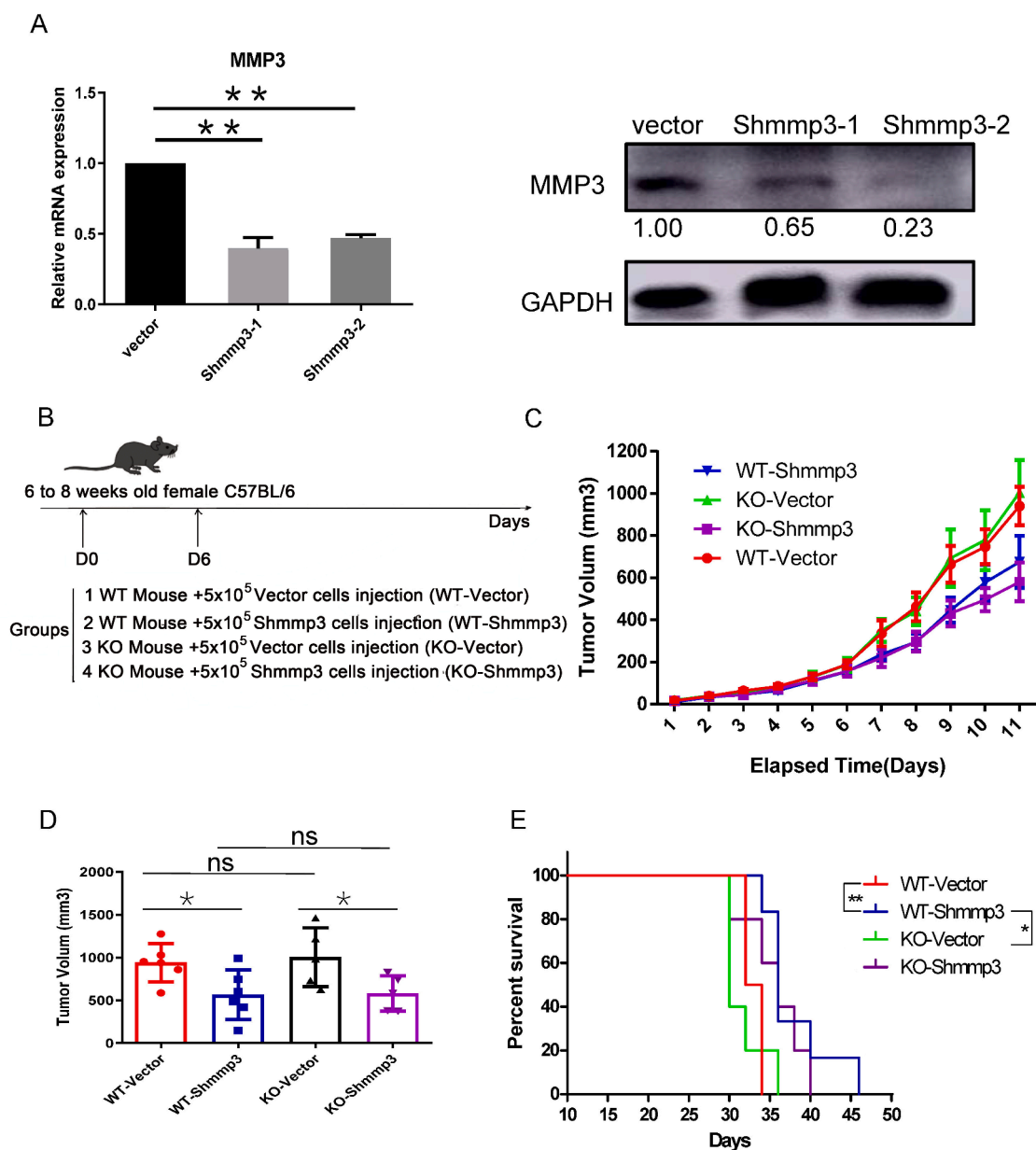


Fig. 2. The tumor cell endogenous MMP3 was crucial in tumor growth. (A) The downregulation of MMP3 in MC-38 cells was confirmed by real-time PCR and Western blot. The relative gray intensity of MMP3 bands in Vector, shmmp3-1# and shmmp3-2# was determined with the comparison of the loading control GAPDH using Image J software. The numbers underneath the blot represent the fold changes compared with that of Vector group (1.00). (B) The schematic of inoculation protocol for different tumor cell and mouse type combinations. The mice were divided into four groups, including (1) the WT mice injected the Vector cells or (2) Shmmp3 knockdown cells on day 0; (3) the MMP3 KO mice injected with Vector cells or (4) Shmmp3 knockdown cells and the injection was performed on day 0. There were 6 mice in WT mouse group and 5 mice KO mouse group. (C) The tumor size change in each mouse group after 6 days post-inoculation. (D) The measurement of tumor size in each mouse group on day 26 of inoculation, Unpaired *t*-test was used for the comparison of mean values between two groups, Error bars represent the standard error of the mean.; (E) The survival analysis tested by log-rank (Mantel-Cox) test for different treatments ($n = 6$). * $P < 0.05$; ** $P < 0.01$.

result in the PBS group. It is noteworthy that in mice treated with NNGH combined with VSV^{M51R}, the tumor volume was significantly reduced, which indicated the synergistic antitumor effect of oncolytic virus and NNGH (Fig. 3D). In accordance with tumor volume, mice in the PBS group died rapidly. Importantly, the combination therapy group had the best survival, as evidenced by either delayed dead time or increased survival rate (1/7, 14.3%) compared with the other three groups (Fig. 3E). Because NNGH is a broad spectrum MMPs inhibitor, to further confirm the effects of MMP3 inhibition with oncolytic virotherapy on colon cancer, we applied lentiviral-mediated shRNA knockdown which was specific to MMP3. Similar results were observed compared with that using NNGH inhibitor (Supplementary data Fig. S1).

The improved efficacy of tumor therapy by using MMP3-specific siRNA together with oncolytic virus

Considering the toxicity of NNGH and the un-safety of lentivirus-mediated knockdown of MMP3 in clinical application [19,33], we further pursued siRNA instead of MMP inhibitors or lentivirus for downregulating MMP3 in mouse tumors. The downregulation of MMP3 was firstly verified by transfection of MMP3-specific siRNAs to MC-38 cells. The MMP3 mRNA level in transfected cells decreased by more than two-folds at 48 h post-transfection; the protein level also decreased, as shown by western blotting (Fig. 4A and B). Mouse tumor models were then established, and at 8 days post-inoculation, they received the

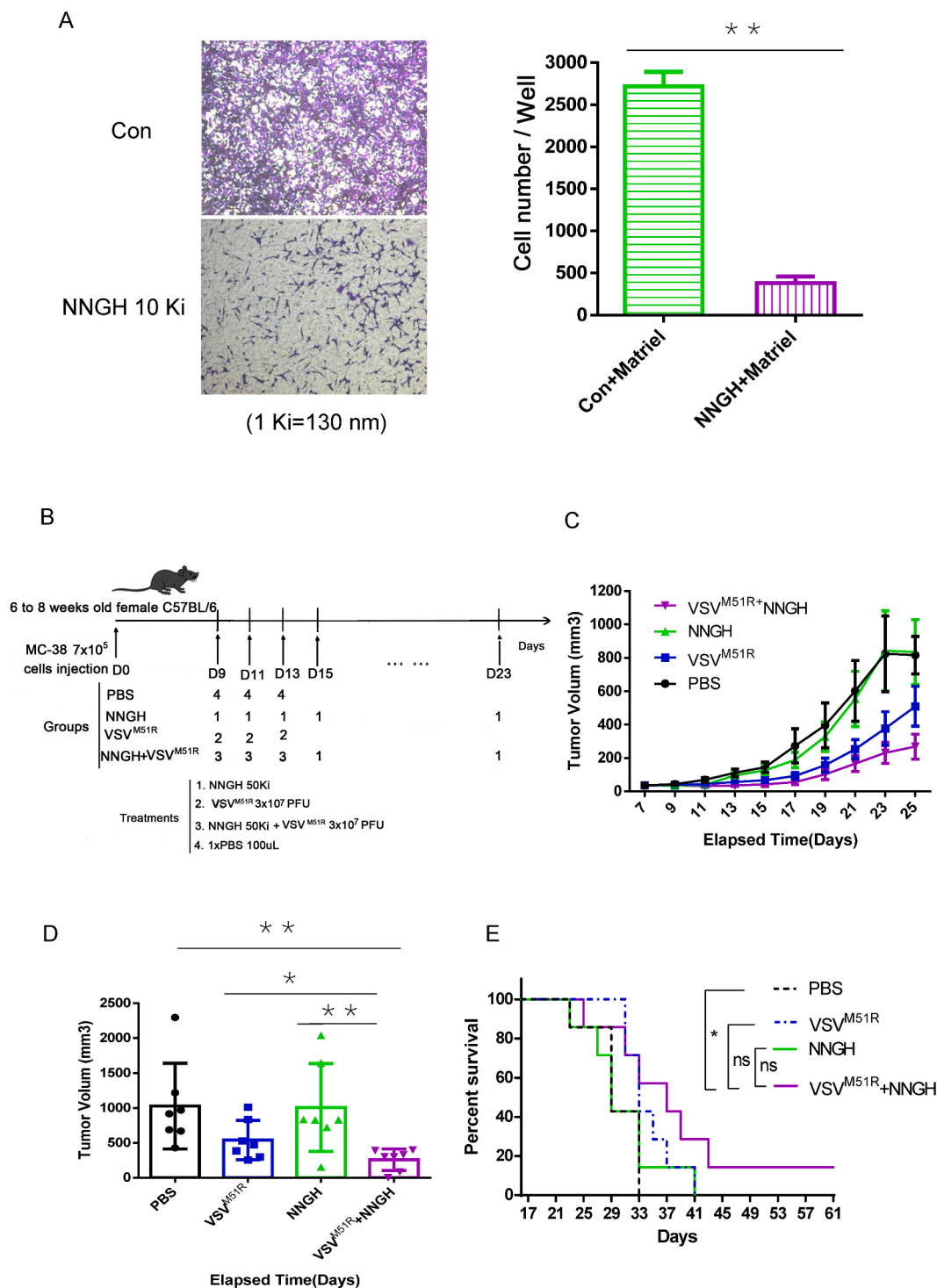


Fig. 3. Combination of MMP3 inhibitor NNGH and VSV^{M51R} virus can effectively suppress tumor growth and increase mouse survival. (A) The NNGH treatment reduced the migration ability of MC-38 in Matrigel invasion assay. Left panel was the fields of low bottom under microscopy; Right panel was the cell number from three independent wells. (B) The schematic of NNGH and VSV^{M51R} virus combination therapy plan in mice ($n = 7$ per group). The treatments of each group were indicated. (C) The measurement of tumor volume in each group 7 days post-implantation. (D) The tumor volume of each group on day 25 post-implantation. Error bars represent the standard error of the mean. Unpaired t -test was used for the comparison of mean values between two groups, Error bars represent the standard error of the mean. (E) The survival analysis tested by log-rank (Mantel-Cox) test for different treatments ($n = 7$). * $P < 0.05$; ** $P < 0.01$.

treatments as indicated in Fig. 4C. Similar to our results in Fig. 3, the tumor growth among these four groups became different on 12 days post-inoculation (Fig. 4D) and it was obvious on 24 days post-inoculation (Fig. 4E). The inhibitory effect of oncolytic virus was significantly improved in the presence of MMP3 siRNA. The average tumor volume was 200 m^3 , which was much lower than that in the

oncolytic virus alone group (500 m^3). Consequently, the group treated with oncolytic virus and MMP3 siRNA had the best survival (2/7, 28.6%), whereas mice in the other three groups all died (Fig. 4F). Taken together, these results indicate that using MMP3 siRNA combined with oncolytic virus would be a beneficial alternative option in tumor therapy.

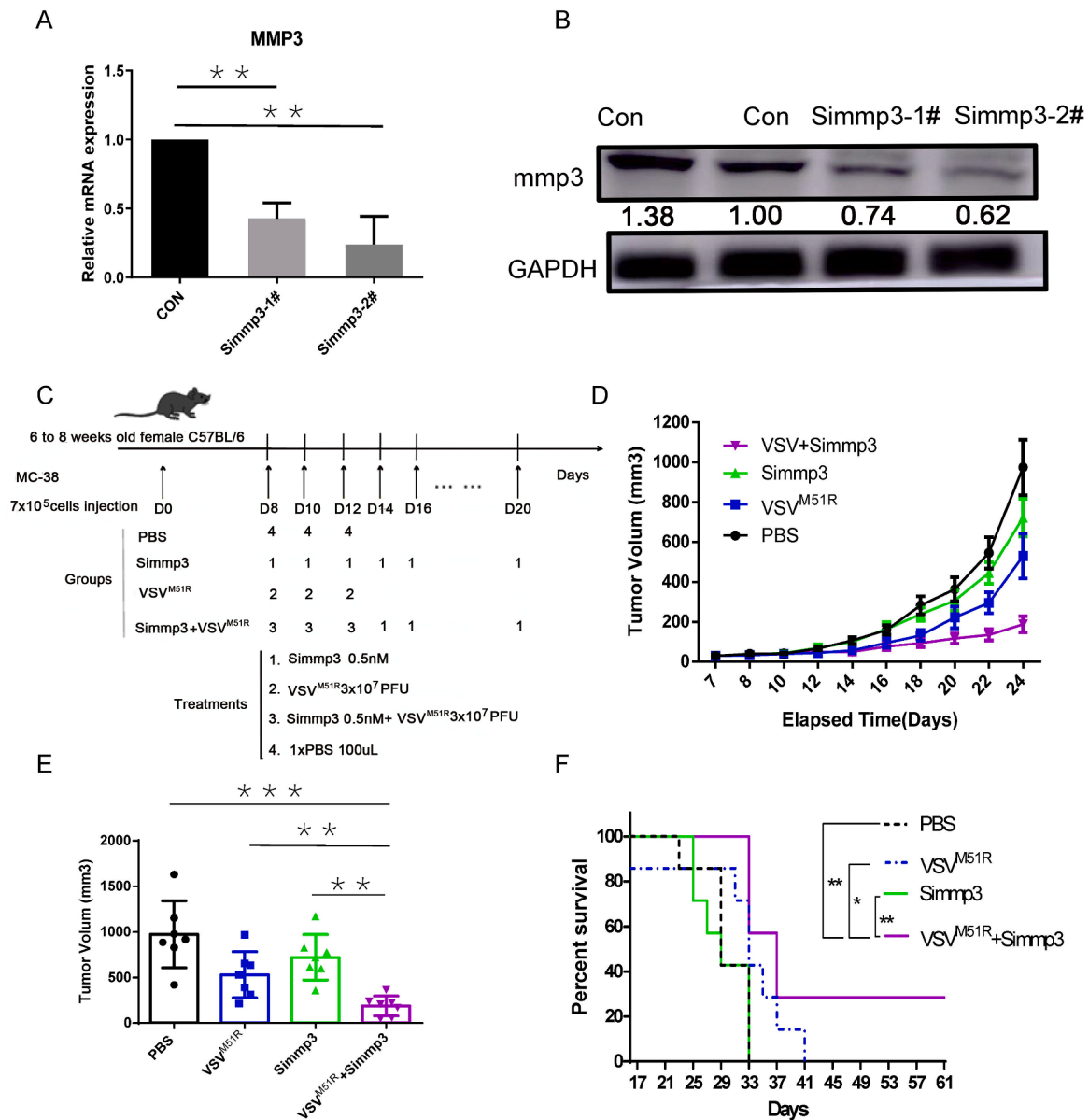


Fig. 4. Combination of MMP3 siRNA and VSV^{M51R} virus can effectively suppress tumor growth and increase mouse survival. The MC-38 cells were transfected with 100 nM MMP3 siRNA Simmp3-1# and Simmp3-2#. The down-regulation of MMP3 was determined by (A) real time-PCR or (B) Western blot 48 h post-transfection. (C) The schematic of MMP3 siRNA and VSV^{M51R} virus combination therapy treatments (n = 7). (D) The change of tumor volume in different mouse groups 7 days post-implantation. (E) The measurement of tumor volume on day 24 post-implantation. Error bars represent the standard error of the mean. Unpaired t-test was used for the comparison of mean values between two groups, Error bars represent the standard error of the mean. **P < 0.01; *** P < 0.001. (F) The survival analysis tested by log-rank (Mantel-Cox) test for different treatments (n = 7). * P < 0.05; **P < 0.01; ***P < 0.001.

Reduced cell proliferation and migration mediated by MMP3 downregulation is dependent on Erk1/2 and NF-κB signaling

Recently, Rh1, a traditional medicine monomer, was shown to inhibit colorectal cancer cell proliferation, migration, and invasion, which was partially due to the inhibition of MMP1 and MMP3 expression, increased TIMP3 expression level, and inactivation of the MAPK signaling pathway [34]. A report also showed that the transcription factor activity of NF-κB was significantly impaired upon MMP3 silencing during DENV infection [35]. Therefore, we then examined the MAPK and NF-κB signaling pathways. The expression of p38, phosphorylated p38 (p-p38), JNK, and phosphorylated JNK (p-JNK) was not changed in MMP3 knockdown MC-38-Shmmp3 cells (Fig. 5A). However, the expression of phosphorylated Erk1/2 (p-Erk1/2) and p65 (p-p65) decreased with MMP3 downregulation, suggesting that MMP3 reduction

may impair Erk1/2 and NF-κB signaling. To further confirm the effect of MMP3 on cell proliferation, we performed EDU cell proliferation experiments. The results indicated that MC-38-Shmmp3 cells had slow proliferation rate. When MC-38-Vector cells were treated with Erk1/2 or NF-κB inhibitors, they exhibited slow growth similar to that after MMP3 knockdown, suggesting that both signaling pathways were important for cell proliferation (Fig. 5B). CCK8 assay also indicated that the mmp3 knockdown MC-38 cells exhibited slow growth after 48 h of culture (supplementary data Fig. S2).

MMPs can degrade the extracellular matrix to make tumor tissues relatively loose, which is beneficial to tumor cell proliferation and invasion [36]. MC-38-Shmmp3 or MC-38-Vector cells were seeded in a 24-well Transwell plate precoated with Matrigel. At 48 h post-seeding, the cells were fixed and stained with 0.5% crystal violet. We found that the migration ability of MC-38-Shmmp3 cells was weaker, as

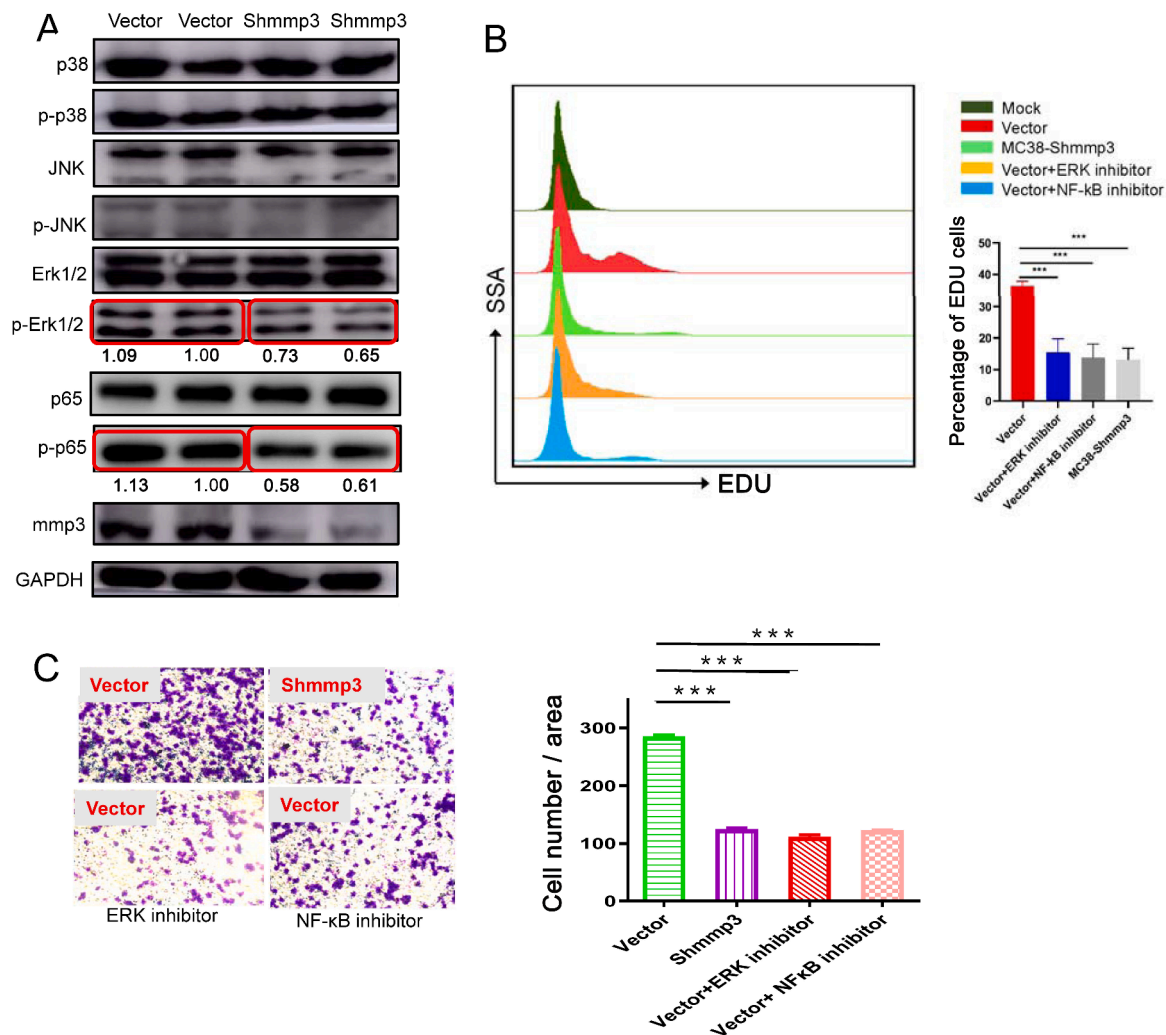


Fig. 5. The tumor cell proliferation and migration were affected by down-regulation of mmp3 through Erk1/2 and NF- κ B signaling pathways. (A) The expression levels of p38, p-p38, JNK, p-JNK and Erk1 / 2 and p-Erk1 / 2 in MAPK and NF- κ B signaling pathways was examined by Western blot in MC-38-Vector and MC-38-Shmmp3 cells. The relative gray intensity of p-Erk1/2 and p-p65 bands in both cells was determined with the comparison of the loading control GAPDH using Image J software. The numbers underneath the blot represent the fold changes compared with that of the Vector group (1.00). (B) The cell proliferation of MC-38-Shmmp3 and MC-38-Vector cells was measured by EDU assay or MC-38-Vector cells 12 h post the inhibitor treatment. (C) Transwell assay of MC-38-Shmmp3 and MC-38-Vector cells with or without inhibitors. Left panel was the fields of low bottom under microscopy; Right panel was the average of cell number from three counted areas. Unpaired *t*-test was used for the comparison of mean values between two groups, Error bars represent the standard error of the mean, Values are means \pm SD from three independent experiments *** *P* < 0.001.

indicated by the significantly reduced number of cells on the membrane (Fig. 5C), and the MC-38-Vector cells treated with Erk1/2 or NF- κ B inhibitors showed the similar trends. These results imply that the reduction of cell migration with MMP3 knockdown might be caused by the impairment of Erk1/2 and NF- κ B signaling in MC-38-Shmmp3 cells as seen in Fig. 5A.

Downregulation of MMP3 reduces tumor cell invasion

Our finding above indicates that the downregulation of MMP3 affects the migration of tumor cells. To further explore the effect of MMP3 downregulation on tumor invasion *in vivo*, we established a lung metastasis model by tail vein injection of tumor cells. Each mouse was injected 3×10^6 MC-38-Shmmp3 or MC-38-Vector cells through the tail vein. At 20 days post-infection, the lungs of the injected mice (*n* = 6) were dissected, and tumor formation was determined (Fig. 6A). Five mice were observed the tumor formation in the lungs in MC-38-Vector group. The represent tumor lesions were indicated by the arrows (Fig. 6B) and there were total 17 lesions in this group. Moreover, tumor

invasion in the small intestine was observed in one mouse, whereas another mouse had a tumor under the skin of the left forelimb. However, in the MMP3 knockdown cell group, only one mouse showed lung tumor invasion with 2 lesions, but it was relatively small (Fig. 6B). HE staining showed that the lungs of the MC-38 Vector-injected mice had obvious tumor lesions (Fig. 6B). These results suggest that targeting MMP3 may also inhibit colon cancer cell invasion in mice.

Discussion

MMPs are a large family of proteases that need Ca^{2+} , Zn^{2+} , and other metal ions as cofactors for their activity [37]. MMPs overexpression has been well documented in multiple types of solid tumors [38]. High levels of MMPs have been correlated with poor overall survival in virtually all solid malignancies, and they affect a series of key cell processes, including cell proliferation, apoptosis, and tissue angiogenesis, through the degradation of matrix proteins in the tumor microenvironment [36, 39]. In the present study, we revealed that targeting MMP3 in oncolytic virus-mediated tumor immunotherapy efficiently inhibited tumor

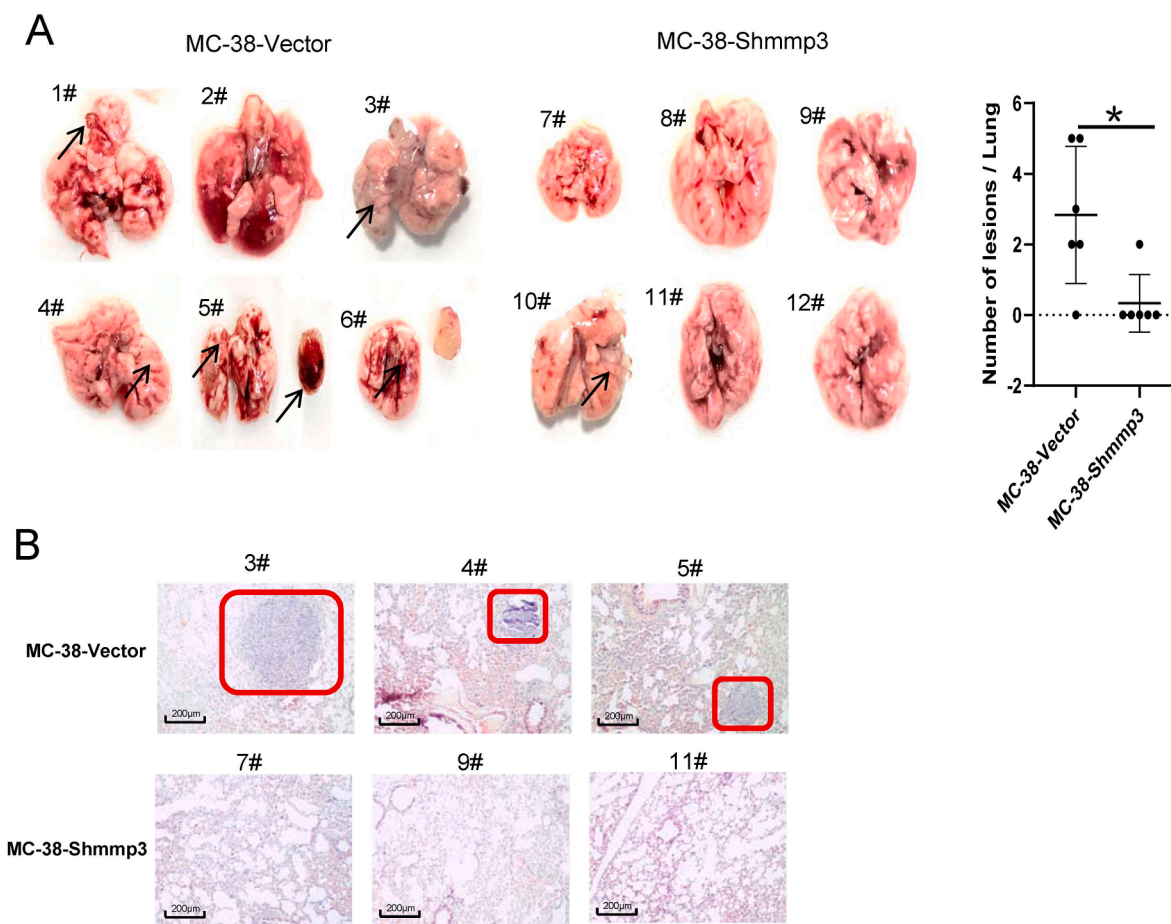


Fig. 6. The tumor cell MMP3 may facilitate tumor invasion in the mouse colon cancer model. (A) Each mouse in group ($n = 6$) was injected with 3×10^6 MC-38-Shmmp3 or MC-38-Vector cells through tail vein for generating metastatic model. The mouse lungs (1-6# in MC-38-Shmmp3 group, 7-12# in MC-38-Vector group) were dissected to observe the lung tumor metastasis 20 days post-injection, and the arrow indicates the lung tumor formations. Right panel was the lesion number of each mouse lung. (B) The mouse lungs in each group were stained with HE, and the representative pathological sections of the lung tumors were labeled with red rectangles (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

growth.

It has long been known that MMPs take an important role in the tumor progression. Although much effort has been paid to the development of MMPis for clinical cancer therapy, few of MMPis got success given their inefficiency and severe side effects [20]. The failure of MMPis in clinical trials can be attributed to three main reasons. First, some MMPs have antitumor effects; thus, the broad-spectrum MMP synthesis inhibitors used in the initial trials may have blocked the activity of these MMPs. Second, some MMPs participate in the early stages of tumor development; thus, they are not effective against the advanced tumors examined in previous studies [20]. Third, MMPis have unpredictable, serious side effects. For example, marimastat has shown great potential in the preclinical setting, targeting various solid tumor types in a metastatic environment. However, owing to side effects such as joint pain, stiffness, and inflammation, their clinical application has to be stopped [40]. In the present study, knockdown of MMP3 was achieved by using not only the chemical MMPi NNGH but also lentiviral-mediated shRNA and MMP3-specific siRNA. We observed that these methods can inhibit tumor growth at 14 days after inoculation, but the effect was weak. This is consistent with previous results suggesting the inefficiency of targeting MMPs alone in cancer therapy. Nevertheless, the most exciting finding here is that the antitumor efficacy of targeting MMP3 in mouse models was greatly improved when combined with an oncolytic virus. The mice receiving MMP3 siRNA and OV combination therapy exhibited significantly reduced tumor size and better survival. Recently, it was reported that many cancer patients benefit from immunotherapy

and targeted therapy, especially with the use of PD-1 immune checkpoint inhibitors [24,41]. Considering the cytotoxicity of NNGH and the un-safety of lentiviral transduction, using siRNA instead of MMPis for MMP3 knockdown along with OVs immunotherapy is promising for future clinical trials. However, when using siRNA in cancer therapy, the development of a potent system for efficient siRNA targeting delivery must be considered [42].

MMP3 is involved in various physiological processes, such as angiogenesis, cell growth, and cell invasion [43]. Studies have reported high levels of MMP3 in the blood, cancer tissues, and urine samples of breast cancer patients [44,45]. Interestingly, we identified that MMP3 from tumor cells, but not that from other tissues, was critical for tumor growth. The tumor size in KO mice was not different from that in WT mice, but tumor volume was reduced in mice inoculated with MMP3 knockdown MC-38 cells. This is in line with our *in vitro* results that cell proliferation is reduced in MMP3 knockdown MC-38 cells. In addition, Erk1/2 and NF- κ B signaling were impaired in MMP3 knockdown cells. In MC-38-Vector cells treated with Erk1/2 or NF- κ B inhibitors, cell proliferation was reduced, similar to that in MMP3 knockdown cells, which suggested that MMP3 may affect cell proliferation through Erk1/2 and NF- κ B signaling. Moreover, the MMP knockdown cells showed decreased migration in Transwell assay and decreased tumor formation in a lung metastasis mouse model, suggesting that targeting MMP3 can be a promising method for the treatment of tumor metastasis.

It has become clear that current oncolytic virus therapeutics on their own are unlikely to be effective in most patients [46]. Several preclinical

and clinical studies investigating the combination of OV with approved chemotherapeutics have been conducted [47]. A previous research in our laboratory also found that the treatment of subcutaneous tumors of non-small cell lung cancer in mice with VSV^{M51R} alone is very limited. However, when these mice were treated with modified VSV^{M51R} expressing PD-L1-scFv, their survival rate significantly improved, suggesting that the combination therapy may provide more benefits than monotherapy [23]. Interestingly, MMP3 inhibition together with VSV^{M51R} greatly increased therapeutic efficacy in the present study. Previous study has shown that upregulation of MMP1 and MMP8 enhances the HSV dissemination in tumor and improve HSV oncolytic efficacy [25]. We found that the viral replication was not changed between MC-38-Vector and MC-38-Shmmp3 cells, indicating that the MMP3 does not have the effects on viral replication either *in vitro* or *in vivo* (data not shown). The improved therapeutic efficacy of combination therapy might come from the superimposition of two different monotherapy including MMP3 inhibition and oncolytic virus. Whether the combination of MMP3 inhibition with other virus, such as HSV or adenovirus, is worthy of investigation in future studies.

In summary, we identified that tumor cell-derived MMP3 is critical for tumor cell growth and migration. Targeting MMP3 using multiple methods together with OV therapy consistently showed great improvement of tumor therapy in mouse colon cancer models, revealing its potential as a novel therapeutic strategy for cancer treatment.

CRedit authorship contribution statement

Minglong Liang: Conceptualization, Formal analysis, Data curation, Writing – original draft. **Jian Wang:** Data curation. **Chuanjian Wu:** Data curation. **Manman Wu:** Data curation. **Jingping Hu:** Data curation. **Jianfeng Dai:** Resources. **Hang Ruan:** Data curation, Formal analysis. **Sidong Xiong:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing, Formal analysis. **Chunsheng Dong:** Conceptualization, Formal analysis, Writing – original draft.

Declaration of Competing Interest

All the authors declare that they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.tranon.2021.101221](https://doi.org/10.1016/j.tranon.2021.101221).

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