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Modulation of the malignant behavior of tongue squamous cell carcinoma cells by matrix metallopeptidase 25 through the NF-κB pathway

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Summary. Objective. Accumulating evidence has implicated matrix metalloproteinases (MMPs) in the progression of human cancers. Matrix metallopeptidase 25 (MMP25) is a membrane-type MMP whose role in tumorigenesis and cancer development is not well understood. Here, we investigated the functions of MMP25 in tongue squamous cell carcinoma (TSCC).

Methods. Gene expression was measured using real-time PCR and western blot. CCK-8 and Transwell assays were used to determine the proliferation, migration, and invasion of TSCC cells. An NK cell co-culture experiment was performed to evaluate the killing of TSCC cells by NK cells.

Results. MMP25 had higher expression levels in TSCC tissues than in adjacent non-cancerous tissues. MMP25-overexpressing and MMP25-silenced TSCC cell lines were established by lentiviral transduction. Overexpression of MMP25 promoted proliferation, migration, and invasion of TSCC cells, whereas knockdown of MMP25 had opposite effects. MMP25 modulated the levels of proliferation- and apoptosis-related proteins (PCNA, cyclin D, cyclin B1, p27, and cleaved caspase 3 and 9) and upregulated two invasion-related MMPs (mature MMP2 and MMP9). Additionally, MMP25 promoted tumor growth of TSCC cells in athymic nude mice. Notably, MMP25 upregulated PD-L1 in TSCC cells, attenuated NK cell killing of TSCC cells, and inhibited the secretion of anti-tumor cytokines (TNF-α and IFN-γ). Furthermore, MMP25 promoted the nuclear translocation of NF-κB p65, suggesting that activation of NF-κB signaling may mediate the pro-tumor functions of MMP25 in TSCC.

Conclusion. This study revealed a novel role for MMP25 in TSCC, highlighting the potential of MMP25 as a therapeutic target in TSCC.

Key words: Tongue squamous cell carcinoma, MMP25, Immune evasion, PD-L1, NF-κB signaling pathway, Tumor progression

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Introduction

Tongue squamous cell carcinoma (TSCC) is the most common form of oral cancer, accounting for more than 40% of all cases (Ion Ciucă Mărăşescu et al., 2018). Surgery is effective in treating early-stage TSCC, however, many patients are diagnosed at a late stage, and individuals with TSCC often experience oral morbidity and require restoration of oral function following surgery (Rivera et al., 2017). Despite significant therapeutic advances over the past two decades, TSCC has a relatively poor prognosis due to its highly invasive behavior, with a five-year survival rate of less than 50% (Seppälä et al., 2016; Johnson et al., 2020; Dolens et al., 2021). Therefore, there is an urgent need to explore promising therapeutic targets for TSCC.

Proteases can process, cleave, or degrade all proteins and are, therefore, involved in almost every biological process (Fortelny et al., 2014). The human matrix metalloproteinase (MMP) family consists of more than 20 members with different domain compositions. Matrix metallopeptidase 25 (MMP25) is a membrane-type MMP (also known as membrane-type matrix metalloproteinase 6, MT-MMP6), which is anchored to the cell membrane by glycosyl-phosphatidyl inositol and is mainly located at intercellular junctions (Kojima et al., 2000; Radichev et al., 2010).

MMP25 showed a higher expression level in gastric cancer than in normal stomach and chronic superficial gastritis, suggesting that the expression of MMP25 is increased as gastric cancer progresses (de la Peña et al., 2014; Wang et al., 2015b). Highly expressed MMP25 was also observed in colon cancer and was found to

promote tumor growth in the xenograft mouse model (Sun et al., 2007). In addition to tumor growth, MMP25 also contributed to lung tropic metastasis of melanoma (Chen et al., 2019), and its pro-metastatic and pro-invasive effects were found to be related to its activation of MMP2 (Nie and Pei, 2003). Interestingly, a compound with inhibitory activity against MMP25 significantly suppressed the invasive ability of glioblastoma cells but did not affect cell viability (Nuti et al., 2011), which makes targeting MMP25 in cancer treatment an attractive therapeutic strategy. However, whether MMP25 has an impact on the malignant phenotypes of TSCC cells is currently unknown.

In addition to its involvement in tumor progression, MMP25 has also been implicated in immune responses. MMP25 regulates the chemotaxis of neutrophils and monocytes (Starr et al., 2012), and knockout of MMP25 significantly impaired innate immunity in mice (Soria-Valles et al., 2016). MMP25 was upregulated on the surface of IL-2-activated NK cells. The Ab-dependent cell-mediated cytotoxicity capacity of NK cells was enhanced by disruption of MMP25 expression (Peruzzi et al., 2013); however, whether MMP25 plays a role in anti-tumor immunity is largely unknown.

In this study, for the first time, we demonstrated the role of MMP25 in the progression of TSCC and elucidated its potential downstream mechanisms.

Materials and methods

Cell lines and cell culture

Two human TSCC cell lines were used in this study. CAL-27 cells (RRID: CVCL_1107) were cultured in DMEM (Cat. No. 12100046; Gibco- ThermoFisher co. Ltd, PA, USA) and UPCI-SCC-090 cells (RRID: CVCL_1899) were cultured in MEM (Cat. No. 41500; Solarbio, Beijing, China). All culture media were supplemented with 10% FBS (Cat. No. 11011-8611; Tianhang, Zhejiang, China). NK-92 cells (RRID: CVCL_2142) were cultured in NK cell culture medium (Cat. No. CM-0530; Procell, Wuhan, China). All cells were cultured at 37°C in a humidified incubator containing 5% CO_2.

Lentiviral transduction and establishment of stable cell lines

Lentiviral vectors pLKO.1-EGFP-puro harboring MMP25 shRNA or PLJM1-EGFP-puro harboring MMP25 CDS were co-transfected with assistant plasmids pSPAX2 and pMD2.G (Fenghui Biotechnology) into HEK293T packaging cells. All plasmids were purchased from Hunan Fenghui Biotechnology Co., Ltd. (Changsha, China). TSCC cells were infected with the corresponding lentiviruses at a 10:1 multiplicity of infection (MOI). Then, 72 hours after infection, the cells were subjected to further experiments. The stable cell lines were established by puromycin selection.

Human TSCC samples

Human TSCC samples and adjacent non-cancerous samples were collected from Tianjin Stomatological Hospital. Written informed consent was obtained from each patient. Research involving human subjects was approved by the Ethics Committee of Tianjin Stomatological Hospital and conducted in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments.

RNA extraction and real-time PCR

Total RNA was extracted from tissues or cells using TRIpure reagent (Cat. No. RP1001; BioTeke Co., Beijing, China). RNA was then reverse transcribed into cDNA using Super M-MLV reverse transcriptase (Cat. No. PR6502; BioTeke) and RNase inhibitor (Cat. No. RP5602; BioTeke). The cDNA was used as a template to run 20 μL real-time PCR reactions using the 2×Taq PCR MasterMix (Cat. No. PC1150; Solarbio) and SYBR Green (Cat. No. SY1020; BioTeke) on an Exicycler real-time PCR system (Bioneer, Daejeon, Korea) with 96-well plates. Results were normalized to the housekeeping gene β-actin (*ACTB*) using the ΔΔCt method. The primer sequences are included in Table 1.

Immunoblotting

Proteins were extracted from cells or tissues using a whole protein extraction kit (Cat. No. WLA019; Wanleibio Co., Ltd., Shenyang, China), resolved by SDS-PAGE, and transferred to PVDF membranes (Cat. No. IPVH00010; Millipore Co., MA, USA). Blots were probed with primary and secondary antibodies and visualized with a chemiluminescence reagent. Antibodies to MMP25 (1:100 diluted, Cat. No. A3032; RRID: AB_2764838), NF-κB p65 (1:1000 diluted, Cat. No. A2547; RRID: AB_2764436), and phospho-NF-κB p65 (1:1000 diluted, Cat. No. AP0124; RRID: AB_2771510) were purchased from the ABclonal Company (Wuhan, China). Antibodies to PCNA (1:500 diluted, Cat. No. WL03213), cyclin D (1:500 diluted, Cat. No. WL01435a), cyclin B1 (1:500 diluted, Cat. No. WL01760), p27 (1:500 diluted, Cat. No. WL04174), cleaved caspase 3 (1:500 diluted, Cat. No. WL02117; RRID: AB_2910623), cleaved caspase 9 (1:500 diluted, Cat. No. WL01551), mature MMP2 (1:500 diluted, Cat.

Table 1. Primers used in real-time PCR.

Primer	Sequences (5' to 3' direction)	Primer length (bp)
MMP25 forward	CGTGGCTGTCCATGAGTTTG	20
MMP25 reverse	GGGTTTCCTTGTGGGCTTG	19
PD-L1 (CD274) forward	GAACTACCTCTGGCACAT	18
PD-L1 (CD274) reverse	CCATCATTCTCCCTTTT	17
β-actin (ACTB) forward	CACTGTGCCCATCTACGAGG	20
β-actin (ACTB) reverse	TAATGTCACGCACGATTTCC	20

No. WL03702), mature MMP9 (1:500 diluted, Cat. No. WL03096; RRID: AB_2921218), and PD-L1 (1:500 diluted, Cat. No. WL02778) were purchased from Wanleibio Co., Ltd. (Shenyang, China).

Cell counting kit-8 (CCK-8) assay

The CCK-8 assay was used to evaluate cell proliferation. Briefly, 72 h after lentivirus infection, TSCC cells were seeded in 96-well plates at a density of 4×10^3 cells/well in DMEM or MEM supplemented with 10% FBS. After different periods (0, 12, 24, 48, 72h) of culture at 37°C in a 5% CO_2 incubator, the culture medium was refreshed by adding 10 μL CCK-8 solution (Cat. No. WLA074; Wanleibio). After 2 hours of incubation at 37°C, the OD was measured at 450 nm using a microplate reader (BIO-TEK, VT, USA).

Transwell migration and invasion assay

Cell invasion and migration were evaluated using the Transwell assay. The invasion assay was performed using a Transwell chamber (Cat. No. 3422; 8-μm pore polycarbonate membrane, Corning, NY, USA) precoated with Matrigel (Cat. No. 354234; Corning). First, 4×10^4 cells suspended in 200 μL serum-free medium were seeded in each well of the upper chamber, while 800 μL complete medium (containing 10% FBS) was placed in the lower chamber. After 24 hours of incubation, invaded cells were fixed with 4% paraformaldehyde (Cat. No. C104188; Aladdin, Shanghai, China), stained with 0.4% crystal violet (Cat. No. 0528; Amresco Corp., Solon, USA), and counted under ×100 magnification. The migration assay was similar to the invasion assay except that the Transwell chamber was not coated with Matrigel and the seeded cell density was 5×10^3/well.

NK cell lysis

To evaluate NK cell lysis of TSCC cells, NK-92 cells were stimulated in the medium supplemented with 500 U/mL IL-2 (Cat. No. GMP-11848-HNAE; SinoBiological, Beijing, China) for 24 hours. Then, 1×10^4 TSCC cells/well (T: Target) were seeded in a 96-well plate and co-cultured with NK-92 cells (E: Effector) at a 5:1 or 10:1 E:T ratio. After 8 hours of co-culture, 200 μL MTT reagent (Cat. No. WLA021; Wanleibio) was added to each well. Four hours later, the OD was measured at 490 nm using a microplate reader. Specific lysis was calculated as $1-(OD_{E:T}-OD_E)/OD_T$. $OD_{E:T}$ indicated the OD value of E: T co-culture; OD_E indicated the OD value of Effector spontaneous; OD_T indicated the OD value of Target spontaneous.

Enzyme-linked immunosorbent assay (ELISA)

After 8 hours of TSCC cell and NK-92 cell co-culture, the levels of anti-tumor immunity cytokines in cell culture supernatants were examined using a TNF-α ELISA detection kit (Cat. No. WLE05; Wanleibio) and an IFN-γ detection kit (Cat. No. EK180; MultiSciences, Hangzhou, China) following the manufacturers' instructions. In brief, cell culture supernatants were centrifuged at 1000 g for 20 min to remove any precipitate.

For TNF-α measurement, the ELISA plate was pre-coated with 10 μg/ml antibody at 4°C overnight. The samples were then added to the plate and incubated at 37°C for 2h. Subsequently, 100 μl capture antibody was added and incubated at 37°C for 1h. After washing, 100 μl HRP-streptavidin was added and incubated at 37°C for 30 min. Next, 100 μl TMB substrate chromogenic solution was added. After 15 min, 50 μl stop solution was added. Finally, the OD was measured at 450 nm using a microplate reader.

For IFN-γ measurement, the samples and 50 μl detection antibody were added to the plate and incubated at room temperature for 2h, shaking at 300 rpm. After washing, 100 μl HRP-streptavidin was added and incubated at room temperature for 45 min. After washing, 100 μl TMB substrate chromogenic solution was added. After 20 min, 100 μl stop solution was added. Finally, the OD was measured at 450 nm (reference wavelength 570 nm) using a microplate reader.

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.1% Triton X-100 (Cat. No. ST795; Beyotime Inc., Shanghai, China) for 30 minutes, and blocked with 1% BSA (Cat. No. A602440-0050; Sangon Biotech, Shanghai, China) for 15 minutes. After washing with PBS, a primary antibody against NF-κB p65 (Cat. No. A2547, Abclonal; RRID: AB_2764436) and a Cy3-conjugated goat anti-rabbit secondary antibody (Cat. No. ab6939, Abcam Co. Cambridge, MA; RRID: AB_955021) were used. DAPI (Cat. No. D106471, Aladdin Ltd., Shanghai, China) was used for DNA staining. Cells were photographed under a fluorescence microscope at ×400 magnification. The percentage of nuclear p65-positive cells was calculated.

Animal experiment and immunohistochemical (IHC) staining

MMP25-overexpressing or MMP25-silenced TSCC cells or their respective control cells were injected subcutaneously into BALB/c athymic nude mice (5×10^6 per mouse). Tumor growth was monitored for 28 days. Tumor size was measured every three days using the formula: volume = 0.5 × long axis × short axis^2, as previously described (Kersemans et al., 2013). All animal experiments were approved by the Ethics Committee of Tianjin Stomatological Hospital and followed the ARRIVE guidelines. The tumor tissue sections were incubated with primary antibodies against MMP25 (Cat. No. DF7151; Affinity Company,

Changzhou, China) and PCNA (Cat. No. 10205-2-AP; Proteintech, Wuhan, China) at 4°C overnight followed by incubation with HRP-conjugated secondary antibodies. Finally, the color was developed using a DAB reagent (Cat. No. DAB-1031; MXB Biotechnologies, Fuzhou, China) and the sections were counterstained using hematoxylin. The integrated optical density (IOD)/Area was calculated.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 9 software. Data were expressed as mean ± standard deviation. Paired or unpaired two-tailed Student's t-test was used to compare the two groups. One-way or two-way ANOVA with *post-hoc* multiple comparisons was used to compare the means of three or more groups. p values <0.05 were considered significant.

Results

MMP25 is highly expressed in TSCC and promotes cancer cell proliferation.

We first evaluated the mRNA expression of MMP25 in 10 pairs of TSCC tissues and adjacent non-cancer tissues using data from the GEO database (GEO accession: GSE160042) (Zhuang et al., 2020) and found that MMP25 expression in TSCC tissues is higher than that in adjacent non-cancerous tissues (Fig. 1A). Next, we evaluated the mRNA expression of MMP25 in 14 pairs of TSCC tissues and adjacent non-cancer tissues collected from Tianjin Stomatological Hospital. As shown in Figure 1B, nine TSCC tissues (9/14, 64.3%) showed higher MMP25 expression than their corresponding adjacent non-cancerous tissues. Additionally, we performed a survival analysis of MMP25 in TSCC using the TCGA Head and neck squamous cell carcinoma (HNSC) samples (127 cases) via the PanCanSurvPlot web portal (https://smuonco.shinyapps.io/PanCanSurvPlot/). The results are shown in Figure 1C. We found that the higher the MMP25 expression the shorter the progression-free interval (PFI), suggesting that MMP25 may promote the progression of HNSC. Considering that TSCC is one of the most common forms of HNSC, it is rational for us to speculate that higher expression of MMP25 also indicates a poorer TSCC prognosis.

We then measured the protein level of MMP25 in the TSCC cell lines CAL-27 and UPCI-SCC-090 and found that CAL-27 showed a higher level of MMP25 than UPCI-SCC-090 (Fig. 1D). Therefore, MMP25 was silenced in CAL-27 and overexpressed in UPCI-SCC-090. Overexpression or knockdown of MMP25 was achieved by lentiviral transduction; 72 h after viral infection, the transduction efficiency was verified by measuring MMP25 expression at the mRNA and protein levels (Fig. 1E,F). As shown by the cell viability curve, cell proliferation was promoted in MMP25-overexpressing cells and suppressed in MMP25-silenced cells (Fig. 1G,H).

MMP25 promotes cell migration and invasion and regulates proliferation, invasion, and apoptosis-related protein levels.

Transwell migration and invasion assays were performed 72h after viral infection to test the migration and invasion capability of TSCC cells. Overexpression of MMP25 increased the migration and invasion ability of TSCC cells (Fig. 2A,B). In contrast, knockdown of MMP25 decreased the number of migrated and invaded TSCC cells (Fig. 2C,D).

To better clarify the molecular traits underlying the pro-tumor functions of MMP25 in TSCC, we then evaluated the levels of proliferation, invasion, and apoptosis-related proteins after MMP25 overexpression or knockdown using the immunoblotting assay. In addition to the well-known proliferation marker PCNA, the G1/S and G2/M cell cycle transition promoters cyclin D and cyclin B are overexpressed in oral squamous cell carcinoma (Lee et al., 2010; Patil et al., 2013). In contrast, the cyclin-dependent kinase inhibitor p27 induces cell cycle exit and inhibits the growth of oral cancer cells (Lloyd et al., 1999; Supriatno et al., 2002). As shown in Figure 3A, the levels of proliferation marker PCNA and pro-cell cycle transition proteins cyclin D and cyclin B1 were increased in MMP25-overexpressing cells (Fig. 3A) but decreased in MMP25-silenced cells (Fig. 3B). The level of the cell cycle inhibitor p27 was decreased by MMP25 overexpression (Fig. 3A) but increased by MMP25 knockdown (Fig. 3B).

In addition to cell proliferation, cell apoptosis also plays an important role in cancer growth. Therefore, we measured the levels of the cleaved core effector Caspase-3 and the upstream Caspase-9 (McDonnell et al., 2003). Consistent with these findings, an increase in the levels of apoptotic proteins (cleaved caspase 3 and cleaved caspase 9) was observed in TSCC cells with MMP25 knockdown (Fig. 3C).

Since MMP2 and MMP9 are vital MMPs involved in extracellular matrix degradation and their expression levels can reflect the invasiveness of TSCC (Fan et al., 2012), we then analyzed the levels of invasion-related proteins. The levels of mature MMP2 and MMP9 were upregulated in MMP25-overexpressing cells but downregulated in MMP25-silenced cells (Fig. 3D,E).

MMP25 promotes tumor growth of TSCC cells in vivo

To determine the effects of MMP25 on the tumor growth of TSCC cells *in vivo*, we generated stable TSCC cells with MMP25 overexpression or knockdown and established a murine xenograft model by subcutaneous injection of TSCC cells. Since the interference efficiency of the two shRNAs was comparable, we chose sh1-MMP25 for the establishment of stable cell lines.

Before injection, the efficiency of MMP25 overexpression or knockdown was validated by real-time PCR (Fig. 4A,B) and immunoblotting (Fig. 4C,D). One week later, tumor volume was monitored every three days. A continuous statistically significant difference between OV-MMP25 and OV-NC or sh-MMP25 and sh-NC was observed from 19 to 28 days (Fig. 4E,F), indicating that MMP25 promotes the tumor growth of

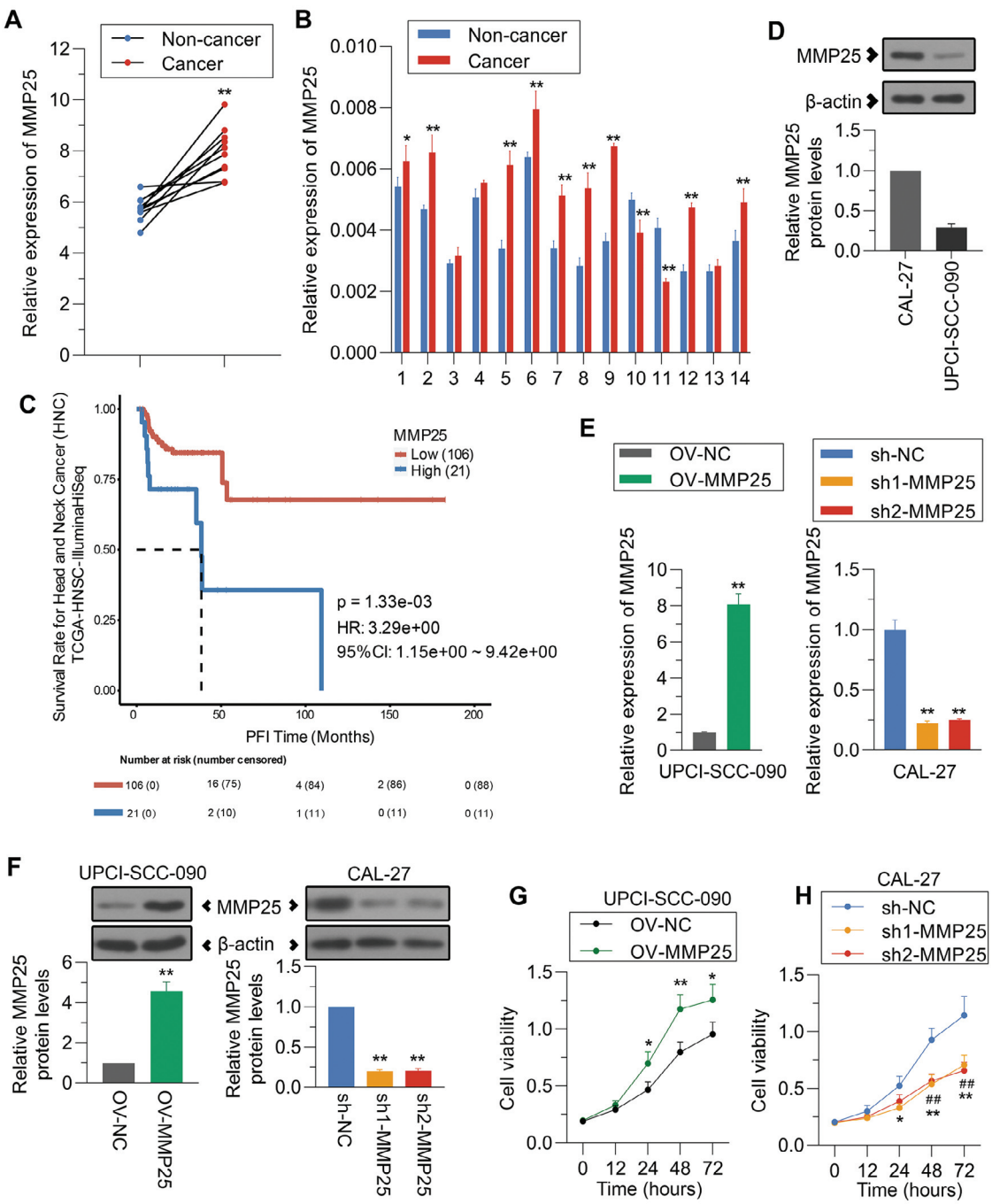

Fig. 1. MMP25 is highly expressed in TSCC and promotes cancer cell proliferation. **A.** MMP25 mRNA expression in 10 pairs of tongue squamous cell carcinoma (TSCC) tissues (cancer) and adjacent non-cancer tissues (non-cancer) was analyzed using data from the GEO database (GEO accession: GSE160042). ***p*<0.01 vs. non-cancer. **B.** MMP25 mRNA expression was analyzed in 14 pairs of TSCC tissues (cancer) and adjacent non-cancer tissues (non-cancer) collected from Tianjin Stomatological Hospital. **p*<0.05 and ***p*<0.01 vs. non-cancer. **C.** The association between MMP25 expression and progression-free interval (PFI) time of TCGA-HNSC patients was analyzed using the PanCanSurvPlot web portal. **D.** MMP25 protein levels in TSCC cell lines (CAL-27 and UPCI-SCC-090) were analyzed by immunoblotting. UPCI-SCC-090 cells were transduced with the lentiviral vector containing the cds of MMP25 to overexpress MMP25 (OV-MMP25). Cells transduced with an empty lentiviral vector (OV-NC) served as controls. CAL-27 cells were transduced with the lentiviral vector containing shRNA against MMP25 to knock down MMP25 (sh1-MMP25 and sh2-MMP25). Cells transduced with a lentiviral vector containing non-targeting shRNA (sh-NC) served as controls. Seventy-two hours after viral infection, MMP25 mRNA expression and protein levels were measured by real-time PCR **(E)** and immunoblotting **(F)**. ***p*<0.01 vs. corresponding control. **G.** Cell viability of UPCI-SCC-090 cells was measured by the CCK-8 assay. **p*<0.05 and ***p*<0.01 vs. OV-NC. **H.** Cell viability of CAL-27 cells was measured by the CCK-8 assay. **p*<0.05 and ***p*<0.01 indicate sh1-MMP25 vs. sh-NC; $^{\#\#}p$<0.01 indicates sh2-MMP25 vs. sh-NC.

TSCC cells *in vivo*. In addition, we performed IHC of MMP25 and PCNA to determine the effect of MMP25 on the proliferation marker *in vivo*. We found that the expression of PCNA was increased upon MMP25 overexpression and decreased upon knockdown (Fig. 4G,H). These findings indicated that MMP25-overexpressing cells showed enhanced proliferation ability *in vivo*.

MMP25 promotes the immune evasion of TSCC cells

Immune evasion is a vital process during tumor progression (Juneja et al., 2017). Higher expression of immune checkpoint PD-L1 in tumor cells is closely

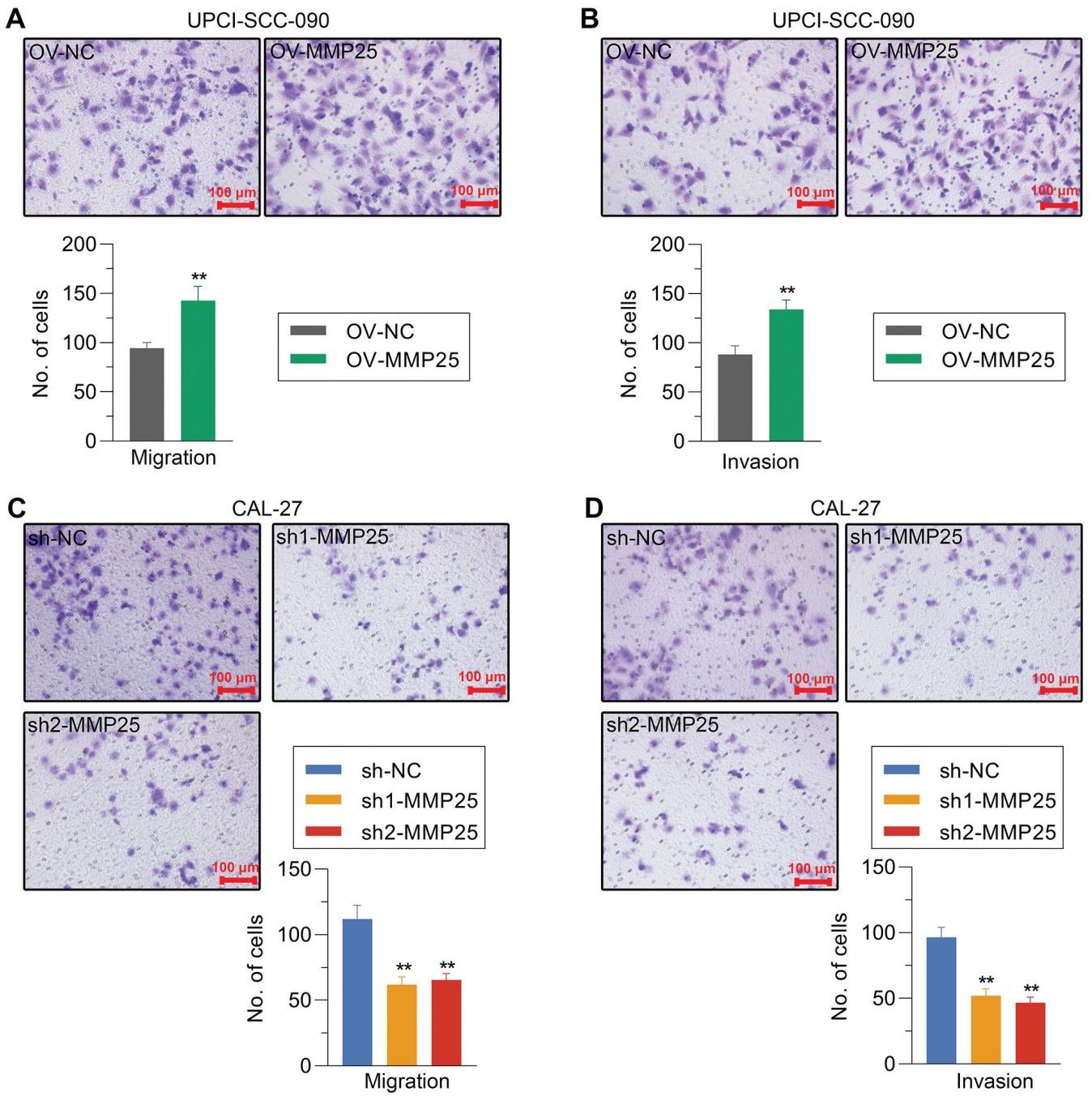

Fig. 2. MMP25 promotes migration and invasion of TSCC cells. Seventy-two hours after viral infection, the migration **(A)** and invasion **(B)** of UPCI-SCC-090 cells were assessed by Transwell assays. ***p*<0.01 vs. OV-NC. Migration **(C)** and invasion **(D)** of CAL-27 cells were evaluated by Transwell assays. ***p*<0.01 vs. sh-NC. Scale bars: 100 μm.

related to the advanced tumor stages of TSCC (Huang et al., 2020). Therefore, we investigated the effects of MMP2 on PD-L1 expression. Compared with control cells, PD-L1 (gene name *CD274*) mRNA expression was increased in MMP25-overexpressing cells (Fig. 5A), whereas it was decreased in MMP25-silenced cells (Fig. 5B). The PD-L1 protein level showed a similar trend (Fig. 5C,D).

The innate immune NK cells also play an important role in anti-cancer immunity via the direct lysis of cancer cells. Besides, the activated NK cells secret multiple anti-tumor cytokines (e.g., IFN-γ and TNF-α) (Dunn et al., 2006; Schuster et al., 2016). Therefore, we evaluated the effects of MMP25 on NK cell anti-tumor activity. According to the results of the NK cell lysis experiment, NK cell-mediated lysis of TSCC cells was

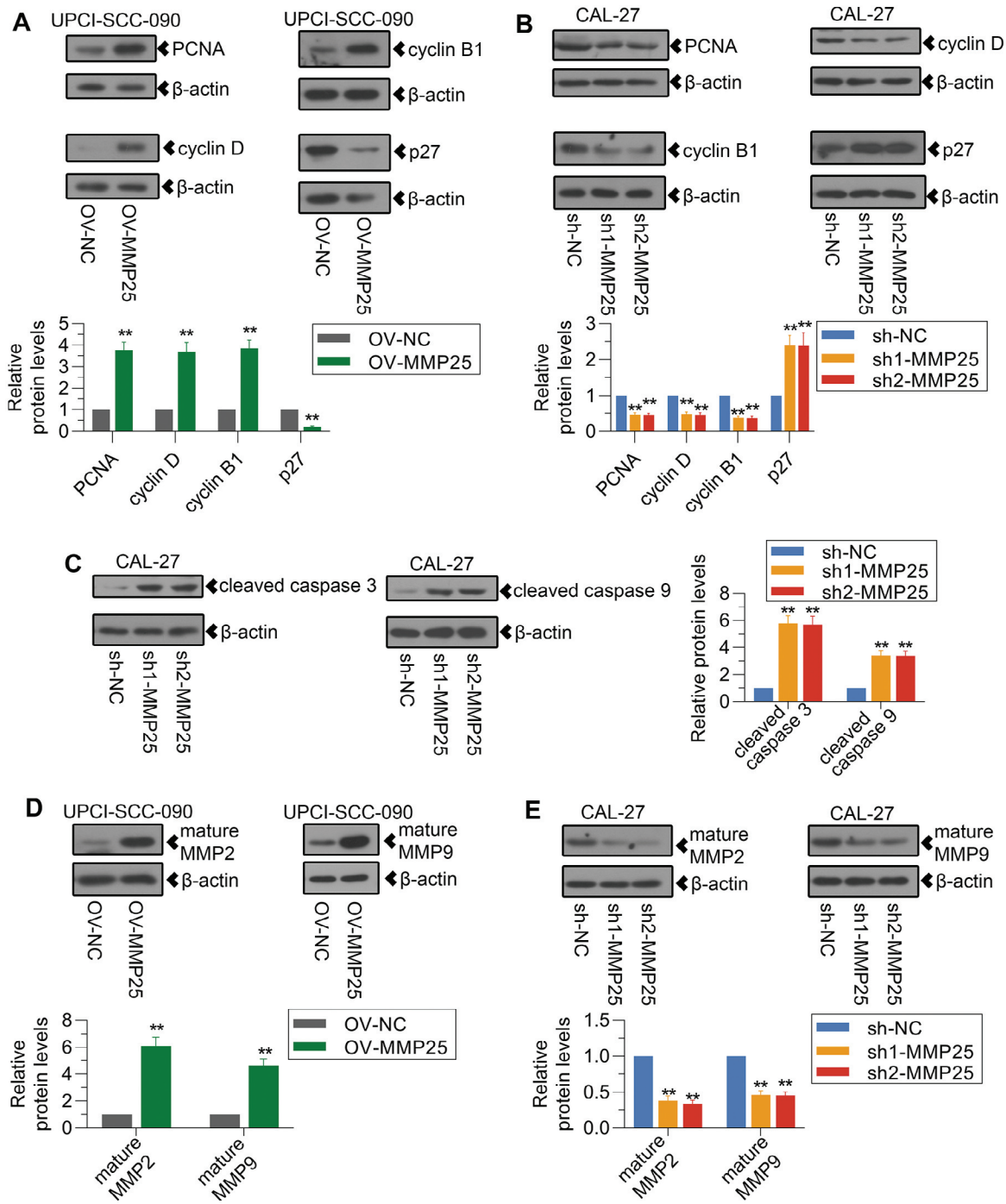

Fig. 3. MMP25 regulates the levels of proliferation-, invasion-, and apoptosis-related proteins in TSCC cells. Seventy-two hours after viral infection, the levels of proliferation-related proteins (PCNA, cyclin D, cyclin B1, and p27) in UPCI-SCC-090 **(A)** and CAL-27 **(B)** cells were evaluated by immunoblotting. The quantification analysis results are shown in the lower panels. **C.** The apoptosis-related proteins (cleaved caspase-3 and cleaved caspase-9) in CAL-27 cells were evaluated by immunoblotting. The quantification analysis results are shown in the right panel. The invasion-related proteins (mature MMP2 and mature MMP9) in UPCI-SCC-090 **(D)** and CAL-27 **(E)** cells were evaluated by immunoblotting. The quantification analysis results are shown in the lower panels. $^{**}p<0.01$ vs. corresponding control.

impaired by MMP25 overexpression (Fig. 5E) but enhanced by knockdown (Fig. 5F). We then measured the levels of anti-tumor immunity cytokines secreted by NK-92 cells after co-culture with TSCC cells. As expected, the levels of TNF-α and IFN-γ were reduced in supernatants from the co-culture of NK-92 cells and MMP25-overexpressing cells (Fig. 5G,H) but increased in supernatants from the co-culture of NK-92 cells and MMP25-silenced cells (Fig. 5I,J).

MMP25 activates NF-κB signaling in TSCC cells

NF-κB signaling is often activated in several types of cancer, and it has been demonstrated that the blockade of NF-κB signaling suppresses the proliferation, invasion, and tumor growth of TSCC cells (Chen et al.,

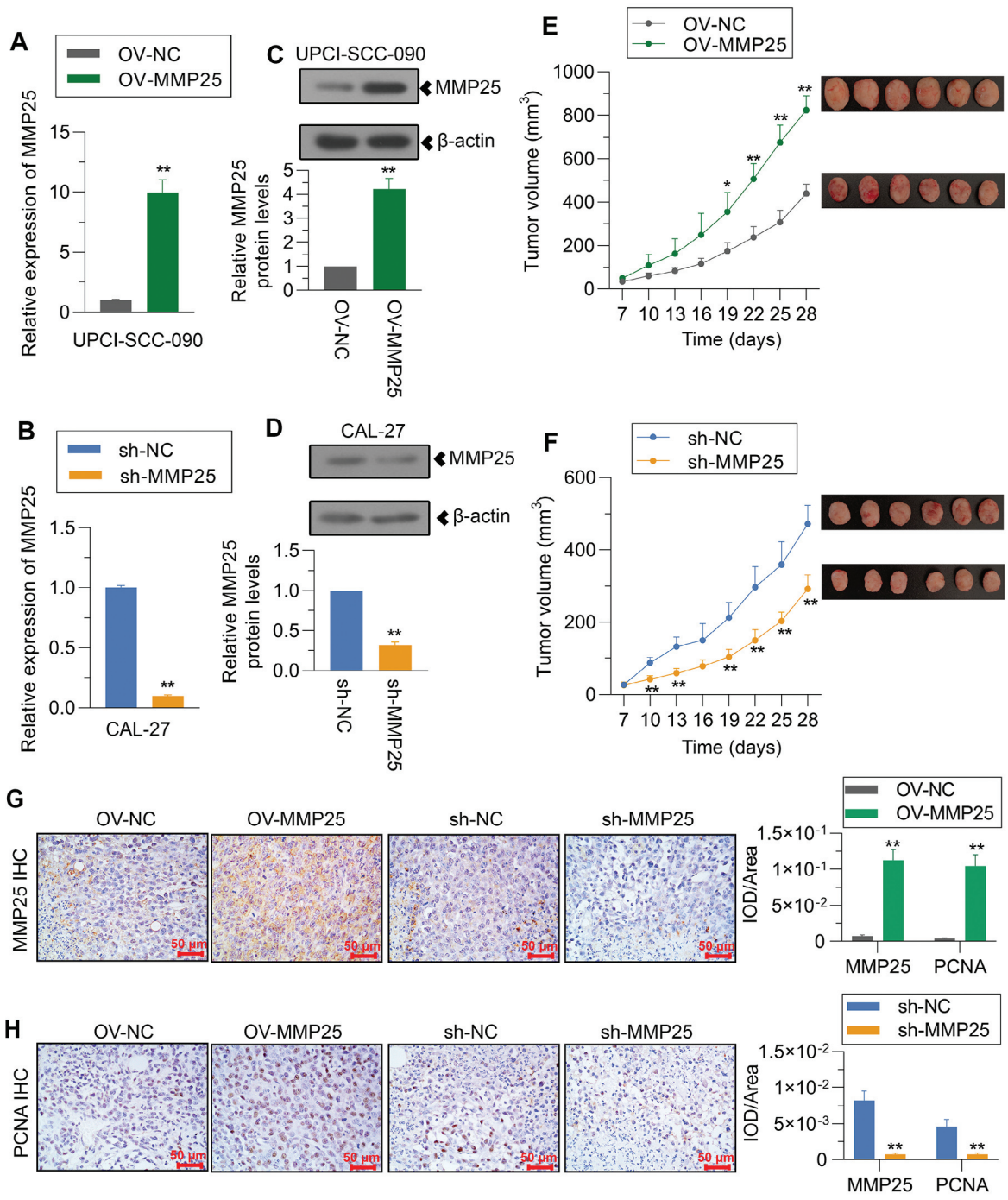

Fig. 4. MMP25 promotes the tumor growth of TSCC cells *in vivo*. Stable cell lines with MMP25 overexpression (OV-MMP25), MMP25 knockdown (sh-MMP25), or their respective controls (OV-NC or sh-NC) were generated by puromycin selection. Mice were injected subcutaneously with the indicated cells. Before injection, the efficiency of MMP25 overexpression or knockdown was validated by real-time PCR **(A, B)** and immunoblotting **(C, D)**. The tumor volume of mice bearing OV-MMP25/OV-NC UPCI-SCC-090 cells **(E)** or sh-MMP25/sh-NC CAL-27 cells **(F)** was calculated. Representative images of IHC staining for MMP25 **(G)** or PCNA **(H)** in tumor sections. The quantification analysis results are shown in the right panels. *p<0.05 and **p<0.01 vs. corresponding control. Scale bars: 50 μm.

2019). Moreover, NF-κB inactivation was observed in bone marrow neutrophils from MMP25-deficient mice (Soria-Valles et al., 2016). Therefore, we speculated that the pro-tumor functions of MMP25 in TSCC are associated with NF-κB signaling activation. We then tested the effect of MMP25 on NF-κB signaling. No obvious difference in the level of total NF-κB p65 was observed between control cells and MMP25-overexpressing or -silenced cells. However, the phosphorylation of NF-κB p65 was promoted by MMP25 overexpression (Fig. 6A) and suppressed by MMP25 knockdown (Fig. 6B). In support of this, a greater nuclear localization of NF-κB p65 was shown in MMP25-overexpressing cells (Fig. 6C) than in MMP25-silenced cells (Fig. 6D). Figure 7 is the schematic illustration of the functions of MMP25 in TSCC.

Discussion

In this study, we demonstrated that MMP25 mediates the promotion of TSCC progression in terms of proliferation, migration, invasion, and immune evasion, and we linked these functions of MMP25 to the activation of NF-κB signaling. This is the first report on the function of MMP25 in TSCC, providing evidence to establish MMP25 as a potential therapeutic target in TSCC.

Increased proliferation is a typical feature of cancer cells and is controlled by the cell-cycle machinery. In the present study, we observed the changes in cell cycle-related proteins (cyclin D, cyclin B, and p27) in response to MMP25 overexpression or knockdown in TSCC cells. Cyclin D and cyclin B promote G1/S and G2/M cell-cycle transitions, respectively. They are overexpressed in oral squamous cell carcinoma and predict lymph node metastasis (Harada et al., 2006; Lee et al., 2010; Patil et al., 2013). In contrast, the cyclin-dependent kinase (CDK) inhibitor p27 induces cell-cycle exit (Lloyd et al., 1999). p27 inhibited the growth of oral cancer cells, and low levels of p27 expression were associated with advanced TNM stage, poor histologic status, and lymph node metastasis in TSCC (Supriatno et al., 2002; Kudo et al., 2005; Gao et al., 2013). In addition, the proliferation marker PCNA increases the affinity of the CDK inhibitor p21 for its E3 ligase to facilitate the degradation of p21, thereby promoting cell-cycle

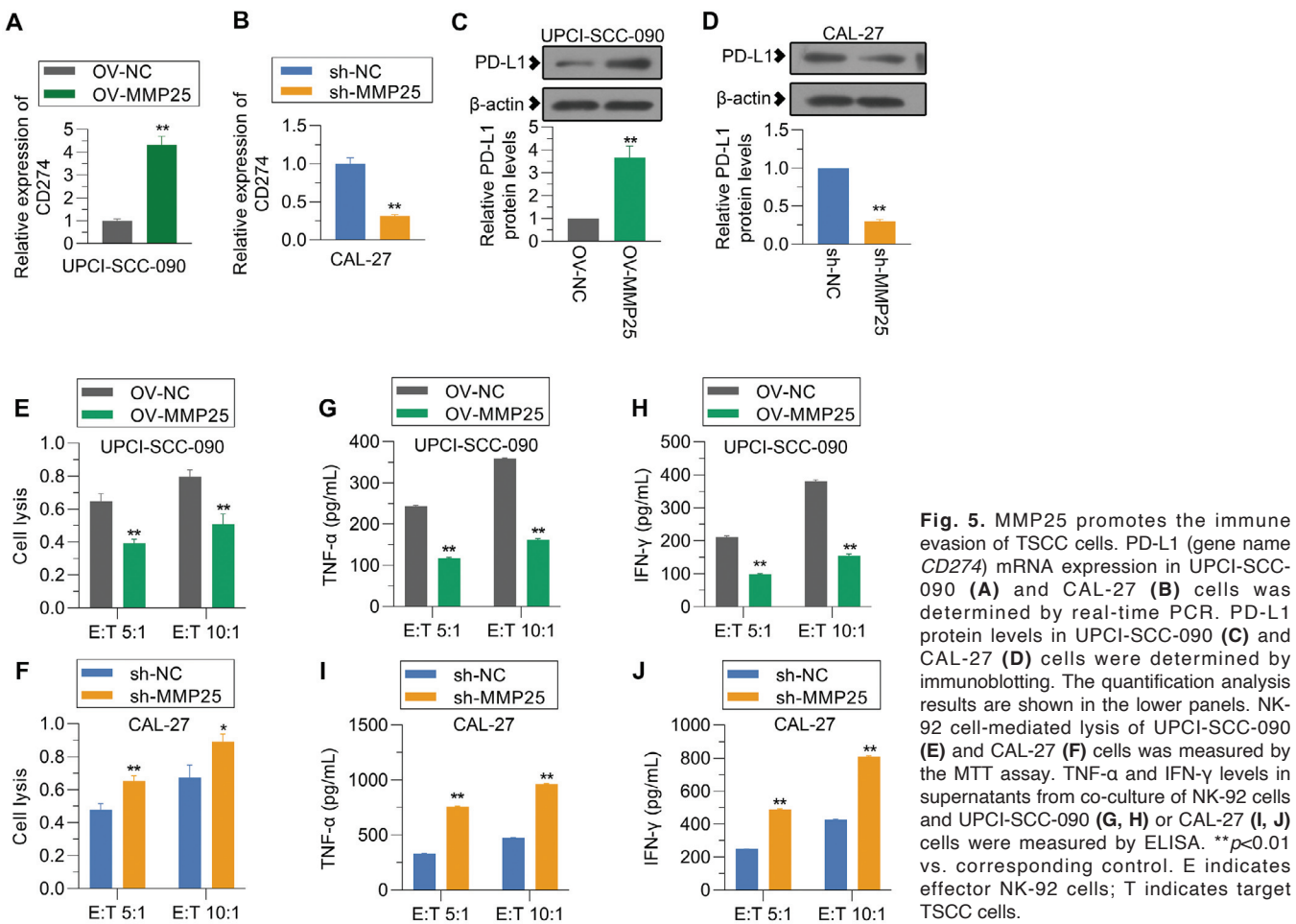

Fig. 5. MMP25 promotes the immune evasion of TSCC cells. PD-L1 (gene name *CD274*) mRNA expression in UPCI-SCC-090 **(A)** and CAL-27 **(B)** cells was determined by real-time PCR. PD-L1 protein levels in UPCI-SCC-090 **(C)** and CAL-27 **(D)** cells were determined by immunoblotting. The quantification analysis results are shown in the lower panels. NK-92 cell-mediated lysis of UPCI-SCC-090 **(E)** and CAL-27 **(F)** cells was measured by the MTT assay. TNF-α and IFN-γ levels in supernatants from co-culture of NK-92 cells and UPCI-SCC-090 **(G, H)** or CAL-27 **(I, J)** cells were measured by ELISA. **p<0.01 vs. corresponding control. E indicates effector NK-92 cells; T indicates target TSCC cells.

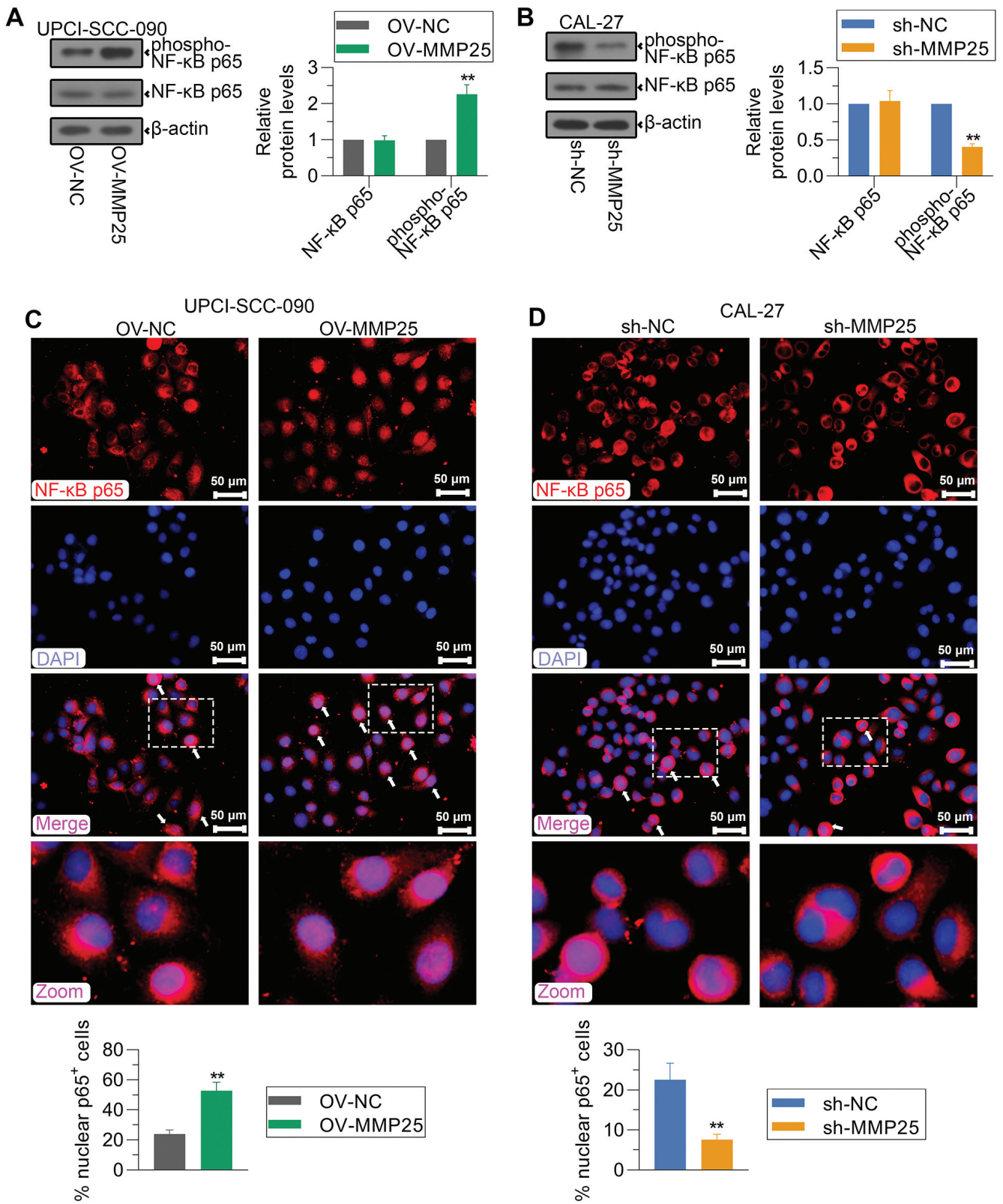

Fig. 6. MMP25 activates NF-κB signaling in TSCC cells. Levels of phospho-NF-κB p65 and NF-κB p65 in UPCI-SCC-090 **(A)** and CAL-27 **(B)** cells were assessed by immunoblotting. The quantification analysis results are shown in the right panels. Representative images of immunofluorescence staining of NF-κB p65 in UPCI-SCC-090 **(C)** and CAL-27 **(D)** cells. The quantification analysis results are shown in the lower panels. The white arrow indicates the nuclear localization of NF-κB p65.

progression (Havens and Walter, 2009). Therefore, we speculated that MMP25 may promote the proliferation of TSCC cells by regulating these cell cycle-related proteins.

In addition to cell proliferation, cell apoptosis is another important factor influencing cancer growth. Caspase-3 is a core effector caspase that initiates the caspase cascade, and it can be activated by caspase-8 and caspase-9 in the extrinsic and intrinsic apoptotic pathways, respectively (McDonnell et al., 2003). In this study, we observed that MMP25 knockdown resulted in increased cleaved caspase-3 and caspase-9, suggesting that MMP25 may inhibit the intrinsic apoptotic pathway to promote TSCC growth; however, whether MMP25 affects the caspase-8-related extrinsic pathway requires further research.

Extracellular matrix degradation is an important step in tumor metastasis, in which the role of MMP2 and MMP9 has been well characterized; their expression levels can reflect the invasiveness of tumors and predict the prognosis of TSCC patients (Fan et al., 2012). In this study, we found that MMP25 significantly increased the levels of mature MMP2/MMP9. Interestingly, MMP9 has been established as a salivary biomarker of oral cancer (Shpitzer et al., 2009), demonstrating the potential of MMPs as non-invasive biomarkers for diagnosis, prognosis, and postoperative monitoring in TSCC.

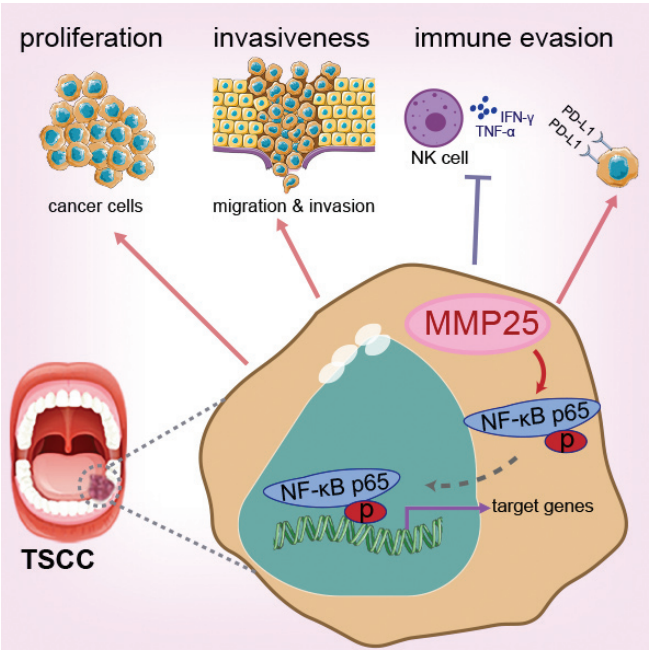

Fig. 7. MMP25 activates the NF-κB pathway to regulate the malignant behavior of TSCC cells. MMP25 increased the proliferation and invasive ability of TSCC cells. MMP25 also increased the expression of immune checkpoint PD-L1 in TSCC cells and helped TSCC cells escape NK cell lysis. MMP25 exerted these pro-tumor functions through the activation of NF-κB signaling.

Proteolytic enzymes are often produced in a latent zymogenic form that lacks enzymatic activity and requires specific cleavage to expose the catalytic domain to achieve catalytic competence (Nagase, 1997). Tissue inhibitor of metalloproteinase (TIMP)-2 binds to the active site of MT1-MMP to form a complex that recruits and binds to pro-MMP2, facilitating its activation (Hernandez-Barrantes et al., 2002). As previously reported, TIMP-2 inhibited the activity of MMP25 (English et al., 2001), and co-transfection of MMP25 and pro-MMP2 caused the activation of pro-MMP2 (Velasco et al., 2000; Nie and Pei, 2003). Therefore, we hypothesized that MMP25 may also activate pro-MMP2 by a mechanism similar to that of MT1-MMP.

Evading the immune system is a necessary step for the development of cancer (Juneja et al., 2017). Expression of the immune checkpoint PD-L1 in tumor cells was found to correlate with the N stage, chemotherapy, and clinical stage of TSCC (Huang et al., 2020). Many immunotherapies have been established targeting the PD-1/PD-L1 axis to activate T cells (Sharpe and Pauken, 2018). Here, in the present study, MMP25 increased PD-L1 expression in TSCC cells, suggesting that MMP25 helped TSCC cells escape from cytotoxic T-lymphocyte responses. In addition to the adaptive immune system involving T cells, the innate immune system also plays an important role in anti-cancer immunity. Beyond their cytotoxic function, activated NK cells produce multiple cytokines (e.g., IFN-γ) to potentiate adaptive immune responses (Martín-Fontecha et al., 2004; Walzer et al., 2005; Dunn et al., 2006; Schuster et al., 2016). Therefore, combining the activation of T and NK cells may further enhance the effects of immunotherapies. Here, we observed that MMP25 suppressed NK cell killing of TSCC cells and prevented NK cells from secreting anti-tumor cytokines. Taken together, these findings suggest that targeting MMP25 may enhance both innate and adaptive anti-tumor immune responses in TSCC.

Lou et al. recently demonstrated that circulating tumor cells in oral cancer induced NK cell functional exhaustion via the killer cell lectin-like receptor subfamily G member 1 receptor to evade NK cell killing (Lou et al., 2022). Peruzzi et al. previously reported that MMP25 downregulated the expression of CD16 in human primary NK cells (Peruzzi et al., 2013). CD16 is an NK cell-activating receptor that can sufficiently trigger the cytotoxic activity of NK cells and promote the production of anti-tumor cytokines and chemokines (Wang et al., 2015a), thereby contributing to the recruitment and activation of tumor-infiltrating immune cells (Morvan and Lanier, 2016; Böttcher et al., 2018). These reports suggest that CD16 may be implicated in MMP25-mediated regulation of the NK cell response, which requires further investigation.

Activation of NF-κB signaling has been identified as a contributor to cancer progression, whereas blockade of NF-κB signaling suppressed the proliferation, invasion, and tumor growth of TSCC cells (Chen et al., 2019).

Soria-Valles et al. previously reported that NF-κB inactivation was observed in bone marrow neutrophils from MMP25-deficient mice (Soria-Valles et al., 2016). Therefore, we hypothesized that activation of NF-κB may mediate the pro-tumor functions of MMP25 in TSCC. In the canonical NF-κB activation pathway, the p65 (also known as RelA) and p50 heterodimers undergo post-translational modifications such as phosphorylation, resulting in increased transcriptional activity, thereby contributing to the activation of target gene transcription after their nuclear translocation (Giridharan and Srinivasan, 2018). In this study, we found that NF-κB activation was promoted in MMP25-overexpressing TSCC cells and inhibited in MMP25-silenced TSCC cells, supporting the positive regulation of MMP25-mediated NF-κB signaling. Furthermore, NF-κB has been shown to increase PD-L1 expression by promoting its transcription (Lee et al., 2005; Kondo et al., 2010), suggesting that the NF-κB pathway may also be implicated in MMP25-mediated immune evasion of TSCC cells.

However, this study has some limitations. Firstly, the molecular mechanism underlying the regulation of NF-κB signaling by MMP25 is not clear. Secondly, we only investigated the effects of MMP25 on NK cell-mediated anti-tumor functions, the effect of MMP25 on cytotoxic T cells remains unclear. In future studies, these issues should be addressed.

In conclusion, our results have demonstrated that MMP25 helps TSCC cells acquire a more malignant phenotype, highlighting the potential of MMP25 as a novel target for TSCC treatment.

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Disclosure of interest. The authors report there are no competing interests to declare.

Ethical statement. Animal experiments were approved by the Ethics Committee of Tianjin Stomatological Hospital and were performed in accordance with the ARRIVE guidelines. Research involving human subjects was approved by the Ethics Committee of Tianjin Stomatological Hospital and conducted in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from each patient. Consent to publish was not applicable.

Data availability statement. The data are available from the corresponding author upon reasonable request.

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