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Nicotinamide *N*-methyltransferase induces cellular invasion through activating matrix metalloproteinase-2 expression in clear cell renal cell carcinoma cells

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***Nicotinamide N-methyltransferase (NNMT)* was recently identified as one clear cell renal cell carcinoma (ccRCC)-associated gene by analyzing full-length complementary DNA-enriched libraries of ccRCC tissues. The aim of this study is to investigate the potential role of NNMT in cellular invasion. A strong NNMT expression is accompanied with a high invasive activity in ccRCC cell lines, and small interfering RNA-mediated NNMT knockdown effectively suppressed the invasive capacity of ccRCC cells, whereas NNMT overexpression markedly enhanced that of human embryonic kidney 293 (HEK293) cells. A positive correlation between the expression of NNMT and matrix metallopeptidase (MMP)-2 was found in ccRCC cell lines and clinical tissues. The treatment of blocking antibody or inhibitor specific to MMP-2 significantly suppressed NNMT-dependent cellular invasion in HEK293 cells. Furthermore, SP-1-binding region of MMP-2 promoter was found to be essential in NNMT-induced MMP-2 expression. The specific inhibitors of PI3K/Akt signaling markedly decreased the binding of SP1 to MMP-2 promoter as shown by chromatin immunoprecipitation assay. We also demonstrated that PI3K/Akt pathway plays a role in NNMT-dependent cellular invasion and MMP-2 activation. Moreover, short hairpin RNA-mediated knockdown of NNMT expression efficiently inhibited the growth and metastasis of ccRCC cells in non-obese diabetic severe combined immunodeficiency mice. Taken together, the present study suggests that NNMT has a crucial role in cellular invasion via activating PI3K/Akt/SP1/MMP-2 pathway in ccRCC.**

Introduction

Renal cell carcinoma (RCC) arises from the renal epithelial cells and represents 3% of all human malignancies (1,2). Several histologic subtypes have been observed, including clear cell, papillary, chromophobe and collecting duct and clear cell renal cell carcinoma (ccRCC) subtype accounts for ~ 70% of RCC patients (3,4). Patients with ccRCC had a very low 5 year survival rate due to the high incidence of cell invasion and the strong resistance to chemotherapy, radiation therapy, hormonal therapy and immunotherapy (5–7), leading to the investigation of the new effective therapy for advanced ccRCC or tumor recurrence.

In the process of cellular invasion, cancer cells penetrate tissue barriers by secreting the proteases, such as matrix metalloproteinases (MMPs), which belong to the family of zinc-dependent endopeptidases and are able to degrade the extracellular matrix (8,9). Among MMPs reported, MMP-2 has the ability to selectively cleave the major component of extracellular matrix (type IV collagen) and is involved in tumor invasion and metastasis in various kinds of malignancies (10). The expression of MMP-2 has been reported to be regulated by several transcription factors and signaling pathways, including transcription factor SP1 and PI3K/Akt pathway (11–16).

Nicotinamide *N*-methyltransferase (NNMT) catalyzes the *N*-methylation reaction of nicotinamide, pyridines and other structural analogs and is involved in the biotransformation of many drugs and xenobiotic compounds (17). Overexpression of NNMT has been observed in various tumors (18–27). NNMT has been considered as a potential tumor marker for ccRCC (28) and a sensitive serum biomarker for the early detection of colorectal cancer and lung cancer (18,29). Previous study demonstrated the correlation between the expression levels of NNMT and tumor progression in hepatocellular carcinoma (24). Wu *et al*. (25) found that NNMT expression is required for the migratory potential of bladder cancer cells. However, the role of NNMT overexpression in the invasive capacity of ccRCC cells is still unclear.

Recently, we identified *NNMT* as a highly upregulated genes by analyzing the gene expression profiles of ccRCC tissue pairs (30,31). In this study, we investigated the relationship of the expression of NNMT to the invasive activity and MMP-2 expression of ccRCC cells. Moreover, the transcription factor and signaling pathway involved in NNMT-induced cellular invasion and MMP-2 expression in ccRCC cells was elucidated.

Materials and methods

Reagents and antibodies

The antibody against NNMT was obtained from Geneway Biotech (Banchiau, TW) and that against actin was purchased from Chemicon International (Temecula, CA). The antibody specific to MMP-2, phospho-Akt and total Akt (for western blot analysis) were obtained from Cell Signaling Technology (Beverly, MA). The validated chemically modified oligonucleotides of small interfering RNA (siRNA) for *NNMT* (5′-AGAUCACACACAUAGGUCACCACUG-3′) and the negative control were obtained from Invitrogen (Carlsbad, CA). LY294002, PD98059, SB202190, SP600125 and mithramycin were bought from Sigma (St Louis, MO). The inhibitors of MMP-2 (OA-Hy; *cis*-9-octadecenoyl-*N*-hydroxylamide) and Akt (Akt inhibitor IV) were obtained from Calbiochem (San Diego, CA).

Cell lines and tumor samples

Tissue pairs of ccRCC tumor and adjacent normal kidney were obtained from 33 patients (9 women and 24 men) by surgery resection at National Taiwan University Hospital under the approval of Institution of Review Board. Chemotherapy had not been administered to patients before surgery. All specimens were immediately frozen by liquid nitrogen and stored at −80°C until use. The ages of the patients ranged from 32 to 76 years old (median age = 59). The stage of ccRCC tumor was determined according to the World Health Organization classification (32). Fourteen patients were at pT1, 8 at pT2 and 11 at pT3. The 786O, 769P, Caki-1, Caki-2, HEK293 and 293FT cells were obtained from American Type Culture Collection (Manassas, VA). All of these cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mmol/l L-glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin (Invitrogen) in 5% CO_2 atmosphere at 37°C.

Gelatin zymography assay

Conditioned media were collected after 2×10^5 cells were incubated in serum-free media for 24 h and subjected to 6% sodium dodecyl sulfate–polyacrylamide gel electrophoresis that contained 0.1 mg/ml gelatin. The volume of conditioned media loaded into the gel was normalized to protein concentration of cell lysate. Sodium dodecyl sulfate was removed by Triton X-100 buffer (50 mmol/l Tris–HCl, pH 7.5, 2.5% Triton X-100), and gels were incubated with develop buffer (50 mmol/l Tris–HCl, pH 7.6, 10 mmol/l $CaCl_2$ and 150 mmol/l NaCl) overnight at 37°C and stained with Coomassie Brilliant Blue. Gelatinase activities were visualized as clear bands.

Abbreviations: ccRCC, clear cell renal cell carcinoma; MMP, matrix metallopeptidase; NNMT, nicotinamide *N*-methyltransferase; PCR, polymerase chain reaction; RCC, renal cell carcinoma; shRNA, short hairpin RNA; siRNA, small interfering RNA.

Western blot analysis

Forty micrograms of protein was resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). The membrane was incubated with primary antibodies specific to MMP-2, NNMT or actin, followed by horseradish peroxidase-conjugated secondary antibodies (Chemicon International). Signals were visualized using enhanced chemiluminescence detection reagent from Millipore. Band intensities were obtained using a UVP BioImaging System (UVP, Upland, CA).

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (PCR) was performed as described previously (30). Briefly, total RNAs were extracted from cell lines and frozen ccRCC tissues using TRIzol reagent (Invitrogen) and treated with RNase-free DNase I (TaKaRa, Shiga, Japan). Two micrograms of RNAs were reverse transcribed to complementary DNA by SuperScript™ II Reverse Transcriptase kit (Invitrogen) at 42°C for 1 h. The primers specific to *NNMT* (forward 5′-TCCTTTAAGGAGATCGTCGTCAC-3′ and reverse 5′-GCTTGACCGCCTGTCTCAAC-3′) and *MMP-2* (forward 5′-CACCCATTTACACCTACACCAAGAAC-3′ and reverse 5′-TGCCAAGGTCAATGTCAGGAGAG-3′) were designed using the Beacon Designer 4 program (Premier Biosoft International, Palo Alto, CA). *TPT1* or *glyceraldehyde-3-phosphate dehydrogenase* was used as the reference gene in ccRCC tissues or ccRCC-derived cell lines, respectively (31). The experiments were repeated in triplicate and the relative levels of gene expression were represented as $\Delta Ct = (Ct_{\text{Gene}} - Ct_{\text{Reference}})$. Fold change of gene expression levels was determined using the $2^{(-\Delta\Delta Ct)}$ method (33).

Plasmid and generation of stable cell lines

The complementary DNA of *NNMT* was amplified by PCR using specific primers (forward, 5′-GAATCAGGCTTCACC-3′; reverse, 5′-TCACAGGGGTCTGCTCAGCTT-3′) and cloned into pcDNA3 vector. HEK293 cells were transfected with pcDNA3-*NNMT* or pcDNA3 vector using LipofectAMINE™ 2000 (Invitrogen) and then treated with 500 μg/ml of neomycin. After neomycin selection for 2 weeks, the stable clones with NNMT overexpression or vector only were obtained.

Immunohistochemical analysis

Immunohistochemical analysis was performed as described previously (31). Briefly, Paraffin-fixed sections were dewaxed by xylene and hydrated by 95, 85 and 75% ethanol sequentially. Antigens were retrieved by heating for 15 min with 10 mM citrate buffer (pH 6.0) in a microwave oven. The sections were treated with 3% hydrogen peroxide to quench endogenous peroxidase reaction and blocked with the normal goat serum (DAKO, Carpinteria, CA). The sections were then incubated with NNMT antibody (1:300 dilution) or MMP-2 antibody (1:50 dilution) at 4°C overnight, followed by horseradish peroxidase-conjugated secondary antibodies (Chemicon International) at 37°C for 60 min. Signals were developed by using the liquid diaminobenzidene substrate-chromogen system (DAKO) and counterstained with hematoxylin. The extent of immunoreactivity was evaluated by a pathologist, and the 0–3 scoring system was applied to represent the extent of staining (0 = negative, ≤5%; 1+ = weak, 6–25%; 2+ = moderate, 26–50%; 3+ = strong, ≥50%).

Construction of human MMP-2 promoter

The full-length (bp −1659 to +57) of *MMP-2* promoter was amplified by PCR with the primers (forward, 5′-CACACCCACCAGACAAGCCT-3′; reverse, 5′-AAGCCCCAGATGCGCAGCCT-3′) as described previously (34). The 5′ serial deletion constructs of *MMP-2* promoter were generated and named as follows: D1, bp −1591 to +57; D2, bp −1259 to +57; D3, bp −562 to +57; D4, bp −161 to +57; D5, bp −139 to +57; D6, bp −64 to +57 and D7, bp −7 to +57 (11). The two SP1-binding elements (bp −94 to −64) in the D3 construct were mutated to 5′-TATCTAGATGATATCTCTAGATGATATCAT-3′ (11). The full-length and deletion mutants of *MMP-2* promoter were cloned into pGL3 luciferase reporter vector. All constructs were verified by nucleotide sequencing.

Luciferase reporter assay

Luciferase activities were determined using a dual licuferase assay kit (Promega, Madison, WI). Cells were transfected with pGL3-*MMP-2* promoter plasmid and pGL3-*Renilla* luciferase control reporter plasmid as an internal control. After incubation for 24 h, the luciferase activities were developed using a dual licuferase assay kit following the manufacturer's instructions (Promega) and measured by a Berthold luminometer (Berthold, Oak Ridge, TN).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay was performed as described previously (31). Chromatin was sonicated and immunoprecipitated with rabbit polyclonal SP1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit immunoglobulin G antibody (Sigma) as the negative control. The cross-links were reversed by 200 m mol/l NaCl at 65°C for 4 h. After extraction and precipitation, DNA was dissolved in 50 μl of ddH_2O. The primers (forward 5′-GCGAGAGAGGCAAGTG-3′ and reverse 5′-CAGCCGCCAGATGTAG-3′) were used to specifically amplify the region containing SP1-binding elements (from −236 to −11) of the *MMP-2* promoter. PCR products were analyzed by 2% agarose gels and visualized by ethidium bromide.

Matrigel invasion assay

Transwell Boyden chambers that contain cell-permeable filters were used for the Matrigel invasion assay (BD Biosciences, Bedford, MA). The upper surface of the filter was coated with 40 μl of Matrigel (1 mg/ml; BD Biosciences). Cells were maintained in serum-free medium for 24 h and then were trypsinized and counted with a hemocytometer. The same number of cells (10^5) was seeded into the matrigel-coated chamber, and the chamber was incubated in complete medium with 10% fetal bovine serum at 37°C for 24 h. The cells on the bottom side of the membrane were fixed with 1% formaldehyde/phosphate-buffered saline for 10 min and stained with 0.2% crystal violet for 60 min. The invaded cells were counted using an inverted contrast light microscope under ×100 magnification.

Lentiviral shRNA infection

The short hairpin RNA (shRNA) specific to *NNMT* gene was obtained from the National RNAi Core Facility, the Institute of Molecular Biology/Genomic Research Center, Academia Sinica. 293FT cells were cotransfected with pCMVΔR8.91 (packaging plasmid), pMD.G (Envelop plasmid) and pLKO.1 vector with *NNMT* shRNA or control (*luciferase*) shRNA for 24 h and then incubated in fresh Dulbecco's modified Eagle's medium to produce lentivirus for 72 h. The supernatants containing lentivirus were collected and concentrated. The 786O cells were infected by the lentivirus containing *NNMT* or *luciferase* shRNA overnight and selected with puromycin for 14 days.

Xenograft experiments

The animal experiments were approved by the Institutional Animal Care and Use Committee of College of Medicine, National Taiwan University. All procedures were performed in compliance with guidelines established by the USA Public Health Service. For tumor growth experiments, 4×10^6 of 786O cells/*NNMT* shRNA or *luciferase* (control) shRNA were subcutaneously injected into the upper portion of the hind limb of non-obese diabetic severe combined immunodeficiency (NOD-SCID) mice ($n = 6$). Tumor size was measured by using calipers every 3 days and calculated by the formula: $V = \pi/6 \times D_l \times D_s^2$, where V is volume, D_l is the largest diameter and D_s is the smallest diameter. For lung metastasis experiments, 1×10^6 of cells were intravenously inoculated into the lateral tail vein. The mice were killed after 6 weeks of injection, and the metastatic nodules of lungs were counted.

Statistical analysis

The two-tailed independent-samples Student's t-test was applied to analyse differences between experimental groups. The 'Pearson' correlation method was used for statistical analysis of the correlation between NNMT and MMP-2 expression levels. The data with P values <0.05 were considered to be statistically significant. All experiments were repeated three times with similar results.

Results

NNMT expression is associated with ccRCC cell invasion

To investigate the involvement of NNMT in the invasive activity of ccRCC cells, expression levels of NNMT were measured, and Matrigel invasion assay was employed to determine the invasiveness of 786O, 769P, Caki-1 and Caki-2 ccRCC cell lines. As shown in Figure 1A, ccRCC cells (786O and Caki-1 cells) with higher NNMT expression displayed a higher invasive activity than those with lower NNMT expression. The siRNA against *NNMT* effectively suppressed both NNMT expression and the invasiveness of 786O and Caki-1 cells (Figure 1B; supplementary Figure S1A is available at *Carcinogenesis* Online). Furthermore, the overexpression of NNMT in human embryonic kidney 293 (HEK293) and 769P cells significantly promoted the invasive activity (Figure 1C; supplementary Figure S1A is available at *Carcinogenesis* Online). These results indicate that NNMT expression has a critical role in the invasive potential of human ccRCC cells.

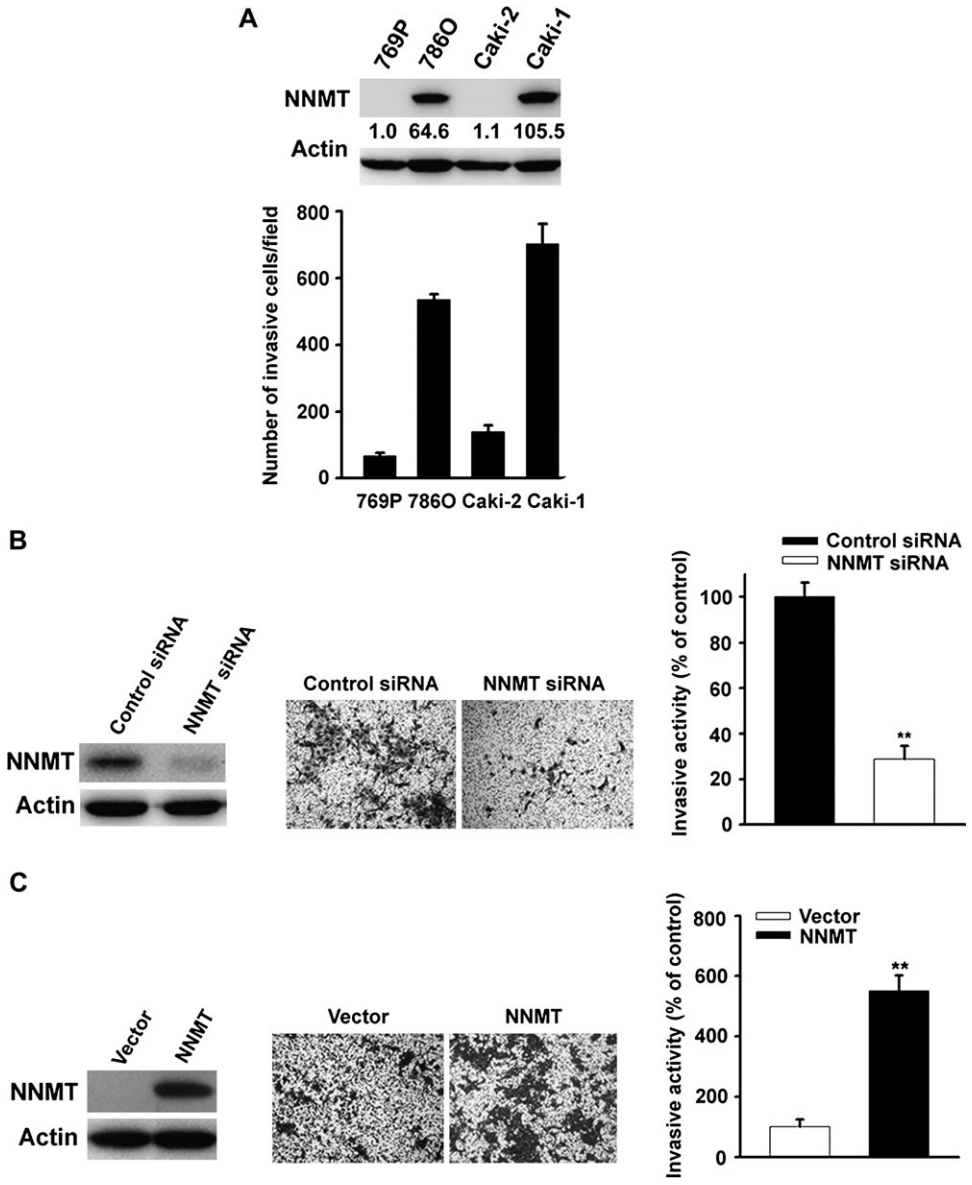

Fig. 1. NNMT expression is associated with the invasiveness of ccRCC cells. (**A**) (upper) The lysates of 769P, 786O, Caki-2 and Caki-1 cells were analyzed by western blot analysis to detect the expression levels of NNMT. Actin was used as the loading control. NNMT band intensities were quantitated and normalized to Actin levels, and the value of 769P cells was considered as 1. (lower) The invasive activity of the indicated ccRCC cells was examined by Matrigel invasive assay. Cells (10^5) were seeded into the Matrigel-coated chamber and incubated at 37°C for 24 h. Photos were taken using an inverted contrast light microscope under ×100 magnification. The invasive activity was determined by counting invaded cells in five randomly selected microscopic fields per well. Data presented are the mean of triplicate replications. (**B**) The 786O cells were transfected with control or *NNMT*-targeting siRNA for 24 h. (left) Expression levels of NNMT in siRNA-transfected cells were examined by western blot analysis. Actin served as the loading control. (middle) Transfected cells were seeded into the Matrigel-coated chamber and incubated at 37°C for 24 h. Photos were taken using an inverted contrast light microscope under ×100 magnification. (right) Data presented are the mean of triplicate replications. Standard deviation is indicated by error bars. $^{**}P < 0.001$ versus control cells. (**C**) (left) The lysates of HEK293 cells with or without NNMT expression were subjected to western blot analysis using anti-NNMT antibody. Actin was used as the loading control. (middle) Cells (10^5) were seeded into the Matrigel-coated chamber and incubated at 37°C for 24 h. Photos were taken using an inverted contrast light microscope under ×100 magnification. (right) Data presented are the mean of triplicate replications. Standard deviation is indicated by error bars. $^{**}P < 0.001$ versus control cells.

MMP-2 activation is involved in NNMT-dependent cellular invasion

MMP-2 has been suggested to play an important role for cancer cell invasion (10). To study whether MMP-2 is crucial for NNMT-dependent cellular invasion, the correlation between the expression of NNMT and MMP-2 in ccRCC cells was examined. Our results showed that 786O and Caki-1 cells with a strong NNMT expression exhibited a higher MMP-2 activation than that of 769P and Caki-2 cells as shown by gelatin zymography and western blot analyses (Figure 2A). Furthermore, we examined the correlation between NNMT and MMP-2 expression in clinical ccRCC tissues. As shown in Figure 2B, MMP-2 expression is positively correlated with NNMT expression in ccRCC tissues as demonstrated by quantitative real-time PCR analysis ($r = 0.47$, $P < 0.01$) and immunohistochemical analysis ($P < 0.05$, the representative cases was shown). Moreover, NNMT-overexpressing HEK293 and 769P cells displayed a significant increase in the enzyme activity and expression of MMP-2 as compared with those of control cells (Figure 2C; supplementary Figure S1B is available at *Carcinogenesis* Online). The treatment of siRNA specific to *NNMT* efficiently suppressed the activation of MMP-2 in 786O and Caki-1 cells (Figure 2C; supplementary Figure S1B is available at *Carcinogenesis*

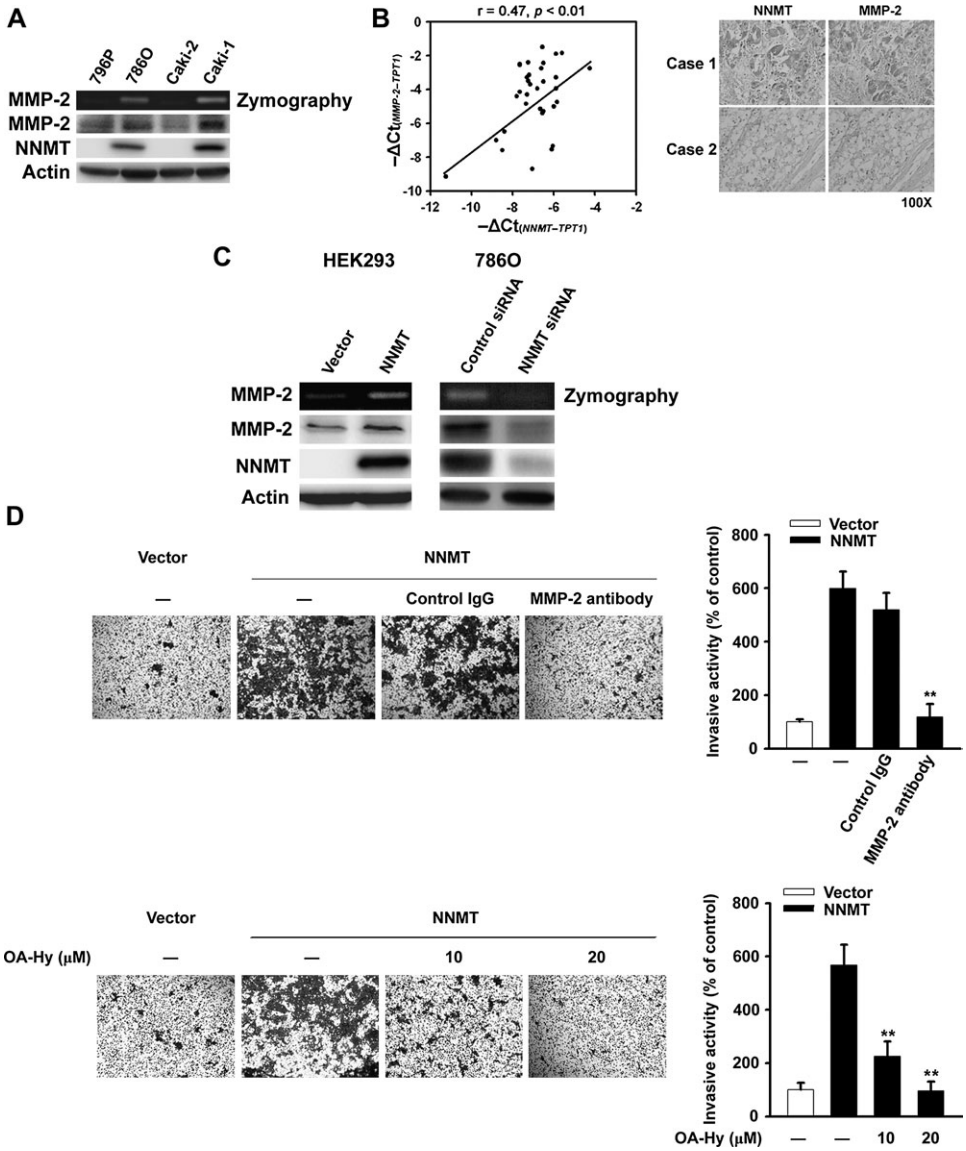

Fig. 2. Correlation between the expression levels of NNMT and MMP-2 in ccRCC cell lines and clinical tissues. (**A**) (upper) MMP-2 enzymatic activity in the conditioned medium and (lower) expression levels of MMP-2 and NNMT of ccRCC cells were detected by gelatin zymography assay and western blot analysis, respectively. (**B**) (left) Expression levels of NNMT and MMP-2 in 33 ccRCC tissues were determined by quantitative real-time PCR analysis. The correlation between expression levels of NNMT and MMP-2 was analyzed by the 'Pearson' correlation method ($r = 0.47$, $P < 0.01$). (right) Immunohistochemical analysis was used to detect NNMT and MMP-2 in ccRCC tissue sections. The representative cases with a positive (case 1) or negative (case 2) immunostaining of NNMT and MMP-2 are shown (see supplementary Figure S2, available at *Carcinogenesis* Online, for enlarged figure). Arrow indicates the cytoplasm immunostaining of NNMT and MMP-2. Photos were taken under ×100 magnification. (**C**) (upper) Gelatin zymography and (lower) western blot analyses were performed to determine MMP-2 activities and expression levels of HEK293 cells with or without NNMT expression and siRNA-transfected 786O cells. Actin served as the loading control. (**D**) Effects of (upper) MMP-2-neutralizing antibody or (lower) MMP-2 inhibitor (OA-Hy) on the invasive activity of HEK293 cells with NNMT expression were examined by Matrigel invasion assay. Data are presented as the mean of triplicate replications. Standard deviation is indicated by error bars; $^{*}P < 0.05$, $^{**}P < 0.001$.

Online). To address the role of MMP-2 in NNMT-dependent cellular invasion, we examined the invasive activity of NNMT-overexpressing HEK293 cells treated with MMP-2 neutralizing antibody (35) or MMP-2-specific inhibitor OA-Hy (36). The results revealed that suppression of MMP-2 activity by MMP-2 neutralizing antibody or OA-Hy markedly inhibited the invasiveness of NNMT-overexpressing HEK293 cells ($P < 0.001$, Figure 2D). These results indicate that NNMT-induced cellular invasion is via MMP-2 activation.

Transcription factor SP1 is involved in NNMT-dependent MMP-2 expression

Previous studies have suggested that several transcription factors participated in the regulation of human MMP-2 expression (11,12,16). To explore which transcription factor is responsible for NNMT-dependent MMP-2 expression, the full-length construct and a serial of successive 5′ deletions (D1–D7 constructs) of *MMP-2* gene promoter were cloned into pGL3 luciferase reporter vector, and luciferase reporter assay was performed to measure the transcriptional activity of *MMP-2* promoter in NNMT-overexpressing HEK293 cells. The results revealed that the *MMP-2* transcriptional activity of NNMT-overexpressing HEK293 cells was elevated ∼2-fold as compared with that of control cells, whereas the deletion of SP1-binding elements (D6) clearly abolished NNMT-dependent *MMP-2* promoter activity (Figure 3A). Furthermore, the *MMP-2* promoter construct with mutations of two SP1-binding elements was generated (11). As shown in Figure 3B, the mutation of SP1-binding elements resulted in a dramatic reduction of *MMP-2*

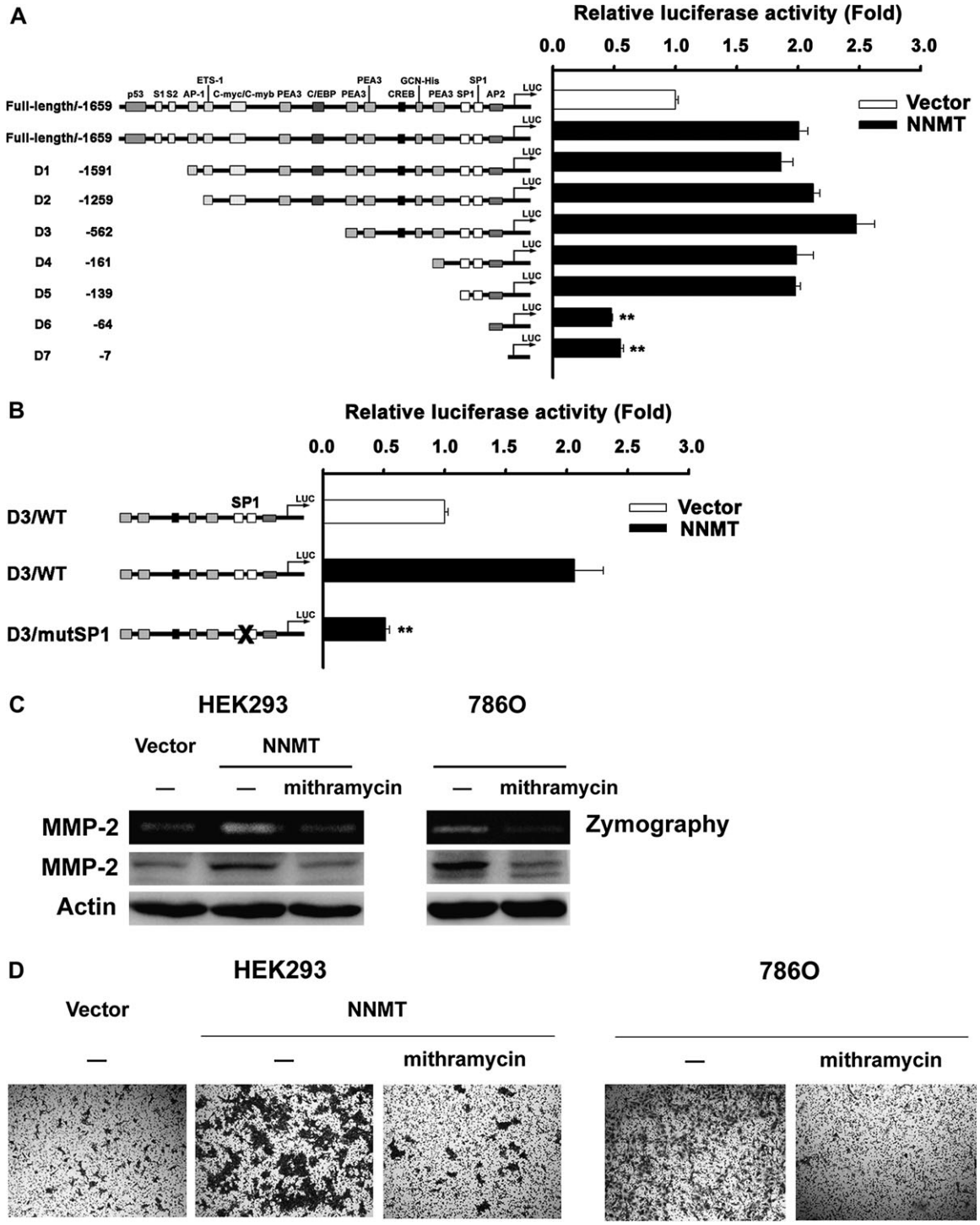

Fig. 3. Transcription factor SP1 is essential for NNMT-dependent MMP-2 activation and cellular invasion. (**A**) The diagrams of the reporter constructs of full-length or various deletion mutants of *MMP-2* promoter (D1–D7) are depicted on the 'left'. The solid lines are the regions cloned upstream of the luciferase gene in the pGL3 luciferase reporter vector. The luciferase activity of cell extracts was analyzed by luciferase reporter assay. Data are presented as the mean of triplicate replications. Standard deviation is indicated by error bars; $^{**}P < 0.001$. (**B**) Effects of the mutation of the two potential SP1-binding elements on *MMP-2* promoter activity were examined by luciferase reporter assay. Data are presented as the mean of triplicate replications. Standard deviation is indicated by error bars; $^{**}P < 0.001$. (**C**) Effects of mithramycin (SP1 inhibitor) on MMP-2 activation were examined. Cells were treated with 25 nmol/l of mithramycin for 18 h, and the activity and expression level of MMP-2 were determined by gelatin zymography and western blot analyses. Actin was applied as the loading control. (**D**) Cells treated with 25 nmol/l of mithramycin were seeded into the Matrigel-coated chamber and incubated at 37°C for 24 h. Photos were taken using an inverted contrast light microscope under ×100 magnification.

promoter activity in NNMT-overexpressing HEK293 cells, indicating that the SP1-binding elements are required for NNMT-dependent activation of *MMP-2* promoter. Moreover, the inhibitor specific to SP1 (mithramycin) displayed a strong inhibitory effect on MMP-2 activation and cellular invasion in NNMT-overexpressing HEK293 and ccRCC cells (Figure 3C and D; supplementary Figure S3 is available at *Carcinogenesis* Online). These results suggest that SP1 is essential for NNMT-dependent MMP-2 activation and cellular invasion.

PI3K/Akt pathway participates in NNMT-dependent MMP-2 activation and cellular invasion

PI3K/Akt pathway has been suggested to act upstream of SP1 to induce MMP-2 activation of cancer cells (12). Therefore, we examined the activation status of Akt in NNMT-overexpressing cells. The results showed that the phosphorylation of Akt was increased in NNMT-overexpressing HEK293 and 769P cells (Figure 4A; supplementary Figure S4A is available at *Carcinogenesis* Online). The knockdown of NNMT expression by treating with *NNMT* siRNA decreased the levels of active Akt in 786O and Caki-1 cells (Figure 4A; supplementary Figure S4A is available at *Carcinogenesis* Online). Additionally, the results of chromatin immunoprecipitation assay demonstrated that the association between SP1 and *MMP-2* promoter was enhanced by NNMT overexpression in HEK293 cells, whereas the inhibitors of PI3K (LY294002) or Akt (Akt inhibitor IV) remarkably reduced it (Figure 4B). Moreover, the treatment with LY294002 or Akt inhibitor IV significantly suppressed MMP-2 activation in NNMT-overexpressing HEK293 and ccRCC cells (Figure 4C; supplementary Figure S4B is available at *Carcinogenesis* Online). NNMT-induced cellular invasion in HEK293 and ccRCC cells was markedly attenuated by LY294002 or Akt inhibitor IV (Figure 4D; supplementary Figure S4C is available at *Carcinogenesis* Online). These results indicate that the activation of PI3K/Akt signaling is required for NNMT-dependent MMP-2 activation and invasive capacity.

NNMT silencing inhibits tumor growth and metastasis of ccRCC cells in vivo

To investigate the effect of NNMT knockdown on ccRCC cells *in vivo*, we generated 786O cells with stable silenced NNMT expression by using the lentivirus containing *NNMT*-specific shRNA. The results revealed that MMP-2 enzymatic activity and expression level of 786O cells with NNMT knockdown were strongly repressed as compared with those of 786O cells with control shRNA (Figure 5A). After injected subcutaneously into the hind limb of –non-obese diabetic severe combined immunodeficiency mice, 786O cells with silenced NNMT expression developed less tumor mass than control cells ($P < 0.001$, Figure 5B). Moreover, NNMT-knockdowned 786O cells were intravenously injected into lateral tail vein of NOD-SCID mice to examine the effect of *NNMT* shRNA on the metastatic activity of ccRCC cells. The results showed that the pulmonary metastasis of NNMT-knockdowned 786O cells was significantly decreased than that of control cells ($P < 0.05$, Figure 5C). These results indicate that NNMT knockdown inhibits the ability of tumor growth and pulmonary metastasis of ccRCC cells.

Discussion

ccRCC cells are characterized by the high invasive and metastatic capacities and resistance to conventional therapies (5–7). To investigate the molecular mechanism of RCC cell invasion is important. In

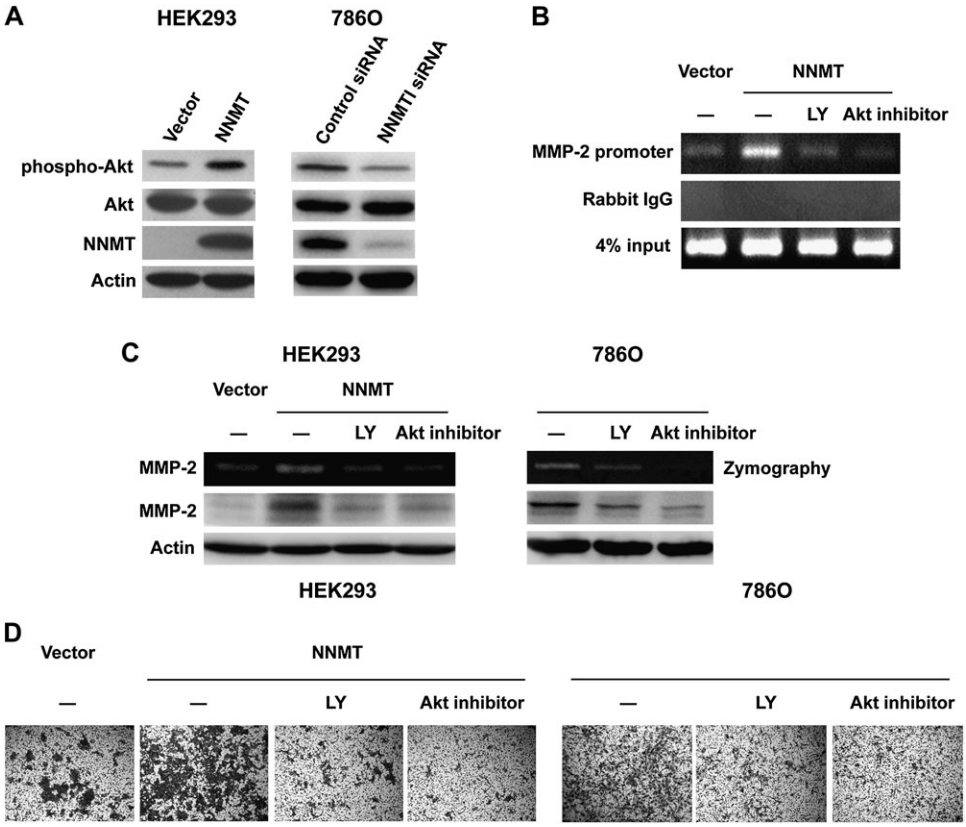

Fig. 4. PI3K/Akt signaling is involved in NNMT-induced MMP-2 activation and cellular invasion. (**A**) Phosphorylated status of Akt in HEK293 cells with or without NNMT expression and siRNA-transfected 786O cells was examined by the antibody specific to phosphorylated Akt. Actin was used as the loading control. (**B**) HEK293 cells with NNMT expression were treated with or without 10 μmol/l of LY294002 (LY) or 1.25 μmol/l of Akt inhibitor IV for 18 h. Chromatin immunoprecipitation assay was performed to determine the effects of LY294002 and Akt inhibitor IV on SP1 binding to *MMP-2* promoter. Rabbit IgG was the negative control and 4% input as positive control. (**C**) Effects of LY294002 and Akt inhibitor IV on MMP-2 activation were examined. Cells were treated with 10 μmol/l of LY294002 or 1.25 μmol/l of Akt inhibitor IV for 18 h, and the activity and expression level of MMP-2 were determined by gelatin zymography and western blot analyses. Actin was applied as the loading control. (**D**) Cells treated with 10 μmol/l of LY294002 or 1.25 μmol/l of Akt inhibitor IV were seeded into the Matrigel-coated chamber and incubated at 37°C for 24 h. Photos were taken using an inverted contrast light microscope under ×100 magnification.

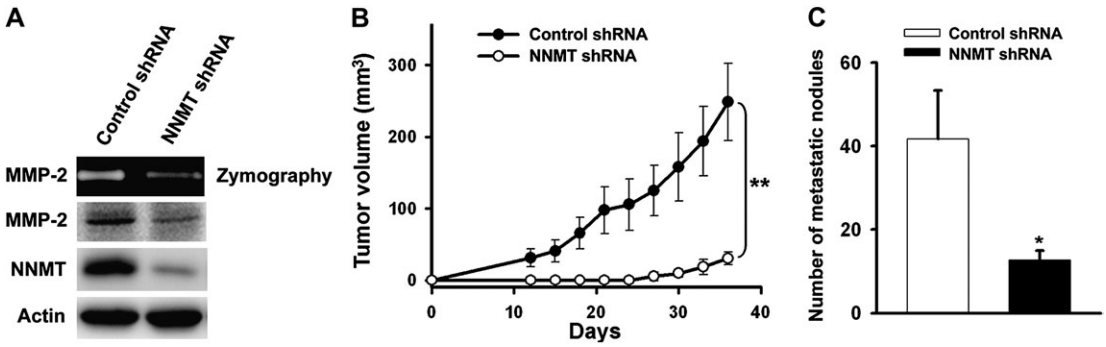

Fig. 5. shRNA-mediated NNMT knockdown suppresses the activities of growth and metastasis of ccRCC cells. (**A**) Gelatin zymography and western blot analyses were performed to determine the activity and expression of MMP-2 in 786O cells with *NNMT* shRNA or control shRNA. Actin was used as the loading control. (**B**) 4×10^6 of 786O cells with *NNMT* shRNA or *luciferase* shRNA were subcutaneously injected into the hind limb of non-obese diabetic severe combined immunodeficiency mice ($n = 6$). Tumor volume was measured every 3 days. Data are presented as the mean of triplicate replications. Standard deviation is indicated by error bars; $^{**}P < 0.001$ versus control cells. (**C**) 1×10^6 of 786O cells with *NNMT* shRNA or *luciferase* shRNA were intravenously injected into the lateral tail vein of non-obese diabetic severe combined immunodeficiency mice ($n = 6$). After 6 weeks of injection, the mice were killed, and the metastatic nodules of lungs were counted. Data are presented as the mean ± Standard deviation; $^{*}P < 0.05$ versus control cells.

this study, we showed for the first time that NNMT overexpression plays a significant role in the invasiveness of ccRCC cells. We further demonstrated that the activation of PI3K/Akt signaling and SP1 induced by NNMT is crucial for the invasive capacity and MMP-2 expression in ccRCC cells.

Enhanced expression of NNMT has been reported in colorectal cancer, pancreatic cancer, gastric cancer, hepatocellular carcinoma, glioblastoma, oral squamous cell carcinoma, papillary thyroid carcinoma, bladder cancer and ccRCC (18–27). Previous studies have shown that NNMT has the potential to be the cancer marker for ccRCC, colorectal cancer and lung cancer (18,28,29). The previous study observed that the aggressive thyroid cancer cell lines show a strong NNMT activity (19). Recently, NNMT expression was suggested to be necessary for cell migration in bladder cancer (25). The present investigation shows that NNMT is strongly expressed in the invasive ccRCC cell lines, but weakly expressed in the primary ccRCC cell lines, indicating an association between the expression of NNMT and the invasiveness of ccRCC cells. This study further demonstrates that siRNA-mediated NNMT knockdown efficiently repressed cellular invasion of aggressive ccRCC cells; conversely, NNMT overexpression strongly induced the invasive capacity in HEK293 and 769P cells. These results indicate that NNMT expression has a critical role in the acquisition of cellular activity in human ccRCC cells.

MMP-2 belongs to a family of zinc-dependent endopeptidases and has the ability to degrade the components of the extracellular matrix, resulting in an important role of MMP-2 in cancer cell invasion and tumor metastasis (37). The activation of MMP-2 was found to be elevated in various tumors, including papillary thyroid carcinoma, bladder cancer glioblastoma and RCC (38–41). In the present study, we observed a correlated expression between NNMT and MMP-2 in clinical samples from ccRCC patients. Moreover, present study demonstrated that NNMT knockdown decreased the MMP-2 expression of invasive ccRCC cells, whereas NNMT overexpression induced that of HEK293 cells, indicating NNMT has the potential to be a novel regulator for the activation of MMP-2. The current investigation also shows that the MMP-2 inhibitor effectively abolished NNMT-dependent cellular invasion, suggesting that the expression of MMP-2 activated by NNMT plays a critical role in NNMT-dependent cellular invasion.

Human *MMP-2* promoter contains various potential *cis*-acting regulatory elements, such as the SP-1 binding elements, leading to a complex mechanism for the regulation of MMP-2 expression (11,15,42,43). SP1 belongs to Specificity Protein/Krüppel-like Factor transcription factor family, which recognizes proximal GC-rich promoter sequences (44). Previous studies showed that the expression of SP1 is elevated in a wide range of human tumors (44). It has been suggested that Akt-mediated phosphorylation of SP1 is important for SP1 DNA-binding activity (45). Recently, SP1 activation has been demonstrated to be involved in the expression of MMP-2 (11,12). For example, SP1 activated by PI3K/Akt pathway is essential for Bcl-w-induced MMP-2 expression in gastric cancer (12). Present investigation revealed that the SP1-binding elements in *MMP-2* promoter were critical for NNMT-dependent MMP-2 activation. We further demonstrated an important role of transcription factor SP1 in MMP-2 expression induced by NNMT. Moreover, this study showed that the inhibition of PI3K/Akt signaling diminished the binding of SP1 to *MMP-2* promoter mediated by NNMT. These observations suggest that NNMT promotes the activation of MMP-2 via a pathway that sequentially involves PI3K/Akt signaling and SP1. However, the mechanism how NNMT is involved in Akt phosphorylation is unclear. Therefore, we performed yeast-two hybrid screening using NNMT as the bait and identified RAN-binding protein 9 (RANBP9)as an NNMT-interacting protein (data not shown). Previous reports showed that RANBP9 functions as an adaptor protein for MET proto-oncogene to enhance Ras activity (46). PI3K/Akt pathway has been demonstrated to be activated by Ras (47), implying a potential mechanism for Akt activation induced by NNMT.

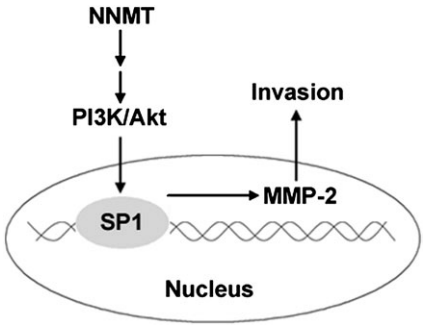

Fig. 6. Proposed mechanism by which NNMT induces cancer cell invasion in ccRCC.

In summary, this study is the first demonstration that NNMT is able to promote cellular invasion through MMP-2 activation. We also showed that activated PI3K/Akt signaling pathway and SP1 are crucial for NNMT-dependent MMP-2 expression and invasive activity (Figure 6). The present observations suggest that NNMT has a novel function on promoting cancer cell invasion and could be a potential therapeutic target for ccRCC.

Supplementary material

Supplementary Figures S1–S4 can be found at http://carcin.oxfordjournals.org/

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