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Honokiol thwarts gastric tumor growth and peritoneal dissemination by inhibiting Tpl2 in an orthotopic model

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Honokiol is known to suppress the growth of cancer cells; however, to date, its antiperitoneal dissemination effects have not been studied in an orthotopic mouse model. In the present study, we evaluated the antiperitoneal dissemination potential of Honokiol in an orthotopic mouse model and assessed associations with tumor growth factor-β1 (TGFβ1) and cells stimulated by a carcinogen, *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG). Our results demonstrate that tumor growth, peritoneal dissemination and peritoneum or organ metastasis of orthotopically implanted MKN45 cells were significantly decreased in Honokiol-treated mice and that endoplasmic reticulum (ER) stress was induced. Honokiol-treated tumors showed increased epithelial signatures such as E-cadherin, cytokeratin-18 and ER stress marker. In contrast, decreased expression of vimentin, Snail and tumor progression locus 2 (Tpl2) was also noted. TGFβ1 and MNNG-induced downregulation of E-cadherin and upregulation of Tpl2 were abrogated by Honokiol treatment. The effect of Tpl2 inhibition in cancer cells or endothelial cells was associated with inactivation of CCAAT/enhancer binding protein B, nuclear factor kappa-light-chain-enhancer of activated B cell and activator protein-1 and suppression of vascular endothelial growth factor. Inhibition of Tpl2 in gastric cancer cells by small interfering RNA or pharmacological inhibitor was found to effectively reduce growth ability and vessel density *in vivo*. Honokiol-induced reversal of epithelial-to-mesenchymal transition (EMT) and ER stress-induced apoptosis via Tp12 may involve the paralleling processes. Taken together, our results suggest that the therapeutic inhibition of Tpl2 by Honokiol thwarts both gastric tumor growth and peritoneal dissemination by inducing ER stress and inhibiting EMT.

Abbreviations: AP-1, activator protein-1; C/EBPβ, CCAAT/enhancer binding protein B; CXCL1, chemokine (C-X-C motif) ligand 1; EGF, epidermal growth factor; EMT, epithelial-to-mesenchymal transition; ER, endoplasmic reticulum; ERK, extracellular signal regulated kinase; HGF, hepatocyte growth factor; HUVEC, human umbilical vein endothelial cell; MEK, mitogen-activated protein kinase kinase; MNNG, *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PET/CT, positron emission tomography–computed tomography; SEM, standard error of the mean; siRNA, small interfering RNA; SVEC, mouse microvascular endothelial cell; TGFβ1, tumor growth factor-β1; Tpl2, tumor progression locus 2; VEGF, vascular endothelial growth factor.

Introduction

Peritoneal dissemination is the primary cause of metastasis from malignancies, which is associated with the loss of epithelial features and the acquisition of motile mesenchymal characteristics and invasive properties (1,2). In gastric cancer patients with unrestrained growth development, peritoneal dissemination and death occur in 20–40% of cases. Outcomes for the treatment of peritoneal dissemination using several well-known therapeutic approaches such as chemotherapy have been poor with 5 year survival rates of 0% (3). Emerging evidence suggests that epithelial-to-mesenchymal transition (EMT) leads to increased tumor formation, tissue invasiveness and tumor dissemination (1,2,4). Recent reports have shown a close relation between EMT and gastric cancer progression. A number of signaling pathways, including developmental transcriptional factors, are involved in regulating the motile-invasion phenotype of tumor cells (5–7). Indeed, hyperactivation of transcriptional factors stimulates the metastatic potential in human cancer. Importantly, upregulation of Snail, Twist or vimentin and decrease in E-cadherin expression in tumors were correlated with poor outcomes (5–9). Previous reports have shown that expression of nuclear β-catenin and vimentin, as well as E-cadherin, was found in most gastric cancer. Of particular note, 70% (83/118) of cases have decreased levels of E-cadherin, indicating the potential role of EMT in gastric cancer metastasis (10,11).

Tumor progression locus 2 (Tpl2) is a serine-threonine kinase with an important physiological role. Activation of Tpl2 regulates the activation of the mitogen-activated protein kinase, which is critically involved in inflammation, oncogenic events and tumor progression (12,13). Indeed, overexpression of Tpl2 was highly correlated with tumorigenesis in human gastric and colon adenocarcinomas, as well as breast cancer (13–15). The higher Tpl2 activity is partially attributed to a variety of signals, including Toll-like receptor ligands, TNFα, interleukin-1β and CD40 ligand, and Tpl2 is also able to transduce additional downstream transcription factor resulting in signaling cascade (16,17). Growth factors, such as transforming growth factor-β, as well as chemical carcinogens, such as *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine, a specific activator of oncogenic Ras, which also stimulated mitogen-activated protein kinase phosphorylation and furthermore induced EMT, have been shown to have strong associations with formation of gastric cancer (18–21). Conversely, there is strong evidence that it may act as a tumor suppressor. Tpl2-deficient mice are highly susceptible to colitis-induced colorectal carcinogenesis (22). In addition, Tpl2-deficient mice develops the enhanced lymphomas in the MHC class I–restricted T-cell antigen receptor transgenic background signals and loss of Tpl2 enhances tumorigenesis and inflammation in two-stage skin carcinogenesis (23). Recently, our report has shown that Tpl2 inhibitor thwarts endothelial cell function and vascular endothelial growth factor (VEGF) expression. However, the mechanism underlying Tpl2 on EMT and cancer peritoneal dissemination remained unknown.

Honokiol is a small-molecule polyphenol isolated from the genus *Magnolia*, which has been shown to be a potential anticancer agent in multiple facets of signal transduction (24–26). A pharmacokinetic study revealed that the half-lives of disposition in rat plasma samples were 49.22 ± 6.68 and 56.24±7.30 min after intravenous injection of 5 mg/kg and 10 mg/kg of Honokiol, respectively (27). Our previous study demonstrated that Honokiol induced endoplasmic reticulum (ER) stress and triggered calpain-II-mediated, glucose-regulated protein-94 cleavage and apoptosis in human gastric cancer cells (28). Honokiol has also been demonstrated to inhibit activation of 15-lipoxygenase-1 and consequently inhibits peroxisome proliferator-activated receptor-γ and COX-2-dependent signals in gastric tumorigenesis (29). In addition, Honokiol-induced calpain/SHP-1 interactions mitigated peritoneal dissemination of gastric cancer in *nu/nu*

mice (30). In addition, such interactions possess anti-inflammatory effects. Interestingly, inhibition of reduced nicotinamide adenine dinucleotide phosphate oxidase-related oxidative stress by Honokiol prevented hyperglycemia-induced endothelial damage, which induced nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB)-regulated COX-2 upregulation, apoptosis and cell death in human umbilical vein endothelial cells (HUVECs) (31). Honokiol could reduce concentrations of glomerular monocyte chemoattractant protein-1 and intracellular adhesion molecule-1 to levels similar to those of type I (alpha1) collagen and fibronectin in experimental mesangial proliferative glomerulonephritis rats (32). Honokiol also improves renal fibrosis by preventing the formation of extracellular matrix and pro-inflammatory factors *in vivo* and *in vitro* (33). Even though several studies found that Honokiol has a direct or indirect impact on gastric cancer cells lines and intraperitoneal inoculation of cancer cells, the antimetastatic potentials of Honokiol have not been investigated in an orthotopic model. The present study was designed to elucidate the role of Honokiol-induced changes in Tpl2 on EMT and peritoneal dissemination in gastric cancer.

Materials and methods

Many of the methods listed here have been published previously but are presented here for clarity (28–31). Honokiol was obtained from Wako Chemical Company (Osaka, Japan), and its purity was determined to be a minimum of 99% by high-performance liquid chromatography.

Cell culture

Human gastric cancer cell lines, AGS (moderately differentiated gastric adenocarcinoma) and MKN 45 cells (poorly differentiated gastric adenocarcinoma) were supplied by the cell bank of Taipei Veterans General Hospital (Taiwan), and SV-40 immortalized mouse microvascular endothelial cells were a kind gift from K.H.L. Cell culture systems were used as described previously (30). HUVECs were obtained from umbilical cord veins by collagenase treatment as described previously (34).

Orthotopic implantation in animals

The gastric cell line MKN 45 was collected at the log phase and 5×10^6 cells were used for implantation (full details of the protocol are provided in Supplementary document 1, available at *Carcinogenesis* Online). Six-week-old male BALB/c nude mice, weighing 25–30 g were used in this study. The MKN45 cells (5×10^6) were suspended in phosphate-buffered saline with a final volume of 100 µl and injected into the stomach region at the point of greatest curvature with an injection rate of 3.3 µl/min using a 26 gauge Hamilton syringe attached to an automated pump and left *in situ* for an additional 5 min to avoid reflux along the injection tract. The mice were evaluated by positron emission tomography–computed tomography (PET/CT) and were then randomly selected for killing at 30, 60, 90 or 120 days after tumor cell inoculation or when moribund and peritoneal dissemination was evaluated by counting the number of tumor nodules in the mesenterium. The body organs were examined for metastasis, and various tissues were processed for histological examination.

Xenograft tumor mouse model

Transfections of small interfering RNA (siRNA)-VEGF and siRNA-Tpl2 into MKN45 cancer cells were performed using Lipofectine. The MKN45 siRNA-VEGF (1×10^6) and MKN45 siRNA-Tpl2 (1×10^6) cells were transected for 24 h and then each of the mice received one of the cell types via intraperitoneal injection to the abdominal cavity for 30 days (full details of the protocol are provided in Supplementary document 1, available at *Carcinogenesis* Online). Quantification of the vessels was estimated by counting five randomly chosen high-power fields.

Positron emission tomography–computed tomography

Imaging studies were performed as described previously (30). Images were interpreted by experienced nuclear medicine physicians with all available clinical information and correlative conventional imaging was used for anatomic guidance.

Immunohistochemistry

Histological examination was performed as described previously (30). Paraffin-embedded specimens were processed for specific anti-Tpl2, or anti-anti-pTpl2 (dilution: 1:250, Cell Signaling Technology) or anti-CD31 (1:250, Santa Cruz Biotechnology) primary antibody immunostaining or routine hematoxylin and eosin staining. After incubation with an appropriate primary antibody enhancer, the slides were incubated with horseradish peroxidase polymer (Laboratory Vision Corporation, Fremont, CA). Reaction products were visualized by immersing the slides in peroxidase-compatible chromogen. Finally, the slides were counterstained with hemalaun.

Tpl2 immunoprecipitation and kinase assay

Measurement of kinase activity was performed as described previously (35). Cells were treated with proangiogenic factors for different periods as indicated, disrupted with lysis buffer and then centrifuged. The lysates containing 500–1000 µg protein were used for immunoprecipitation with an antibody against Tpl2 and then incubated at 4°C overnight. After the addition of protein G Plus agarose beads, the mixture was continuously rotated at 4°C. The beads were washed three times with kinase buffer, resuspended in kinase buffer and incubated for an additional 30 min. Next, myelin basic protein and [γ-^{32}P]adenosine triphosphate solution were added, and the mixture was incubated. A 20 µl aliquot was transferred onto p81 paper and washed with 0.75% phosphoric acid. The radioactive incorporation was determined using a scintillation counter. Experiments were done in triplicate.

Luciferase reporter assay

Cells at 60% confluence were cotransfected with 0.2 µg of the promoter-reporter construct CCAAT/enhancer-binding protein B (C/EBPβ), NF-κB and activator protein-1 (AP-1), and 0.1 µg of a thymidine kinase promoter-driven Renilla-luciferase vector (pRLTK; Promega, Mannheim, Germany). After incubation, cells were lysed and processed using the Dual Luciferase Kit (Promega) as described by the manufacturer. Luciferase activity was normalized to Renilla firefly activity for transfection efficiency and recorded by a luminometer (LKB, Rockville, MD).

Measurement of cell proliferation

Cell proliferation was measured using the Cell Titer 96 AQueous cell viability assay kit. After the indicated duration of incubation with the appropriate medium, 20 µl of a novel tetrazolium compound, MTS/PMS, an electron coupling reagent mixture was added per well, and cells were incubated for 1 h before absorbance at 490 nm was measured.

Cell invasion assay

Cell invasion was monitored using cell culture polycarbonate inserts with 8.0 µm pore size (Becton Dickinson Labware, Franklin Lakes, NJ). After exposure to different stimuli, cells were trypsinized, washed, resuspended in RPMI and loaded into the insert. RPMI supplemented with 2.5% fetal bovine serum was placed in the plate well as a cell migration stimulus. Cells were allowed to migrate for 8–12 h at 37°C through an 8 µm polyethylene terephthalate track-etched membrane that had been precoated with Matrigel basement membrane matrix (BD Biosciences) at 9.6 mg/ml and subsequently blocked with 1% bovine serum albumin in phosphate-buffered saline. Then, cells from the upper side of the membrane were removed using a cotton swab, and the remaining cells on the bottom side of the membrane were fixed with 70% ethanol, stained with 0.1% crystal violet staining and counted in five random fields. The mean of the number was used to quantitate the migration. The experiments were done in triplicate wells.

Western blot analysis and immunoprecipitation

Protein levels were analyzed by western blot as described previously (30,34). Antibodies against glyceraldehyde 3-phosphate dehydrogenase, pE-cadherin, E-cadherin, cytokeratin-18, Snail, vimentin, N-cadherin, caspase7, calpain-II, (Santa Cruz Biotechnology), Tpl2, pTpl2, p-elf2A (New England BioLabs) and β-actin (Sigma) were used.

VEGF assay

VEGF concentrations were measured with a human VEGF ELISA kit (R&D Systems).

Statistical analysis

Values were presented as mean ± standard error of the mean (SEM). Analysis of variance, followed by Fisher's least significant difference test, was performed for all data. A value of $P < 0.05$ was considered statistically significant.

Results

Honokiol suppresses primary tumor growth and spontaneous in vivo *metastasis of gastric tumors*

Previous reports have shown that Honokiol suppresses the growth of gastric cancer cells (28–30). In this study, we investigated whether

Honokiol could suppress the peritoneal dissemination of gastric cancer along with primary tumor growth in the stomach. To investigate the antiperitoneal dissemination potential of Honokiol *in vivo*, mice were implanted orthotopically with MKN45 cells into the gastric wall of nude mice. First, we analyzed the tumor progression by PET/CT imaging and assessed the tumor burden (Figure 1A), and finally two animals were randomly selected to be killed and subjected to postmortem examination for macroscopic and histological analyses (data not shown). Four animals in each group were then randomly assigned to either with or without treatment with 5 mg/kg Honokiol, and tumor growth was monitored using PET/CT imaging every 2 weeks for up to 4 months (Figure 1B and Supplementary Figure 1A and B, available at *Carcinogenesis* Online). Our results show that Honokiol substantially reduced the primary body weight by 68% compared with that of the control tumor group (Supplementary Figure 1C, available at *Carcinogenesis* Online). Honokiol was well endured by the mice as indicated by the increase of body weight for up to 7 months, high activity levels of the mice, as well as the

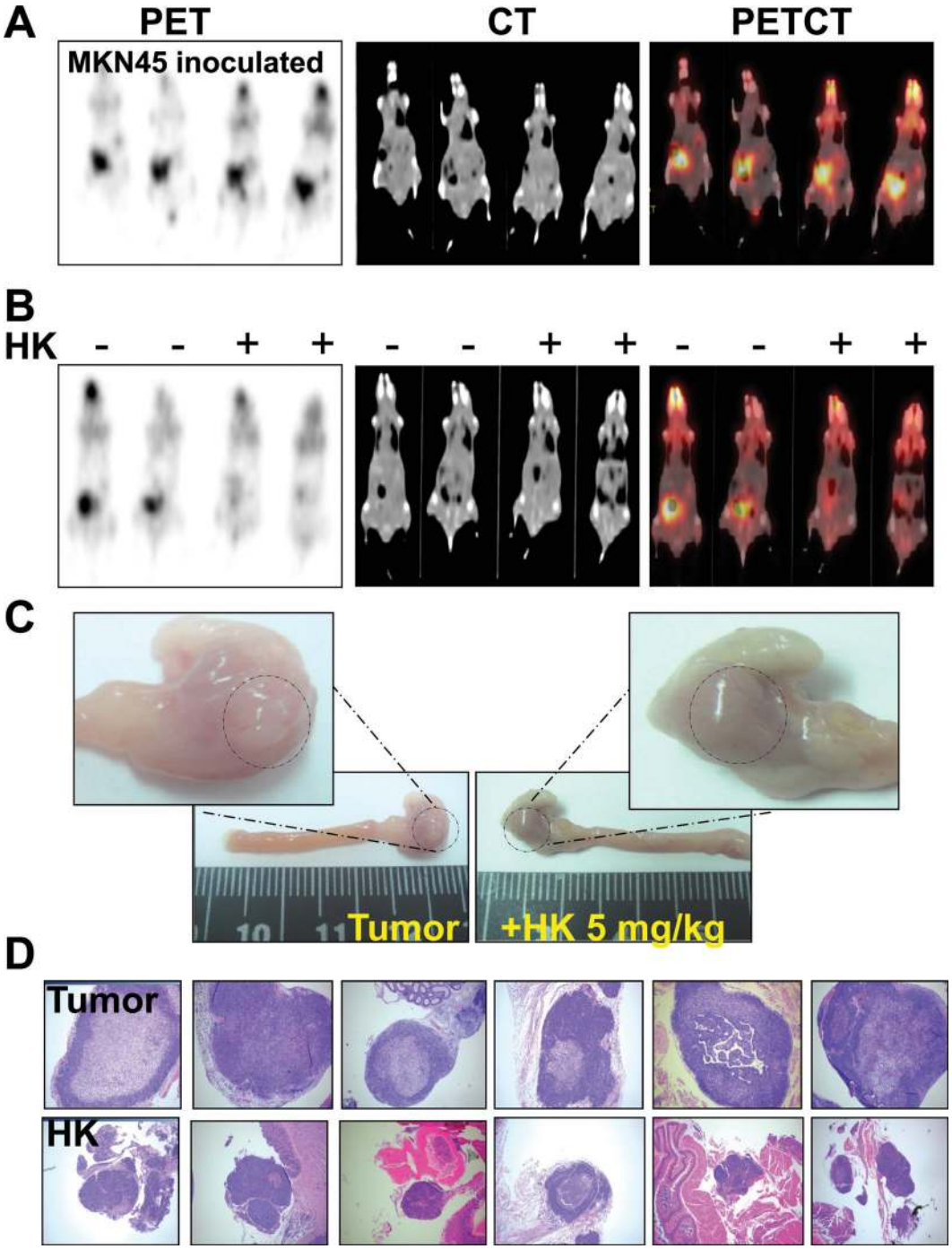

Fig. 1. Honokiol restrain primary tumor growth of gastric cancer tumors. Mice were orthotopically implanted with MKN45 cells and treated with Honokiol (5 mg/kg, twice/weekly, intraperitoneally) for 60 days. (**A**) Tumor growth was monitored by PET/CT imaging of control and (**B**) Honokiol-treated mice twice a week by tomography imaging station. Tumor growth detection of control and Honokiol-treated mice. (**C**) Macroscopic and (**D**) microscopic hematoxylin and eosin staining appearance of the tumor growth of stomach of nude mice after orthotopic implantation of MKN45 cells. At the end of the experiment, stomach along with the primary tumor from the control and Honokiol-treated mice were carefully excised and weighed. The results are presented as a bar graph ($n = 10$). * $P < 0.05$ in Supplementary Figure 1, available at *Carcinogenesis* Online; HK, Honokiol.

absence of signs of acute or delayed toxicity. Moreover, Honokiol-treated mice developed a tumor mass in the gastric wall of much smaller size compared with that in control mice. Both macroscopic and microscopic imaging studies disclosed the tumor growth in the stomach of nude mice after orthotopic implantation at day 60 as shown in Figure 1C and D. Quantification of tumor volume is detailed in Supplementary Figure 1D, available at *Carcinogenesis* Online. Tumor growth is mostly restricted to the stomach without peritoneal dissemination. As shown in Figure 2A and B, in the control group, tumors migrated to the abdominal cavity away from the stomach, whereas Honokiol-treated tumors (5 mg/kg/twice/week) showed less migration, indicating significant inhibition of tumor cell migration by Honokiol. The orthotopical model was applied using postmortem examinations at different time points to assess the vulnerability of peritoneal metastasis. Eight out of 10 mice in the Honokiol group showed no metastatic lesions whereas, in the control

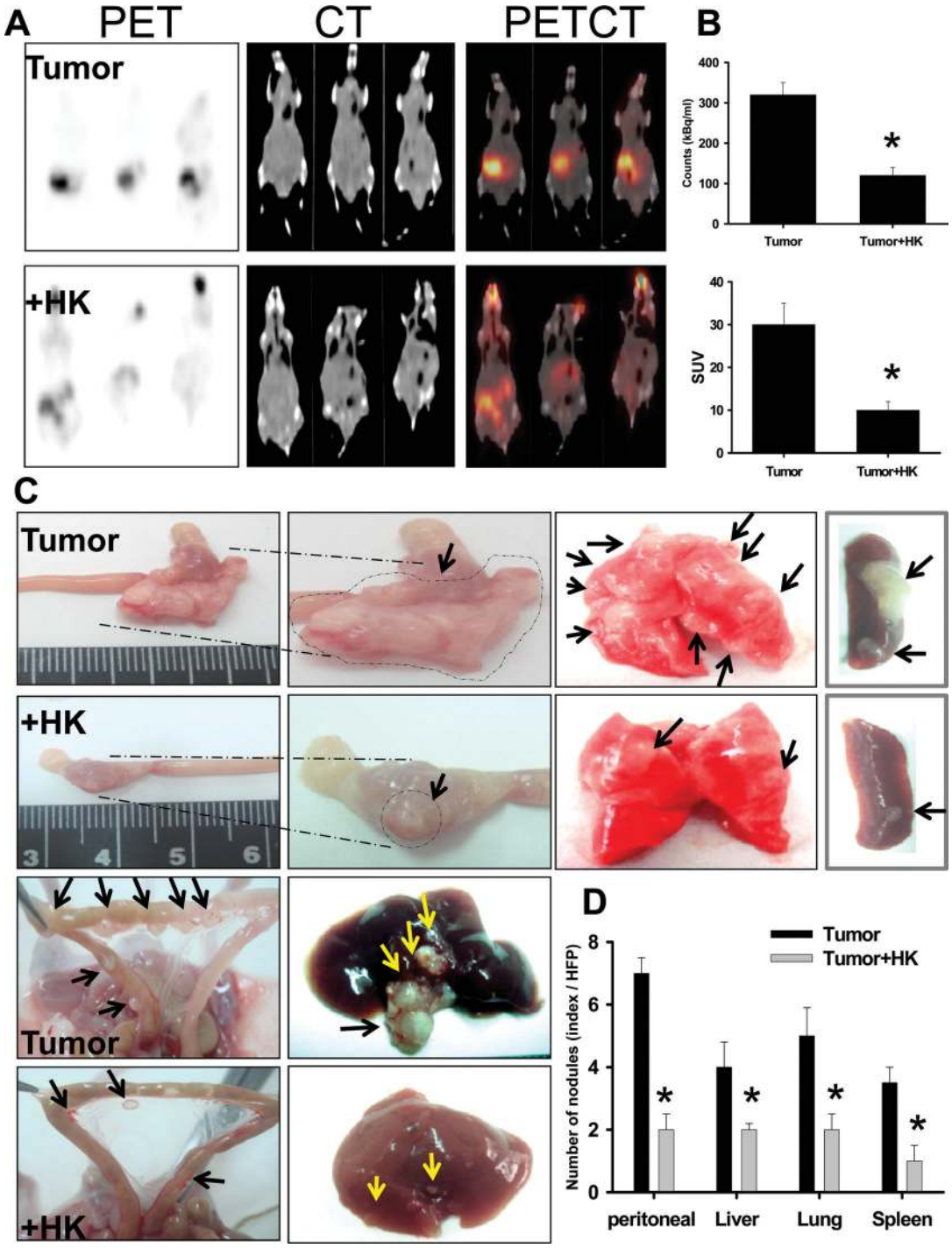

Fig. 2. Honokiol inhibits *in vivo* metastasis of gastric tumors. Mice were orthotopically implanted with MKN45 cells and treated with Honokiol (5 mg/kg, twice/weekly, intraperitoneally). Tumor burden was measured twice a week by PET/CT imaging. (**A**) A representative image showing peritoneal dissemination of animals. (**B**) Randomized grouping of Honokiol-treated mice. A representative image showing stomach burden of control and Honokiol-treated animals. Distance of the secondary tumors that migrated from stomach was measured by (**C**) macroscopic (upper panel, tumor group; lower panel, tumor group treated with Honokiol) and (**D**) quantifications of numbers of metastases in different organs (counts/field) were calculated. All data are presented as mean ± SEM ($n = 6$). *$P < 0.05$ compared with control. HK, Honokiol.

group, 9 out of 10 mice demonstrated metastatic lesions. Compared with the control group, Honokiol-treated mice exhibited reduced peritoneal dissemination of MKN45 cells to various organs such as liver, spleen, lung, intestinal mesentery nodules, abdominal cavity, bone marrow and lymph nodes, indicating that Honokiol inhibits the peritoneal dissemination from implanted tumors to multiple organs in the abdominal cavity (Figure 2C). The peritoneum is usually the most common site of metastasis and recurrence in gastric cancer. As shown in Figure 2B, metastatic organs from Honokiol-treated mice showed no or modest specific uptake values, whereas control mice organs demonstrated strong radioactivity, indicating that Honokiol markedly inhibits the peritoneal dissemination of gastric tumors to the abdominal cavity. Quantification of Honokiol-inhibited peritoneal metastasis of gastric tumor cells is shown in Figure 2D.

Honokiol inhibits Tpl2 expression and activity of tumor burden and tumor perivasculatures in vivo

Cancer growth and peritoneal metastasis are angiogenesis dependent, which has been reported to be associated with Tpl2 (12,13,36–38). Due to the strong association of peritoneal metastasis with expression of angiogenesis, the objective of the present study was to evaluate the antiangiogenesis potential of Honokiol. Inhibition of gastric tumor by Honokiol *in vivo* prompted us to examine whether the antiperitoneal metastasis effect of Honokiol was due to the inhibition of Tpl2 expression. We further examined microvessel density in tumors in response to Honokiol treatment by immunohistologic staining of CD31 antibody. As shown in Figure 3A and B, Honokiol significantly inhibited tumor angiogenesis as indicated by reduced microvessel density in tumor mass. We next tested the hypothesis that Tpl2 inhibition can prevent angiogenesis. We determined the effects of Tpl2 inhibition on tumor burden and tumor perivasculatures *in vivo*. As shown in Figure 3C and D, treatment of Honokiol for 60 days significantly reduced the Tpl2 expression as determined by immunostaining. The attenuation of growth in the presence of Tpl2 inhibitor, which was used as the positive control, was also found in our previous study (data not shown). Moreover, Tpl2 expression also reduced the perivasculature of surrounding mass *in vivo* (Figure 3E). We further isolated tumor mass and evaluated the protein expression by western blotting using antibody probe with Tpl2 and detected Tpl2 kinase activity *in vivo* (Figure 3G and H). Honokiol-induced ER stress marker p-elf2α served as the positive control. These results indicate that there were high levels of Tpl2 protein and kinase activity in the tumor mass. Honokiol exposure caused a substantial reduction in Tpl2 production and kinase activity.

Tpl2 inhibition reduced CXCL1- and MNNG-induced cancer growth and prevented epidermal growth factor-induced proliferation of HUVECs

Proto-oncogene Tpl2 plays an obligatory role in the transduction of cell proliferation and angiogenesis signals (12,15,17,22,36,39–41). We further evaluated the effects of Tpl2 inhibition on tumor growth signal. As shown in Supplementary Figure 2A (available at *Carcinogenesis* Online), treatment of AGS or SCM-1 gastric cancer cells with chemokine (C-X-C motif) ligand 1 (CXCL1) or *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (data not shown) for 24 h significantly stimulated the tumor growth as determined by MTS assay. The increase in growth was attenuated in the presence of Tpl2 inhibitor and Honokiol. To exclude non-specific effects of a pharmacological inhibitor, we ablated the Tpl2 message by transient transfection of cancer cells with siRNA-Tpl2. Transfection with siRNA-Tpl2 but not scrambled siRNA caused efficient ablation of Tpl2 protein. Consistent with the aforementioned results, transfection of HUVECs or mouse microvascular endothelial cells with siRNA-Tpl2 significantly inhibited epidermal growth factor (EGF)-induced endothelial cell growth (Supplementary Figure 2B, available at *Carcinogenesis* Online). The inhibitory activity of Tpl2 inhibitor on CXCL1, MNNG or EGF-induced cell proliferation was also assessed by western blotting using antibody probe with proliferating cell nuclear antigen and Ki67 (data not shown), a proliferation marker. Treatment with CXCL1, MNNG or EGF increased the proliferating cell nuclear antigen expression, whereas Tpl2 inhibition significantly reduced it. These results suggest that Tpl2 mediates CXCL1, MNNG or EGF-induced cancer cells and endothelial cell growth.

Honokiol inhibits EMT marker in vitro *via Tpl2 inhibition*

The EMT programmed activation in cancer cells enables these cells to complete the initial steps of invasiveness in the metastatic cascade, which involve specifically, local invasion, intravasion survival in the circulation and extravasion. We then examined whether Tpl2 regulated mesenchymal (metastatic) properties controlled by Honokiol. In addition, whether Honokiol induced epithelial cell signature and induced apoptosis was not determined. We found that Honokiol significantly downregulated Snail, vimentin, N-cadherin and Tpl2, and upregulated cytokeratin-18 and E-cadherin expression but not phosphorylation of E-cadherin. Honokiol-induced cleavage of caspase7 and ER stress-related marker was also found. Honokiol induced cancer cells SCM-1 and MKN45 in a dose- and time-dependent manner (Figure 4A). Cancer cells may relay Tpl2 activity to promote EMT progression. Furthermore, we investigated whether inhibition of Tpl2 suppresses EMT markers. Our results show that treatment with Tpl2 inhibitors or transfection with siRNA-Tpl2 significantly was consistent with the finding that Honokiol treatment upregulated cytokeratin-18 and E-cadherin expression but downregulated Snail and vimentin. A conventional ER stress agent (tunicamycin) reduced the expression of the EMT marker, Tpl2, and induced p-elf2α phosphorylation (Figure 4B). Therefore, Honokiol is unique in its ability to induce epithelial cell signature via a classic ER stress–Tpl2 depression mechanism.

Honokiol inhibits TGFβ1- or MNNG-induced EMT by Tpl2 constraint in gastric cancer cells

EMT was a key initial step during tumor invasion. The TGF-β superfamily proteins can pioneer and possess EMT during tumor invasion and various other biological systems (18–20). Carcinogen-induced tumor formation also leads to invasive cancer and lymph node metastasis (21,42). Hence, we attempted to establish whether Honokiol would inhibit TGFβ1- or MNNG-induced invasion in gastric cancer cells. As shown in Figure 5A, AGS cells treated with TGFβ1 (10 ng/ml) or MNNG (0.1 μM) had slim morphology, including projecting and spreading cells and were scattered (data not shown) compared with control cells. However, Honokiol exposure substantially inhibited TGFβ1- or MNNG-induced cell morphological changes in AGS cells. Importantly, TGFβ1 and MNNG enhanced the invasion of AGS cells in Boyden's chamber by 2.5- and 2.3-fold, respectively, compared with control cells and were associated with EMT. In contrast, Honokiol treatment remarkably reduced TGFβ1- and MNNG-induced cell invasion associated with EMT markers in AGS (Figure 5B). These results suggest that Honokiol markedly thwarted TGFβ- or MNNG-induced mesenchymal characteristics and increased the expression of epithelial signature markers.

Knockdown of Tpl2 reduced C/EBPβ, NF-κB and AP-1 activation

VEGF is a key regulator of tumor angiogenesis, a prerequisite for the growth of disseminated tumor, includes numerous transcription factors such as C/EBPβ, NF-κB and the AP-1 binding site, which are involved in initiating and activating the transcription of a gene directly (43–50). To determine whether knockdown of Tpl2 abolished transcriptional activity of AGS cancer cells, or HUVECs, the expression of transcriptional activity was measured by luciferase reporter assay (Supplementary Figure 3A and B, available at *Carcinogenesis* Online). Inhibition of Tpl2 or siRNA-Tpl2 effectively prevented C/EBPβ, NF-κB and AP-1 activation, suggesting an important role of Tpl2 in transcriptional activity in cancer cell or HUVECs in response to VEGF. In addition, TGFβ1 or MNNG treatment decreased C/EBPβ, NF-κB and AP-1 luciferase reporter activity in AGS cells pretreated with Tpl2 inhibitors or siRNA-Tpl2 transfection compared with that

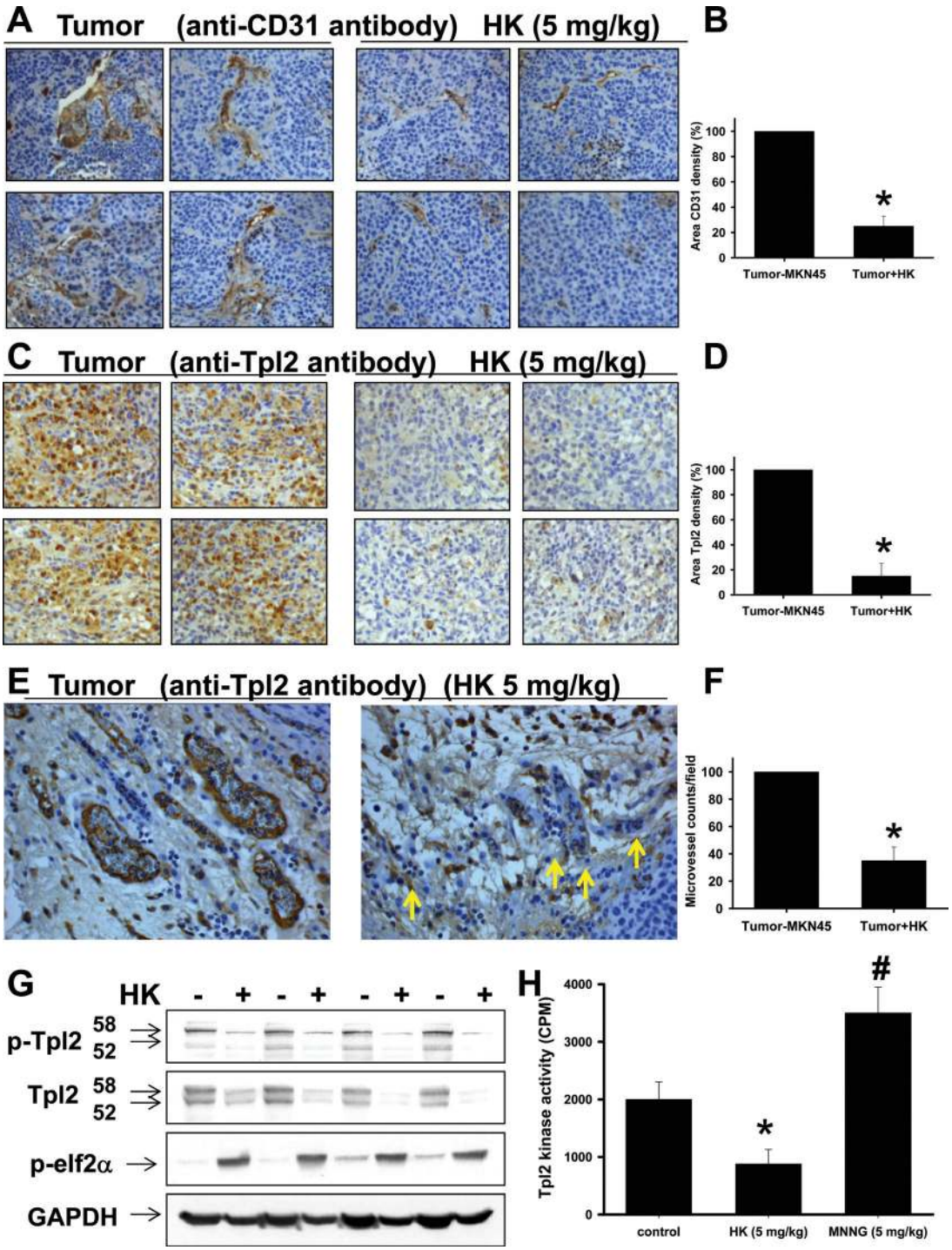

Fig. 3. Honokiol inhibits Tpl2 expression and activity of tumor burden and tumor perivasculatures *in vivo*. (**A**) Immunohistochemistry analysis of tumor mass stained with anti-CD31 (upper panel), (**C**) anti-Tpl2 (lower panel) and (**E**) perivasculature of tumor margin. Right panel shows relative quantification was evaluated by densitometry (**B**, **D** and **F**). Data are presented as mean ± SEM ($n = 4$). *$P < 0.05$ compared with control. Quantitation of tumor perivasculature indicated that Tpl2 inhibition of tumor growth and angiogenesis were abolished. Microvessel density of CD31 in each section was measured in five images from each treatment. Original magnification, ×100. (**G**) Tumors from control and treated mice were homogenized and an equal amount of protein was subjected to western blot. Results shown are representative of four independent experiments ($n = 4$). (**H**) Tumor Tpl2 activity was quantified using kinase activity and presented as a bar diagram. Carcinogen MNNG (5 mg/kg)-induced Tpl2 kinase activity served as the positive control. Values are mean ± SEM of four separate tumors. *$P < 0.05$; #$P < 0.01$ compared with control; HK, Honokiol.

in control scrambled RNA cells (Supplementary Figure 4, available at *Carcinogenesis* Online). The results showed that C/EBPβ, NF-κB and AP-1 are downstream regulators of Tpl2 and are also highly associated with VEGF. We also confirmed statistically increased VEGF production compared with that of either treatment alone (Supplementary Figure 3C, available at *Carcinogenesis* Online). These data clearly indicated that knockdown Tpl2 reduced C/EBPβ, NF-κB and AP-1 activation resulting in downregulation of VEGF expression and thus, this finding may have potential in the development of potent antiangiogenesis and antiperitoneal dissemination treatment stratagems.

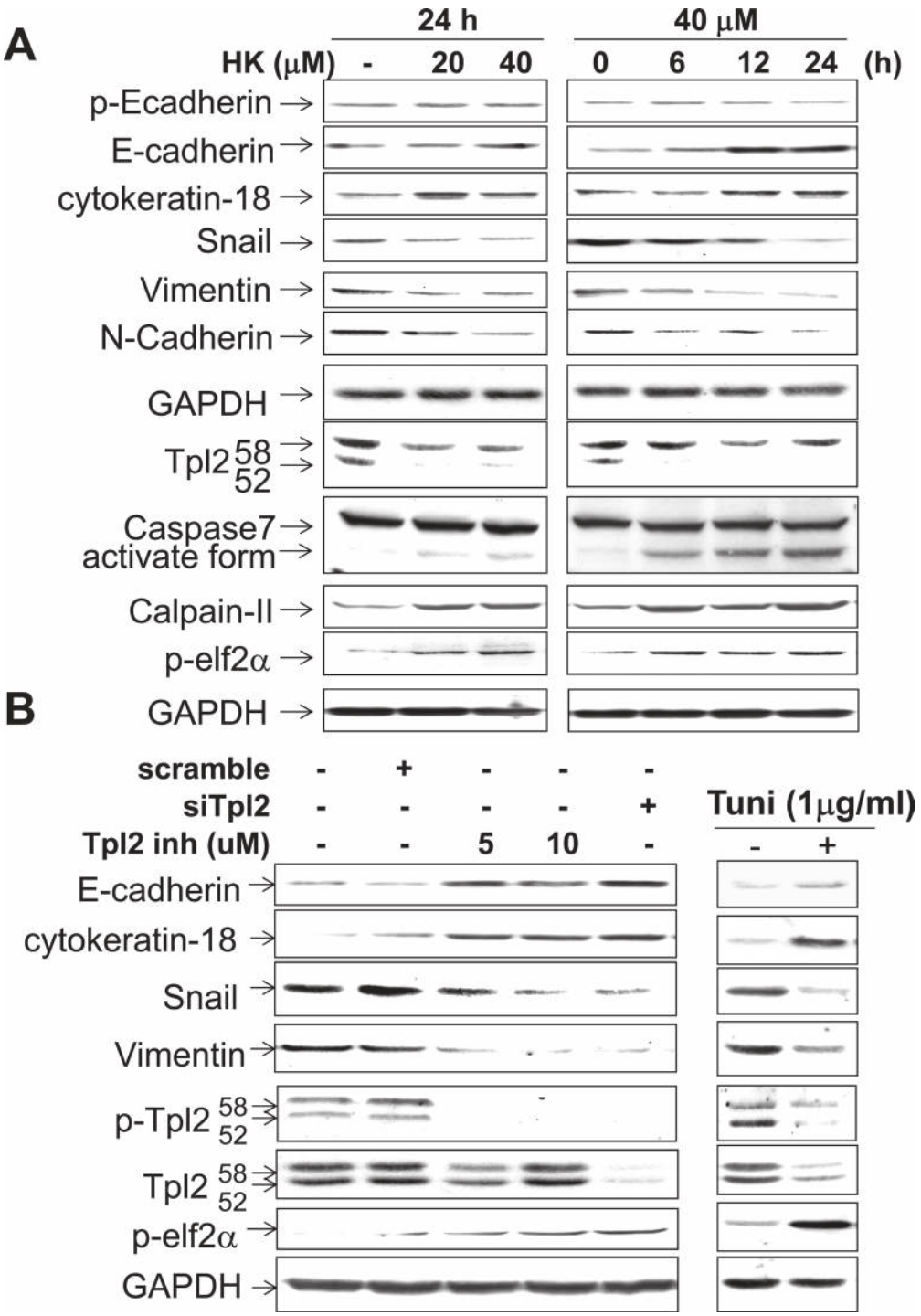

Fig. 4. Honokiol inhibits EMT marker *in vitro* via Tpl2 inhibition. (**A**) SCM-1 and MKN45 cancer cells were treated with Honokiol either in a dose- or time-dependent manner, and EMT markers (E-cadherin, cytokeratin-18, N-cadherin, vimentin, Snail), ER stress marker (caspase7 and calpain) and Tpl2 were evaluated by western blot. (**B**) Cancer cells were pretreated with Tpl2 inhibitor or transfected with siRNA-Tpl2 or scrambled siRNA followed by stimulation with Honokiol for 24 h. The results shown are representative of at least four independent experiments. HK, Honokiol.

Inhibition of gastric cancer growth or peritoneal dissemination in vivo *by siRNA-Tpl2 and siRNA-VEGF*

As has been shown above, the growth or peritoneal dissemination of cancer cells can be reduced by treatment with Honokiol and by Honokiol-induced inhibition of Tpl2. This enhancement might be correlated with angiogenesis in parallel with VEGF and Tpl2 expressed by cancer cells and endothelial cells. The proangiogenic capabilities of endothelial cells are dependent upon the expression of VEGF, which can be induced via a C/EBPβ-, NF-κB- and AP-1-dependent pathway. Therefore, we demonstrated that dampening of the antiangiogenesis effect on cancer cells could be inhibited by siRNA-VEGF and siRNA-Tpl2 (Figure 6A). Similar results were found for tumor volume and weight (Figure 6B and C). We also assessed the density of newly formed microvessels in tumor tissue sections via immunohistochemistry for CD31 (Figure 6D). The profile for peritoneal dissemination was similar. Our data demonstrate that suppression of Tpl2 or VEGF expression can reduce microvessel formation in tumor tissues (Figure 6D and E). These data support the hypothesis that peritoneal

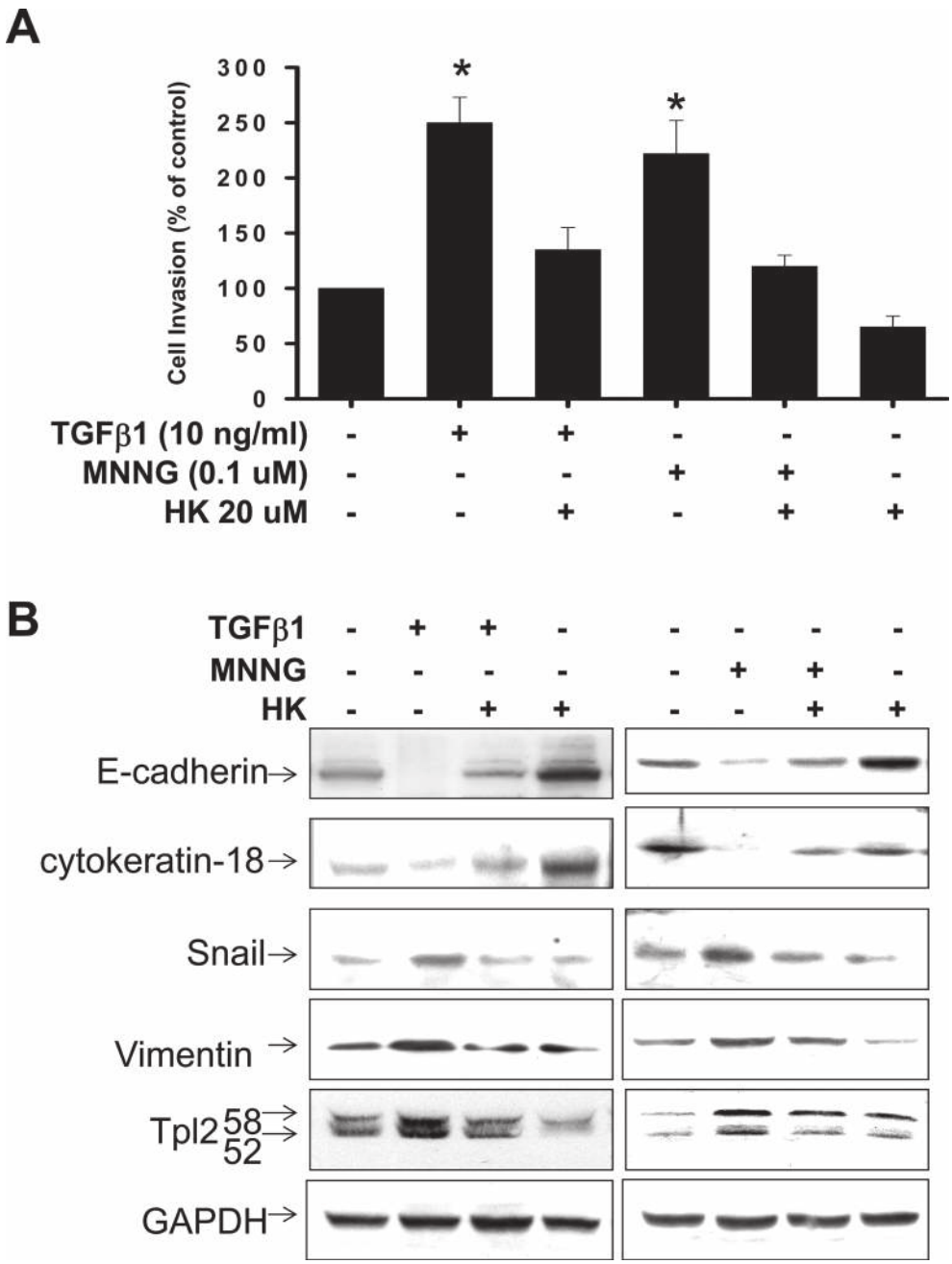

Fig. 5. Honokiol inhibits TGFβ1- or MNNG-induced EMT by Tpl2 constraint in gastric cancer cells. AGS cells were serum starved and treated with or without TGFβ1 or MNNG. (**A**) After 48 h of treatment, cells were evaluated for invasion by Boyden's Transwell assay or (**B**) EMT markers were evaluated by western blot. (**B**) AGS cells were treated with Honokiol for 24 h and whole-cell lysates were evaluated for phosphorylation and expression of Tpl2. The results shown are representative of at least six independent experiments. HK, Honokiol.

dissemination mediated by angiogenesis via the Tpl2/VEGF axis plays an essential role in the progression of gastric cancer cells *in vivo*.

Discussion

In the present study, we investigated the role of Honokiol in gastric tumor growth and peritoneal dissemination *in vivo* and *in vitro*. Our results showed that Honokiol conspicuously reduced peritoneal dissemination and organ metastasis of orthotopically implanted MKN45 cells in nude mice through the activation of apoptosis. Moreover, Honokiol markedly thwarted the development of mesenchymal characteristics and increased the expression of epithelial signature markers *in vivo* and *in vitro*, demonstrating that Honokiol targets EMT to prevent peritoneal dissemination of gastric tumors along with primary tumor growth. In this study, we also established that Honokiol targets tumorigenesis inducer TGFβ1 or carcinogenesis inducer MNNG in Tpl2 signaling and controls multiple transcription factor signaling pathways to reduce Snail expression leading to the induction of E-cadherin, which consequently suppresses peritoneal dissemination. Honokiol also blocks endothelial cell growth by targeting Tpl2.

An EMT is a biological process that results in the upregulation of transcriptional modulators such as C/EBPβ, NF-κB, AP-1, Snail, Twist, β-catenin and Smad, as well as the loss of adherence molecules, and the acquisition of new moieties important for cell growth and movement (1,2,5,20,48). However, although factors

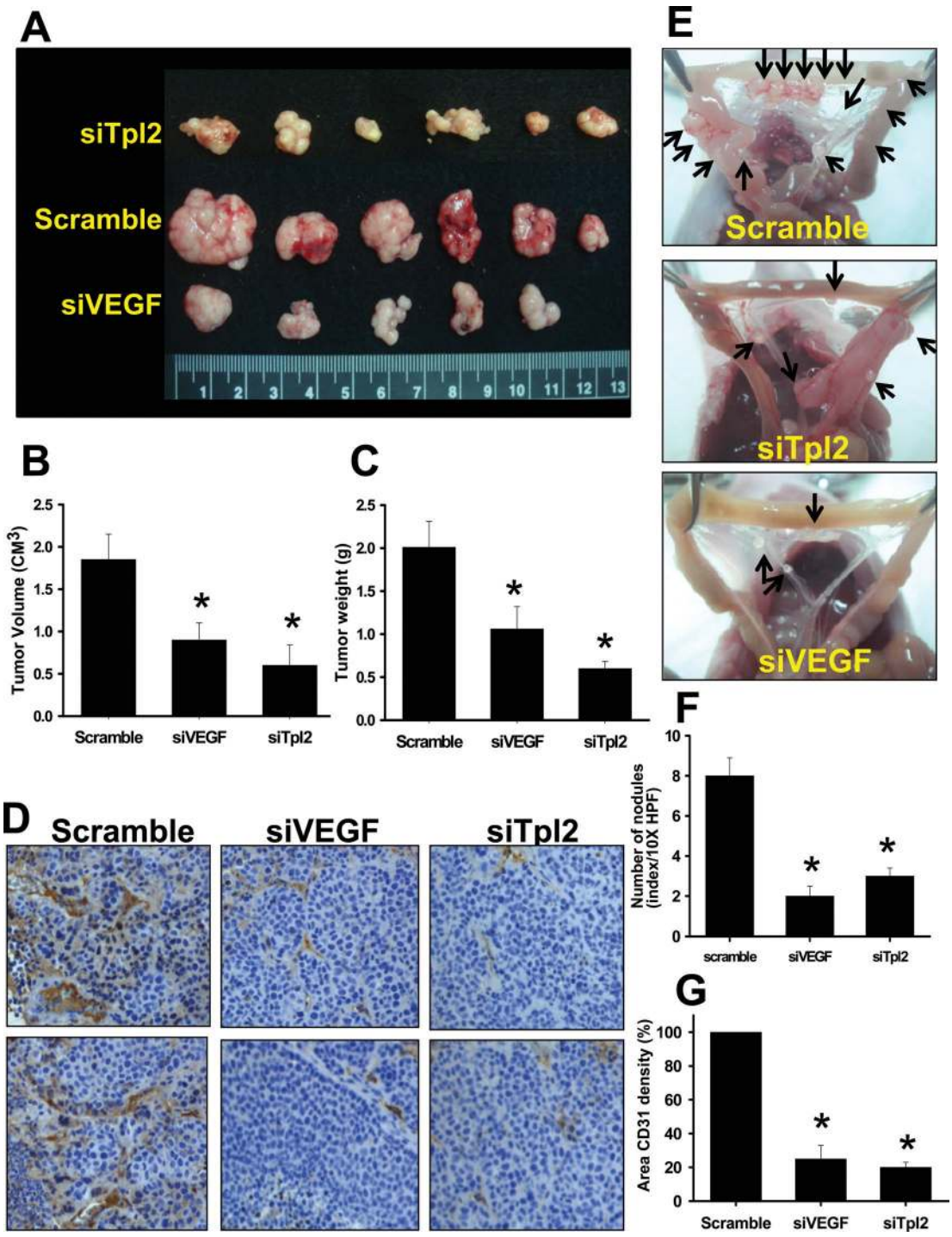

Fig. 6. Inhibition of gastric cancer growth favored *in vivo* by siRNA-Tpl2 and siRNA-VEGF. Transfections of siRNA-Tpl2 and siRNA-VEGF into MKN45 were performed using Lipofectamine. Twenty-eight days after implantation in subcutaneous administration to nu/nu mice, the animals were euthanized and their tumors were dissected. (**A**) Photographic illustration of tumors. (**B**) Mice tumor volume and (**C**) tumor weight of MKN45 tumors. (**D**) CD31 immunostaining showing the numbers of newly formed vessels in gastric cancer tissues: control scrambled, siRNA-Tpl2, siRNA-VEGF. (**E**) Photographic illustration of metastatic peritoneal nodules. (**F**) Quantifications of nodules number and (**G**) stained with anti-CD31 of tumor vasculature. Quantification of the vessels was estimated by counting five random chosen high-power fields. $^*P < 0.05$.

relay available transcription factors to cancer cells, none of them accounts for the actual targeting of Tpl2. Tpl2 proto-oncogene is a serine-threonine protein kinase that plays an important role in the transduction of mitogen-activated protein kinase pathways, nuclear factor-activated T cells and NF-κB signaling in a variety of cell types, and promotes cell proliferation (51). Tpl2 phosphorylation is required for the physiological activation of Tpl2 by external signals, such as Toll-like receptor, lipopolysaccharide and death receptor signals (16,17). Overexpression of Tpl2 has been found in breast cancer, gastric cancer and colon adenocarcinoma (13–15). Moreover, oncogenic kinase Tpl-2 expressed transforming ability in cell cultures and in oncogenic animals. Tpl-2 has been reported to confer a growth advantage to affected cell clones and has also been associated with progression in 22.5% of the tumors (13–15). Paradoxically, Tpl2 fully expressed its tumor-suppressive function via intrinsic signaling in intestinal myofibroblasts. Tpl2-deficient

intestinal myofibroblasts upregulated hepatocyte growth factor (HGF) production and became less sensitive to the negative regulation of HGF by TGFβ3. Importantly, Tpl2-deficient mice are highly susceptible to colitis-induced colorectal carcinogenesis probably by enhancing susceptibility to carcinogenesis via fibroblast-specific upregulation of HGF production due to Tpl2 inhibition. Collectively, these observations provide evidence that Tpl2 has a critical role for Tpl2 in the regulation of HGF production may apply in several other solid tumors where TGF-βR/HGF deregulation has been causally associated with tumorigenesis (22). In addition, *Tpl2* ablation promotes inflammation in $Apc^{min/+}$ mice by downregulating the expression of interleukin-10 in myeloid cells in the intestinal mucosa and by interfering with the development and function of regulatory T cells, suggesting that Tpl2 also has a critical role in regulating systemic inflammation and in the susceptibility to intestinal tumorigenesis. Serebrennikova *et al*. proposed that the positive feedback initiated by inflammatory signals originating in developing polyps is more robust in $Apc^{min/+}$/$Tpl2^{-/-}$ mice. This may be because these mice have a higher intestinal polyp burden as a result of the enhancement of tumor initiation. Despite well-established mechanistic differences in the pathophysiology of the Apc^{min} and AOM/DSS models, a stromal cell-intrinsic Tpl2-dependent mechanism such as the foregoing study may be commonly contributing to the development of tumorigenesis in the two models (52). Knockout of Tpl2 in mice has been shown to induce a heightened inflammatory state in response to 12-*O*-tetradecanoylphorbol 13-acetate, possibly contributing to their increased susceptibility to development of chemically induced skin carcinogenesis, indicating that Tpl2 may serve more as a tumor suppressor than as an oncogene (23). In addition, Tpl-2 is known to exert inflammatory functions. Tpl2 knockout mice produce low levels of TNF-α when exposed to lipopolysaccharide and they are resistant to lipopolysaccharide/D-galactosamine-induced pathology, implying that endogenous Tpl2 may be involved in innate and adaptive immunity rather than in proliferative signals (53). Although previous studies suggest that Tpl2 possesses different functions, the roles of Tpl2 in peritoneal dissemination and in regulating EMT progression remain poorly understood. The present study showed that Tpl2 inhibition by Honokiol markedly diminished tumor peritoneal dissemination and reduced mesenchymal characteristics in a mouse primary tumor model. Other evidence demonstrates that Tpl2 inhibition by Honokiol or gene silencing by RNAi causes tumor burden changes and inhibits tumor growth within 4 weeks. Combination of gene-silencing Tpl2 and VEGF will be predicted markedly effective in the future work. These changes are consistent with the results of PET/CT imaging studies and nodule counts in the present study. Therefore, inhibition of constitutively high levels of Tpl2 activity may offer new targets for therapeutic intervention to abolish tumor invasion and block metastasis.

Recently, Johannessen *et al*. (54) have shown that Tpl2 expression may potentiate extracellular signal regulated kinase (ERK) activation in a mitogen-activated protein kinase kinase (MEK)-independent manner. Importantly, Tpl2 may confer *de novo* resistance to RAF kinase inhibition (55). The kinase pathway comprising RAF, MEK and ERK is activated in most human tumors, such as high frequency in melanomas, colon and lung (56). In addition, tumor cells carrying the serine–threonine protein kinase B-RAF (BRAF) mutations are much more reliant on MEK–ERK signaling, and oral BRAF inhibitors induce substantial tumor regression in patients with BRAF (V600E) metastatic melanoma (57). However, single-agent targeted therapy is almost invariably followed by relapse due to acquired drug resistance. Hence, resistance to RAF inhibition can be achieved by multiple MAP3K-dependent mechanisms of MEK/ERK reactivation but might be intercepted through combined therapeutic modalities for mitogen-activated protein kinase pathway inhibition (e.g. RAF/MEK or RAF/Tpl2 combinations). Our findings therefore raise the possibility that Honokiol-inhibited Tpl2 expression may prove a drug target of novel resistant therapeutics.

ER stress activates a signaling network called the unfolded protein response, a signaling pathway for adaptive response. The unfolded protein response initially exerts a protective effect by upregulation of specific ER stress-regulated genes and inhibition of general protein translation. However, severe or prolonged ER stress results in cell death via apoptotic signaling, which could be used as a therapeutic stratagem in cancer therapy (58–60). Emerging evidence indicates that ER stress plays a pivotal role in the inhibition of angiogenesis *in vivo*. Our previous study has shown that Honokiol induces ER stress resulting in human gastric cancer cell apoptosis and inhibits tumorigenesis through a calpain-mediated, glucose-regulated protein-94 cleavage (28). Moreover, we also found direct evidence that the ER were dilated and fragmented in Honokiol-treated gastric cancer cells and HUVECs. Meanwhile, our group demonstrated for the first time that calpain/SHP-1 interaction has a direct effect via inhibition by Honokiol of STAT-3 phosphorylation (30). These results indicate that Honokiol may be useful in inducing ER stress and may exert its effect in parallel with antiangiogenesis in gastric cancer therapy. This finding is consistent with the results reported by Banerjee *et al.* (61), which showed that unfolded protein response is required in *nu/nu* mice microvasculature for treating breast tumor with tunicamycin, thereby reducing angiogenesis *in vivo*. Cancer growth and peritoneal metastasis are angiogenesis-dependent processes. Hence, these results indicate that Honokiol targets Tpl2 to induce a reversal of EMT and that ER stress-induced apoptosis might involve parallel processes in gastric cancer cells and endothelial cells.

Our data, taken together with the findings of previous studies, enabled the authors to integrate Honokiol regulated-Tpl2 into the transcriptional network that controls peritoneal dissemination in pathological conditions. A schematic potential molecular mechanism of Honokiol targets is presented in Supplementary Figure 5, available at *Carcinogenesis* Online. This is the first report to our knowledge to demonstrate that Honokiol is capable of thwarting the regulatory effect of Tpl2 on mesenchymal characteristics activity in EMT progression. Tpl2 expression level is increased in cancer cells. Decreased Tpl2 expression blocks C/EBPβ, NF-κB and AP-1 transcriptional activity and degrades Snail expression. Furthermore, reduced expression of Tpl2 increases the epithelial signature, as well as that of key markers such as E-cadherin and cytokeratin-18. Hence, Honokiol may have potential in the development of pharmaceutical drugs for the treatment of peritoneal dissemination-dependent human diseases such as tumors.

Supplementary material

Supplementary document 1 and Figures 1–5 can be found at http://carcin.oxfordjournals.org/

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