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Molecular Cancer Research

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Genomics

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Tpl2 Kinase Impacts Tumor Growth and Metastasis of Clear Cell Renal Cell Carcinoma

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Abstract

Due to the innate high metastatic ability of renal cell carcinoma (RCC), many patients with RCC experience local or systemic relapses after surgical resection. A deeper understanding of the molecular pathogenesis underlying advanced RCC is essential for novel innovative therapeutics. *Tumor progression locus 2* (*Tpl2*), upregulated in various tumor types, has been reported to be associated with oncogenesis and metastatic progression via activation of the MAPK signaling pathway. Herein, the relevance of *Tpl2* in tumor growth and metastasis of RCC is explored. Inspection of The Cancer Genome Atlas (TCGA) indicated that Tpl2 overexpression was significantly related to the presence of metastases and poor outcome in clear cell RCC (ccRCC), which is the most aggressive subtype of RCC. Moreover, expression of Tpl2 and CXCR4 showed a positive correlation in ccRCC patients. Depletion of Tpl2 by RNAi or activity by a *Tpl2* kinase inhibitor in human ccRCC cells remarkably suppressed MAPK pathways and impaired *in vitro* cell proliferation, clonogenicity, anoikis resistance, migration, and invasion capabilities. Similarly, orthotopic xenograft growth and lung metastasis were significantly inhibited by *Tpl2* silencing. Furthermore, Tpl2 knockdown reduced CXCL12-directed chemotaxis and chemoinvasion accompanied with impaired downstream signaling, indicating potential involvement of Tpl2 in CXCR4-mediated metastasis. Taken together, these data indicate that Tpl2 kinase is associated with and contributes to disease progression of ccRCC.

Implications: *Tpl2* kinase activity has prognostic and therapeutic targeting potential in aggressive clear cell renal cell carcinoma. *Mol Cancer Res; 11(11); 1375–86. ©2013 AACR.*

Introduction

Renal cell carcinoma (RCC) is the most common kidney tumor, whose incidence and mortality increases continuously over the past 30 years (1, 2). Although surgical intervention is effective for the majority of patients with a localized disease, approximately 30% of patients are diagnosed with metastatic diseases initially (3). In addition, a similar portion of patients with RCC who had radical surgery experiences relapses and systemic metastases (4). Despite improved understanding of dysregulated molecular pathways in RCC, there has been no significant improvement in the therapeutic strategies against advanced RCC with metastatic lesions; the 5-year survival rate of metastatic patients with RCC is about 10% (5, 6).

RCC represents a spectrum of heterogeneous histologic and genetic subtypes (7). Among pathologic subtypes, clear cell RCC (ccRCC) accounts for approximately 80% of all RCC and shows worse clinical outcomes compared with other subtypes including papillary, chromophobe, and collecting duct cell RCC; more than 90% of metastatic RCCs are ccRCCs (8, 9). Molecularly, 33% to 57% of ccRCCs harbor Von Hippel–Lindau (VHL) gene mutation, however, the clinical implications of this mutation are still controversial (10). Therefore, the identification of prognostic markers and novel therapeutic targets for ccRCC is of crucial importance for highly responsive therapeutic options for RCC.

Dysregulation of mitogen-activated protein kinase (MAPK) kinases (MKK) signaling enhances tumorigenesis and tumor metastasis (11). *Tumor Progression Locus 2 (Tpl2)*, which is known as *MAP3 kinase 8 (MAP3K8)*, plays essential roles in tumor necrosis factor, interleukin-1 (IL-1), CD40, Toll-like receptor and G protein–coupled receptor-mediated MAPK and c-*jun*-NH_2-kinase (JNK) pathways (12). Tpl2 has been reported to be overexpressed in various human tumors and to promote cell transformation, proliferation, migration, and invasion by the activation

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of extracellular signal–regulated kinase (ERK), Rac1, and focal adhesion kinase (FAK; refs. 12–18).

Tpl2 maybe a novel target for ccRCC as its mRNA levels are significantly elevated in ccRCC compared with normal kidneys (12), and elevated MKK or ERK activity has been detected in human RCC cases (19, 20). However, little information exists about prognostic relevance and functional significance of Tpl2 in oncogenesis and metastatic conversion of ccRCC. Herein, we report that elevated Tpl2 expression in ccRCC is associated with the metastasis and poor prognosis and provide the first *in vitro* and *in vivo* evidence for its roles in the tumorigenesis and metastasis of ccRCC using short hairpin RNAs (shRNA)–targeting *Tpl2* (shTpl2) and a specific Tpl2 kinase inhibitor (KI).

Materials and Methods

The Cancer Genome Atlas dataset for RCC

RNA sequencing data, VHL mutation status, and clinicopathologic data for 468 ccRCCs and 73 papillary RCCs (papRCC) were downloaded from The Cancer Genome Atlas (TCGA) data portal (http://cancergenome.nih.gov/) and the cBioPortal for Cancer Genomics (http://www.cbioportal.org/).

RCC cell line expressing shTpl2

Human ccRCC cell lines (caki-1, ACHN, A498, 786-O, and 769-P) were obtained from the American Type Culture Collection (ATCC). Cells were maintained in RPMI-1640 (for caki-1, 786-O, and 769-P) or minimum essential medium (MEM; for ACHN and A498) media (Gibco) supplemented with 10% FBS (Invitrogen) in a humidified incubator containing 5% CO_2 at 37°C. *VHL* gene mutation status of each ccRCC cell line was referred from previous report (21). Caki-1 cells that stably express either nontargeting short hairpin RNA (shRNA; caki-1-shCon) or shTpl2 [caki-1-shTpl2-1 (Pf. Jeong at Medical College of Wisconsin, Milwaukee, WI) and caki-1-shTpl2-2 (Applied Biological Materials Inc.)] were established by lentiviral transduction and puromycin selection (2 μg/mL; Gibco).

Western blot analysis and quantitative reverse transcription PCR

Cells were lysed in the radioimmunoprecipitation assay buffer (RIPA) lysis buffer [15 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris (pH 8.0)] supplemented with 1× phosphatase inhibitors (PhosStop; Roche Diagnostics) and a 1× protease inhibitor cocktail (Complete Mini; Roche Diagnostics). After centrifugation at 10,000× *g* for 5 minutes, the supernatant was harvested. Amount of protein was analyzed using the BCA Protein Assay Kit (Thermo). Protein was separated on SDS-PAGE for 2 hours at 100V. The proteins on the gel were transferred onto polyvinylidene difluoride membranes (Whatman) for 1 hour at 100V and then probed with antibody. The antibodies against total Tpl2 were obtained from Santa Cruz Biotechnology and Life Technologies. Antibodies to detect total MAP–ERK kinase (MEK), phosphorylated MEK (p-MEK), total ERK1/2, phosphorylated ERK1/2 (p-ERK), total JNK, phosphorylated JNK (p-JNK), total protein kinase B (Akt), and phosphorylated Akt (p-Akt) were from Cell Signaling Biotechnology. Internal control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Santa Cruz Biotechnology. For quantitative reverse transcription PCR (qRT-PCR), mRNA was extracted using a RNeasy Kit (Qiagen) and then used for subsequent reverse transcription using a SuperScript III First-Strand Synthesis SuperMix (Invitrogen). Resulting cDNA was used for quantitative PCR (qPCR) using SYBR Green PCR Master Mix and gene-specific primers; human *Tpl2*, forward = 5′-CAAGTGAAGAGCCAGCAGTTT-3′, reverse = 5′-GCAAGCAAATCCTCCACAGTTC-3′; human *GAPDH*, forward = 5′-CATCATCTCTGCCCCCT-CT-3′, reverse = 5′-GGTGCTAAGCAGTTGGTGGT-3′.

Cell proliferation assay

Human ccRCC cells were seeded to 96-well plates at a density of 1×10^5 cells/mL in 100 μL 10% FBS/RPMI or MEM. After 1, 24, 48, or 72 hours, cell viability was determined with an EZ-Cytox Cell Viability Assay Kit (Daeil Lab.) according to the manufacturer's instruction. For pharmacologic Tpl2 inhibition, the Tpl2 KI, 4-(3-chlor-4fluorophenylamino)-6-(pyridin-3-yl-methylamino)-3-cyano-[1,7]-naphthylridine was purchased from Calbiochem (22). The naphthyridine-3-carbonitriles, which are Tpl2 inhibitors, are reported to be quite specific for Tpl2 with IC_{50} of 12 nmol/L to 50 nmol/L. This affinity is at least 2,000- to 10,000-fold more than those for other protein kinases such as Raf-1, MEK, p38, CaMKII, and so on (23). Alternatively, RCC cells were treated with different concentrations of TKI (dimethyl sulfoxide, DMSO only, 1, 2.5, 5, or 10 μmol/L) for 1, 24, 48, and 72 hours, and cell proliferation inhibition was analyzed using the same assay.

Cell-cycle analysis

Cells were fixed by 70% ethanol at 4°C for 30 minutes and then stained in PBS with RNase (100 μg/mL) and propidium iodide (40 μg/mL; BD Biosciences) at 37°C for 30 minutes. Cell cycle was analyzed using a FACSCalibur (BD Biosciences).

Focus forming assay

Caki-1 control cells (shCon) or Tpl2 stable knockdown cells (shTpl2-1 and shTpl2-2; 300 cells/well) were seeded in 6-well plates and maintained in 10% FBS/RPMI for 3 weeks. In another experiment, caki-1 cells were cultured with Tpl2 KI (DMSO only, 2.5 or 5 μmol/L) for 3 weeks. The number of viable colonies per well was counted after staining with 0.2% crystal violet.

Anoikis evaluation

Cells undergoing death by anoikis were identified and quantified using the CytoSelect 24-well Anoikis Assay Kit (Cell Biolabs) according to the manufacturer's instructions.

In vitro Transwell migration and invasion assay

After 24 hours serum starvation, 1×10^5 cells suspended in 300 μL serum-free RPMI or MEM media were loaded

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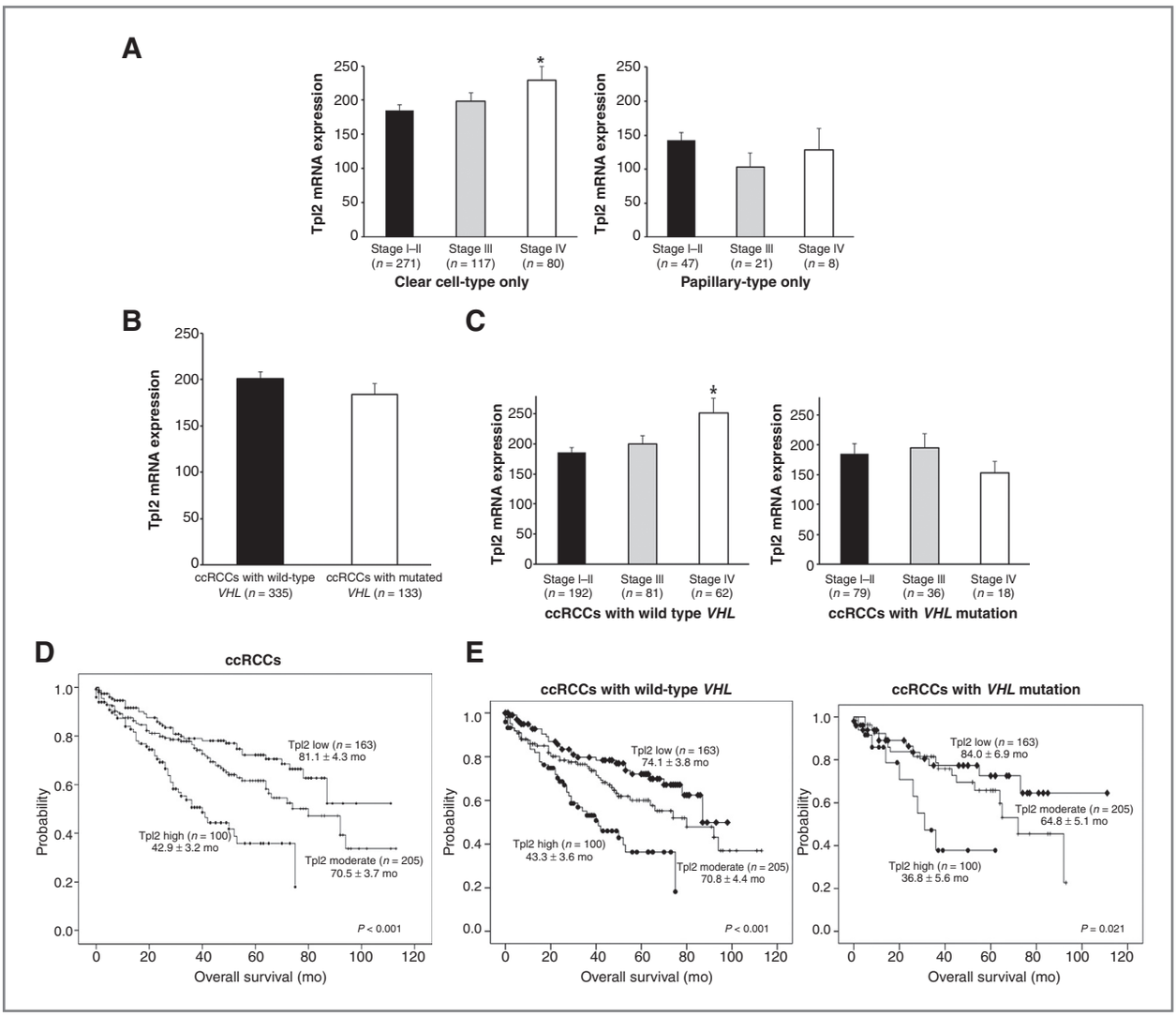

Figure 1. Upregulation of Tpl2 in human primary ccRCCs is correlated with metastasis and worse clinical outcome. A, the differential mRNA expression of Tpl2 in different stages of ccRCCs and papRCCs derived from TCGA RNA sequencing dataset. *, $P < 0.05$. Column = mean, error bar = SEM. B, comparison of Tpl2 mRNA expression of ccRCCs with *VHL* gene mutation status. Column = mean, error bar = SEM. C The differential mRNA expression of Tpl2 in different stages of ccRCCs with wild-type *VHL* or mutated *VHL* gene. *, $P < 0.05$. Column = mean, error bar = SEM. D, Kaplan–Meier graphs representing the probability of cumulative OS in patients with ccRCC stratified according to Tpl2 level in their primary tumors. High, moderate, and low expression of Tpl2 were designated as more than mean + SD, mean − SD approximately to mean + SD and less than mean − SD, respectively. The log-rank test was used to analyze statistical significance. E, survival analysis was stratified according to the VHL mutation status.

into a top insert of 24-well microchemotaxis chambers (Costar). Next, 10% FBS/RPMI or serum-free RPMI supplemented with 200 ng/mL recombinant human chemokine (C–X–C motif) ligand 12 (CXCL12; Prospec) was added to the bottom chamber as a chemoattractant. Cells were allowed to migrate through an 8-μm pore polycarbonate filter or invade through the filter precoated with 100 μg of Matrigel (BD Bioscience) for 24 hours. The upper side of the membrane was scraped with a cotton swab to remove cells that had attached but not migrated or invaded and the migrating or invading cells attached to the lower membrane surface were fixed with 4% paraformaldehyde and stained with hematoxylin and eosin (H&E). The degree of migration/invasion was assessed in each well by counting the number of cells in 10 randomized fields at ×100 magnification. The degree of migration and invasion was adjusted by the cell viability assay to correct for cell proliferation effects of 10% FBS or 200 ng/mL CXCL12 treatment (corrected migration or invasion index = counted migrating or invading cell number/percentage of viable cells; refs. 24, 25).

Spheroid counting and invasion assay in three-dimensional system

The three-dimensional (3D) culture was carried out, as described previously (26). Briefly, caki-1-shCon, caki-1-shTpl2-1, or caki-1-shTpl2-2cells (2×10^3 cells/well) were

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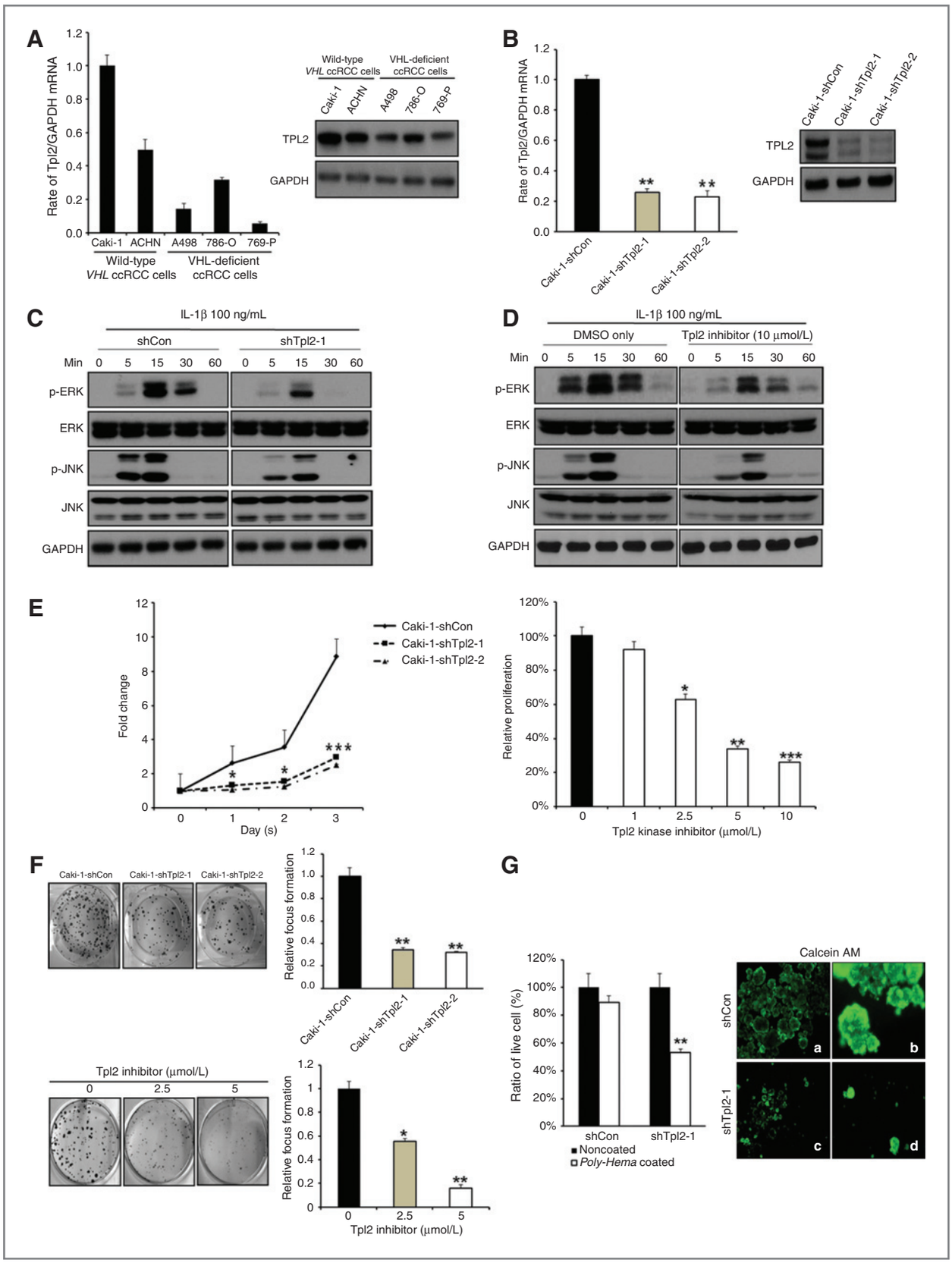

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seeded in lipid-based low-cell adhesion 96-well plates (Lipidure) in 10% FBS/RPMI on day 0. On day 1, 3, 6, and 9, morphology of spheroid was observed, spheroids were dissociated into single cells by Accumax buffer (Millipore), and then cell numbers were counted. For spheroid cell counting assay, on day 3, culture media was changed by 2% Matrigel (BD Bioscience) containing 10% FBS/RPMI media. The number of secondary spheroid was counted on day 9.

Orthotopic xenografts implantation and *in vivo* metastasis assay

All animal experiments were approved by the appropriate Institutional Review Boards of the Samsung Medical Center (Seoul, South Korea) and conducted in accord with the "National Institute of Health Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80–23, revised in 1996). A total of 1×10^6 caki-1-shCon or caki-1-shTpl2-1cells in 40 μL Hank's Balanced Salt Solution (Gibco) were injected into subcapsular area of the left kidney of female 6- to 8-week-old BALB/c-nu mice. Four weeks after the implantation, kidneys and lungs were harvested and assessed for tumor weight. Tumor weight was calculated by subtracting the weight of the right normal kidney (or lung) from that of the left tumor-bearing kidney (or lung). When right kidney (or lung) was heavier than left, the weight of the tumor was recorded as zero. For *in vivo* experimental metastasis assay, 2×10^5 caki-1-shCon or caki-1-shTpl2-1 cells were injected into the tail vein of female 6- to 8-week-old BALB/c-nu mice. Four weeks later, pulmonary nodules were counted both macroscopically and microscopically. Harvested organs were processed for paraffin-embedded sections, which were stained with H&E to confirm pathologically the presence of orthotopic renal or lung metastatic tumors. In addition, to examine effects of Tpl2 silencing on MAPK signaling *in vivo* tumors, immunohistochemistry (IHC) was conducted. Paraffin-embedded orthotopic renal xenografts sections (4 to 6 μm thick) were mounted on slides, which were used to determine Tpl2, p-ERK and p-JNK expression levels. The antibodies used were from the companies indicated in parentheses: anti-Tpl2 (Santa Cruz Biotechnology); anti-p-ERK and anti-p-JNK antibodies (Cell Signaling Technology).

Statistical analysis

All data are presented as mean ± SD or mean ± SEM for Fig. 1). For comparison of Tpl2 mRNA expression levels between different clinical stages, ANOVA was used. Overall survival (OS) curves were estimated by the Kaplan–Meier method, and the resulting curves were compared using the log-rank test. Significance of difference between the experimental groups and controls was assessed by the Student *t* test. Difference is significant if *P* value is < 0.05.

Results

Clinical implications of Tpl2 in RCC

To understand clinical relevance of Tpl2 in RCC, we investigated whether elevated expression of Tpl2 in RCC portends poor clinical outcomes. With the mRNA sequencing data including Tpl2 and CXCR4 retrieved from TCGA (http://cancergenome.nih.gov), *VHL* mutation status and clinicopathologic data of patients with RCC were obtained. Stage IV metastatic ccRCCs ($n = 80$) have significantly higher level of Tpl2 mRNA than stage I and II localized ccRCCs ($n = 271$; Fig. 1A; $P = 0.043$), whereas this expressional difference was not observed in papRCCs ($n = 76$; Fig. 1A). Although level of Tpl2 mRNA expression of *VHL* wild-type ccRCCs ($n = 335$) was not different that of *VHL* mutant ccRCCs ($n = 133$; Fig. 1B), significant elevation of Tpl2 mRNA in metastatic ccRCC was identified only in the *VHL* wild-type ccRCCs (Fig. 1C). Kaplan–Meier plots of survival for patients with ccRCC showed that OS was significantly decreased with upregulation of Tpl2 (Fig. 1D; Log-rank test, $P < 0.001$). This correlation between upregulation of Tpl2 and worse prognosis was observed in not only *VHL* wild-type ccRCCs ($P < 0.001$) but also *VHL* mutant ccRCCs ($P = 0.021$; Fig. 1E). In contrast, papRCC patients with high level of Tpl2 mRNA showed similar clinical outcome compared with those with low or moderate level of Tpl2 mRNA (Supplementary Fig. S1). These analyses suggest that Tpl2 would play a role in the progression of ccRCC, not in papRCC.

Impaired proliferation, clonogenicity, and anoikis resistance of human RCC cells by the depletion of Tpl2

To establish a functional role for Tpl2 in ccRCC, expression of Tpl2 in various kinds of ccRCC cell lines [*VHL* wild-type ccRCC cells = caki-1, ACHN; *VHL* deficient (mutated or hypermethylated) ccRCC cells = A498, 786-O, 769-P]. Levels of Tpl2 mRNA and protein of *VHL* wild-type ccRCC cells were higher than those of VHL deficient ccRCC cells (Fig. 2A). As correlation between Tpl2 expression and worse clinical outcome of ccRCC was more obvious in *VHL* wild-type ccRCC, Caki-1 cells that show higher expression of Tpl2 than the other *VHL* wild-type ccRCC cell line ACHN (Fig. 2A), were selected to test functional roles of Tpl2

Figure 2. Depletion of Tpl2 activity–impaired proliferation, colony formation, and anoikis resistance ability of human ccRCC caki-1 cells. A, relative Tpl2 mRNA and protein level were analyzed by qRT-PCR (left) and immunoblot analysis (right), respectively, in five different human ccRCC cell lines. GAPDH = internal control (for qRT-PCR) and loading control (for immunoblot analysis). B, relative Tpl2 mRNA and protein level were analyzed by qRT-PCR (left) and immunoblot analysis (right), respectively, in caki-1 cells with Tpl2-silencing shRNA (shTpl2-1 or shTpl2-2) or nontargeting shRNA (shCon). GAPDH = internal control (for qRT-PCR) and loading control (for immunoblot analysis). C, IL-1β (100 ng/mL)-mediated MAPK signaling activation was analyzed by Western blot analysis in caki-1 cells with shTpl2-1 or shCon. GAPDH = loading control. D, alteration in IL-1β–induced MAPK signaling activation by the *in vitro* treatment of a Tpl2 KI was analyzed by Western blot analysis in caki-1 cells. GAPDH = loading control. E, suppressed proliferation of caki-1 cells by *Tpl2* gene silencing and Tpl2 KI treatment. F, evaluation of colony formation in accordance with Tpl2 knockdown inhibition and Tpl2 KI treatment. G, anoikis resistance of caki-1 shCon and shTpl2-1 cells was examined. a and c = ×20 magnification; b and d = ×60 magnification. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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in ccRCC. Stable knockdown of Tpl2 and appropriate control cells were established in Caki-1by lentiviral transduction of sh-Tpl2-1, shTpl2-2, and sh-Con. The knockdown efficiency was verified by qRT-PCR and Western blot analysis (Fig. 2B).

Because of the major function of Tpl2 is to activate downstream MAPKs in response to IL-1 stimulation (27), measuring the phosphorylation levels of MAPKs including MEK, ERK, and JNK could therefore reflect the inhibition of Tpl2. Both shRNA-mediated knockdown of Tpl2 and *in vitro* Tpl2 KI treatment suppressed of the activation of IL-1β–induced Tpl2-mediated MAPK downstream signaling pathways in caki cells (Fig. 2C and D, respectively). These MAPK downstream signaling pathways are known to be crucial at the intersection of several oncogenic pathways that regulate cell proliferation and survival (6, 7). *In vitro* proliferation of caki-1 cells was significantly reduced in Tpl2 knockdown cells (both in shTpl2-1 and shTpl2-2) compared with control cells (Fig. 2E) and the suppressed proliferation was accompanied with a significant delayed cell-cycle progression; increase in G_1 (shTpl2-1 = 54.2%, shCon = 37.8%) and decrease in S (shTpl2-1 = 35.4%, shCon = 46.4%) phase cell ratio (Supplementary Fig. S2). Dose-dependent inhibition of proliferation was also observed by the Tpl2 KI (Fig. 2E; $P = 0.021$ at 2.5 μmol/L, $P = 0.0004$ at 5 μmol/L and $P = 0.0002$ at 10 μmol/L). Moreover, the clonogenicity of caki-1 cells was significantly diminished by *Tpl2* gene silencing (all $P = 0.005$) and pharmacologic inhibition (37% reduction at 2.5 μmol/L, $P = 0.008$; 83% reduction at 5 μmo/L, $P = 0.002$; Fig. 2F).

Generally, in the absence of the anchorage, epithelial cells undergo anoikis, a form of apoptosis by disruption of the cell adhesion and cell-extracellular matrix (ECM) interactions (28). The ability of transformed cells to survive under anchorage independent (anoikis-resistance) is correlated with the metastatic potential of a tumor. To elucidate the role of Tpl2 activity and metastasis further, the cell viability after detachment was evaluated. Caki-1 Tpl2 knockdown cells showed significantly suppressed anoikis-resistance compared with control cells at 24 hours after detachment (Fig. 2G, $P = 0.02$). Taken together, these findings showed that Tpl2, presumably by its capacity to control MAPK downstream signaling activity including MER, ERK, and JNK, participates in the proliferation, cell-cycle progression, clonogenicity, and anoikis-resistance of ccRCC cells.

Tpl2 in the migration and invasion of RCC cells

Enhanced migratory and invasive capabilities independent of cell proliferation are required in the local invasion, intra-, and extravasation steps of metastasis. Recent studies reported that activation of ERK, Rac1, and FAK–mediated Ca^{2+} signaling regulated by Tpl2 induces cell migration and invasion (18) The wound healing assay showed that significant inhibition of cell motility occurred in the Tpl2 knockdown cells in comparison with control cells (data not shown). In addition, *in vitro* Transwell migration/invasion assay revealed that Tpl2 silencing significantly decreased the number of caki-1 cells migrating through the uncoated membrane or invading the Matrigel coated membrane compared with control (Fig. 3A; $P < 0.05$ for both shTpl2-1 and shTpl2-2). Similarly, the addition of the Tpl2 inhibitor to caki-1 cells dose dependently reduced their capacity to migrate and invade (Fig. 3B; all $P < 0.05$). Taken together, Tpl2 could contribute to the migration and invasion of ccRCC cells.

Effects of *Tpl2* silencing on Caki-1 cells in 3D culture

Although two-dimensional (2D) monolayer cell cultures represent epithelial cancers, the loss of physiologic ECM on artificial plastic surfaces limits several *in vivo* pathophysiologic properties of cancer cells. To obtain more reliable evidence of the role of Tpl2 in tumorigenic and metastatic potential of caki-1 cells, cell proliferation, sphere formation, and invasion abilities were evaluated in a 3D culture system. In contrast with the results from 2D cell proliferation assay, Tpl2 silencing showed no significant effect on the proliferation of caki-1 cells (data not shown). Initially, caki-1 control and Tpl2 knockdown cells formed round spheroids without branching structures in the 3D culture system. Round spheroids composed with caki-1 control cells subsequently underwent a spontaneous morphologic transformation, cell detachment from sphere and/or formation of branching structures (Fig. 3C). In contrast, caki-1 Tpl2 knockdown cells showed less and delayed cell detachment and impaired secondary sphere formation after detachment (Fig. 3C; shTpl2-1, $P = 0.022$; shTpl2-2, $P = 0.015$), which were accompanied with suppressed ERK activity (Supplementary Fig. S3). These data strongly supported the roles of Tpl2 in RCC metastasis in addition to previous results from 2D culture system.

Effects of *Tpl2* silencing on other ccRCC cells

To rule out the possibility that those *in vitro* effects of Tpl2 were specific in caki-1 ccRCC cells, *in vitro* effects of Tpl2 activity depletion via Tpl2 KI on proliferation and migration/invasion were also tested with *VHL* wild-type ACHN and *VHL*-deficient 786-O/769-P ccRCC cells. Tpl2 KI treatment induced significantly decreased proliferation (Fig. 4A) and migration/invasion (Fig. 4B and C) abilities in all three *VHL* wild-type and deficient ccRCC cells.

In vivo effects of *Tpl2* silencing in caki-1 orthotopic and lung metastasis models

In RCC orthotopic xenograft models established by subrenal injection of 1×10^6 caki-1-shCon ($n = 9$) or shTpl2-1 ($n = 9$), *Tpl2* gene silencing effectively suppressed primary tumor growth of caki-1 cells (Fig. 5A, $P = 0.004$). Moreover, no spontaneous lung metastasis was detected in Tpl2 knockdown group, while all animals harboring caki-1-shCon showed microscopic lung metastases (Fig. 5A, arrows, $P < 0.001$). When the role of Tpl2 in the RCC metastasis was separately evaluated in an experimental lung metastasis model via tail vein injection of 2×10^5 caki-1-shCon ($n = 10$) or caki-1-shTpl2-1 ($n = 10$), Tpl2 knockdown

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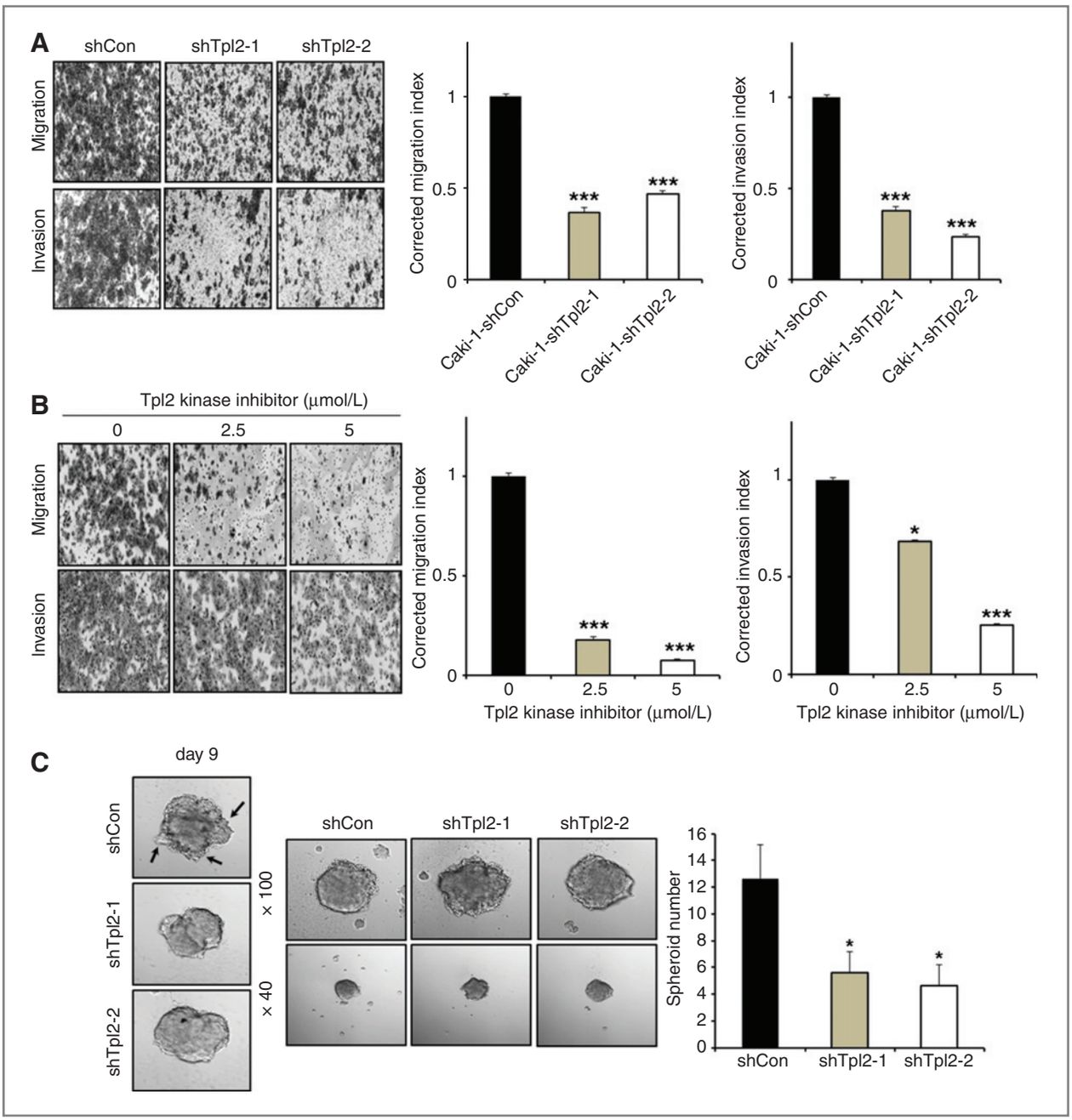

Figure 3. Suppression of Tpl2 activity–inhibited cell migration and invasion of caki-1 cells. A and B, migration and invasion was determined using Boyden Transwell chambers after silencing *Tpl2* with shRNA (A) or following treatment with 0, 2.5 or 5 μmol/L Tpl2 KI (B) in caki-1 cells. Representative pictures of the bottom surface are shown. An average of ten random microscopic fields is presented. C, inhibitory effects of Tpl2 silencing on cell detachment from sphere (extraneous growth, black arrow) and invasion abilities of caki-1 cells in 3D culture. A 3D culture experiment was triplicated showing similar reults. Day on which the cells were seeded in 3D culture plate = day 0. For 3D invasion assay, 3 days after seeding cells on the lipid-based low-cell adhesion plate, the culture media was exchanged with matrix-containing media (2% Matrigel). Photographs were taken on day 9. The number of invading secondary spheroids in the entire well was counted. *, $P < 0.05$; ***, $P < 0.001$.

showed meaningful reduction of lung metastatic foci (Fig. 5B, arrows, $P = 0.005$) and the increase of lung weight by lung metastasis (data not shown). IHC analysis of orthotopic xenografts confirmed the downregulation of Tpl2 and subsequent decreased in phosphorylation of ERK and JNK in shTpl2-1 group, compared with those of control group (Fig. 5C). Consistent with the clinical implication revealed by the analysis of TCGA database, our *in vivo* data suggested that Tpl2 is crucial in the tumorigenesis and progression of ccRCC.

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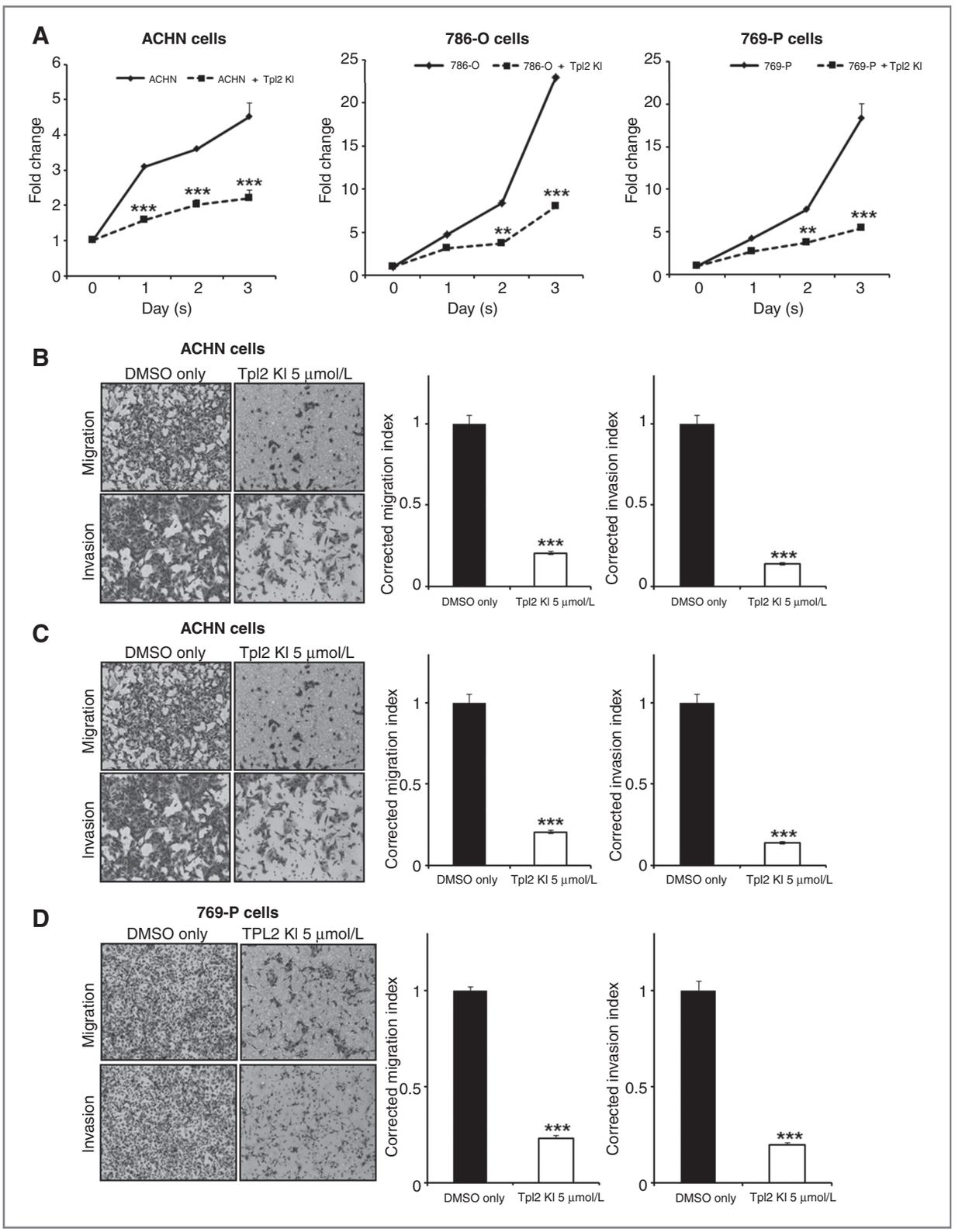

Figure 4. Inhibitory effects of Tpl2 depletion on cell proliferation, migration, and invasion in various ccRCC cell lines. A, Three different ccRCC cells with wild-type *VHL* (ACHN) and deficient *VHL* (786-O and 769-P) were treated with 10 μmol/L Tpl2 KI for 72 hours. Cell proliferation was assessed. B and D, migration and invasion was determined using Boyden Transwell chambers following treatment with 5 μmol/L Tpl2 KI in ACHN cells (B), 786-O cells (C) and 769-P cells (D). **, $P < 0.01$; ***, $P < 0.001$.

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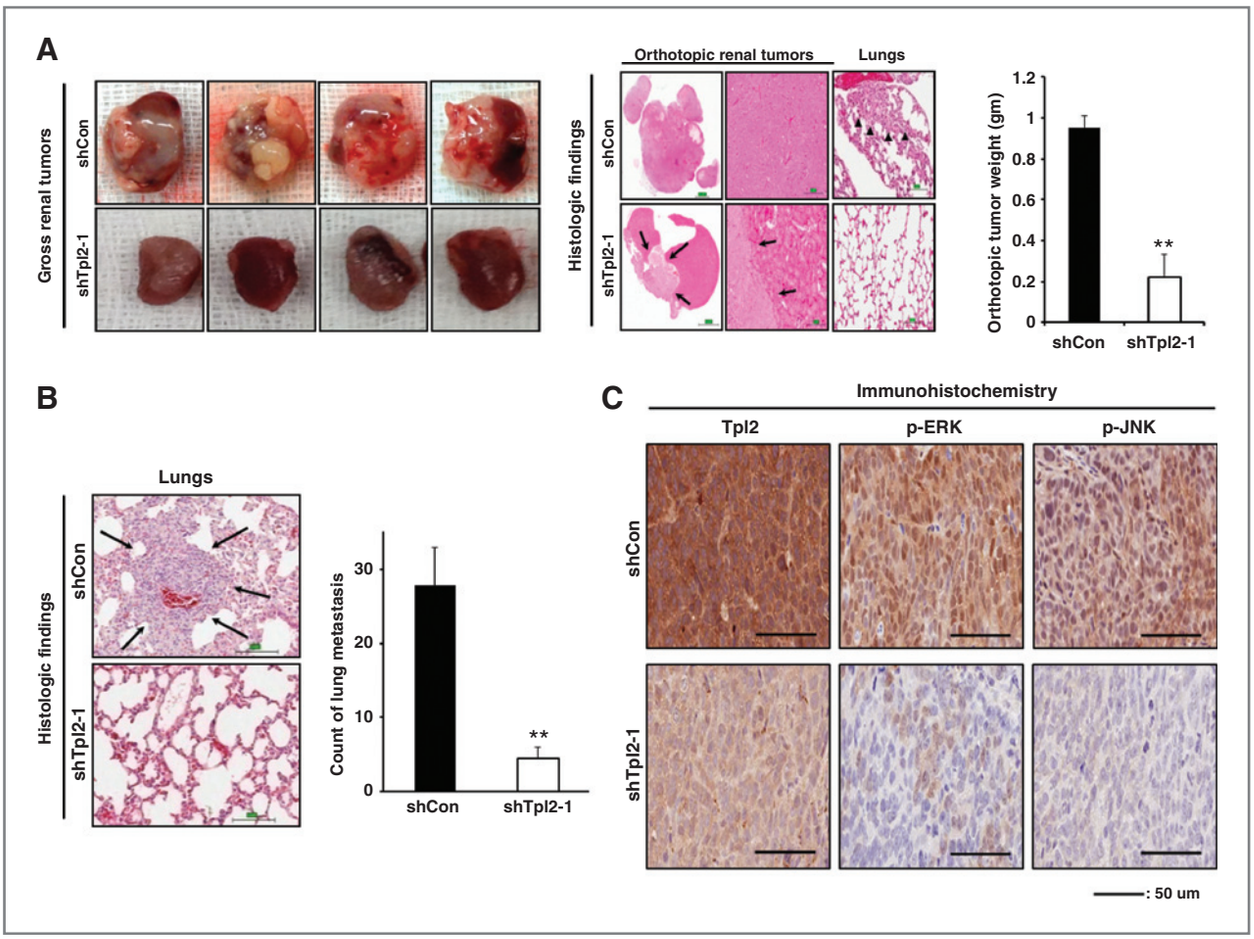

Figure 5. Targeting Tpl2-suppressed orthotopic RCC xenograft tumorigenesis and lung metastasis. A, antitumor efficacy of shRNA-mediated Tpl2 knockdown in caki-1 orthotopic xenografts model. Primary tumor growth and spontaneous lung metastasis were evaluated in an orthotopic RCC model by injecting 1×10^6 caki-1 cells stably expressing shCon ($n = 9$) or shTpl2-1 ($n = 9$) under the subrenal capsule of female BALB/c-nude mice. Representative photographs of orthotopic tumors and H&E images of renal tumors and spontaneous lung metastasis are shown. Arrows, renal tumors; arrow heads, lung micrometastasis. B, *in vivo* antimetastatic effects of Tpl2 silencing in caki-1 lung metastasis model. Experimental lung metastasis was evaluated by injecting 2×10^5 caki-1-shCon ($n = 10$) or shTpl2-1 ($n = 10$) cells into the tail vein of female BALB/c-nude mice. All mice were euthanized on week 4 after injection or earlier if the mice seemed moribund. Representative illustrations of lung micrometastasis are shown. Arrows, lung micrometastasis. **, $P < 0.01$. Column = mean, error bars = SD. C, immunohistochemical analysis of Tpl2, p-ERK, and p-JNK expression in orthotopic renal xenografts. Scale bars = 50 μm.

Decrease in CXCL12-induced downstream signaling activation and chemotaxis/chemoinvasion by Tpl2 silencing

Previously, it was reported that chemokine receptor type 4 (CXCR4)–toll-like receptor 2 (TLR2) complex mediated activation of MEK/ERK signaling pathway which is implicated in tumor progression and metastasis of pancreatic cancer (29). As CXCL12/CXCR4 signaling is closely related with the progression of ccRCC, correlation between Tpl2 and CXCR4 using dataset of TCGA (ccRCC, $n = 335$) was analyzed and, interestingly, expression of Tpl2 and CXCR4 showed significant positive correlation (Fig. 6A; Rs = 0.336, $P = 0.0095$). Therefore, the effects of the Tpl2 knockdown on the CXCR4-mediated downstream signaling pathways and *in vitro* migration/invasion potentials were examined. *In vitro* a CXCR4 ligand, CXCL12 (200 ng/mL), stimulation activated CXCR4 (Akt, MEK, and ERK) and Tpl2 (MEK and ERK) downstream signaling components of caki-1 cells (Fig. 6B). Those activations by CXCL12 (200 ng/mL) were significantly impaired by *Tpl2* gene silencing (Fig. 6B). Concomitantly, CXCR4-dependent chemotaxis and chemoinvasion were suppressed significantly in both caki-1-shTpl2-1 and caki-1-shTpl2-2 compared with caki-1-shCon (Fig. 6C, $P = 0.004$ and $P = 0.008$, respectively). These data indicate that endogenous Tpl2 could regulate CXCR4-dependent metastatic potential in ccRCC.

Discussion

In the past 10 years, progresses in the understanding of RCC biology have resulted in the successful clinical development of antiangiogenic- and mTOR-targeted drugs.

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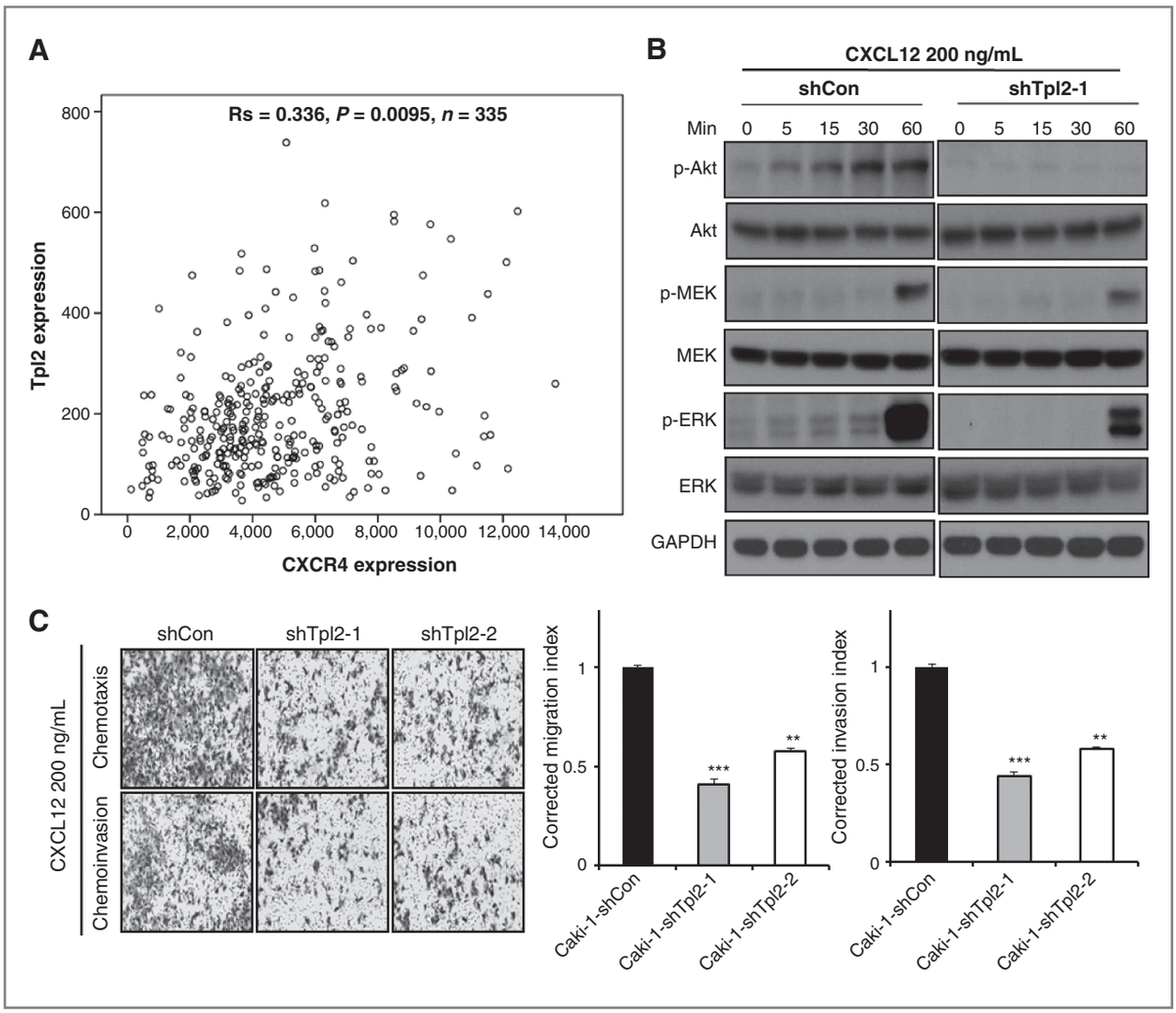

Figure 6. Depletion of Tpl2 activity–suppressed activation of CXCL12-mediated downstream signaling and chemotaxis/chemoinvasion to CXCL12 in caki-1 cells. A, correlation between Tpl2 and CXCR4 mRNA expression level in ccRCC samples (Rs = 0.336, $P = 0.0095$) was analyzed using data available in TCGA datasets. Rs = spearman rank correlation coefficient. B, suppression of CXCL12-mediated signal transduction by Tpl2 silencing in caki-1 cells. After serum starvation for 24 hours, cells were incubated with CXCL12 (200 ng/mL) for indicated time intervals. C, silencing of Tpl2 expression with shRNA-impaired CXCL12/CXCR4–directed chemotaxis and chemoinvasion of human ccRCC caki-1 cells. The *in vitro* migration and invasion activity of caki-1 cells stably expressing shCon, shTpl2-1, or shTpl2-2 was measured after 24 hours with Boyden Transwell chamber assays with CXCL12 (200 ng/mL) as a chemoattractant.

However, the majority of patients with RCC still succumb to the disease, as current targeted therapies and other adjuvant chemotherapeutic regimens showed limited the therapeutic effects. Moreover, useful biomarkers for advanced RCC need to be elucidated further (30). Progression and metastasis can be summarized in a series of steps starting with local invasion, followed by intravasation, survival in the circulation, extravasation, initiation of micrometastasis at a distant site, and vascularization of the new metastatic tumor (31). While overexpression of Tpl2 has been observed in different types of human tumors (12) and a recent article reported significant upregulation of Tpl2 in metastatic prostate cancer samples (32), there is little evidence about the role of the *Tpl2* oncogene in RCC. Herein, we showed that Tpl2 promotes ccRCC tumorigenesis and metastasis *in vitro* and *in vivo* by regulating diverse steps including cell-cycle progression that leads to increased proliferation, anoikis resistance, clonogenicity, migration, and invasion. Several antitumor and antimetastatic activities by *Tpl2* silencing in human RCC cells would be resulted from impaired TPL2–mediated MAPK signaling activation and blocking effects on CXCR4-dependent axes including AKT and ERK pathways. These results suggest that targeting of Tpl2, which has a multifaceted role in ccRCC growth and metastatic progression could be an effective therapeutic strategy for ccRCC.

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Interestingly, prognostic implication of *Tpl2* upregulation was observed only in ccRCCs but not in papRCCs. In addition, those clinical meanings were stronger in *VHL* wild-type ccRCCs than in *VHL* mutant ccRCCs. RCC is heterogeneous pathologically and molecularly. The most frequent is ccRCC, which accounts for approximately 80% of all RCCs and has been reported to have most aggressive clinical outcomes among various pathologic subtypes. Frequently, ccRCCs (>70%) are characterized by somatic mutations or hypermethylation of the *VHL* gene. Although somatic mutation of the *VHL* gene is among the most frequent genetic alterations observed in ccRCCs, recent study reported that absence of VHL alteration is associated with advanced stages and diminished survival in sporadic ccRCC (33, 34). Regarding those correlations, Tpl2 is associated with most aggressive subtypes of RCC and could contribute to the aggressiveness of RCC, indicating a value as a novel prognostic factor and therapeutic target in ccRCC.

The CXCR4 receptor is involved in the metastatic process of RCC and its overexpression predicts a worse prognosis in RCC (35–38). CXCR4 and CXCL12 have multiple roles in tumor growth and metastasis, cancer cell-tumor microenvironment interactions, and angiogenesis, activating the Akt/MAPK signaling pathway (39–41). More importantly, Tpl2 knockdown trapped the CXCR4-dependent biologic axes and downregulated the Akt and ERK signaling pathways in our study. This indicates that *Tpl2* silencing is a powerful therapeutic approach for controlling RCC growth and metastasis by interfering with the cross-talk involved in CXCR4-mediated downstream signaling. Furthermore, precise mechanistic studies elucidating the interrelationships between Tpl2 and CXCR4-mediated signaling are needed.

To the best of our knowledge, this is the first report showing that MAPK pathway activation and cross-talk with CXCR4/CXCL12 axis downstream signaling depend on Tpl2 contributes to both tumorigenesis and metastasis of ccRCC cells. Our data showed that Tpl2 is a pivotal member of several intracellular signaling pathways including MEK/ERK and MKK4/JNK. Activation of MAPK signaling pathways including ERK and JNK plays a crucial role in tumorigenesis, metastasis, and angiogenesis of RCC, and overexpression of MAP2K is observed in human RCC cases (42–44). The Raf/MEK/ERK signaling pathway, which is overactivated in RCC, regulates cell-cycle progression, apoptosis resistance, cellular motility, angiogenesis, and drug resistance (45). In addition, JNK is selectively activated in ccRCC specimens and, induces epithelial–mesenchymal transition, suggesting that JNK represents a novel molecular target that is selectively activated in and drives the growth of ccRCCs (46). In addition to previously reported anticancer effects of combined sorafenib and ERK inhibitor or simvastatin treatment suppressing Akt or ERK signaling pathway (47, 48), our findings can serve as the preclinical foundation for a combination targeting both the Tpl2/MEK/ERK and JNK pathways and the Ras/Raf/MEK/ERK pathway may lead to new therapeutic intervention strategies for ccRCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H.W. Lee, K.M. Joo, H.J. Cho, H.J. Cho, D.-H. Nam
Writing, review, and/or revision of the manuscript: H.W. Lee, K.M. Joo, S.I. Seo, J.-I. Lee, D.-H. Nam
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.J. Seol, S. Kim
Study supervision: K.M. Joo, H.J. Seol, S.I. Seo, B.C. Jeong, D.-H. Nam

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