Expression of the PTTG1 Oncogene Is Associated with Aggressive Clear Cell Renal Cell Carcinoma

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Abstract

The pituitary tumor transforming gene (PTTG1) is a recently discovered oncogene implicated in malignant progression of both endocrine and nonendocrine malignancies. Clear cell renal cell carcinoma (ccRCC) is cytogenetically characterized by chromosome 3p deletions that harbor the ccRCC-related von Hippel-Lindau, PBRM1, BAP1, and SETD2 tumor suppressor genes, along with chromosome 5q amplifications where the significance has been unclear. PTTG1 localizes to the chromosome 5q region where amplifications occur in ccRCC. In this study, we report a functional role for PTTG1 in ccRCC tumorigenesis. PTTG1 was amplified in ccRCC, overexpressed in tumor tissue, and associated with high-grade tumor cells and poor patient prognosis. In preclinical models, PTTG1 ablation reduced tumorigenesis and invasion. An analysis of gene expression affected by PTTG1 indicated an association with invasive and metastatic disease. PTTG1-dependent expression of the RhoGEF proto-oncogene ECT2 was observed in a number of ccRCC cell lines. Moreover, ECT2 expression correlated with PTTG1 expression and poor clinical features. Together, our findings reveal features of PTTG1 that are consistent with its identification of an oncogene amplified on chromosome 5q in ccRCC, where it may offer a novel therapeutic target of pathologic significance in this disease. Cancer Res; 72(17); 4361–71. ©2012 AACR.

Introduction

Renal cell carcinoma (RCC) is the most common type of kidney cancer and accounts for approximately 39,000 new cases and 13,000 deaths per year within the United States (1). Clear cell renal cell carcinoma (ccRCC) is the most common subtype of renal cell carcinoma and accounts for approximately 75–80% of cases (2). ccRCC is characterized by a recurrent set of chromosomal abnormalities that includes deletions of chromosomes 3p, 9q, and 14q and amplifications of chromosome 5q and 7 (3). Deletion of chromosome 3p removes one allele of the von Hippel-Lindau (VHL) tumor suppressor gene, whereas somatic mutation typically inactivates the remaining allele. Biallelic loss of VHL is the most frequently reported genetic abnormality in ccRCC, occurring in the majority of cases (4, 5) and leading to stabilization of the HIF proteins and subsequent transcription of HIF target genes (6–8). Amplification of chromosome 5q is the second most common cytogenetic change associated with ccRCC, yet the significance of this abnormality is not understood (3, 9–11). Multiple cytogenetic studies have concluded that genetic alterations of chromosome 5q, particularly 5q33.2–35, are associated with the progression of ccRCC (10, 12–15). Despite the frequency of this aberration, few candidate oncogenes within this region of copy number gain have been implicated in ccRCC (16).

Pituitary tumor transforming gene (PTTG1) is a recently discovered oncogene that resides on 5q33.3 and has been implicated in the development and progression of many malignancies (17). Upregulation of PTTG1 has been correlated with aggressive disease and poor prognosis in hepatocellular carcinoma, prostate cancer, esophageal cancer, and glioma, among others (18–22). PTTG1 overexpression has also been associated with the invasiveness of thyroid and colorectal cancers (23, 24). The oncogenic potential of PTTG1 was initially shown by its ability to transform NIH3T3 fibroblasts upon overexpression. This was later substantiated in HEK293 cells in which overexpression of PTTG1 resulted in increased cell proliferation, anchorage-independent growth in soft agar, and tumor formation in nude mice (25). Although its potent transforming ability is now well established both in vitro and in vivo, its precise oncogenic mechanism remains unclear as PTTG1 seems to be a multi-functional protein with several possible means of promoting tumorigenesis.
As a securin protein, PTTG1 regulates sister chromatid separation during mitosis and overexpression can interfere with this process, leading to aneuploidy (26). Other studies have suggested an important role for PTTG1 in influencing the tumor microenvironment, in part by increasing the expression and secretion of the important proangiogenic factors (25, 27–29). PTTG1 involvement in cell-cycle progression and proliferation, p53-mediated apoptosis, and DNA repair has also been reported (27, 30–32). The key to PTTG1 oncogenic effects likely lies in its role as a transcriptional activator. In 2000, Pei and colleagues showed that the activation of the MAPK pathway led to phosphorylation of the PTTG1 transcription domain, initiating PTTG1 nuclear translocation and transcriptional activity (33), and direct downstream targets identified thus far include basic fibroblast growth factor and c-MYC (27, 34). Mutation of the double PXPF motif required for transactivation has been shown to abrogate the transforming effects of PTTG1 expression (17, 35, 36).

Although overexpression of PTTG1 has now been observed in many cancer types, there is little information about the role of the PTTG1 oncogene in kidney cancer. Here, we show that PTTG1 overexpression in ccRCC is associated with patient survival and assess its putative role as a promoter of disease progression. In addition, we show that this 5q oncogene is genetically amplified in ccRCC and contributes to the oncogenic transformation of renal cancer cells, as well as, the progression of ccRCC from localized to invasive disease. We also show that PTTG1 regulates the expression of the Rho-GEF ECT2, another novel proto-oncogene that exhibits significant overexpression in aggressive ccRCC.

Materials and Methods

DNA copy number analysis

Tissue was collected from Spectrum Health Hospital of Grand Rapids, MI, and the Cooperative Human Tissue Network under the VARI Institutional Review Board. All tissue was snap-frozen in liquid nitrogen immediately after nephrectomy and stored at –80°C. Genomic DNA was extracted using a JetQuick Maxiprep Kit (Genomed) per the manufacturer’s instructions. Single nucleotide polymorphism (SNP) mapping assay was carried out according to Affymetrix’s protocol. The SNP intensity data were analyzed using Affymetrix GeneChip Genotyping analysis software (GTYPE) version 4.0 and examined largely with the exception that a divide-and-conquer algorithm was used to segment the copy number data (see Supplementary Methods; ref. 37). The SNP intensity data have been deposited at the Gene Expression Omnibus (GEO 25399).

PTTG1 and ECT2 expression analysis

Expression data were generated from the Affymetrix HG-U133 Plus 2.0 platform as previously described (15). A Cox Proportional Hazards model using cancer-specific survival and the expression level of each examined gene as a continuous predictor. The significance values (P values) from the Cox models are reported in the figures and the text. To visualize the relationship between gene expression levels and cancer-specific survival, Kaplan–Meier survival analysis was carried out. The gene expression values were turned into dichotomous variables (high and low) using an optimal cut procedure (see Supplementary Methods). To identify differentially expressed genes following PTTG1 knockdown, expression profiles from treated and control samples were generated from the Affymetrix HG-U133 Plus 2.0 using RNA analysis and data were subsequently unlogged. Genes with expression values less than 20 were considered to be not expressed in the indicated cell line. Fold-change of each gene was defined to be the ratio of expression intensity of that gene in the treated sample over that in the control sample. If the ratio was positive, otherwise, the fold change was defined to be the negative of inverse value of the ratio. The gene expression data have been deposited at the Gene Expression Omnibus (GEO 25493).

Fluorescent in situ hybridization

ISH probes were prepared from purified BAC clones CTD-2155B8 (5q33.3; Invitrogen) and RP11-254G13 (5q11.2; BACPAC Resource Center, bacpac.chori.org). The BAC clone DNA was labeled with SpectrumGreen or SpectrumOrange (Abbott Molecular Inc.), by nick translation. Tumor touch preparations were prepared by imprinting thawed tumors onto glass slides. Images were analyzed using FISHView EXPO v6.0 software (ASI) and 100 to 200 interphase nuclei were scored for each sample.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissues were micromicrotome cut into 3-micron sections, deparaffinized, and rehydrated. Samples were boiled in citrate buffer, cooled, and subsequently incubated with 3% hydrogen peroxide to quench endogenous peroxidase activity. The sections were blocked with goat serum and incubated with human anti-PTTG1 antibody (Invitrogen) or anti-ECT2 antibody (Sigma Aldrich) overnight followed by incubation with biotinylated anti-rabbit IgG for 1 hour at room temperature. Subsequently, the slides were stained with an avidin–biotin complex to visualize antigen–antibody complex, counterstained with hematoxylin, dehydrated, and mounted. Substitution of TBS for primary antibody was used as a negative control. Sections were imaged and sent to pathologist (Dr. James Resau) for semiquantitative analysis. Stain intensity, either nuclear or cytoplasmic, was scored on a scale of 0 (low) to 4 (high). Statistical significance of variable stain intensity between low-grade ccRCC (Fuhrman grades 1 and 2) and high-grade ccRCC (Fuhrman grades 3 and 4) was assessed by Wilcoxon rank-sum test.

Cell culture

Caki-1, ACHN, A-498, HK2, SW-156, and 786-O RCC cell lines were obtained from the American Type Culture Collection (ATCC). SN12C cells were kindly provided by Dr. George Vande Woude (Van Andel Research Institute). UO-31, TK-10, and RXF395 cells were obtained from the National Cancer Institute. HK2 cell lines were obtained in November 2011 and characterized by short-tandem repeat analysis by ATCC. All other cell lines were obtained before the new requirements for publica tion and therefore, have not been authenticated. Cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM;
Invitrogen) supplemented with 10% FBS (Invitrogen), in a humidified incubator containing 5% CO₂ at 37°C. Human umbilical vascular endothelial cells (HUVEC) were obtained from Clonetics and maintained in Clonetics EBM-2 medium supplemented with EGM-2 SingleQuots or EGM-MV SingleQuots (Lonza).

**Immunoblotting**

Total protein was extracted from cell lines using HNTG lysis buffer supplemented with protease-inhibitor cocktail (Roche). Briefly, cells were scraped and incubated on ice in lysis buffer for 30 minutes and subsequently centrifuged at 20,000 rpm for 20 minutes at 4°C to isolate soluble proteins. This fraction was heat denatured and used for SDS-PAGE and subsequent transfer to a polyvinylidene difluoride membrane. Target proteins were identified using rabbit polyclonal anti-PTTG1 (Invitrogen), mouse monoclonal anti-β-Actin (Abcam) or rabbit polyclonal anti-ECT2 (Santa Cruz Biotechnology) primary antibodies, and appropriate secondary reagents.

**Quantitative real-time PCR**

RNA was isolated from cells using Trizol Reagent (Invitrogen) using a standard guanidinium thiocyanate-phenol-chloroform extraction method. Taq-Man probes specific for PTTG1, ECT2, and β-actin were obtained from Applied Biosystems. RNA was reverse transcribed and amplified for quantitative real-time PCR using the qScript One-Step SYBR Green qRT-PCR Kit (Quanta Biosciences) according to protocol. Analysis was carried out using the comparative-Ct method normalizing target mRNA levels to β-actin mRNA. A control human RNA sample (Applied Biosystems) was amplified and used as a calibrator for this analysis.

**RNA interference**

PTTG1 was transiently silenced in vitro using two separate siRNAs (Invitrogen), and ECT2 was silenced using a combination of 3 siRNAs (Invitrogen). A nontargeting siRNA (Invitrogen) was used as a negative control. siRNA was transfected into ccRCC cell lines with lipofectamine-2000 (Invitrogen) using standard lipid transfection methods. Cells were allowed to recover from lipid reagent permeabilization following transfection by incubation in DMEM 10% FBS overnight before usage. Stable knockdown of PTTG1 was achieved using the pLKO.1 lentivirus-based short hairpin RNA (shRNA) delivery system (38, 39). Plasmids carrying shRNA sequence against PTTG1 (clones TRCN0000015104, TRCN0000015105, TRCN-0000015106, TRCN0000015103, TRCN0000015107) were purchased from OpenbioSystem™ (Thermo Scientific), whereas a negative control plK0.1 vector carrying a nontargeting shRNA sequence was purchased from Addgene. The generation of lentivirus particles was achieved by cotransfecting plK0.1Ic0ne with second generation packaging vectors, psPAX2 (Addgene plasmid 12250), and pMD2.G (Addgene plasmid 12259) into HEK293FT cells (Invitrogen). Virus packaging, transduction, and selection of stably transduced cells were done according to a manufacturer recommended protocol (Addgene).

**Plasmid construction**

PTTG1 from a human cDNA pool (Clontech) was amplified by PCR using forward primer (5′-4TCTCGATCCACATGCTC-3′) and reverse primer (5′-AGA ATTCCAAATATCTAGTGCACAGAACAGG-3′). The resulting PTTG1 PCR product was inserted into pcDNA6/myc-His B vector (Invitrogen) at the Bam HI and Eco RI restriction sites.

**Immunofluorescence**

Cells were fixed and permeabilized using 1:1 methanol/acetone solution at –20°C for 3 minutes and subsequently blocked with 10% donkey serum in PBS with 1% bovine serum albumin for 1 hour. Cells were then incubated with rabbit polyclonal anti-PTTG1 antibody (Invitrogen) or anti-β-actin antibody (Abcam) diluted 1:100 in PBS with 1% bovine serum albumin for 2 hours at room temperature. Cells were then incubated with FITC-conjugated and TRITC-conjugated secondary antibodies (Cell Signaling), counterstained with DAPI, and imaged.

**Colony formation assay**

Cells with or without PTTG1 were tested for their anchorage-independent growth potential. Parental lines 786-O and SN12C and cell lines transduced with shPTTG1 or control vector were counted and 1 × 10⁵ cells were plated in 2× DMEM, 20% FBS, mixed with equal volume 0.7% agarose, and plated on top of a base layer of 0.8% agar, and plated on top of a base layer of 0.8% agar and DMEM, 10% FBS in a single well of a 6-well plate. For each cell line, 6 replicates were plated. Cells were incubated at 37°C and fed DMEM with 20% FBS twice weekly for 3 weeks until colonies became visible. The plates were methanol fixed and stained in crystal violet for imaging.

**Subcutaneous xenograft**

Cells with and without PTTG1 knockdown were tested for their tumorigenic ability in nude mice. Two different parental lines, 786-O and SN12C, and their derivatives shPTTG1-786-O, con shPTTG1-SN12C, or control-vector transduced cells were cultured and 3 × 10⁵ resuspended in 0.2 mL DMEM with 10% FBS were injected subcutaneously into the right flank of each animal. Tumor volume was caliper measured every other day and animal weight was measured weekly until tumor burden necessitated sacrifice.

**Proliferation assays**

Cells were seeded into 96-well plates at an approximate concentration of 500 cells per well, 12 wells per treatment. The cells were allowed to adhere overnight before cell viability was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-(4-sulphophenyl)-2H-tetrazolium assay according to the manufacturer’s instruction (MTS; Promega). This was repeated every 24 hours for 4 days. The results are presented as fold change from initial measurement (t = 0).

**Invasion assays**

Cells transfected with anti-PTTG1 siRNA or a nontargeting control siRNA were serum starved for 24 hours in culture. Subsequently, 5 × 10⁴ (1 × 10⁵ for Caki-1 cells) were
resuspended in 200 μL serum-free-media and placed onto BioCoat Growth Factor Reduced Matrigel invasion chambers (BD Biosciences; Pharmingen), which were then placed into a 24-well plate containing 800 μL DMEM plus 10% FBS as a chemoattractant. Cells were allowed to invade through the chambers for 16 hours before the chambers were swabbed and invading cells were methanol fixed and stained with crystal violet for imaging and counting. Two replicates per treatment were used and the experiment was carried out in duplicate.

ECT2 rescue experiment

HK-2 cells were transfected with empty vector pcDNA6 or PTTG1 construct. The stable transfected cells were established and maintained in 20 μg/mL blasticidin (Sigma). Then the stable cell lines were transduced with lentivirus expressing shRNA targeting ECT2 or a control construct prior subjected to cell proliferation assay and colony formation assay.

Results

5q amplification results in frequent PTTG1 copy number gain

The SNP arrays were used to identify regions of copy number change associated with the pathogenesis of ccRCC (37). Consistent with previous reports, we observed deletion of chromosome 3p to be the most frequent cytogenetic abnormality in ccRCC. Deletion of some portion of chromosome 3p was seen in 39 of 43 (91%) cases tested by SNP 100 K microarray (data not shown), a frequency consistent with previous reports (12, 37). The second most common chromosomal aberration was amplification of the long arm of chromosome 5 (Fig. 1A). We observed copy number gain of this region in 26 of 43 (60%) cases and identified 5q33.2–35 as a refined region of copy number gain, amplified in all cases showing 5q amplification. Similar results about the minimal region of amplification have been obtained by others using similar SNP profiling studies (16, 40). Notably, we observed a high degree of variability in amplicon size. Some cases showed gain of all of chromosome 5, whereas other events were restricted to the terminal end of chromosome 5q. Polyploidy of chromosome 5 was observed in 9 of 43 tumors. Smaller amplification events, typically involving approximately half of 5q, were more common than whole chromosome gain. The smallest amplicon detected by SNP microarray showed copy number gain from 5q33.5 to 5qter, spanning just 3 cytobands. In all cases with 5q gain, PTTG1 is located within the minimal region of amplification (Fig. 1A). We noted that the oncogenes, EGR1 and CSF1R, are located in the region of PTTG1 amplification and are overexpressed in ccRCC. However, expression of these genes is not associated with overall survival (Supplementary Table S1 and data not shown).

As PTTG1 is a known oncogene that maps within the region of chromosome 5q amplification, we examined the copy number status and expression levels of this gene in ccRCC in greater detail. Examination of the copy number status of PTTG1 locus using the Broad Institute’s Genomic Identification of Significant Targets in Cancer (GISTIC; http://www.broadinstitute.org/igv/GISTIC) module shows copy number gain at the PTTG1 locus in 60% of cases tested (data not shown), the same frequency of amplification observed in our SNP array study. To further investigate copy number gain at the PTTG1 locus at 5q33.3, interphase FISH was carried out on ccRCC tumor tissue touch-prep samples (Supplementary Table S2, Fig. 1D). FISH analysis showed between 2 and 9 copies of the PTTG1 gene in ccRCC samples, confirming frequent amplification at this locus. More than 2 copies of the PTTG1 oncogene were observed in 9 of 11 tumors tested by FISH. Some tumors showed large-scale amplification, as detected by increased copy number of a control probe located at 5q11.2 immediately adjacent to the centromere. In contrast, other tumors showed increased copy number of only the PTTG1 probe at 5q33.3, indicative of smaller amplification events (Fig. 1D).

PTTG1 is overexpressed in ccRCC at both the mRNA and protein levels

Next, we examined the expression of PTTG1 in ccRCC. We observed a significant increase in PTTG1 mRNA levels in carcinoma tissue specimens relative to nondiseased kidney as measured by gene expression microarrays (Fig. 1B and C and Supplementary Fig. S1). Elevated PTTG1 mRNA expression is associated with high grade (Fuhrman grade 3 and 4) versus low grade (Fuhrman grade 1 and 2; \( P = 0.04 \)), high tumor stage \( (P = 0.002) \), and decreased patient postoperative survival \( (P = 0.008) \). Expression of PTTG1 does not follow a bi-modal expression pattern in which a subset of samples contains extremely high levels of PTTG1 and is associated with decreased survival rates. Rather, increased expression of PTTG1 is associated with a proportional decrease in survival probability (Supplementary Fig. S1), suggesting PTTG1 plays a role in the progression of this disease and the acquisition of an aggressive phenotype.

PTTG1 overexpression was confirmed by immunohistochemical staining of ccRCC tumors and adjacent normal kidney (Fig. 2). Elevated PTTG1 protein levels were seen in ccRCC sections relative to normal kidney with the strongest staining observed in high-grade tumors. There was a significant difference \( (P < 0.05) \) in staining intensity between low-grade tumors (Fuhrman grades 1 and 2) and high-grade tumors (Fuhrman grades 3 and 4). These findings are consistent with recent reports of PTTG1 gene and protein expression patterns that have identified PTTG1 in a gene signature of ccRCC pulmonary metastases and showed that PTTG1 protein expression is associated with high nuclear grade, tumor stage, and tumor size (41, 42). Furthermore, we observed predominately nuclear staining with some high-grade cases exhibiting both strong nuclear and cytoplasmic staining, consistent with a previous report of both PTTG1 nuclear positivity and cytoplasmic mislocalization in malignant esophageal squamous cell carcinoma (ESCC) tissues (22).

PTTG1 influences ccRCC tumorigenesis both in vitro and in vivo

In order to study the importance of the PTTG1 oncogene in vitro and in vivo, we examined the level of PTTG1 protein expression in several ccRCC and other kidney-derived cell lines (Fig. 3A). Compared with tumor-derived cell lines,
nontransformed HUVEC and HK2 immortalized renal proximal tubular epithelial cells showed low expression of PTTG1. In contrast, 7 of 9 (78%) ccRCC cell lines examined have easily detectable levels of PTTG1. Notably, one of the 2 cell lines that did not show high levels of PTTG1 (SW-156) is not tumorigenic in vivo (data not shown). Two PTTG1-positive cell lines, SN12C and 786-O, were selected for further study. SN12C contains a wild-type allele of VHL (VHL wild-type) whereas 786-O contains a mutated allele of VHL (VHL null).

To establish a functional role for PTTG1 in ccRCC, the endogenously expressed oncogene was targeted using RNA
interference in SN12C and 786-O cells. Stable knockdowns were established in these cell lines by lentiviral transduction of shRNA sequences targeting PTTG1. The knockdown efficiency over multiple passages was verified by immunoblotting (Fig. 3b). PTTG1 knockdown cells were then tested for their proliferative and colony-formation abilities relative to both the parental line and a vector-transduced control cell line. There was only a marginal effect on the proliferative ability of 786-O cells and no significant effect on SN12C cells before and after PTTG1 knockdown. Matrigel-coated Boyden chamber invasion assays showed that siRNA-mediated depletion of PTTG1 on both cell proliferation and colony formation (Fig. 7).

Due to the reduction of ECT2 expression following PTTG1 knockdown, we also investigated the effects of directly targeting the ECT2 proto-oncogene and MMP1 metalloproteinase, 2 genes involved in cellular motility and invasion, suggested that PTTG1 may play a role in promoting the cellular locomotion/invasion phenotype as opposed to a proliferative phenotype (Fig. 3C). To examine the role PTTG1 function has on this aspect of ccRCC cell behavior, we compared the invasive ability of several ccRCC cell lines before and after PTTG1 knockdown. Matrigel-coated Boyden chamber invasion assays showed that siRNA-mediated depletion of PTTG1 could reduce the invasive potential of all 4 cell lines tested when compared with transfection with a nontargeting siRNA (P < 0.05; Fig. 6).

Identification of PTTG1-associated genes

To identify potential proteins that are influenced by PTTG1, we carried out gene expression analysis on mock-transfected cell lines and cell lines in which PTTG1 expression was diminished by siRNA transfection. The efficiency of siRNA-mediated PTTG1 knockdown was analyzed by both immunoblotting and immunofluorescence, which revealed almost complete ablation of PTTG1 (Supplementary Fig. S2).

Subsequent gene expression microarray analysis indicated that expression of several genes was affected by PTTG1 downregulation, including the Rho GTPase-activating proto-oncogene ECT2 (Supplementary Table S3). ECT2 is a guanine exchange factor (GEF) for the Rho family of GTPase’s (Rho, Rac, CDC42), and is capable of activating these proteins. Activation of Rho and subsequent Rho-dependent actin polymerization is critical for regulating actin dynamics in both cellular locomotion and cellular division (cytokinesis). PTTG1 suppression led to an approximately 2-fold decrease in ECT2 signal as measured by gene expression microarray and we confirmed this finding at both the mRNA and protein levels by quantitative RT-PCR (qRT-PCR) and immunoblotting. siRNA inhibition of PTTG1 resulted in an approximate 4-fold decrease in ECT2 transcript in 786-O and Caki-1 ccRCC cells as measured by qRT-PCR (Fig. 5A). This PTTG1-mediated regulation of ECT2 expression was seen at the protein level in 4 cell lines tested (786-O, Caki-1, ACHN, and SN12C; Fig. 5B and C). This relationship between PTTG1 and ECT2 is also supported by expression levels in ccRCC tumors. Similar to PTTG1, expression of ECT2 is associated with high Fuhrman grade and poor survival (Fig. 5D and E). In addition, there is a strong association between PTTG1 and ECT2 expression (Fig. 5F and G). This statistically significant (P < 0.001) clinical correlation strengthens the direct relationship observed between the two genes at both transcript and protein levels in vitro.

PTTG1-mediated upregulation of the ECT2 proto-oncogene and MMP1 metalloproteinase, 2 genes involved in cellular motility and invasion, suggested that PTTG1 may play a role in promoting the cellular locomotion/invasion phenotype as opposed to a proliferative phenotype (Fig. 3C). To examine the role PTTG1 function has on this aspect of ccRCC cell behavior, we compared the invasive ability of several ccRCC cell lines before and after PTTG1 knockdown. Matrigel-coated Boyden chamber invasion assays showed that siRNA-mediated depletion of PTTG1 could reduce the invasive potential of all 4 cell lines tested when compared with transfection with a nontargeting siRNA (P < 0.05; Fig. 6).

Due to the reduction of ECT2 expression following PTTG1 knockdown, we also investigated the effects of directly targeting the ECT2 oncogene in ccRCC cells. Moreover, ECT2 knockdown in HK-2 cells rescued the effect of overexpression of PTTG1 on both cell proliferation and colony formation (Fig. 7). However, in contrast to PTTG1, siRNA inhibition of ECT2 expression resulted in a dose-dependent growth inhibition of ccRCC cells that promptly acquire a spindle-shaped morphology (Supplementary Fig. S1). Flow-cytometric analysis revealed that cells lacking ECT2 accumulate in G2/M phase, consistent with ECT2 role in cytokinesis. Subsequent immunofluorescent staining showed frequent multinucleation resulting from a failure to complete cytokinesis (Supplementary Fig. S3), as previously described in glioma cells (49).
Figure 3. Knockdown of PTTG1 prevents colony formation in soft agar. PTTG1 protein levels were determined by Western blotting in the indicated cell lines (A) and cell lines stably transduced to express control or shRNA sequences targeting PTTG1 (B). Cell number was measured using an MTS assay in 786-O cells and SN12C cells transfected with control or PTTG1-targeting siRNA. D and E, representative images (D) and quantification (E) of 786-O and SN12C cells and cells transfected with control or PTTG1-targeting shRNAs after culture in soft agar for 3 weeks (*, *P < 0.01).
Discussion

Although copy number gain of chromosome 5q constitutes the second most frequent chromosomal aberration in ccRCC, the significance of this abnormality in the development and progression of the disease has not been explained to date. We show that PTTG1, a recently described oncogene, is located in this region of amplification. PTTG1 is both overexpressed in poor prognosis renal tumors and shown to play an important functional role in vitro. Following PTTG1 knockdown, the effect on ccRCC cell proliferation is modest; however, ccRCC cells exhibit renewed anchorage dependence. As growth in soft agar is a hallmark of neoplastic transformation, it seems that efficient targeting of the endogenously overexpressed PTTG1 oncogene can cause ccRCC cells to revert to a non-transformed phenotype. When these cells are injected into mice, they show a significant reduction in tumorigenesis. The tumors that eventually develop in these mice show restored PTTG1 expression, indicative of selection for cells expressing PTTG1, underscoring the importance of this oncogene in ccRCC pathogenesis (data not shown).

PTTG1 overexpression in human tumor samples is correlated with high Fuhrman grade and high tumor stage.
indicative of more aggressive tumors and more invasive tumors, respectively. This clinical observation was corroborated by cellular studies in vitro, which showed PTTG1-dependent overexpression of 2 important activators of invasion, the MMP1 metalloproteinase and the ECT2 Rho-GTPase GEF, a proto-oncogene discovered based upon its transforming abilities. Furthermore, suppression of PTTG1 significantly reduces the invasive phenotype of these cells in vitro. Due to its overexpression in high grade, high stage, and poor prognosis tumors, as well as, clinical correlation to PTTG1 expression, we examined the relationship between PTTG1 and ECT2 expression.

At the protein level, ECT2 expression is strongly influenced by PTTG1 expression in ccRCC cells in vitro, and these genes are coordinately overexpressed in a subset of high stage, poor prognosis ccRCC tumors clinically. It is interesting to note that Ito and colleagues identified a number of other Rho family members and Rho-interacting proteins as probable PTTG1 downstream targets in ESCC (22). However, whether PTTG1 lies upstream, downstream, or part of a complex with ECT2 remains to be examined in greater detail. The association between ECT2 and PTTG1 implies that PTTG1 promotion of the invasive/anchorage independent growth contributes more to the tumorigenic phenotype than an effect on cellular proliferation. Hirata and colleagues implicated ECT2 in esophageal carcinoma invasion, suggesting that the PTTG1 and ECT2 oncogenes...
may be coordinately activated in ESCC much like we have observed in ccRCC (44).

In the ongoing search for molecular points of therapeutic intervention for various malignancies, transcriptional activators such as PTTG1 have generally not been successful as drug targets, though this has been a point of contention (45). In addition to the targeted inhibition of kinases, it has previously been shown that GEFs, such as ECT2, represent viable targets for the development of novel antivascular cancer therapeutics (46). The development of resistance to monotherapy and incomplete response rates has necessitated the identification of additional targets for anticancer drug development for use in either personalized therapeutic regimens or combination therapies. Herein, we have identified PTTG1 as a potential candidate oncogene in ccRCC, contributing to both tumorigenesis and the invasive phenotype of ccRCC cells, and furthermore, identified a downstream effector of this oncogene in the Rho-GEF ECT2.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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References

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Wondergem, Z. Zhang, J. Koeman, D. V. Hehl, K. A. Furge, B. T. Teh

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PTTG1 and Aggressive Renal Cancer

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