Posttranscriptional Inhibition of Protein Tyrosine Phosphatase Nonreceptor Type 23 by Staphylococcal Nuclease and Tudor Domain Containing 1: Implications for Hepatocellular Carcinoma

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Oncoprotein staphylococcal nuclease and tudor domain containing 1 (SND1) regulates gene expression at a posttranscriptional level in multiple cancers, including hepatocellular carcinoma (HCC). Staphylococcal nuclease (SN) domains of SND1 function as a ribonuclease (RNase), and the tudor domain facilitates protein–oligonucleotide interaction. In the present study, we aimed to identify RNA interactome of SND1 to obtain enhanced insights into gene regulation by SND1. RNA interactome was identified by immunoprecipitation (IP) of RNA using anti-SND1 antibody from human HCC cells followed by RNA immunoprecipitation sequencing (RIP-Seq). Among RNA species that showed more than 10-fold enrichment over the control, we focused on the tumor suppressor protein tyrosine phosphatase nonreceptor type 23 (PTPN23) because its regulation by SND1 and its role in HCC are not known. PTPN23 levels were downregulated in human HCC cells versus normal hepatocytes and in human HCC tissues versus normal adjacent liver, as revealed by immunohistochemistry. In human HCC cells, knocking down SND1 increased and overexpression of SND1 decreased PTPN23 protein. RNA binding and degradation assays revealed that SND1 binds to and degrades the 3′-untranslated region (UTR) of PTPN23 messenger RNA (mRNA). Tetracycline-inducible PTPN23 overexpression in human HCC cells resulted in significant inhibition in proliferation, migration, and invasion and *in vivo* tumorigenesis. PTPN23 induction caused inhibition in activation of tyrosine-protein kinase Met (c-Met), epidermal growth factor receptor (EGFR), Src, and focal adhesion kinase (FAK), suggesting that, as a putative phosphatase, PTPN23 inhibits activation of these oncogenic kinases. *Conclusion:* PTPN23 is a novel target of SND1, and our findings identify PTPN23 as a unique tumor suppressor for HCC. PTPN23 might function as a homeostatic regulator of multiple kinases, restraining their activation. (*Hepatology Communications* 2019;3:1258-1270).

Staphyloccocal nuclease and tudor domain-containing 1 (SND1) has been identified as an oncogene and potential molecular target in multiple cancers, including breast,[1] prostate,[2] colorectal,[3] glioblastoma,[4] and hepatocellular carcinoma (HCC).[5,6] In the context of HCC, we generated and

characterized a transgenic mouse with liver-specific overexpression of SND1 (Alb/SND1) that developed spontaneous HCC with variable penetrance, thereby establishing SND1 as a *bona fide* driver oncogene.[6] It was demonstrated that SND1 overexpression resulted in expansion of tumor-initiating cells and created a proinflammatory milieu by activation of nuclear factor kappa B, thereby facilitating development of HCC.[6]

SND1 protein structure comprises five independent motifs: four repeats of staphylococcal nucleases (SNs) and a fusion motif of tudor and SN domains. Tudor domains have been structurally characterized to allow protein–protein and protein–oligonucleotide interactions.[7] SNs function as ribonuclease (RNase) and can be inhibited by the specific chemical inhibitor 3′, 5′-deoxythymidine bisphosphate (pdTp).[8] SND1 regulates gene expression at transcriptional as well as posttranscriptional levels.[9] In prostate cancer, SND1 promotes alternative splicing of the oncogenic splice variant of clusters of differentiation (CD)44.[10] In HCC, SND1 is a functional RNase in the RNA-induced silencing complex where it regulates microRNA-mediated gene expression in a protumorigenic manner.[5] SND1 can also bind to messenger RNA (mRNA) transcripts to increase its stability and translation efficiency, as demonstrated by its interaction with angiotensin II type 1 receptor.[11] In HCC, this interaction promotes extracellular signal-regulated kinase (ERK) activation and transforming growth factor beta signaling, thereby significantly increasing angiogenesis and epithelial–mesenchymal transition (EMT).[12]

We demonstrated that pdTp markedly inhibits *in vitro* and *in vivo* growth of human HCC cells, indicating that enzymatic activity of SND1 plays an important role in mediating its oncogenic function.[5,6] To obtain a comprehensive insight into the RNA targets of SND1, we focused on identifying SND1 RNA interactome, which unraveled protein tyrosine phosphatase nonreceptor type 23 (PTPN23 [or HD-PTP]) to be a major target of SND1.

PTPN23 was identified as an inhibitor of Ha-Ras-mediated transformation of cardiomyocytes[13] and characterized as a tumor suppressor in testicular cancer[14] and breast cancer.[15] Genetic deletion of PTPN23 is nonviable, and loss of heterozygosity results in c-Myc-driven B-cell lymphoma and lung adenoma.[16] PTPN23 is a functional component of the endosomal sorting cargo trafficking complex (ESCRT)[17] and thus facilitates lysosomal degradation of epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR).[18,19] PTPN23 inhibits endothelial migration through inhibition of Src kinase[20] and focal adhesion kinase (FAK).[21]

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Although PTPN23 has been identified as a potential tumor suppressor, its role in HCC has not been investigated. In this study, we analyzed the mechanism of PTPN23 regulation by SND1 and unraveled molecular and phenotypic changes following PTPN23 overexpression.

Materials and Methods

RNA IMMUNOPRECIPITATION SEQUENCING

RNA immunoprecipitation (RIP) using lysates from QGY-7703 cells and control immunoglobulin G (IgG) and anti-SND1 antibody (rabbit polyclonal, HPA002632; Prestige Antibodies Powered by Atlas Antibodies from Sigma) was performed using the Magna RIP RNA Binding Protein Immunoprecipitation kit (Millipore) according to the manufacturer's protocol. RNA was extracted from the immunoprecipitates using an miReasy kit (QIAGEN). Independent RNA samples, three from control IgG and five from anti-SND1 antibody, were subjected to RNA sequencing (RNA-Seq). All samples were aligned with their reference genome (University of California Santa Cruz [UCSC]-hg19) using TopHat2, and the Bam files from alignment were processed using HTSeq-count to obtain the counts per gene. Counts were read into R software using the DESeq package, and plot distributions were analyzed using reads per kilobase million (RPKM) values. Pairwise tests were performed between control IgG and anti-SND1 antibody. Data were filtered based on low count or low RPKM value (<45 percentile).

CELL CULTURE AND CLONING

Primary human hepatocytes, Hc3716-hTERT, and human HCC cells were cultured as described.[22-24] QGY-7703 cells were obtained from Fudan University, China, where the cell line was developed. Generation and characterization of SND1-overexpressing clones of Hep3B cells and SND1 knockdown clones of QGY-7703 cells have been described.[5] Tetracycline-inducible PTPN23-overexpressing clones in QGY-7703 and HepG3 cells were created using the T-Rex system (Invitrogen). The full-length human PTPN23 open reading frame was obtained from Origene, amplified by polymerase chain reaction

(PCR) using a 5′ primer containing a HindIII site and Kozak sequence along with a 3′ primer containing an XhoI site, and cloned into the pcDNA4/TO/myc-His plasmid (K1030-01; Invitrogen). Both cell lines were transfected with pcDNA6/TR plasmid, which encodes the Tet repressor, and pcDNA4-PTPN23. QGY-7703 clones were selected using 90 μg/mL zeocin and 30 μg/mL blasticidin for 2-3 weeks. HepG3 clones were selected with 30 μg/mL zeocin and 10 μg/mL blasticidin for 2-3 weeks. QGY-7703 clones were maintained on 30 μg/mL zeocin and 10 μg/mL blasticidin, whereas HepG3 clones were maintained on 10 μg/mL zeocin and 5 μg/mL blasticidin in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. We used 4 μg/mL of tetracycline for inducing PTPN23 expression. HCC cell lines were treated with 200 μM of pdTp for 24 hours.

RNA GEL SHIFT ASSAY

PTPN23 full length and 3′-untranslated region (UTR) were cloned into pGEM-T Easy vector by 3′-T end ligation. [^{32}P]uridine-5′-triphosphate from Perkin Elmer was used in the MAXIScript kit (AM1308-AM1326; Ambion) along with these plasmids to generate in vitro transcribed RNA, as per the manufacturer's protocol. Radiolabeled transcripts were separated and purified on a 4% nondenaturing gel. SND1 protein or nonspecific control protein (matrix metalloproteinase 9) was synthesized in vitro using TNT T7 Quick Coupled Transcription/Translation Systems (L1171; Promega). In vitro translated protein was incubated with purified radiolabeled transcript at 37°C for 30 minutes. Reaction mixtures were separated on a 4% nondenaturing gel using Tris-boric acid–ethylene diamine tetraacetic acid buffer (LC-860; National Diagnostics). The gel was dried on filter paper and exposed to autoradiography.

RNA DEGRADATION ASSAY

Total RNA was purified from QGY-7703 cells using the QIAGEN miReasy kit. In vitro translated SND1 protein was incubated with 10 μg RNA at 37°C for 15 minutes, 30 minutes, 1 hour, and 2 hours. In vitro translated nonspecific protein control (monoacylglycerol lipase) was incubated with 10 μg RNA at 37°C for 2 hours. RNA was purified from each reaction mixture using the QIAGEN miReasy kit. The complementary DNA (cDNA) library was synthesized using the cDNA

synthesis kit (Life Technologies). Basal cell adhesion molecule (BCAM), laminin subunit beta 3 (LAMB3), and PTPN23 mRNA levels were measured by real-time PCR using an ABI ViiA7 fast real-time PCR system and Taqman gene expression assays according to the manufacturer's protocol (Applied Biosystems).

XENOGRAFT STUDY

We suspended 1×10^6 cells of each QGY-Rep-Pt5, QGY-Rep-Pt6, and parental QGY-7703 cell line in 50 μL of sterile phosphate-buffered saline, mixed with 50 μL of Matrigel, and injected subcutaneously in flanks of 2-month-old male NOD scid gamma (NSG) mice. Macroscopic tumors were observed after 4 days. A test group of mice was fed doxycycline-containing chow (#2919; ENVIGO), and a control group of mice was fed regular chow. Tumor dimensions were measured using a Vernier caliper twice a week for up to 4 weeks. Tumors were harvested and tumor weights were measured. Tumor volume was measured using the formula (width^2 × length)/2.

STATISTICAL ANALYSIS

Data are represented as mean ± SEM and analyzed for statistical significance using one-way analysis of variance followed by the Newman-Keuls test as a post hoc test. $P < 0.05$ was considered significant.

Results

RIP-SEQ IDENTIFIES PTPN23 AS AN SND1-INTERACTING mRNA

To identify SND1 RNA interactome, we immuno-precipitated and purified RNAs from lysates of QGY-7703 cells (that endogenously overexpress SND1), using control IgG and anti-SND1 antibody (Fig. 1A).
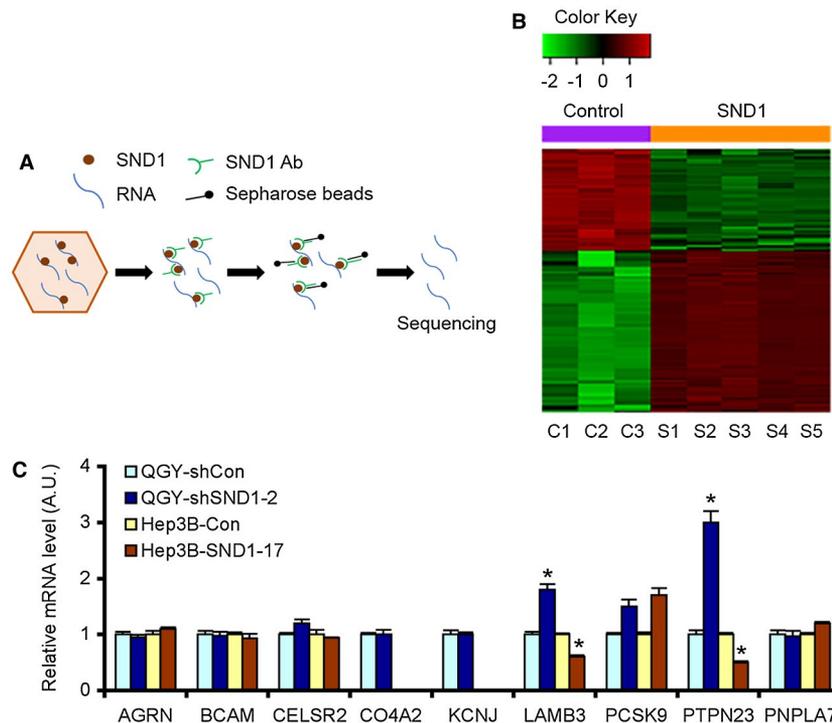
FIG. 1. RIP-Seq identifies PTPN23 as an SND1-interacting mRNA. (A) Schematic representation of the RIP-Seq protocol. (B) Heatmap shows clustering of genes identified in SND1-IP. C) Taqman qRT-PCR was performed for the indicated genes in the indicated clones. GAPDH was used as the control. Data represent mean ± SEM; *$P < 0.01$ versus control. Abbreviations: Ab, antibody; AGRN, agrin; A.U., arbitrary units; C, control IgG; CELSR2, cadherin epidermal growth factor LAG seven-pass G-type receptor 2; COL4A2, collagen type IV alpha 2 chain; KCNJ, potassium voltage-gated channel; PCSK9, proprotein convertase subtilisin/kexin type 9; PNPLA7, patatin-like phospholipase domain containing 7; S, SND1 antibody.

Independent RNA samples, three from control IgG and five from anti-SND1 antibody, were subjected to RNA-Seq. Using an adjusted P value of <0.01, average expression level >0 (log2 transformed RPKM level) and 4-fold absolute fold change, 370 transcripts were up-regulated and 232 transcripts were down-regulated in the SND1 group compared to the control (Fig. 1B). The 370 transcripts that were enriched in the SND1 group should interact with the SND1 protein. The 232 transcripts that were enriched in the control group were false positives; this was quite high, and consequently subsequent analyses were performed with stringent parameters. For further analysis, we focused on those genes that showed significant changes with >10-fold enrichment in the SND1 group over the control and have relevance to cancer pathophysiology. These highly stringent parameters identified nine genes, the levels of which were analyzed in clones of QGY-7703 cells expressing either control (Con) short hairpin (sh)RNA or SND1 shRNA (QGY-shCon and QGY-shSND1-2) and control and SND1-overexpressing clones of Hep3B cells (Hep3B-Con and Hep3B-SND1-17) by quantitative reverse-transcription PCR (qRT-PCR). These clones have been extensively characterized in our previous publications.[5,12,25] Agrin (AGRN), BCAM, cadherin epidermal growth factor LAG seven-pass G-type receptor 2 (CELSR2), and patatin-like phospholipase domain containing 7 (PNPLA7) mRNA expressions were not affected by changes in SND1 levels (Fig. 1C). Potassium voltage-gated channel (KCNJ) and collagen type IV alpha 2 chain (COL4A2) mRNAs were detected only in QGY-7703 clones without showing any difference. Proprotein convertase subtilisin/kexin type 9 (PCSK9) mRNA levels were increased both following SND1 knockdown and overexpression, making the observation difficult to interpret (Fig. 1C). LAMB3 and PTPN23 mRNAs were increased following SND1 knockdown and decreased following SND1 overexpression, indicating a potential direct regulation by SND1 (Fig. 1C).

PTPN23 has been identified as a putative tumor suppressor phosphatase inhibiting activation of oncogenic Src and FAK.[16,20,21] A role of PTPN23 in human HCC has not been studied yet. Immunohistochemical analysis in an HCC tissue microarray containing 40 primary HCC, 10 metastatic HCC, and nine normal adjacent liver samples revealed PTPN23 expression in normal adjacent liver with a gradual decrease in staining with the stages of HCC (Fig. 2A). We previously analyzed expression of SND1 by immunohistochemistry in the same transcription-mediated amplification[5] and compared PTPN23 and SND1 expression levels. A significant negative correlation was observed between SND1 and PTPN23 protein levels with the stages of HCC (Fig. 2B). PTPN23 protein levels were lower in multiple human HCC cells compared to primary human hepatocytes and telomerase reverse transcriptase immortalized human hepatocytes (Hc3716-hTERT) (Fig. 2C). Immunofluorescence analysis identified PTPN23 in the cytoplasm of primary human hepatocytes, and at the same laser exposure, PTPN23 was almost undetectable in QGY-7703 human HCC cells (Fig. 2D).

PTPN23 protein and mRNA levels were significantly higher in multiple SND1 knockdown clones of QGY-7703 cells versus control clones (Fig. 3A,B). PTPN23 protein levels were markedly lower in SND1-overexpressing clones of Hep3B cells as well as in liver of a transgenic mouse with hepatocyte-specific overexpression of SND1 (Alb/SND1)[6] compared to the respective controls (Fig. 3A). These studies suggest that SND1 might directly regulate PTPN23 levels. We treated human HCC cell lines QGY-7703, HepG3, and HuH-7 with SN inhibitor pdTp; this resulted in up-regulation of PTPN23 mRNA and protein (Fig. 3C,D). In our previous study, we established subcutaneous xenografts of QGY-7703 cells in nude mice and treated the mice with pdTp, which significantly inhibited tumor growth.[6] Analysis of these tumors showed up-regulation of the PTPN23 protein and mRNA in the pdTp-treated group compared to the vehicle-treated group (Fig. 3E,F). These findings indicate that nuclease activity of SND1 is required to modulate PTPN23 levels.

SND1 BINDS TO AND DEGRADES PTPN23 mRNA

To check direct binding and degradation of PTPN23 mRNA by SND1, we first performed an RNA gel shift assay using *in vitro* translated SND1 protein and ^{32}P-labeled *in vitro* transcribed full-length PTPN23 mRNA. SND1 protein, but not nonspecific control protein, caused a mobility shift of the PTPN23 band when the reaction was performed at 37°C (Fig. 4A). No band shift was observed when the reaction was performed at 4°C (Fig. 4B). We repeated the assay using only the 3′-UTR of PTPN23 as a probe;
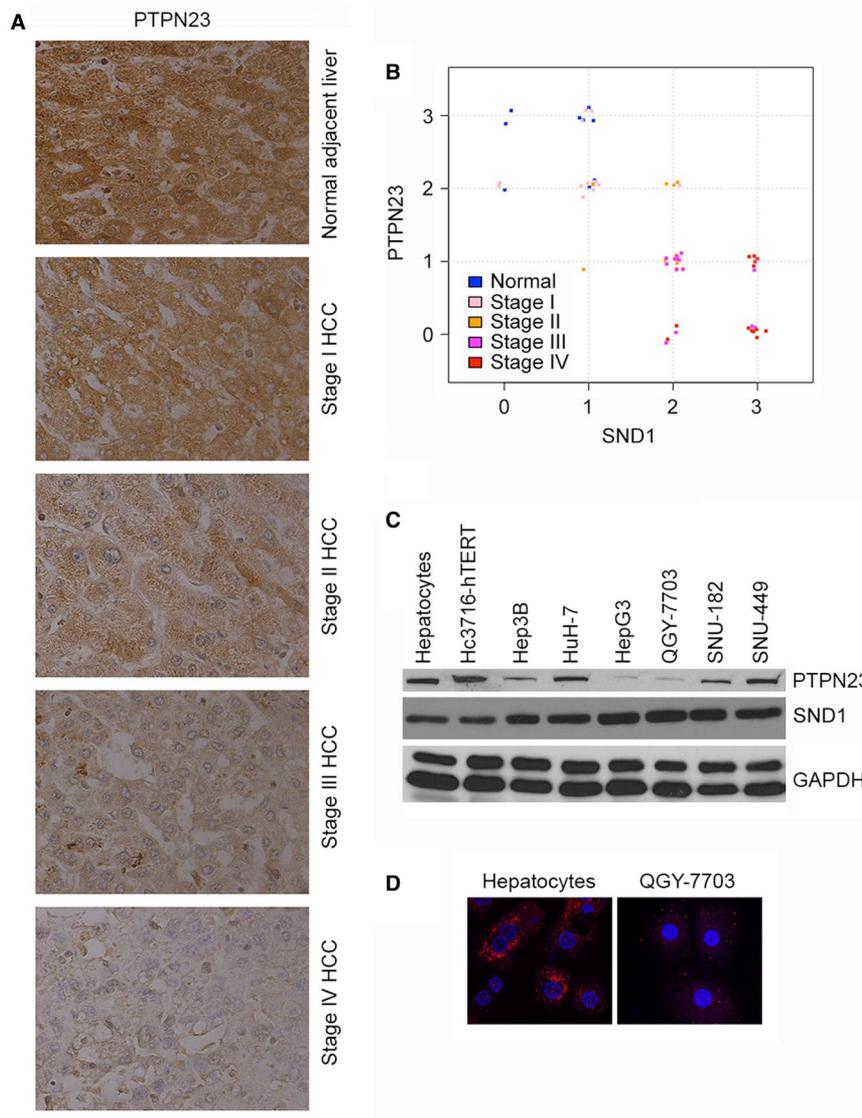
FIG. 2. PTPN23 is down-regulated in human HCC. (A) Immunohistochemistry for PTPN23 in normal adjacent liver and different stages of HCC. Magnification ×400; scale bar, 20 μm. (B) An ordinal logistic regression was conducted to assess the strength of association between SND1 and PTPN23. Pearson's χ^2 goodness of fit test with 9 degrees of freedom was performed; $P = 1.02 \times 10^{-7}$. (C) Western blot for PTPN23 in the indicated cells. GAPDH was used as the loading control. (D) Immunofluorescence for PTPN23 in the indicated cells.

this also showed a mobility shift following incubation with SND1 protein, indicating that SND1 binds to the 3′-UTR of PTPN23 mRNA (Fig. 4A). To confirm that the SND1 protein–PTPN23 mRNA interaction results in degradation of PTPN23 mRNA, we performed an *in vitro* RNA degradation assay in which *in vitro* translated SND1 protein was incubated with total RNA from QGY-7703 cells at 37°C for 15 minutes to 2 hours, followed by purification of RNA and analysis of relative abundance of specific RNA by Taqman qRT-PCR. In addition to PTPN23, we chose BCAM as a negative control and LAMB3 as a positive control. Congruent to our initial observation, incubation with SND1 protein did not alter the levels of BCAM mRNA, but there was a significant decrease in the levels of LAMB3 and PTPN23 mRNAs, the effect on PTPN23 mRNA being more pronounced than that on LAMB3 mRNA (Fig. 4C). No significant
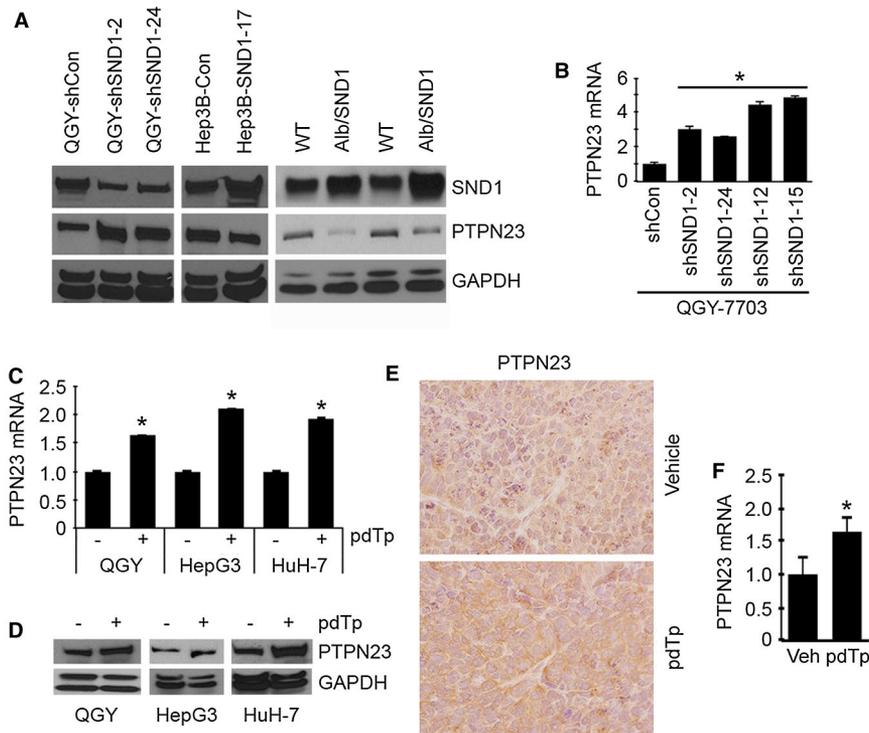
FIG. 3. SND1 inhibition increases PTPN23 levels. (A) Western blot for PTPN23 and SND1 in the indicated clones as well as in the livers of wild-type and Alb/SND1 mice. GAPDH was used as the loading control. (B) Analysis of PTPN23 mRNA levels by Taqman qRT-PCR in the indicated clones. GAPDH was used for normalization. (C,D) The indicated cells were treated with 200 µM pdTp for 24 hours. PTPN23 mRNA levels (C) and protein levels (D) were measured by Taqman qRT-PCR and western blot, respectively. GAPDH was used for normalization and as the loading control. (E) Formalin-fixed paraffin-embedded sections from QGY-7703 xenografts treated or not with 0.8 mg/kg pdTp were stained for PTPN23. Magnification ×400. (F) Analysis of PTPN23 mRNA levels by Taqman qRT-PCR in QGY-7703 xenografts treated or not with 0.8 mg/kg pdTp. GAPDH was used for normalization. For graphs, data represent mean ± SEM; $*P < 0.01$ versus control. Abbreviations: veh, vehicle; WT, wild type.

change was noted in LAMB3 or PTPN23 mRNA levels in the presence of a nonspecific protein (Fig. 4C).

PTPN23 OVEREXPRESSION REDUCES PROLIFERATION AND DAMPENS PRO-ONCOGENIC SIGNALING IN HCC CELLS

To evaluate the effect of PTPN23 overexpression on human HCC cells, we established tetracycline-inducible PTPN23-overexpressing clones in QGY-7703 and HepG3 cells. A tetracycline-dependent increase in PTPN23 protein levels was confirmed in QGY-Rep-Pt5 and QGY-Rep-Pt6 and HepG3-Rep-Pt4 and HepG3-Rep-Pt8 clones (Fig. 5A). A colony-formation assay showed significant inhibition in proliferation of these clones following tetracycline treatment (Fig. 5B). Because PTPN23 may function

as a phosphatase, we used lysates from untreated and tetracycline-treated QGY-Rep-Pt5 and QGY-Rep-Pt6 cells to probe a receptor tyrosine kinase array, which identified inhibition of activation of several key kinases (data not shown) that were validated by western blot analysis (Fig. 5C). Tetracycline treatment resulted in a decrease in phosphorylated EGFR, tyrosine-protein kinase Met (c-Met), ERK, and Src, without a change in total levels, in both QGY-7703- and HepG3-inducible PTPN23-overexpressing clones (Fig. 5C). All these oncogenic kinases are key regulators of cell proliferation, thereby indicating their involvement in mediating PTPN23-dependent inhibition of proliferation. It should be noted that tetracycline treatment did not affect proliferation, migration, and activation of the aforementioned kinases in parental HepG3 and QGY-7703 cells (Supporting Fig. S1A-E), indicating that the observations are specific for PTPN23.
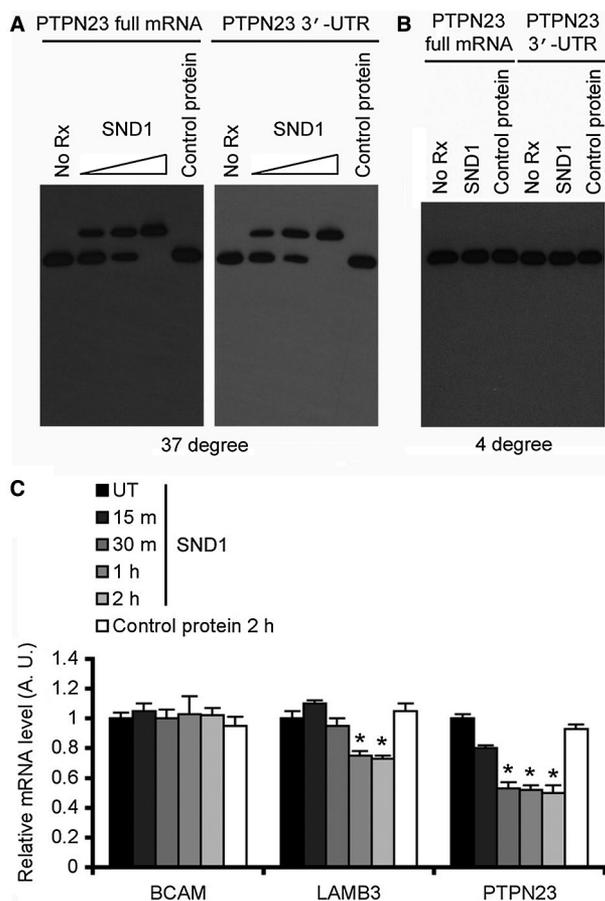
FIG. 4. SND1 degrades PTPN23 mRNA. (A) RNA gel shift assay using radioactive full-length PTPN23 mRNA and PTPN23 3′-UTR as probes and *in vitro* translated control protein (MMP9) and increasing concentration of SND1 protein at 37°C for 30 minutes. (B) RNA gel shift assay using radioactive full-length PTPN23 mRNA and PTPN23 3′-UTR as probes and *in vitro* translated control protein (MMP9) and SND1 protein at 4°C for 30 minutes. (C) *In vitro* translated SND1 or control protein (MGLL) was incubated with 10 μg total RNA from QGY-7703 cells at 37°C for the indicated times. RNA was purified and Taqman qRT-PCR was performed for the indicated genes. GAPDH was used for normalization. For graphs, data represent mean ± SEM; *P < 0.01 versus control. Abbreviations: A.U., arbitrary units; h, hour; m, minutes; MGLL, monoacylglycerol lipase; MMP9, matrix metalloproteinase 9; No Rx, No treatment, UT, untreated.

PTPN23 OVEREXPRESSION INHIBITS MIGRATION, INVASION, AND EMT IN HCC CELLS

c-Met and Src play a significant role in regulating migration and invasion of cancer cells. Additionally, in our initial kinase array, we also identified phosphorylation of FAK, a key regulator of cellular adhesion and motility, to be down-regulated following PTPN23 overexpression. Migration, determined by the wound-healing assay, and invasion, determined by the Matrigel invasion assay, showed significant inhibition following tetracycline treatment of both QGY-7703 and HepG3 clones, demonstrating a role of PTPN23 in regulating these processes (Fig. 6A,B). These changes were associated with disorganization of the actin cytoskeleton network (Fig. 6C) and inhibition in activation of FAK and β-catenin (Fig. 6D). Changes in cell motility are preceded by EMT.[26] Indeed, following tetracycline treatment, a pronounced inhibition in Snail and Slug, transcription factors regulating EMT, and N-cadherin and vimentin, markers for EMT, was observed in both QGY-7703- and HepG3 PTPN23-overexpressing clones (Fig. 6D). Collectively, these findings indicate that PTPN23 inhibits multiple kinases in human HCC cells, resulting in abrogation of major hallmarks of cancer, such as proliferation, migration, and invasion.

PTPN23 INHIBITS *IN VIVO* GROWTH OF HUMAN HCC CELLS

To confirm that *in vitro* phenotypes following PTPN23 overexpression are retained *in vivo,* we established subcutaneous xenografts of inducible PTPN23-overexpressing clones of QGY-7703 cells that were fed either regular chow or a doxycycline-containing diet. Over a period of 4 weeks, a significant inhibition in tumor growth was observed in doxycycline-fed mice compared to mice fed regular chow (Fig. 7A-D). Doxycycline treatment did not affect growth of parental QGY-7703 cells (Fig. 7C,D). Doxycycline treatment resulted in an increase in PTPN23 expression in the tumor tissue that was associated with a decrease in the proliferation marker proliferating cell nuclear antigen (PCNA) and an increase in the apoptosis marker cleaved caspase 3 (Fig. 7E). Following doxycycline treatment, a pronounced inhibition in phosphorylation of EGFR, c-Met, Src, FAK, and ERK was observed in the tumor tissue compared to regular chow (Fig. 7F). Doxycycline treatment did not affect these signaling pathways in parental QGY-7703 xenografts (Supporting Fig. S1F).

Discussion

Although SND1 has been implicated to function as a coactivator for transcription factors,[27,28] localization
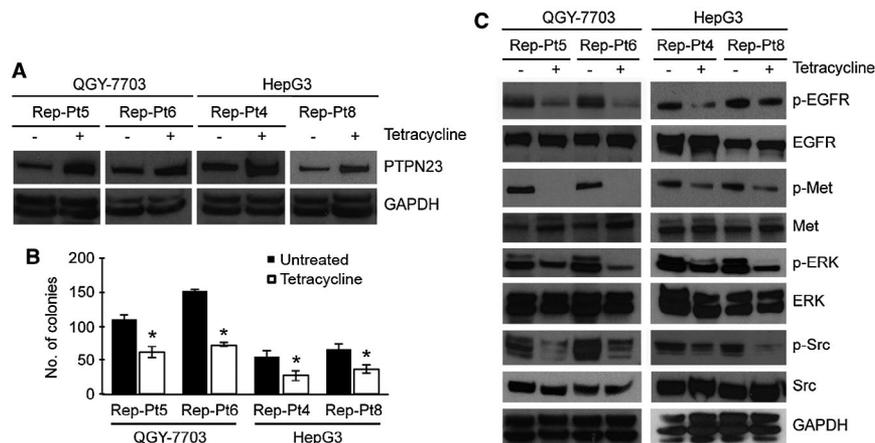
FIG. 5. PTPN23 overexpression reduces proliferation and dampens pro-oncogenic signaling in human HCC cells. (A) PTPN23-overexpressing inducible clones were treated with 4 μg/mL of tetracycline for 24 hours, and PTPN23 levels were analyzed by western blot. GAPDH was used as the loading control. (B) Colony formation assay using the indicated clones for 2 weeks. Data represent mean ± SEM; *P < 0.01 versus untreated. (C) The indicated clones were treated with 4 μg/mL of tetracycline for 48 hours, and western blot was performed for the indicated proteins. GAPDH was used as the loading control. Abbreviation: p-, phosphorylated.
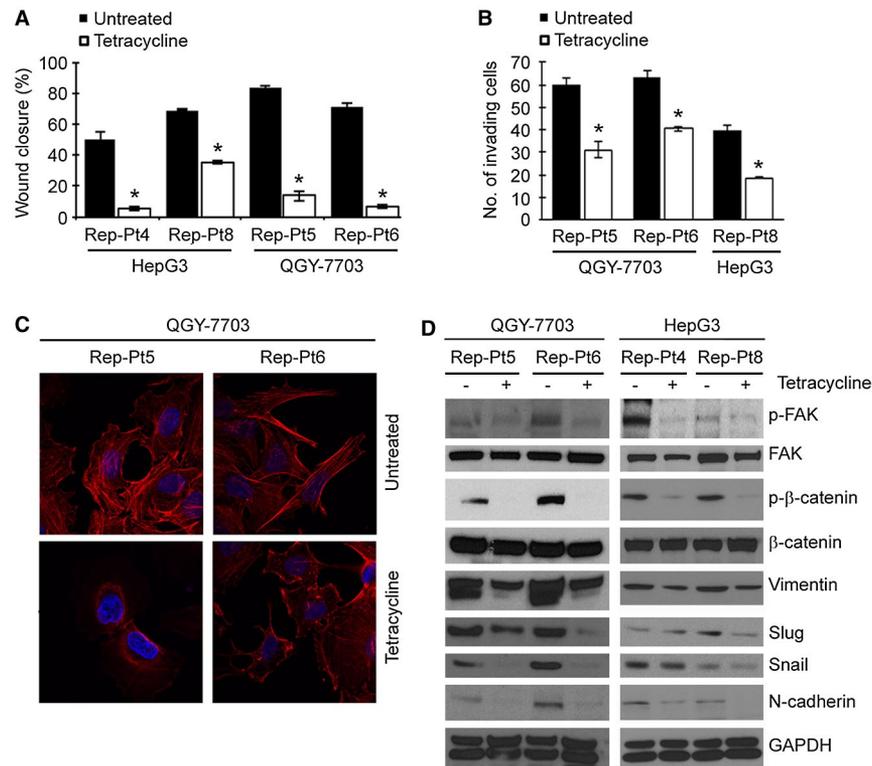
FIG. 6. PTPN23 overexpression inhibits migration, invasion, and EMT in HCC cells. PTPN23-overexpressing inducible clones were treated or not with 4 μg/mL of tetracycline, and (A) migration by wound healing assay and (B) invasion by Matrigel invasion assay were performed. Data represent mean ± SEM; *P < 0.01 versus untreated. (C). The indicated clones were treated or not with 4 μg/mL of tetracycline for 48 hours, and actin cytoskeleton was stained. Magnification ×1,000. (D) The indicated clones were treated with 4 μg/mL of tetracycline for 48 hours, and western blot was performed for the indicated proteins. GAPDH was used as the loading control.
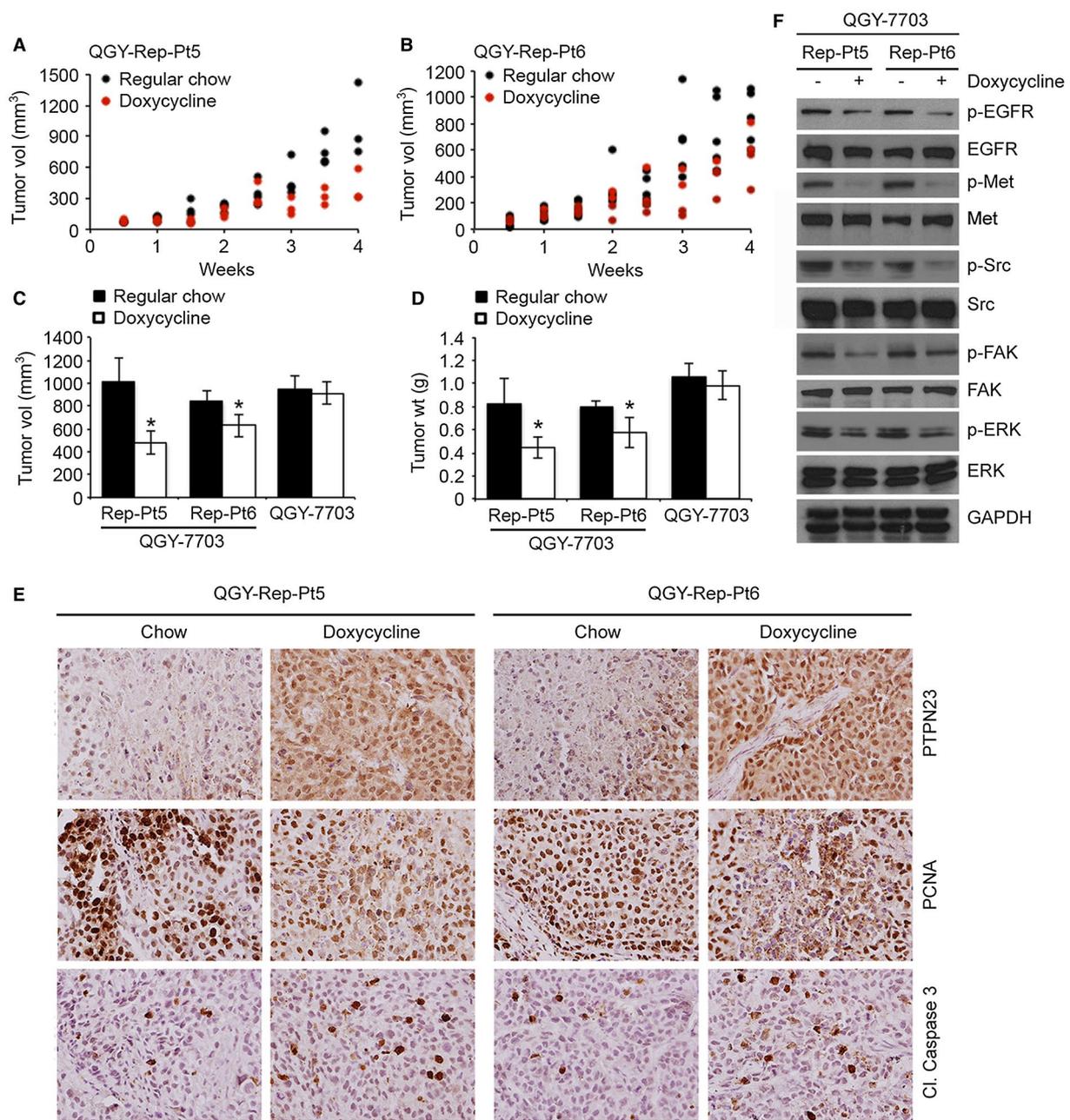
FIG. 7. PTPN23 induction inhibits *in vivo* growth of human HCC cells. Subcutaneous xenografts from parental QGY-7703 and QGY-Rep-Pt5 and QGY-Rep-Pt6 clones were established in male NSG mice, which were fed regular chow or doxycycline–containing chow for 4 weeks. (A,B) Representative twice weekly measurement of tumor volume in QGY-Rep-Pt5 (n = 3, A) and QGY-Rep-Pt6 (n = 4, B) clones. Each circle represents one mouse. (C) Tumor volume and (D) tumor weight at the end of the study. Data represent mean ± SEM; *P < 0.05 versus untreated. (E) Formalin-fixed paraffin-embedded sections from the tumors of the indicated groups were immunostained for PTPN23, PCNA, and cleaved caspase 3. Magnification ×400; scale bar, 20 μm. (F) Western blot for the indicated proteins in the tumors of the indicated groups at the end of the study. GAPDH was used as the loading control. Abbreviations: Cl, cleaved; PCNA, proliferating cell nuclear antigen; vol, volume; wt, weight.

of SND1 protein almost exclusively in the cytoplasm of HCC cells[5] indicates that posttranscriptional gene regulation is a major mechanism by which the nuclease SND1 exerts its function. Of interest along with its nuclease function, SND1 has also evolved to bind to specific mRNAs and increase mRNA

stability.[11] However, the factors that determine whether SND1 binds to specific RNAs to degrade it or stabilize it remain to be identified. Because tudor domains are known to interact with oligonucleotides, a unique identifier sequence tag in mRNAs might function as a message for SND1. Within the cytoplasm, SND1 can be part of subcellular compartments, such as endoplasmic reticulum membrane or spliceosome assembly, which are actively involved in posttranscriptional gene regulation events. The site of SND1–mRNA interaction within such compartments could also determine the effect of SND1 protein on target mRNA transcripts. We hypothesized that identification of SND1 RNA interactome coupled with expression analysis will provide comprehensive insight into posttranscriptional gene regulation by SND1. We identified 370 mRNAs that were specifically and significantly enriched in SND1 immunoprecipitation (IP) compared to control IP. Additionally, a large number of RNAs were found to be enriched in control IP, indicating that this assay tends to generate false positives and negatives. As such, in our further analysis we used extremely stringent parameters with a highly significant *P* value with at least 10-fold enrichment in SND1 IP. This approach identified nine transcripts. Further analysis of these nine transcripts showed that only two, PTPN23 and LAMB3, displayed meaningful and reciprocal changes with SND1 levels. Specificity of this interaction might be increased by ultraviolet-crosslinking of RNA and protein prior to IP, thereby reducing false positives. Among the nine genes studied, we did not identify one that showed a positive correlation with SND1, suggesting a potential regulation of mRNA stability by SND1. More in-depth analysis of the data with comparison of global expression data is required to obtain a comprehensive insight into the SND1 RNA interactome; these analyses are currently in progress.

Nevertheless, our studies have unraveled a novel oncogenic mechanism of SND1 in driving hepatocarcinogenesis. We show that SND1 specifically inhibits expression of PTPN23, a tumor suppressor gene, by degrading its mRNA transcript. SND1 binds to the 3′-UTR of PTPN23 mRNA, and SND1-mediated cleavage at this site might cause destabilization of RNA structure, resulting in degradation. Previous studies documented Src and FAK kinases as targets of the phosphatase PTPN23.[20,21] Our analysis of inducible PTPN23-overexpressing clones reveal that in addition to Src and FAK, PTPN23 overexpression results in inactivation of EGFR, c-Met, ERK, and β-catenin. Whether PTPN23 actively dephosphorylates all these kinases remains to be determined. The effect on ERK could be indirect because it is downstream of EGFR. Similarly, the effect on β-catenin could also be indirect because it is downstream of c-Met and FAK.[29-31] c-Met activation is also initiated by integrin-mediated activation of Src and FAK.[31,32] Thus, it might be possible that PTPN23 might directly dephosphorylate key upstream kinases, resulting in inactivation of the downstream kinase cascade and generating profound changes in proliferation, cell motility, and disorganization of actin cytoskeleton. The interaction between PTPN23 and SH2/SH3 domain containing proteins growth factor receptor bound protein 2 (Grb2) and GRB2-related adaptor protein 2 (GrpL) has been illustrated.[33] This interaction might facilitate interaction between PTPN23 and receptors, such as EGFR and c-Met as well as FAK.

Studies have shown that fibroblast growth factor-mediated induction in Src kinase activity phosphorylates and inhibits PTPN23 phosphatase activity.[20,34] Src is also known to be autophosphorylated when under an active protein conformation state. Thus, a decrease in Src activation when PTPN23 protein is overexpressed indicates a positive feedback loop wherein PTPN23 is capable of maintaining its activity by inhibiting an active conformation state of Src.

A role of PTPN23 in the ESCRT complex has been identified in which it regulates trafficking and lysosomal degradation of EGFR and PDGFR.[17-19] We observed pronounced inhibition in EGFR phosphorylation following induction of PTPN23 expression. However, no change was observed in the levels of total EGFR. These observations argue that PTPN23 functions predominantly as a phosphatase rather than regulating turnover of EGFR protein in HCC cells.

HCC is a highly virulent disease with no effective treatment for advanced disease.[35] The only U.S. Food and Drug Administration-approved therapeutics, sorafenib and regorafenib, are multikinase inhibitors that provide survival benefit of less than 3 months.[36,37] The observation that PTPN23 has the ability to inhibit multiple kinases that are known to play key roles in promoting HCC suggests that PTPN23 overexpression might be a potential way to counteract HCC, which is supported by

the observation that doxycycline-mediated induction of PTPN23 resulted in significant inhibition of HCC xenografts in NSG mice. HCC develops on a cirrhotic liver from viral hepatitis and alcoholic or nonalcoholic steatohepatitis.[35] Compromised liver function in patients with HCC renders small molecule inhibitors either relatively ineffective or toxic because of improper metabolism. As such, gene-based therapies might have a lasting impact on the HCC disease process. Nanoparticle or virus-based therapies are showing promise in clinical trials in patients with HCC[38,39] and might be used as a vehicle for delivering PTPN23 in these patients.

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