Genome-Wide RNAi Screen Identifies PMPCB as a Therapeutic Vulnerability in EpCAM⁺ Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is a genetically heterogeneous disease for which a dominant actionable molecular driver has not been identified. Patients with the stem cell-like EpCAM⁺AFP⁺ HCC subtype have poor prognosis. Here, we performed a genome-wide RNAi screen to identify genes with a synthetic lethal interaction with EpCAM as a potential therapeutic target for the EpCAM⁺AFP⁺ HCC subtype. We identified 26 candidate genes linked to EpCAM/Wnt/β-catenin signaling and HCC cell growth. We further characterized the top candidate PMPCB, which plays a role in mitochondrial protein processing, as a bona fide target for EpCAM⁺ HCC. PMPCB blockage suppressed EpCAM expression and Wnt/β-catenin signaling via mitochondria-related reactive oxygen species production and FOXO activities, resulting in apoptosis and tumor suppression. These results indicate that a synthetic lethality screen is a viable strategy to identify actionable drivers of HCC and identify PMPCB as a therapeutically vulnerable gene in EpCAM⁺ HCC subpopulations.

Significance: This study identifies PMPCB as critical to mitochondrial homeostasis and a synthetic lethal candidate that selectively kills highly resistant EpCAM⁺ HCC tumors by inactivating the Wnt/β-catenin signaling pathway.

Introduction

Cancer initiation and progression is a result of an accumulation of multiple somatic alterations of driver genes, which are ideal druggable targets (1). However, only few targets have proven to be beneficial to patients. This is in part due to the identification of cancer drivers that are historical events, which represent genetic alterations required during tumor initiation but are not important for tumor maintenance (2). Moreover, low frequency somatic mutations, often referred to as passenger mutations, are abundant but their role in carcinogenesis is elusive, making them unsuitable for molecular targeting. The current challenge is to effectively identify drivers that serve as cancer vulnerabilities (3). RNAi and genome-wide CRISPR/Cas9 screens have evolved as powerful tools to identify novel targets in a high-throughput manner while addressing functional phenotypes (4–6). In addition, synthetic lethality screens have been used to identify vulnerabilities or the “Achilles heel” of cancers, which have been developed into therapies that target cancers associated with TP53 loss, RAS activation, or ARID1A mutation (6–8). Large-scale RNAi screening projects such as the Project DRIVE provide vast information to aid in the development of novel therapeutics across cancer histologies, including hepatocellular carcinoma (HCC; ref. 9). Although both the RNAi and CRISPR-based methods have been successfully utilized to identify novel targets, RNAi methods are better suited for synthetic lethal screens, because the RNAi machinery is cytoplasmic, gene knockdowns are not biased by chromatin conformation, locus accessibility, or cell ploidy (10).

HCC is the most common histology of primary liver cancer, representing the fifth most prevalent and second most lethal cancer in the world (11). Similar to other solid tumors, HCC is biologically and molecularly heterogeneous and refractory to most treatment modalities, including targeted therapies (12). The major barrier to improve outcomes of HCC patients is our poor understanding of tumor heterogeneity and inability to subdivide HCC patients into biologically and genomically unique subgroups carrying specific actionable targets (12).
Transcriptome-based technologies have been effective in identifying stable HCC molecular subtypes with unique tumor biology, although defining key driver genes among different tumor subtypes has been challenging because transcriptome-based studies usually generate hundreds of candidates (13–16). Large-scale RNAi and CRISPR screens have been utilized to circumvent the shortcoming of these transcriptomic-based techniques.

EpCAM is a biomarker to define progenitor cells of the liver and other organs; it has been shown to activate Wnt signaling during hepatic development (17, 18). Pathophysiologically, EpCAM expression is associated with a stem cell–like HCC subtype with poor prognosis, and EpCAM+ HCC cells have been demonstrated as cancer stem cells (CSC; ref. 19). EpCAM+ HCCs contain an activated Wnt/β-catenin signaling whose expression is transcriptionally activated by β-catenin (CTNNB1, refs. 20, 21). Moreover, EpCAM functionally promotes cell proliferation by shuttling from the cell surface to the nucleus to regulate Wnt/β-catenin and MYC signaling pathways (22). Furthermore, EpCAM is required for the formation of EpCAM+ HCC organoids, which promotes tumorigenicity and metastasis in mice (23). To identify critical genes that interact with EpCAM in EpCAM+ CSCs, we applied System Biosciences’ (SBI) genome-wide shRNA libraries to screen genes that functionally depend on EpCAM expression, i.e., synthetic lethal interaction in the HCC cell line that has features of HCC CSCs and EpCAM-APF+ HCC subtypes (13, 24). We identified 16 candidate genes and confirmed their association with EpCAM expression and HCC prognosis. We also validated the top candidate gene, mitochondrial processing peptidase subunit beta (PMPCB), as a bona fide EpCAM-dependent gene and a vulnerable target of EpCAM+ HCC cell subpopulations.

Materials and Methods

Cell line, plasmids, and reagents

HUH7, HUH1, and MHCC97H cells were cultured in Dulbecco's modified Eagle medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, and 1-glutamine (PSG). Hep3B cells were cultured in Minimum Essential Medium (Life Technologies) supplemented with 10% FBS, PSG, nonessential amino acids, and sodium pyruvate. pLKO.1-PMPCB shRNA, pLKO.1-EpCAM shRNA, and pLKO.1-eGFP shRNA lentivector were purchased from GE Healthcare. R980-M15-663, a lentiviral vector with CAG promoter–driven firefly luciferase and eGFP expression was purchased from the Protein Expression Laboratory, Frederick National Laboratory for Cancer Research. Lentivirus particles were generated using the Translentiviral shRNA Packaging System (GE Healthcare) or the Lenti-vpak packaging kit (Origene). pCMV6-PMPCB-DDK plasmid was purchased from Origene. M50 Super 8 × TOPFlash, M51 Super 8 × FOPFlash, and pCI-neo beta catenin S33Y plasmids were purchased from Addgene. InSolution Caspase Inhibitor VI (Z-VAEDMK), GSK-3 Inhibitor IX (BIO), and GSK-3 Inhibitor IX Control (MeBIO) were purchased from Millipore. All cell lines were tested for Mycoplasma and authenticated via STR analysis (August 2015). Cells were passaged less than 15× after the first thaw from liquid nitrogen.

SBI shRNA library packaging

The GeneNet h50K siRNA library from SBI was packaged according to the manufacturer’s recommendations. Briefly, ten 15-cm plates were seeded at 7 × 10⁶ 293T cells (SBI) per plate, resulting in a confluency of 60% to 80% 24 hours later. Each plate was transfected with 5 µg of pooled library plasmids and pPACKH1 packaging plasmids (22.5 µg; SBI) using Lipofectamine (Thermo Fisher Scientific). Forty-eight hours after transfection, the supernatant containing virus was harvested, clarified, and concentrated using PEG-IT (SBI) at a ratio of one part PEG-IT to five parts supernatant overnight at 4°C. The supernatant + PEG-it solution was centrifuged for 20 minutes at 4°C and the pelletted virus + PEG-it was resuspended in 2.4 mL of PBS. Virus was stored at −80°C in 25 µL aliquots.

Viral preparation was titrated using the Global UltraRapid Lentiviral Titering Kit (SBI) according to the protocol. The optimal MOI of 0.51 to achieve a transduction efficiency of ~40% in the target cells (less than one copy per cell) was determined by flow cytometry using a GFP-expressing viral construct with the same backbone as the shRNA library.

shRNA library screen

HUH7 EpCAM+ cells were plated in six 10-cm plates (5 × 10⁵ cells per plate) and allowed to grow overnight. The GeneNet shRNA library virus particles were quickly thawed in a 37°C water bath and transferred to ice until used. Virus was transduced at an MOI of 0.5 per cell using 1 × TransDux (SBI), total volume 3 mL per plate, in 5 plates. The sixth plate was mock transduced using the cell medium and 1 × TransDux. Twenty-four hours after transduction 2 µg/mL puromycin was added to select for transduced cells. Three to 5 days after transduction, puromycin-surviving cells were magnetic activated cell sorted (MACS) for EpCAM+ cell-surface expression. RNA was purified from EpCAM-positive cells and the separate EpCAM+ population as well as nontransduced cells.

Recovering shRNA templates from selected cells

SBI provides a comprehensive protocol and specific reagents to amplify and label shRNA targets with biotin for detection when hybridized to Affymetrix Arrays. Please refer to SBI protocol "GeneNet Lentiviral shRNA libraries" version 5.062810 for specific details. In brief, RNA was reverse transcribed using a specific cDNA synthesis primer. shRNA inserts were amplified using 2 rounds of PCR. The second nested round included a biotinylated primer to allow for subsequent Affymetrix array hybridization. PCR products were purified using the QIAquick PCR Purification II protocol (Qiagen) and further treated with Lambda exonuclease to remove the sense nonbiotinylated strand. Biotinylated shRNA target (10 µg) was hybridized to the Affymetrix Human Genome U133 Plus 2.0 Array according to the manufacturer's protocol.

DNA isolation and qRT-PCR analysis

Total RNA was extracted using TRIzol (Life Technologies) according to the manufacturer's protocol. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Gene-expression level was quantified by qRT-PCR using 7900 HT Fast Real-Time PCR System (Life Technologies). TaqMan Gene-Expression Assays (Life Technologies) including primers and a probe specific for each gene used for the analyses were as follows: PMPCB, Hs00188704_m1; EpCAM, Hs00901885_m1; MYC, Hs00153408_m1; cyclin D1, Hs00765553_m1. Eukaryotic 18S rRNA Endogenous Control (Life Technologies) was used for the normalization of the endogenous mRNA level.
Mitochondrial respiration analysis

Mitochondrial respiration was measured using the XF Cell Mito Stress Test Kit (Seahorse Bioscience) according to the manufacturer’s protocol. Oxygen consumption ratio was measured using XF24 analyzer under baseline conditions with 2.5 μmol/L oligomycin or 0.5 μmol/L carbonyl cyanide-p-trifluoromethoxyphenylhydrazine (FCCP) to assess maximal oxidative capacity.

Animal study

Four-week-old athymic nude mice (Crl:NU-Foxn1nu) were purchased from Charles River Laboratories, Inc. The animal study protocol was approved by the National Cancer Institute–Bethesda Animal Care and Use Committee. Cells were stably transfected with the luciferase and GFP expression vector. Three million cells were suspended with 200 μL of DMEM and Matrigel High Concentration (Corning: 1:1) and subcutaneously injected into the flanks of mouse. Every 2 weeks, luciferase signals were measured using IVIS Lumina Series III (PerkinElmer) after intraperitoneal injection of 3 mg. D-Luciferin (PerkinElmer). Luciferase signals were quantified using Living Image Software (PerkinElmer). Mice were sacrificed 4 weeks after the injection and the size of tumors collected was recorded. Tumors were fixed with 10% formalin, followed by embedding with paraffin.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney U test (nonparametric parameters such as patient data points) or Student t test for differences between 2 groups and χ² test or Fisher’s exact test to test for enrichment. P < 0.05 was considered significant. Cox–Mantel log-rank test was performed for survival analyses. All tests were performed in GraphPad Prism (v7.0).

Results

A genome-wide RNAi screen to determine EpCAM-dependent genes

To identify EpCAM-dependent genes, we utilized an RNAi screen using the GeneNet h50K shRNA library, which is composed of ~200,000 lentiviral shRNAs and more than 47,000 transcript targets (Fig. 1A; Supplementary Fig. S1). After days 3 and 5, we enriched EpCAM<sup>high</sup> or EpCAM<sup>low</sup> cells from the HUH7 cells, which were previously transduced, using MACS. We chose HUH7 cells for the screening because it is a heterogeneous stem-like HCC patient (24). Following enrichment, EpCAM<sup>high</sup> or EpCAM<sup>low</sup> cell populations were then analyzed via microarray analyses using Affymetrix Chip. After data quality control and data normalization, differential gene expression analyses between EpCAM<sup>high</sup> and EpCAM<sup>low</sup> cells were compared using multiple t tests with Bonferroni correction. Only genes with P values < 0.05, differential expression >1.5-fold change, independently targeted by at least 2 separate shRNAs, and occurred in both time points (days 3 and 5) were considered potential candidates. These analyses resulted in 76 candidate genes, including 26 genes that were depleted (significantly lost in EpCAM<sup>+</sup>) and 50 genes that were enriched (significantly enriched in EpCAM<sup>−</sup>) in the screen (Supplementary Tables S1 and S2). Because we are interested in synthetic lethal targets against EpCAM, only depleted targets (26 candidate genes with decreased in expression between EpCAM<sup>high</sup> vs. EpCAM<sup>low</sup>) were considered for downstream studies.

Validation of the RNAi screen strategy

By selecting candidate targets that are differentially expressed between EpCAM<sup>+</sup> and EpCAM<sup>−</sup> cells with at least 1.5-fold change, essential genes that are important for cell survival for either cell population were not considered. In addition, we also determined the effect of the screen on EpCAM expression and found that EpCAM, which ranked number 3 on our top 26 depleted targets, was significantly reduced (Supplementary Table S1). Furthermore, we also investigated the signaling pathways associated with either lists via Ingenuity Pathway Analysis (IPA, v.9.0). We found that the 26 depleted candidates were a part of the Wnt/β-catenin signaling pathways (Fig. 1B). Accordingly, WNT16 (Wnt ligand) and CSNK1A1 (casein kinase 1α) are known to be functionally linked to CTNNB1 signaling and stem cell maintenance, consistent with our previous findings that Wnt/β-catenin signaling regulates EpCAM (24–26). In contrast, the 50 candidate genes that may antagonize EpCAM expression were associated with various signaling pathways; all of which have no known direct functional connection with EpCAM (Fig. 1C). The top gene network associated with EpCAM revealed several key signaling pathways, including CTNNB1, nuclear factor kappa B (NF-kB), and transforming growth factor β-1 (TGFB1; Fig. 1D). Together, these data indicate that the screen worked as expected.

To determine the relationship between the 76 candidates and EpCAM expression, we first performed unsupervised clustering analysis of these genes in 247 HCC samples (27). These genes separated HCC patients into 2 clusters, where C1 was enriched with EpCAM<sup>high</sup> HCC cases and C2 was enriched with EpCAM<sup>low</sup> HCC cases (Fig. 2A), indicating that the 76 candidates are associated with EpCAM expression. To further test whether the 76 candidates are clinically relevant, we performed Kaplan–Meier survival analysis between the 2 clusters. We found no significant prognostic difference between the C1 and C2 clusters (Supplementary Fig. S2A–S2B, left), consistent with our observation that EpCAM status alone is not a good prognostic marker (13).

EpCAM expression is associated mainly with cells with an epithelial-like HCC origin (13, 20, 24). Thus, we sought to determine whether the 76 candidate genes associated with EpCAM<sup>−</sup> HCC patients perform better in only epithelial-like HCC. Because elevated CDH1 expression and low VIM expression is associated with epithelial tumors, we first calculated the E-cadherin/vimentin (CDH1/VIM) expression ratio (log(OD<sub>CDH1</sub>)−log(OD<sub>VIM</sub>)). Using the median ratio as the cutoff, we classified all HCC cases with a CDH1/VIM ratio above the median as epithelial-like tumors. Accordingly, the 76 candidate genes separated epithelial-like HCC samples into 2 subgroups C1 and C2 with statistical significance in overall survival in 2 independent cohorts (Fig. 2B). Moreover, when we used the 76-candidate gene list to separate mesenchymal HCC (low CDH1, high VIM expression), we observed no significant difference in overall survival (Supplementary Fig. S1A–S1B, right). To further confirm the 76 candidate genes are prognostic in epithelial-like HCC, we performed IHC analyses of EpCAM, VIM, CDH1, and neural cell adhesion 1 (NCAM1) in a cohort of 145 HCC patients. Due to the presence of VIM in normal hepatic stellate cells (Fig. 2C), we used another mesenchymal marker, NCAM1, which is not normally expressed in liver cells to avoid possible misclassification. First, we identified epithelial-like HCC by considering all cases with < 25% NCAM1 and >50% CDH1 as epithelial-like HCC, which resulted in 82 HCC cases (Fig. 2C). From the 82 epithelial-like HCC cases, we stratified them into 2 groups,
EpCAM$^{+}$ (C1) versus EpCAM$^{-}$ (C2). We considered HCC cases as EpCAM$^{+}$ if at least 50% of the given area is positively stained for EpCAM$^{+}$ HCC (all others were considered EpCAM$^{-}$). We then preformed Kaplan–Meier curve analyses between C1 (EpCAM$^{+}$) and C2 (EpCAM$^{-}$) and found a significant difference in overall survival and disease-free progression (Fig. 2D). Taken together, the above results reaffirmed our confidence about the effectiveness of our strategy to positively identify genes that are clinically linked to EpCAM expression, which may serve as potential therapeutic vulnerabilities in EpCAM$^{+}$ HCC.

PMPCB is dependent on EpCAM

Among the 26 depleted candidates, peptidase, PMPCB ranks as the top (based on fold change) differentially altered shRNA-targeted gene between EpCAM$^{+}$ high cells and EpCAM$^{-}$ low cells during the screen (Supplementary Table S1). Because PMPCB is a synthetic lethal marker in EpCAM$^{+}$ HCC, we investigated whether increased PMPCB expression is associated with poor OS in EpCAM$^{+}$ high compared with EpCAM$^{+}$ low HCC in the Liver Carcinogenesis Section (LCS) and Laboratory of Experimental Carcinogenesis (LEC) cohorts. First, we used the median gene expression of PMPCB as a cutoff, considering PMPCB$^{+}$ high HCC cases as any HCC case with a PMPCB gene expression greater than the median. Independently, we considered EpCAM$^{+}$ high HCC cases as any HCC cases with an EpCAM gene-expression value greater than the median. We next identified only PMPCB$^{+}$ high HCCs and separated HCC cases into PMPCB$^{+}$EpCAM$^{+}$ high or PMPCB$^{+}$EpCAM$^{-}$ low subgroups. Finally, we performed Kaplan–Meier curve analyses in the LCS and LEC cohorts and found that PMPCB$^{+}$ high EpCAM$^{+}$ high HCC had a poor overall survival compared with PMPCB$^{+}$ high EpCAM$^{-}$ low HCC in the LCS ($P = 0.003$) and the LEC ($P = 0.045$) cohorts (Supplementary Fig. S2C).

To functionally validate PMPCB as an EpCAM-dependent gene, we designed 2 independent lentiviral shRNAs that...
differ from those in the SBI library to increase the on-target effects of RNAi-mediated PMPCB silencing. We found both shPMPCB-1 and shPMPCB-2 effectively reduced PMPCB mRNA levels as well as EpCAM mRNA levels in HUH7 cells (Fig. 3A). Similar results were found in HUH1 cells (Supplementary Fig. S3A). Moreover, EpCAM⁺ cell populations upon PMPCB knockdown were reduced compared with control eGFP shRNA in both HUH7 and HUH1 cells, similar to the degree of shEpCAM-induced activity determined by flow cytometry (Fig. 3B; Supplementary Fig. S3B). These results indicate that EpCAM expression depends on PMPCB.

Figure 2.
Validation of 76 candidate gene signature in the HCC cohort. A, Hierarchical clustering analysis of 247 HCC cases (LCS cohort) based on 76 candidate genes. EpCAM row, black (EpCAM⁺); gray bars, EpCAM⁻; CDH1/VIM row, the black and white bars indicate the cases with >1 and <1 of CDH1/VIM expression ratio, respectively. B, Kaplan–Meier plot of epithelial-like cases from the LCS (left) or LEC (right) cohort based on the dendrogram classification in the clustering analysis. P values are calculated from Cox log-rank analysis. Only samples with clinical data are shown. C, IHC of CDH1, VIM, EpCAM, and NCAM1 protein expression in the validation cohort. a–d, ×100; scale bars, 50 μm; b and d, ×200; scale bars, 20 μm. D, Kaplan–Meier curve analyses of epithelial-like HCC cases. EpCAM⁺ cases are C1, and EpCAM⁻ cases are C2. P values are calculated from Cox log-rank analysis.
To determine whether the tumorigenic features of EpCAM^+^ stem-like HCC cells depend on PMPCB, we first determined their abilities to self-renew using 3D HCC organoid assays upon PMPCB silencing (23). We found that shPMPCB can effectively inhibit colony formation and 3D spheroid formation (Fig. 3C and D; Supplementary Fig. S3C-S3D). Furthermore, cell proliferation in both HUH7 and HUH1 cells was significantly reduced upon PMPCB inhibition (Fig. 3E; Supplementary Fig. S3E) and the overexpression of PMPCB cDNA can partially rescue HUH7 cells.
after shPMPCB-induced growth inhibition (Supplementary Fig. S3F). We next determined whether the knockdown of PMPCB in HCC cells had an effect on CD133+ or CD90+ CSC populations. Accordingly, we found no significant effect on CD133 and CD90 subpopulations, indicating that PMPCB effects are specific to EpCAM (Fig. 3F; Supplementary Fig. S3G). Together, these results demonstrate PMPCB contributes to EpCAM+ HCC cell survival.

Because the PMPCB gene encodes the β subunit of mitochondrial processing peptidase (MPP), which consists of MPP-α and MPP-β, and plays a critical role in the maintenance of mitochondrial functions, we hypothesized that the loss of PMPCB has an impact on mitochondrial function (28). To test this hypothesis, we measured oxygen consuming reaction (OCR), ATP levels, and ROS in HUH7 and HUH1 cells after shPMPCB treatment. We found a significant reduction of OCR and ATP levels and an induction of ROS levels following shPMPCB knockdown in both HCC cell lines (Fig. 3G–I; Supplementary Fig. S3H–S3J). We next determined the effect of shPMPCB on the MHCC97H EpCAM− HCC cell line. We found the knockdown of PMPCB effectively inhibited cell proliferation in the MHCC97H cells (Supplementary Fig. S3K). Interestingly, we also observed an increase in ROS levels in EpCAM− HCC cells following shPMPCB knockdown (Supplementary Fig. S3L). These results suggest PMPCB plays a key role in the maintenance of mitochondrial function in HCC cells regardless of EpCAM status.

To further explore EpCAM+ HCC cells’ dependence on PMPCB, we measured apoptosis following PMPCB knockdown using flow cytometry. We found a significant increase in 7-AAD− positive cells in both EpCAM+ HUH7 and HUH1 but not in MHCC97H cells after PMPCB knockdown (Fig. 4A and B), suggesting that EpCAM+ HCC but not EpCAM− HCC cells are dependent on PMPCB for cell survival. Moreover, the number of 7-AAD− positive cells in EpCAM+ HCC following PMPCB knockdown was significantly reduced after the treatment of caspase inhibitor Z-VAD (Fig. 4C). Taken together, our results suggest that EpCAM+ HCC cells depend on PMPCB to maintain stemness.

Impact of PMPCB in tumorigenicity
To evaluate the effect of shPMPCB on tumorigenicity in vivo, we introduced a luciferase reporter stably expressed in HUH7 cells, which we named HUH7-Luc. We then treated HUH7-Luc cells with PMPCB or eGFP shRNA and inoculated these cells into nude mice subcutaneously. Compared with control mice, in vivo imaging analysis revealed no detectable luciferase signal in shPMPCB 4 weeks after inoculation (Fig. 5A). Upon tumor dissection, we found that tumors in the control group were significantly larger than those in the shPMPCB group (Fig. 5A, bottom, and Fig. 5B). Histologically, tumor cells in the shPMPCB were less dense compared with those in the control group (Fig. 5C). We also found tumors in the PMPCB knockdown group had an increase in TUNEL-positive cells compared with those in the control group (Fig. 5D and E). These data indicate that shPMPCB suppressed tumor development by inducing cellular apoptosis in vivo.

Role of PMPCB in regulating Wnt/β-catenin signaling
Because EpCAM is a transcriptional target of Wnt/β-catenin signaling, we determined the effect of shPMPCB on the Wnt/β-catenin signaling pathway as a potential mechanism of PMPCB dependence in EpCAM+ HCC cells (20). We also tested whether EpCAM+ HCC cells with an active Wnt/β-catenin signaling are affected by PMPCB in Hep3B cells, which contains...
Figure 5. 
In vivo tumor-suppressive effect of PMPCB shRNA. A, Live luminescence imaging of nude mice 4 weeks after the subcutaneous inoculation of HUH7-Luc cells with eGFP shRNA or shPMPCB (top; n = 5, cells were injected at both sides of each mouse). Macroscopic images of tumors resected immediately after live imaging (bottom). B, Average volume of tumors resected. C, Representative microscopic (hematoxylin and eosin stain) images of tumors with eGFP shRNA and shPMPCB (a and c, ×40 (scale bars, 200 μm); b and d, ×200 (scale bars, 20 μm)). D, TUNEL staining of tumors with eGFP shRNA (a and c) and shPMPCB (b and d). [a and b, ×100 (scale bars, 50 μm)/L; c and d, ×400 (scale bars, 20 μm)]. E, Apoptotic index of tumors with eGFP shRNA or PMPCB shRNA. All graphs are represented as mean ± SD (triplicates). * P < 0.05, Student t test.
nonmutated active Wnt/β-catenin signaling (29). We found that PMPCB knockdown resulted in a significant reduction of TCF4 activities as determined by the TOP/FOP TCF4 reporter assay in both wild-type and active Wnt/β-catenin signaling (Fig. 6A, top left; Supplementary Fig. S4A). Although the knockdown of PMPCB reduced the expression of downstream Wnt/β-catenin signaling targets, such as EpCAM, MYC, and CCND1 in HUH7 and HUH1 cells, PMPCB knockdown had no effect on the same targets in Hep3B cells (Fig. 6A; Supplementary Fig. S4B–S4E). Interestingly, the knockdown of PMPCB decreased colony formation, spheroid formation, and cell proliferation in Hep3B cells (Supplementary Fig. S4F–S4H). We next tested PMPCBs’

Figure 6.
Role of PMPCB in the Wnt/β-catenin pathway. A, Relative TCF4 reporter activity on HUH7 cells (top left). Relative mRNA expression of EpCAM (top right), MYC (bottom left), and CCND1 (bottom right) in HUH7 cells. All graphs are represented as mean ± SD (triplicates). *P < 0.05, Student t test. B, Representative Western blot analysis for whole lysates of HUH7 and HUH1 cells. CTR, cells with eGFP shRNA; KD, cells with PMPCB shRNA. C, Flow-cytometric analysis of HUH7 cells with eGFP shRNA (top left), PMPCB shRNA (bottom left), or PMPCB shRNA with mutant CTNNB1 expression (top right) stained with anti-EpCAM antibody and 7-AAD. The proportion of 7-AAD-positive cells is shown as a bar graph (bottom right). D, Representative flow-cytometric analysis of HUH7 cells with eGFP shRNA and MeBIO (top left), eGFP shRNA and BIO (top right), PMPCB shRNA and MeBIO (bottom left), or PMPCB shRNA and BIO (bottom right) stained with anti-EpCAM antibody and eFlour 520.
important role in mitochondria function in Hep3B cells and found the knockdown of PMPCB decreased ATP levels and increased ROS production and observed no changes in EpCAM or CD133+ CSC populations (Supplementary Fig. S4-I). Furthermore, PMPCB knockdown in Hep3B cells had no effect on apoptosis (Supplementary Fig. S4L). Together, these data indicate that PMPCB affects mitochondria function but not EpCAM+ cells with an active β-catenin signaling.

We next examined the levels of key proteins of canonical Wnt/β-catenin signaling, including LRPs6, EZD5 (Frizzled5), DVL2, and CTNNB1 after PMPCB shRNA. We also determined CCND1 and MYC, downstream targets of Wnt/β-catenin, by Western blotting analysis (25). Consistent with the RT-PCR data, HUH7 and HUH1 cells with shPMPCB expressed significantly less CCND1 and MYC protein expression compared with control, whereas Hep3B cells showed no significant change in CCND1 or MYC protein expression compared with control (Fig. 6B, Supplementary Fig. S5A). We observed no change in the phosphorylation of CTNNB1 at threonine 41 and serine 45 (pCTNNB1Thr41/Ser45), EZD5, or DVL2 following PMPCB knockdown across all 3 cell lines, suggesting that PMPCB does not affect nuclear localization of CTNNB1 (Fig. 6B). Moreover, total CTNNB1 levels in the cytosol and nuclei fractions were not affected by PMPCB knockdown in all 3 HCC cell lines (Supplementary Fig. S5B). However, the knockdown of PMPCB decreased the phosphorylation of LRPs6 and CTNNB1 (pCTNNB1-S675) in all 3 HCC cell lines (Fig. 6B, Supplementary Fig. S5A). To investigate PMPCB-mediated induction of apoptosis is through Wnt/β-catenin signaling, we constitutively activated mutant CTNNB1 (S33Y) in HUH7 shPMPCB knockdown cells (Supplementary Fig. S5C). We found that CTNNB1 (S33Y) could partially rescue PMPCB knockdown-induced apoptosis (Fig. 6C). GSK-3β, an important member of the CTNNB1 destruction complex, plays an important role in degrading CTNNB1 and subsequent Wnt signaling. We thus determined the effects of BIO, a GSK-3β inhibitor, on apoptosis induced by PMPCB knockdown in HUH7 cells. Indeed, the inhibition of GSK-3β and subsequent stability of CTNNB1 decreased shPMPCB-induced apoptosis (Fig. 6D). Because shPMPCB had no significant effect on apoptosis or Wnt/β-catenin downstream targets such as EpCAM, MYC, and CCND1 in Hep3B cells, we hypothesize that the overactive Wnt/β-catenin signaling can overcome the knockdown effect of shPMPCB. To test our hypothesis, we inhibited CTNNB1 using pimozide, a potent Wnt/β-catenin inhibitor following PMPCB overexpression (29). We found that PMPCB overexpression partially rescued pimozide-induced apoptosis and increased EpCAM+ cells (Supplementary Fig. SSD–SSE). Furthermore, PMPCB overexpression partially rescued EpCAM gene expression (Supplementary Fig. S5E). Together, these data indicate that PMPCB helps maintain CTNNB1 stability through LRPs6 and CTNNB1 phosphorylation in HCC cells (Fig. 6D; refs. 30–32). In HUH1 and HUH7, PMPCB is synthetic lethal to EpCAM+ HCC cells but not in Hep3B cells, which has an overactive Wnt/β-catenin signaling that can overcome PMPCB knockdown.

PMPCB regulates Wnt/β-catenin signaling through FOXO

It is known that the interaction between Forkhead box O (FOXO) transcription factors and CTNNB1 is enhanced upon ROS production, decreasing TCF activity and downregulation of the Wnt/β-catenin signaling (33–35). Moreover, FOXO activity is closely related to the induction of apoptosis (36, 37). Because PMPCB knockdown resulted in an increased ROS production, we determined the levels of FOXO1 and FOXO3a. FOXO family members found physiologically in the liver (38, 39). We found FOXO1 and FOXO3a were elevated upon PMPCB knockdown in both HUH7 and HUH1 in the nuclear fractions but not in cytoplasmic fractions (Fig. 7A, left; Supplementary Fig. S6). In Hep3B cells, we observed no significant changes in FOXO1 but an increase in FOXO3A in the nuclear fractions (Fig. 7A, right). Moreover, the phosphorylation of FOXO1 at Thr24 (p-FOXO1) and FOXO3 at Thr32 (p-FOXO3a) was markedly reduced in HUH7, HUH1, and Hep3B cells upon PMPCB knockdown and significantly increased in the nuclear fractions (Fig. 7A; Supplementary Fig. S5C). However, this activity was not observed in the EpCAM+ MHCC97H cells (Fig. 7B and C). Together, these data suggest that PMPCB knockdown–mediated ROS production reduces the phosphorylation of FOXOs, thereby enhancing its nuclear translocation and interaction with CTNNB1 to down-regulate the transcription activity of the Wnt/β-catenin signaling pathway (40, 41).

Discussion

HCC is one of the most difficult malignancies to treat with rising incidence and mortality rates in the United States (42). Recent setbacks of multiple targeted therapies in clinical phase III trials reinforce an urgency to better define candidate targets for HCC prior to drug development (43). The heterogeneous nature of HCC and lack of well-defined dominant driver genes have contributed significantly to these failures. Furthermore, whole-genome sequencing and exome sequencing analyses of HCC revealed a complex mutational landscape with vast intratumor heterogeneity, complicating the ability to efficiently identify specific druggable targets essential for the fitness of cancer cells within a defined tumor subtype (44, 45). Moreover, because each tumor may contain thousands of somatic genome alterations accumulated over time, it is unlikely that one single targeted therapy would benefit most HCC patients (12). Synthetic lethality screens have been used to identify the “Achilles’ heel” of cancers, aiming on identifying therapies with greater effectiveness and fewer adverse effects (46). In the current study, we used a synthetic lethality screen and have successfully identified 26 genes that may be functionally linked to the survival of EpCAM+ HCC cells as potential targets for an aggressive EpCAM+ AFP+ HCC subtype (13). Consistent with EpCAM+ AFP+ subtype–related prognosis, we showed the expression of these candidate genes is associated with EpCAM and HCC prognosis. Moreover, pathway analysis revealed that they are enriched with genes linked to Wnt/β-Catenin signaling, similar to the data found in previous studies (13, 24). Among them, we validated the top-ranked gene, PMPCB, as a bona fide therapeutic vulnerability where it functionally interacts with EpCAM/Wnt/β-Catenin/FOXO signaling. Thus, PMPCB and its downstream signaling partners may be exploited as therapeutic targets for EpCAM+ HCC.

Stem cell–surface markers are useful for the identification of stem-like cancer cells including circulating tumor cells and potential candidates for molecular targeted therapy. For example, miR-34a can inhibit prostate CSCs and metastasis by directly repressing CD44, a prostate CSC marker (47). MRX34, a double-stranded mimic of miR-34a, is currently being evaluated clinically for the treatment of metastatic cancer (48). Because EpCAM is expressed in stem cells and functionally linked to CSCs of various
cancer types, including liver cancers, several antibody-based therapies targeting EpCAM are being developed as therapeutics in colon, pancreatic, and ovarian cancers (23, 24, 49–53). However, targeted therapy using anti-EpCAM antibody for HCC has not been established. Moreover, such therapies are only effective if EpCAM is highly expressed by cancer cells. Thus, a drug-discovery strategy that exploits synthetic lethal interactions between EpCAM and another potential target may prove to be effective in EpCAM$^+$AFP$^+$ HCCs. In this study, we utilized an RNAi screening technology to identify EpCAM-dependent genes and identified 76 candidate genes whose expressions are closely associated with EpCAM expression. Although CRISPR/Cas9 screens have evolved to significantly improve the specificity for large-scale screens, RNAi screens remain optimal for identifying synthetic lethal targets (10). In addition, RNAi remains the standard for analyzing the function of gene splice variants and utilized extensively for the identification of novel targets against cancer drivers and synthetic lethal relationships (9, 10). Because any large-scale analyses have inherent rates of false positives and negatives, we used different strategies to minimize the risk of reporting false positives. First, we selected only candidate genes targeted by at least 2 independent shRNAs occurring in 2 different time points and performed functional studies in 3 HCC cell lines with variable EpCAM status. Second, the expression of candidate genes is associated with EpCAM expression patterns in HCC specimens and HCC prognosis, analogous to the EpCAM$^+$AFP$^+$ HCC subtype. Third, we validated an on-target activity of the potential candidate, using 2 additional shRNAs that are different from the original screen platform. Finally, to further demonstrate the requirement of the potential candidate in EpCAM-dependent apoptosis, we performed rescue experiments to exclude shRNA off-target effects.

Our screen identified PMPCB, which encodes the β-subunit of MPP and plays a critical role in the maintenance of mitochondrial functions. It has been demonstrated that altered MPP leads to mitochondria abnormality and dysfunction, which have been implicated in multiple cancers (54, 55). Consistent with the important role of MPP in mitochondrial
dysfunction, we observed that PMPCB silencing leads to mitochondrial dysfunction in both EpCAM⁺ and EpCAM⁻ HCC cells. However, only EpCAM⁺ HCC cells underwent apoptosis, suggesting that while both cell populations may immediately respond to ROS production, only EpCAM⁺ cells underwent ROS-induced FOXO activation and subsequent attenuation of CTNNB1 nuclear activity. These data suggest that PMPCBs’ inhibition and subsequent mitochondrial dysfunction is especially sensitive to EpCAM⁺ cells as these cells are more dependent on the Wnt/β-catenin pathway for survival. This dependence is most evident in HCC cells with a constitutive Wnt/β-catenin signaling such as the Hep3B cells. Although the PMPCB/FOXO/Wnt/β-catenin pathway is similarly affected in EpCAM⁺ HCC cells with active Wnt/β-catenin signaling (induction of ROS and increased FOXO nuclear activity), we did not observe significant effects on EpCAM expression or apoptosis following PMPCB knockdown. It is likely that a constitutive Wnt/β-catenin signaling can overcome PMPCB-ROS-induced FOXO attenuation of Wnt/β-catenin activity, indicating that PMPCB is downstream of the Wnt/β-catenin pathway. Thus, it is likely that PMPCB is a synthetic lethal target for EpCAM⁺ HCC subpopulations dependent on the Wnt/β-catenin pathway.

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