EPHB2 activates β-catenin to enhance cancer stem cell properties and drive sorafenib resistance in hepatocellular carcinoma

Hoi-Wing Leung[1]*, Carmen Oi-Ning Leung[1]*, Eunice Y. Lau[2], Katherine Po Sin Chung[1], Etienne H. Mok[1], Martina Mang Leng Lei[1], Rainbow Wing Hei Leung[1], Man Tong[3], Vincent W. Keng[1], Cong Ma[1], Qian Zhao[1], Irene Oi-Lin Ng[4,5],
Stephanie Ma[3,5], Terence K. Lee[1,6#]

[1] Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University; [2] Department of Clinical Oncology, Queen Elizabeth Hospital, [3] School of Biomedical Sciences, Li Ka Shing Faculty of Medicine, The University of Hong Kong; [4] Department of Pathology, Queen Mary Hospital, The University of Hong Kong; [5] State Key Laboratory of Liver Research, The University of Hong Kong [6]State Key Laboratory of Chemical Biology and Drug Discovery, The Hong Kong Polytechnic University

*contributed equally

[#]Corresponding author:
Dr. Terence K.W. Lee, Room 805, Block Y, Department of Applied Biology and Chemical Technology, Lee Shau Kee Building, The Hong Kong Polytechnic University, Hong Kong. Tel: (852) 3400-8799; Fax: (852) 2364-9932; Email: terence.kw.lee@polyu.edu.hk

Running title: EPHB2 forms a Wnt/β-catenin positive signaling loop in HCC.

Keywords: β-catenin, CSCs, drug resistance, EPHB2, TCF1

Abbreviations: beta-catenin, CTNNB1; cancer stem cells, CSCs; EPH Receptor B2, EPHB2; epithelial cell adhesion molecule, EpCAM; gene set enrichment analysis, GSEA; hepatocellular carcinoma, HCC; hydrodynamic tail vein injection, HTVI; immunohistochemistry, IHC; immunoprecipitation, ChIP; immunofluorescence, IF; nonalcoholic steatohepatitis, NASH;

quantitative reverse transcription polymerase chain reaction, qPCR; patient-derived tumor xenografts, PDTXs; Wild-type, WT

Competing interests: The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Abstract

The survival benefit derived from sorafenib treatment for patients with hepatocellular carcinoma (HCC) is modest due to acquired resistance. Targeting cancer stem cells (CSC) is a possible way to reverse drug resistance; however, inhibitors that specifically target liver CSCs are limited. In this study, we established two sorafenib-resistant, patient-derived tumor xenografts (PDX) that mimicked development of acquired resistance to sorafenib in HCC patients. RNA-sequencing analysis of sorafenib-resistant PDXs and their corresponding mock controls identified EPHB2 as the most significantly upregulated kinase. EPHB2 expression increased stepwise from normal liver tissue to fibrotic liver tissue to HCC tissue and correlated with poor prognosis. Endogenous EPHB2 knockout showed attenuation of tumor development in mice. EPHB2 regulated the traits of liver CSCs; similarly, sorted EPHB2High HCC cells were endowed with enhanced CSC properties when compared with their EPHB2Low counterparts. Mechanistically, EPHB2 regulated cancer stemness and drug resistance by driving the SRC/AKT/GSK3β/β-catenin signaling cascade, and EPHB2 expression was regulated by TCF1 via promoter activation, forming a positive Wnt/β-catenin feedback loop. Intravenous administration of rAAV-8-shEPHB2 suppressed HCC tumor growth and significantly sensitized HCC cells to sorafenib in an NRAS/AKT-driven HCC immunocompetent mouse model. Targeting a positive feedback loop involving the EPHB2/β-catenin axis may be a possible therapeutic strategy to combat acquired drug resistance in HCC.

Significance: This study identifies a EPHB2/β-catenin/TCF1 positive feedback loop that augments cancer stemness and sorafenib resistance in HCC, revealing a targetable axis to combat acquired drug resistance in HCC.

Introduction

Hepatocellular carcinoma (HCC) ranks the 6th most commonly diagnosed cancer with a poor prognosis (1). The first-line treatments for HCC are liver transplantation and surgical resection. However, most HCCs are inoperable, as patients typically are diagnosed at advanced stages. HCC treatment recently entered a new era with the development of molecular-targeted therapies, as sorafenib improved survival in advanced HCC patients (2). However, the survival benefit of sorafenib is modest, with median survival times 2.8 and 2.3 months longer than those of the placebo arm found in two large-scale trials (2,3). Therefore, studies on the mechanism underlying sorafenib resistance are urgently needed. We and others have shown that sorafenib-resistant HCC cells are endowed with enhanced CSC properties (4-6). These data strongly support the hypothesis that targeting the signaling pathways that mediate the functions of liver CSCs may be a strategy to reverse drug resistance in HCC.

To mimic this clinical situation, many researchers have employed *in vitro* establishment of sorafenib-resistant cells. However, the translational potential of this model is limited because the model is not representative of the true clinical situation. For this purpose, we employed *in vivo* models established with patient-derived tumor xenografts (PDTXs) derived directly from two samples from HCC patients and developed sorafenib resistance *in vivo* by administration of several rounds of sorafenib treatment. Using this strategy, we were able to enrich liver CSCs in these sorafenib-resistant PDTXs, as evidenced by an increase in self-renewal. Using RNA-sequencing analysis, we compared the expression profiles of sorafenib-resistant PDTXs and their corresponding mock controls. This analysis showed that EPH Receptor B2 (EPHB2) showed remarkably higher expression in the liver CSC-enriched sorafenib-resistant PDTXs than in their mock counterparts, suggesting a role for EPHB2 in liver CSCs.

EPHB2 belongs to the Eph receptor family of receptor tyrosine kinase transmembrane glycoproteins and was first identified as a tumor suppressor because it has been shown to exhibit significantly downregulated expression in a variety of cancers, including colon (7) and head and neck cancers (8), mediated by chromosomal aberrations and promoter methylation. A number of studies have shown that EPHB2 is overexpressed and plays crucial roles in tumor growth,

migration, and chemoresistance (9,10). However, the clinical relevance and functional role of EPHB2 in HCC remain uncharacterized. In mouse and human HCCs, we found that EPHB2 became overexpressed in a stepwise manner from healthy liver tissue to cirrhotic liver tissue and then HCC tissue. High EPHB2 expression in patients was significantly correlated with poor patient survival. Interestingly, EPHB2 expression was markedly upregulated in our established sorafenib-resistant PDTXs. By overexpression and knockdown approaches, EPHB2 was found to be critically involved in the regulation of liver CSCs. Likewise, $EPHB2^{High}$ HCC cells were shown to possess enhanced liver CSC properties. EPHB2 was found to regulate cancer stemness and drug resistance via a Wnt/β-catenin positive feedback loop. Targeting EPHB2 with rAAV-8-shEPHB2 suppressed HCC tumor growth and sensitized HCC cells to sorafenib in an immunocompetent mouse model. In summary, targeting the TCF1/EPHB2/β-catenin pathway may be a promising strategy for HCC treatment.

Materials and Methods

Plasmids

Lentiviral expression ORF clone, CMV, EPHB2 (EX-E2415-Lv105) was purchased from Genecopoeia (Rockville, MD, USA). Δ-45-β-catenin and WT-β-catenin were obtained from Dr. Stephanie Ma (University of Hong Kong).

Chemicals

Doxorubicin was purchased from EBEWE Pharma. Sorafenib was purchased from LC Laboratories.

Human HCC cell lines

MHCC-97L (Liver Cancer Institute, Fudan University, China), Huh7 and PLC/PRF/5 (Japan Cancer Research Bank, Tokyo, Japan), were maintained in DMEM containing high glucose (Gibco BRL) with 10% heat-inactivated fetal bovine serum (Gibco BRL), 100 mg/mL penicillin G, and 50 μg/mL streptomycin (Gibco BRL) at 37°C in a humidified atmosphere containing 5% CO_2. These three HCC cell lines were established from male origin. All cell lines used in this study were obtained between 2013 and 2016, regularly authenticated by morphologic observation and AuthentiFiler STR (Invitrogen) and tested for the absence of mycoplasma contamination (MycoAlert, Lonza). Experiments were performed within 20 passages after cell thawing.

Human tissue specimens for mRNA expression analysis

Paired patient HCC and adjacent noncancerous liver tissue specimens were collected at the time of surgical resection at Queen Mary Hospital, Hong Kong, from 1991 to 2013. These samples were obtained from patients with their written consent. The use of human clinical specimens was approved by the Institutional Review Board (IRB) of the University of Hong Kong/Hospital Authority Hong Kong West Cluster.

Lentiviral-based transfection into HCC cells

For suppression of EPHB2 in HCC cells, shRNA sequences against human EPHB2 were cloned to pLKO.1 vector (Addgene). Transduced cells were selected with 2 μg/mL puromycin.

Supplementary Table S1 lists the sequences of the EPHB2 and NTC used. For overexpression, Stable HCC cells ectopically overexpressing EPHB2 were selected with 2 μg/mL puromycin.

RNA extraction and quantitative PCR (qRT-PCR) analysis

Total RNA was isolated using TRIzol reagent according to the manufacturer's protocol (Invitrogen). Complementary DNA (cDNA) was synthesized using PrimeScript RT Reagent Kit (Takara) according to the manufacturer's instructions and then subjected to qPCR with BrightGreen 2x qPCR Master mix (Applied Biological Materials) using QuantStudio 7 Flex Read Time PCR System (Applied Biosystems) with primers specific to the sequences of genes of interest which were provided in Supplementary Table S2. Relative expression differences were calculated using $2^{-\Delta\Delta CT}$method with reference to HPRT/GAPDH.

Chromatin immunoprecipitation (ChIP) assay

The cells were processed for ChIP assays according to Upstate CHIP protocol. Briefly, cells were cross-linked for 10 minutes with 1% formaldehyde and lysed. Lysate pellets were resuspended and enzymatic digested with micrococcal nuclease for 5 minutes. Protein-DNA complexes were immunoprecipitated using TCF-1 antibody (Cell Signaling Technology) or Normal Rabbit IgG (Invitrogen) bound to protein A agarose, eluted, and digested with proteinase K. For qPCR analysis of the ChIP DNA samples before amplicon generation, QIAquick-purified immunoprecipitates were dissolved in 30 μl of water. Standard PCR reactions using 2 μL of the immunoprecipitated DNA were performed with a SYBR Green PCR kit (Applied Biosystems). The EPHB2 promoter region (CTGTTAATGATG) in the TCF-1 precipitated chromatin was amplified by qPCR primers (forward primer sequence CACTCCACAGAGTTCCGAGC and reverse primer sequence CCTGCTCTGACAGCCTTCAT. The amplicon is at -1014bp downstream of TSS. Calculation of TCF1 occupancy on the EPHB2 promoter was performed according to the ChIP-qPCR primer assay data analysis template from Sigma-Aldrich.

RNA-sequencing

Total RNA of PLC/PRF/5 of non-target control (NTC) and EPHB2 knockdown (shEPHB2-2) groups and sorafenib-resistant PDTXs (PDTX#1 and #5) and their corresponding mock controls were extracted using TRIzol Reagent (Life Technologies) according to manufacturer's protocol. The quality of total RNA was checked by Agilent 2100 bioanalyzer (Agilent Technologies Inc.) to have OD260/280 ratio of between 1.8-2.0 and RNA integrity number (RIN) value higher than 8.0. The RNA samples which met the quality assessment were then subjected to Illumina Solexa sequencing using Hiseq 1500 sequencer (Illumina) for performing HiSeq sequencing run (pair-end sequencing of 101bp). Each sample had an average throughput of 10.8Gb and a total throughput of 21.5Gb. An average of 94% of the bases achieved a quality score of Q30 where Q30 denotes the accuracy of a base call to be 99.9%. Expression estimation and tests for differential expression were processed by Cufflinks v2.1.1. All data was expressed as fragments per kilobase of exon per million fragments mapped (FPKM) values and fold changes in transcript levels relative to the NTC group from PLC/PRF/5. RNA-sequencing data is available publicly at GSE168783 and GSE168839.

Hydrodynamic tail vein NRAS+AKT HCC mouse model

6-to-8-week old male wild-type C57BL/6 mice were used and the procedure was performed as previously described (11). In brief, 7.5μg of plasmids encoding human AKT1 (myristylated AKT1 or myr-AKT1) and human neuroblastoma Ras viral oncogene homolog (N-RasV12) along with sleeping beauty (SB) transposase in a ratio of 25:1 were diluted in 2ml saline (0.9% NaCl), filtered through 0.22μm filter and injected into the lateral tail vein of C57BL/6 mice in 5-7s. The constructs used in this study showed long term expression of genes via hydrodynamic injection. To examine the role of EPHB2 in liver carcinogenesis using this model, eSpCas9-2A-GFP (PX458 against mouse EPHB2 (CGGCCCTGTAGTAGCCGTTG, Genscript) or eSpCas9-2A-GFP against NTC (Genscript) will also be injected together with the AKT1, N-RasV12 and sleeping beauty (SB) transposase. The study protocol was approved by and performed in accordance with the Committee of the Use of Live Animals in Teaching and Research at the University of Hong Kong and the Hong Kong Polytechnic University.

Intravenous injection of rAAV8-shEPHB2 in hydrodynamic injection model

6-8-week-old male wild-type C57BL/6J mice were used and the procedure was performed as previously described (11). In brief, 7.5μg of plasmids encoding human AKT1 (myristoylated AKT1 or myr-AKT1) and human neuroblastoma Ras viral oncogene homolog (N-RasV12) along with sleeping beauty transposase in a ratio of 25:1 were diluted in 2ml saline (0.9% NaCl), filtered through 0.22μm filter and injected into the lateral tail vein of C57BL/6J mice in 5-7 seconds. The constructs used in this study showed long term expression of genes via hydrodynamic injection. Upon injection of plasmids for two weeks, the mice were injected with rAAV8-shEPHB2/rAAV8-NTC at the dosage of 1×10^{12} vg/mouse intravenously (Vigene Biosciences, USA). The sequence of shRNA against mouse EPHB2 is 5'-ACGAGAACATGAACACTAT-3'. After one week, we started to treat mice with sorafenib at 30mg/kg for 21 days. At this point, the mice were separated into the following four subgroups: (i) DMSO with rAAV8-NTC; (ii) DMSO and rAAV8-shEPHB2; (iii) rAAV8-NTC and sorafenib (30mg/kg); and (iv) rAAV8-shEPHB2 and sorafenib. The mice were sacrificed at day 21 after sorafenib treatment. The study protocol was approved by and performed in accordance with the Committee of the Use of Live Animals in Teaching and Research at the University of Hong Kong and the Hong Kong Polytechnic University.

Statistical analysis

Statistical significance of qPCR, sphere formation assay, flow cytometry analysis, expression of liver CSC markers were determined by Student's t test using Microsoft Office Excel software (Microsoft Corporation). The displayed results showed the means and the standard deviations, and those with p values less than 0.05 were considered statistically significant (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Kaplan-Meier survival analysis was used to analyze disease-free survival and a log-rank test was used to determine the statistical significance; these analyses were carried out using SPSS 20 software.

Inducible knockdown, sphere formation assay, limiting dilution assay, Annexin V apoptosis assay, flow cytometric analysis, immunoprecipitation, luciferase reporter assay, western blot analysis, immunofluorescence staining, immunohistochemistry, TUNEL assay, cell sorting, NASH-HCC model, tumorigenicity assay

The methods have been described in Supplementary Materials and Methods.

Result

EPHB2 exhibited upregulated expression in sorafenib-resistant HCC cells and was sporadically expressed in human HCC specimens with clinical significance

In an attempt to identify critical kinases involved in sorafenib resistance, we developed two sorafenib-resistant HCC cell lines from two PDTXs (PDTX#1 and PDTX#5)(4), which were enriched for an increase in self-renewal and tumorigencity (Figure 1A-C). We have analyzed four tumor samples derived from these two sorafenib-resistant HCC cell lines and their corresponding mock counterparts by RNA-sequencing analysis. Upon analysis, we found 67 genes to be commonly upregulated in the two sorafenib-resistant PDTXs, among which we found EPHB2 to be the most significantly upregulated kinase (Figure 1D). Quantitative PCR analysis confirmed this observation, as elevated expression of EPHB2 was indeed found in the sorafenib-resistant PDTXs compared with their corresponding mock controls (Figure 1E). In addition to these two PDTXs, we also observed similar upregulation of EPHB2 expression in sorafenib-resistant Huh7, PLC/PRF/5 and MHCC-97L cells (11) (Supplementary Figure S1). In an analysis of dataset (GSE25097), EPHB2 expression was significantly upregulated in tumor specimens, with stepwise upregulation from normal to cirrhotic to tumor tissues, suggesting an oncogenic role for EPHB2 in liver carcinogenesis (Figure 1F). Similarly, we found stepwise increases in the mRNA expression of EPHB2 from normal to fibrotic to HCC tissues in the STAM^{TM} nonalcoholic steatohepatitis (NASH) HCC mouse model (Figure 1G). Interestingly, along with EPHB2 overexpression, a similar stepwise increase in the expression of liver CSC markers, including CD133, CD90 and CD24, was previously reported upon fibrosis and subsequent HCC development (12). Next, we examined the prognostic significance of EPHB2 expression in HCC patients. The cutoff value of T/N > 6 was used to divide HCC patients according to their EPHB2 expression. Patients with high EPHB2 expression were significantly correlated with poor disease-free survival (p=0.041) (Figure 1H). Collectively, these data suggest that EPHB2 overexpression may be associated with liver carcinogenesis and sorafenib resistance via regulation of liver CSCs.

Endogenous EPHB2 knockout in the liver of an immunocompetent mouse model attenuates heparocarcinogenesis

Firstly, we performed endogenous knockout of EPHB2 in an immunocompetent mouse model to investigate the tumor-promoting role of EPHB2 in HCC. Using the hydrodynamic tail vein injection (HTVI) delivery approach, we induced HCC tumors in C57BL/6 mice using a combination of activated forms of myr-AKT and N-RasV12 proto-oncogenes and SB transposase (13)(Figure 2A). A stepwise increase in EPHB2 mRNA expression was noted in livers harvested from mice injected with the empty vector (EV) control alone to livers with early (1 to 2 weeks postinjection) to advanced HCC (2 to 4 weeks postinjection) (Figure 2B). Using the same model, we examined the role of EPHB2 in tumor formation by hydrodynamic injection of sgEPHB2 together with myr-AKT, N-RasV12 and sleeping beauty transposase (Figure 2C). Upon suppression of EPHB2 expression, the number of tumors and survival of mice were compared between the sgEPHB2 and sgNTC groups, and endogenous EPHB2 knockout significantly attenuated the size and number of tumor nodules compared to sgNTC treatment (Figure 2D&E). In addition, EPHB2 knockout mice tended to show longer survival ($p=0.0888$) than mice injected with sgNTC controls (Figure 2F). HTVI of shEPHB2 is efficiently transducted to the livers of mice, as evidenced by the intense GFP signal and suppression of EPHB2 by qPCR analysis (Figure 2G&H). The above findings support a key role for EPHB2 in promoting HCC growth.

EPHB2 regulates the cancer stemness and drug resistance of HCC cells

To further examine whether EPHB2 functionally drives self-renewal, tumor formation and drug resistance, we performed EPHB2-knockdown and EPHB2-overexpression experiments using lentiviral-based overexpression and knockdown approaches. Western blot analysis showed successful establishment of knockdown clones derived from PLC/PRF/5 and MHCC-97L cells and overexpression clones derived from Huh7 cells (Figure 3A). In a sphere formation assay performed with PLC/PRF/5 or MHCC-97L cells, knockdown of EPHB2 expression significantly reduced the number and size of primary spheres and their second passages. Accordingly, EPHB2 overexpression was shown to enhance the sphere-forming ability of Huh7 cells (Figure 3B). Similar findings were observed in limiting dilution assay (Supplementary Figure S2). In line with

this finding, knockdown of EPHB2 expression reduced the size and number of tumors formed by both PLC/PRF/5 cells and MHCC-97L cells (Figure 3C, Table S3). Conversely, EPHB2 overexpression in Huh7 cells significantly enhanced tumorigenicity, with an increase in the estimated CSC frequency (Figure 3C, Supplementary Table S3). We consistently found that shEPHB2 HCC cells exhibited decreases in the percentages of cells expressing CD90 and CD47, while EPHB2-overexpressing cells showed increased expression of these markers (Figure 3D). In addition, OCT4, one of the core pluripotent genes, was consistently changed upon alteration of EPHB2 expression (Supplementary Figure S3). Next, we found that knockdown of EPHB2 expression enhanced the number of apoptotic cells in response to both doxorubicin treatment and sorafenib treatments (Figure 3E). Conversely, EPHB2-overexpressing Huh7 cells exhibited reduced cell death when treated with doxorubicin or sorafenib (Figure 3E). To further confirm the role of EPHB2 knockdown in chemosensitization, we employed lentiviral-based doxycycline-inducible knockdown of EPHB2 expression in PLC/PRF/5 cells. In this experiment, we added doxycycline at the same time as sorafenib administration. Consistently, inducible suppression of EPHB2 led to sensitization of the PLC/PRF/5 cells to sorafenib treatment (Supplementary Figure S4A-B).

EPHB2^{High} HCC cells possess enhanced CSC properties

Next, we examined whether EPHB2^{High} HCC populations possess enhanced liver CSC properties by separating MHCC-97L cells into EPHB2^{High} and EPHB2^{Low} populations by a cell sorting approach. Upon analysis, purities ranged from 99% to 100% for EPHB2^{Low} cells and 85% to 95% for EPHB2^{High} cells (Figure 4A). Western blot analysis further confirmed successful separation of MHCC-97L cells into EPHB2^{High} and EPHB2^{Low} populations (Figure 4B). By sphere formation assay, EPHB2^{High} cells generated significantly larger spheres and more spheres in the first and second passages (Figure 4C), and this finding supported the greater self-renewal capability of EPHB2^{High} HCC cells. Furthermore, EPHB2^{High} cells showed higher tumorigenic ability, when compared with EPHB2^{Low} cells purified from MHCC-97L cells. A significant difference in tumor incidence was observed between the EPHB2^{High} and EPHB2^{Low} cells (Figure 4D). As few as 500 EPHB2^{High} cells were sufficient for consistent tumor development in NOD/SCID mice (Figure

4D). In parallel with this finding, EPHB2^{High} cells exhibited higher expression of CD90 and CD47 (Figure 4E). Lastly, EPHB2^{High} cells were more refractory to the effects of sorafenib and doxorubicin than EPHB2^{Low} cells by Annexin V staining (Figure 4F).

Wnt/β-catenin signaling pathway is the downstream effect of EPHB2 mediated CSC functions

To determine the major downstream effector of EPHB2, we employed RNA-sequencing analysis to compare the gene expression profiles of shEPHB2-2 and nontarget control (NTC)-transfected cells derived from the PLC/PRF/5 cell line. By gene set enrichment analysis (GSEA), we found several pathways to be downregulated, with over 60% of genes related to the Wnt/β-catenin signaling pathway being significantly altered (Figure 5A-C). To confirm this finding, we examined the effect of EPHB2 alternations on the transactivation activity of β-catenin. A TOP/FOP reporter assay showed that the transactivation activity of β-catenin was suppressed when EPHB2 was repressed, while this activity was elevated in EPHB2-overexpressing cells (Figure 5D). Consistent with the results for the transactivation activity of β-catenin, the expression of known downstream targets of the Wnt/β-catenin pathway, including Cyclin D1 and CMYC, showed similar trends in response to alterations in EPHB2 expression (Figure 5D). Consistently, higher β-catenin transactivation activity was observed in EPHB2^{High} Huh7 cells than in EPHB2^{Low} cells (Supplementary Figure S5A-B). By immunofluorescence (IF) staining, increased cytoplasmic and nuclear β-catenin protein levels were observed in EPHB2 OE cells, and the opposite effect was observed upon EPHB2 suppression (Figure 5E). Next, we examined the mediator linking EPHB2 and the Wnt/β-catenin signaling pathway by identifying the direct substrate of EPHB2. By immunoprecipitation analysis, we found that EPHB2 physically interacted with SRC (Figure 5F). Upon interaction, EPHB2 was able to phosphorylate SRC, as evidenced by the corresponding changes in phosphorylation at Y416 upon suppression or overexpression of EPHB2, which subsequently led to changes in the AKT/GSK3β/β-catenin signaling pathway (Figure 5G). In a TCGA cohort analysis, we found that the Wnt/β-catenin signaling pathway was the only pathway that was significantly enriched in HCC patients with high EPHB2 expression, which further confirm Wnt/β-catenin as the major effector of EPHB2 signaling in our *in vitro* finding (Figure 5H).

β-catenin/TCF-1 pathway regulates the expression of EPHB2, forming a Wnt/β-catenin positive feedback loop

EPHB2 is known to be a Wnt target gene in colorectal cancer (14); however, the mechanism remains unclear. To examine whether Wnt/β-catenin alterations affect EPHB2 expression, we analyzed the impact of introducing constitutively active β-catenin (Δ45β-cat) into Huh7 cells. Constitutively active β-catenin is mutated at serine 45 and, as a result, cannot be phosphorylated or undergo ubiquitination and degradation (15). Wild-type (WT) β-catenin was also overexpressed as a control. Upon activation of β-catenin transactivation activity (Figure 6A&B), we found that both the mRNA and protein levels of EPHB2 were upregulated (Figure 6C). To further confirm this finding, we administered Wnt3a-supplemented medium to activate the Wnt/β-catenin pathway, and EPHB2 mRNA and protein levels were consistently upregulated (Figure 6D). Echoing the above *in vitro* observations, we found that the EPHB2 mRNA level was consistently upregulated in an HCC mouse model (Fah-/-) with hydrodynamic injection of constitutively active β-catenin-S33Y, together with shp53 in Fah/SB11 mice (16) (Figure 6E). Based on these *in vitro* and *in vivo* observations, we postulated that Wnt/β-catenin transcriptionally activates EPHB2 expression. *In silico* analysis (PROMO 3.0) identified one putative TCF1 binding site (CTGTTAATGATG) at the position of -1014bp to -1003bp upstream of the TSS of the EPHB2 gene (Figure 6F). Upon suppression of TCF1 in EPHB2-knockdown HCC cells, we found that there was a significant decrease in the fold enrichment of TCF1 binding relative to that in NTC cells (Figure 6G&H), suggesting that TCF1 physically binds to and activates the promoter of EPHB2, which forms a Wnt/β-catenin positive feedback loop. Intriguingly, high EPHB2 expression significantly co-occurred with beta-catenin (CTNNB1) driver mutations (Figure 6I), which further confirms the role of Wnt/β-catenin in driving EPHB2 expression in HCC clinical samples.

Intravenous administration of rAAV-8-shEPHB2 sensitized HCC cells to sorafenib treatment in an immunocompetent mouse model

Currently, EPHB2 kinase inhibitors are not commercially available. Therefore, we examined the therapeutic targeting of EPHB2 by intravenous injection of rAAV-8-EPHB2 alone or in combination with sorafenib in an NRAS/AKT-induced HCC mouse model (11). Two weeks after hydrodynamic injection of plasmids encoding the activated forms of NRAS and AKT, we intravenously injected the mice with either rAAV8-shEPHB2 or rAAV8-NTC. After one week, we started to treat the mice with sorafenib at 30 mg/kg for 21 days. At this point, the mice in a group of 5-6 were separated into the following four subgroups: (i) DMSO and rAAV8-NTC, (ii) DMSO and rAAV8-shEPHB2, (iii) rAAV8-NTC and sorafenib (30 mg/kg), and (iv) rAAV8-shEPHB2 and sorafenib (Figure 7A). Firstly, we found that rAAV8-NTC/ rAAV8-shEPHB2 were efficiently delivered to the livers of mice in these four groups, as evidenced by the intense GFP signal (Figure 7B). The efficacy of the combined drug treatment was evaluated by calculating the liver weight to body weight ratio was compared among the groups. We found that rAAV8-shEPHB2/sorafenib produced significantly maximal suppression of tumor growth, indicating that shEPHB2 treatment can synergize with sorafenib treatment and is effective against liver tumors *in vivo* (Figure 7C-D), which is accompanied with increase in number of apoptotic cells by TUNEL assay (Supplementary Figure S6) . Immunohistochemistry (IHC) staining showed the efficient suppression of EPHB2 in the livers of rAAV8-shEPHB2 transduced groups (Figure 7E).

Discussion

Liver CSCs have been identified by several cell-surface antigens, including CD133 (17), CD90 (18), CD13 (19), CD24 (20) and epithelial cell adhesion molecule (EpCAM) (21). However, there are no druggable kinase inhibitors that show specific targeting against the cell surface of liver CSCs. Identification of kinases in liver CSCs could offer a new paradigm for HCC treatment by developing specific kinase inhibitors to target these cells. In an attempt to identify specific cell-surface kinases crucial for the regulation of liver CSCs, we established sorafenib-resistant PDTXs with increased self-renewal. By RNA-sequencing analysis, we found that EPHB2 was one of the most commonly upregulated kinases in the two sorafenib-resistant PDTX models.

EPHB2, belongs to the Eph receptor family of receptor tyrosine kinase transmembrane glycoproteins. These receptors bind ligands called ephrins and are involved in diverse cellular processes, including motility, division, and differentiation (22). A distinguishing characteristic of Eph-ephrin signaling is that both the receptors and the ligands are competent to transduce a signaling cascade, resulting in bidirectional signaling (23). Two ligands of EPHB2, EFNB1 and EFNB2, have been reported (24). The role of EPHB2 in cancer remains controversial, and it is still unclear whether EPHB2 acts as a tumor suppressor gene or oncogene. We found that EPHB2 was overexpressed in a stepwise manner, increasing from healthy tissue to cirrhotic tissue and then to HCC tissue. This result is consistent with a previous report showing the role of EPHB2 in the development of cirrhosis in a CCL4-induced fibrosis model (25), which at least in part involves activation of stellate cells (26). In addition, EPHB2 overexpression was significantly correlated with poor patient survival.

EphB2 was first determined to play a critical role in regulating the homeostasis of stem cells in intestinal crypts (27). Subsequently, EPHB2 was found to regulate cell movement in intestinal crypts through a kinase-independent PI3K signaling mechanism (9). Recently, a role for EPHB2 in liver CSCs has been suggested. In colon cancer, EphB2-sorted intestinal stem cell-like tumor cells display a robust tumor-initiating capacity in immunodeficient mice and long-term self-renewal potential (28). Although these findings suggest a potential role for EPHB2 in regulating CSCs, the

molecular mechanism by which EPHB2 regulates CSC properties is poorly understood. Through overexpression and knockdown approaches, we showed that EPHB2 regulates the self-renewal, tumorigenicity and drug resistance of HCC cells, and expression of liver CSC markers, including CD90(18) and CD47(29). By using a cell sorting approach, we consistently found that EPHB2^{High} HCC cells contained enhanced liver CSC populations. This result is consistent to previous report showing the role of EPHB2 in CSCs in colon cancer (28). Mechanistically, we found that Wnt/β-catenin signaling is the downstream signaling pathway in EPHB2-mediated liver CSC functions, as evidenced by the transactivation activity of β-catenin and its downstream targets. This result was supported by TCGA analysis showing enrichment of Wnt/β-catenin signaling in patients with high EPHB2 expression. By immunoprecipitation, we found that EPHB2 directly interacts with Src and induces phosphorylation at Y416. This result is consistent with a previous report demonstrating the interaction between these two proteins (30,31). Through Src activation, EPHB2 drives activation of the AKT/GSK3β/β-catenin signaling cascade. EPHB2 is a known Wnt target gene in colorectal cancer (28), but the exact mechanism remains unclear. Through bioinformatic analysis, we found one putative TCF1 binding site upstream of the transcription start site in the EPHB2 promoter. Using a ChIP assay, we found a physical interaction between TCF1 and the EPHB2 promoter. Upon knockdown of EPHB2 expression, we found a consistent decrease in TCF1 expression, leading to a decrease in the site occupancy of TCF1 binding in knockdown cells relative to NTC cells. Consistent with this finding, HCC tumors with a β-catenin driver mutation exhibited high EPHB2 expression, further confirming the role of β-catenin/TCF1 as an upstream activator of EPHB2 signaling.

Finally, we examined the therapeutic potential of targeting EPHB2 alone or in combination with sorafenib in an immunocompetent mouse model by intravenous administration of rAAV-8-shEPHB2. rAAV-8 was previously shown to efficiently introduce transgenes *in vivo* with a specific tropism for the liver (32). When compared with those in the rAAV8-NTC group, mice injected with rAAV8-shEPHB2 showed suppression of tumor growth in terms of the liver/body ratio. rAAV8-shEPHB2 showed maximal suppression of HCC tumor growth compared with sorafenib or rAAV8-

shEPHB2 alone. Based on these data, suppression of EPHB2 could sensitize immunocompetent HCC mouse model to sorafenib.

In conclusion, we have demonstrated that EPHB2 regulates cancer stemness and drug resistance via the TCF1/EPHB2/β-catenin positive feedback loop. Targeting EPHB2 alone or in combination with other targets may be a novel therapeutic strategy for the treatment of HCC.

Acknowledgements: We thank the University Research Facility in Life Sciences at the Hong Kong Polytechnic University for providing and maintaining the equipment and technical support needed for flow cytometry and imaging work as well as the Centralized Animal Facility at the Hong Kong Polytechnic University for supporting our animal studies. We thank Mr. Chi-Ho Lin (University of Hong Kong) for his help to deposit the RNA sequencing data to GEO database. This study was supported by the RGC General Research Fund (15103817 to T.K. Lee), the Theme-based Research Scheme project (T12-704/16-R to T. K. Lee, S. Ma, and I.O.L. Ng), Project of Strategic Importance (PolyU)(FAST-5 to T. K. Lee, Q. Zhao, C. Ma), and Research Impact Fund (R5050-18F to T.K. Lee).

References

1. Bray F, Ferlay J, Soeriomataram I, Siegel RL, Torre LA, Jermal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68:394-424.
2. Llovet JM, Di Bisceglie AM, Bruix J, Kramer BS, Lencioni R, Zhu AX, et al. Design and endpoints of clinical trials in hepatocellular carcinoma. J Natl Cancer Inst 2008;100:698-711.
3. Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, et al. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. Lancet Oncol 2009; 10: 25-34.
4. Lo J, Lau EY, Ching RH, Cheng BY, Ma MK, Ng IO, et al. NF-κB mediated CD47 upregulation promotes sorafenib resistance and its blockade synergizes the effect of sorafenib in hepatocellular carcinoma. Hepatology 2015; 62:534-45.
5. Wilson GS, Hu Z, Duan W, Tian A, Wang XM, McLeod D, et al. LCSCs Efficacy of using cancer stem cell markers in isolating and characterizing liver cancer stem cells. Stem Cells Dev 2013; 22:2655-64.
6. Xin HW, Ambe CM, Hari DM, Wiegand GW, Miller TC, Chen JQ, et al. Label-retaining liver cancer cells are relatively resistant to sorafenib. Gut 2013; 62:1777-86.
7. Alazzouzi H, Davalos V, Kokko A, Domingo E, Woerner SM, Wilson AJ, et al. Mechanisms of inactivation of the receptor tyrosine Kinase EPHB2 in colorectal tumors. Cancer Res 2005; 65:10170-3.
8. Lefeuvre M, Gunduz M, Nagatsuka H, Gunduz E, Al Sheikh Ali M, et al. Fine deletion analysis of 1p36 chromosomal region in oral squamous cell carcinomas. J Oral Pathol Med 2009; 38:94-8.
9. Genander M, Halford MM, Xu NJ, Eriksson M, Yu Z, Qiu Z, et al. Dissociation of EphB2 signaling pathways mediating progenitor cell proliferation and tumor suppression. Cell 2009; 139:679-92.
10. Nakada M, Anderson EM, Demuth T, Nakada S, Reavie LB, Drake KL, et al. The phosphorylation of ephrin-B2 ligand promotes glioma cell migration and invasion. Int J Cancer 2010; 126:1155-65.

11. Leung CO, Tong M, Chung KP, Zhou L, Che N, Tang KH, et al. Overriding adaptive resistance of sorafenib through combination therapy with SHP2 blockade in hepatocellular carcinoma. Hepatology 2020; 72:155-68.
12. Lau EY, Lo J, Cheng BY, Ma MK, Lee MF, Ng KY, et al. Cancer-associated fibroblasts regulate tumor initiating cell plasticity in hepatocellular carcinoma through c-Met/FRA1/HEY1 signaling. Cell Rep 2016; 15: 1175-89.
13. Chan LH, Zhou L, Ng KY, Wong TL, Lee TK, Sharma R, et al. PRMT6 regulates RAS/RAF binding and MEK/ERK-mediated cancer stemness activities in hepatocellular carcinoma through CRAF methylation. Cell Rep 2018, 25: 690-701.
14. Clevers H, Batlle E. EphB/EphrinB receptors and Wnt signaling in colorectal cancer. Cancer Res 2006; 66:2-5.
15. Liu C, Li Y, Semenov M, Han C, Baeg GH, Tan Y, et al. Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell 2002; 108:837-47.
16. Keng VW, Sia D, Sarver AL, Tschida BR, Fan DH, Alsinet C, et al. Sex Bias occurrence of hepatocellular carcinoma in Poly7 molecular subclass is associated with EGFR. Hepatology 2013; 57:120-30.
17. Ma S, Chan KW, Hu L, Lee TK, Wo JY, Ng IO, et al. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. Gastroenterology 2007; 132:2542-56.
18. Yang ZF, Ho DW, Ng MN, Lau CK, Yu WC, Ngai P, et al. Significance of CD90+ cancer stem cells in human liver cancer. Cancer Cell 2008; 13:153-66.
19. Haraguchi N, Ishii H, Mimori K, Tanaka F, Ohkuma M, Kim HM, et al. CD13 is a therapeutic target in human liver cancer stem cells. J Clin Invest 2010; 120:3326-39.
20. Lee TK, Castilho A, Cheung VC, Tang KH, Ma S, Ng IO. CD24+ liver tumor-initiating cells drive self-renewal and tumor initiation through Stat3-mediated nanog regulation. Cell Stem Cell 2011; 9:50-63.
21. Yamashita T, Budhu A, Forgues M, and Wang XW. Activation of hepatic stem cell marker EpCAM by Wnt-beta-catenin signaling in HCC. Cancer Res 2007; 67: 10831-9.
22. Pasquale EB. Eph receptors and ephrins in cancer: bidirectional signalling and beyond. Nat Rev Cancer 2010; 10:165-80.

23. Pasquale EB. Eph-ephrin bidirectional signaling in physiology and disease. Cell 2008; 133, 38–52.
24. Kataoka H, Tanaka M, Kanamori M, Yoshii S, Ihara M, Wang YJ, et al. Expression profile of EFNB1, EFNB2, two ligands of EPHB2 in human gastric cancer. J Cancer Res Clin Oncol 2002; 128:343-8.
25. Mimche PN, Brady LM, Bray CF, Lee CM, Thapa M, King TP, et al. The receptor tyrosine kinase EphB2 promotes hepatic fibrosis in mice. Hepatology 2015; 62:900-14.
26. Mimche PN, Lee CM, Mimche SM, Thapa M, Grakoui A, Henkemeyer M, et al. EphB2 receptor tyrosine kinase promotes hepatic fibrogenesis in mice via activation of hepatic stellate cells. Sci Rep 2018;8:2532.
27. Batlle E, Henderson JT, Beghtel H, van den Born MM, Sancho E, Huls G, et al. Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. Cell 2002; 111:251-63.
28. Merlos-Suárez A, Barriga FM, Jung P, Iglesias M, Céspedes MV, Rossell D, et al. The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse. Cell Stem Cell 2011; 8:511-24.
29. Lee TK, Cheung VC, Lu P, Lau EY, Ma S, Tang KH, et al. Blockade of CD47-mediated cathepsin S/protease-activated receptor 2 signaling provides a therapeutic target for hepatocellular carcinoma. Hepatology 2014; 60:179-91.
30. Alapin JM, Dines M, Vassiliev M, Tamir T, Ram A, Locke C, et al. Activation of EphB2 Forward Signaling Enhances Memory Consolidation Cell Rep 2018;23:2014-25.
31. Zisch AH, Kalo MS, Chong LD, Pasquale EB. Complex formation between EphB2 and Src requires phosphorylation of tyrosine 611 in the EphB2 juxtamembrane region. Oncogene 1998; 16:2657-70.
32. Pan XY, You HM, Wang L, Bi YH, Yang Y, Meng HW, et al. Methylation of RCAN1.4 mediated by DNMT1 and DNMT3b enhances hepatic stellate cell activation and liver fibrogenesis through Calcineurin/NFAT3 signaling. Theranostics 2019; 9:4308-23.

Figure Legends

Fig. 1. EPHB2 expression was upregulated in sorafenib-resistant PDTXs with clinical significance. (A) Sorafenib-resistant PDTX#1 and PDTX#5 were established by administering sorafenib orally at 100 mg/kg for three rounds and five rounds, respectively. **(B)** The dose response curves showed that there was no tumor reduction upon sorafenib administration, when compared with their corresponding mock controls. **(C)** Sorafenib PDTXs exhibited enhanced abilities in sphere formation (****p<0.0001, t test) and tumor initiation in NOD/SCID mice (500 cells were injected subcutaneously). **(D)** Venn diagram showed 67 upregulated genes common in two sorafenib-resistant PDTXs with 2-fold cut off (p<0.05). EPHB2 was found to be the most significant kinase commonly upregulated in two sorafenib-resistant PDTXs **(E)** By qPCR analysis, we confirmed the upregulation of EPHB2 expression in sorafenib-resistant PDTXs. **(F)** By analysis of dataset GSE25097, we found upregulation of EPHB2 mRNA expression in a cohort of 243 paired HCC and nontumor samples (****p<0.0001, t test). In addition, EPHB2 expression was upregulated in cirrhotic samples compared with healthy donor samples (**p<0.01, t test) **(G)** In a STAM^{TM} nonalcoholic steatohepatitis (NASH) HCC mouse model in which HCC develops from liver fibrosis, we found a stepwise increase in the expression of EPHB2 from the normal to the fibrotic and HCC stages (n=3, **p<0.01 and ***p<0.001, t test). **(H)** Patients with high EPHB2 expression (T/N > 6) showed shorter disease-free survival than those with low EPHB2 expression (T/N< 6) (p=0.041, log-rank test).

Fig. 2. Endogenous EPHB2 knockout in the liver of an immunocompetent HCC mouse model attenuates hepatocarcinogenesis. (A) Schematic representation of the hydrodynamic tail vein injection (HTVI) model established with C57BL/6 mice**. (B)** qPCR analysis of EPHB2 expression in mice that received HTVI of either an empty vector (EV) control (CTRL) or NRAS, AKT and sleeping beauty (SB) transposase for HCC induction with samples collected at various time points. The analysis showed that EPHB2 expression was increased in a stepwise manner from early to advanced HCC. n = 15 in total (EV n = 3, 1 W n = 3, 2 W n =3, 3 W n = 3, and 4 W n = 3). **(C)** Strategy for testing the functional significance of EPHB2 in hepatocarcinogenesis. NRAS, AKT and SB transposase were delivered together with either NTC/sgEPHB2 by HTVI for HCC induction. Mice

were sacrificed at 7 weeks post plasmid injection. **(D)** Representative images of dissected livers at the end of the experiment. Scale bar = 1 cm**. (E)** The number of tumor nodules (diameter≤0.25 cm) was significantly lower in mice that were administered sgEPHB2 than in those administered NTC. n= 7 per group (*p<0.05, t test). **(F)** Kaplan-Meier survival curves showing the tumor-free survival rate of each annotated group. n = 5 per group (p=0.0888). **(G)** Two representative livers (#1 & #2) from each group showed **i**ntense GFP signal in mice injected with either sgNTC or sgEPHB2. Scale bar = 2 cm**. (H)** qPCR analysis of EPHB2 expression in livers injected with NTC/sgEPHB2 compared with livers from naïve mice (*p<0.05, t test).

Fig. 3. EPHB2 regulates the cancer stemness and drug resistance of HCC cells. (A) Two different shEPHB2 sequences were used (shEPHB2-1 and shEPHB2-2). Western blotting showed the successful knockdown of EPHB2 expression in PLC/PRF/5 and MHCC-97L cells. Successful overexpression of EPHB2 (EPHB2 OE) in Huh7 cells was also confirmed. **(B)** Knockdown of EPHB2 expression in PLC/PRF/5 or MHCC-97L cells reduced the size and number of primary spheres and their secondary passages (*p<0.05 and **p<0.01, t test), while overexpression of EPHB2 in Huh7 cells increased the size and number of primary spheres and their secondary passages (*p<0.05, t test, scale bar = 100 μm). **(C)** Knockdown of EPHB2 expression in the PLC/PRF/5 and MHCC-97L cells suppressed tumorigenicity compared with NTC treatment. Representative photos show the results of injecting 50000 or 200 cells derived from PLC/PRF/5 or MHCC-97L cells. Overexpression of EPHB2 led to increased Huh7 cell tumorigenicity. Representative photographs show the results of injecting $6x10^5$ cells (scale bar = 1 cm). CSC frequency of each group was calculated. **(D)** Knockdown of EPHB2 expression decreased the expression of CD90 and CD47 in both PLC/PRF/5 cells and MHCC-97L cells compared with NTC cells (*p<0.05, **p<0.01, and ***p<0.001, t test). Overexpression of EPHB2 increased the expression of CD90 and CD47 in Huh7 cells (*p<0.05 and **p<0.01, t test). **(E)** Compared to NTC cells, shEPHB2 cells derived from PLC/PRF/5 or MHCC-97L cells showed a higher percentage of annexin V-positive cells in response to treatment with doxorubicin at 0.5 μg/mL and 1 μg/mL or sorafenib from 5 μM to 20 μM. Contour diagrams for lower doses of doxorubicin and sorafenib were shown. Compared to EV control cells, EPHB2 OE

cells showed a lower percentage of annexin V-positive cells in response to treatment with doxorubicin at 0.5 μg/mL or sorafenib at 15 μM (*p<0.05 and **p<0.01, t test).

Fig. 4. EPHB2^{High} HCC cells possessed stem/progenitor cell traits. (A) By cell sorting, MHCC-97L cells were separated into EPHB2^{High} and EPHB2^{Low} populations. Postsorting analysis showed that the EPHB2^{High} cells were 88.8% positive, while the EPHB2^{Low} cells were 0% positive. **(B)** Western blot analysis showed high expression of EPHB2 in EPHB2^{High} HCC cells. **(C)** In a sphere formation assay, *in vitro* self-renewal was found to be significantly enhanced in EPHB2^{High} MHCC-97L cells (*p<0.05, t test). **(D)** EPHB2^{High} HCC cells showed increased tumorigenicity compared with their low-expression counterparts. Representative images of 500 cells, 1000 cells, and 5000 cells are shown (scale bar = 1 cm). CSC frequency of each group was calculated. **(E)** EPHB2^{High} MHCC-97L cells showed increased expression of CD90 and CD47 (*p<0.05, t test). **(F)** An Annexin V staining assay performed with sorted MHCC-97L cells demonstrated that EPHB2^{High} HCC cells were more chemoresistant to treatment with doxorubicin at 0.5 μg/mL and 1 μg/mL or sorafenib at 15 μM and 20 μM than EPHB2^{Low} cells (*p<0.05 and **p<0.01, t test). Contour diagrams for lower doses of doxorubicin and sorafenib were shown.

Fig. 5. Wnt/β-catenin is the downstream effector of EPHB2 signaling in HCC. (A&B) RNA sequencing was performed to compare the genetic profiles of shEPHB2-2- and NTC control-transfected PLC/PRF/5 cells. By GSEA, we found that Wnt/β-catenin was enriched in shEPHB2-2 cells with a normalized ES score of -1.63 (FDR q-value of 0.019). **(C)** The heatmap shows the alterations in genes in the Wnt/β-catenin pathway (red represents upregulation; green represents downregulation). **(D)** The effect of EPHB2 alteration on the transactivation activity of β-catenin was examined with a TOP/FOP assay and by assessing the expression of β-catenin downstream genes. Compared with NTC treatment, EPHB2 knockdown significantly decreased the transactivation activity of β-catenin, as measured by a TOP/FOP assay and assessment of the expression of β-catenin downstream genes, including Cyclin D1 and CMYC (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, t test), while opposite effects were observed in EPHB2 OE cells (*p<0.05, **p<0.01, and ***p<0.001, t test). **(E)** IF staining of β-catenin showed reductions in the

total expression and nuclear expression of β-catenin in EPHB2-knockdown HCC cells (scale bar = 25 μm). EPHB2 OE cells exhibited increased nuclear and cytoplasmic expression of β-catenin. Arrows indicate nuclear expression of β-catenin. **(F)** By immunoprecipitation**,** we found that EPHB2 interacts with SRC through a protein interaction (IP: EPHB2; IB: SRC). **(G)** Upon EPHB2 knockdown in MHCC-97L or PLC/PRF/5 cells, the expression of pSRC (Y416), AKT (Ser473), pGSK3β (Ser9) and β-catenin was downregulated, while the levels of these proteins were upregulated in EPHB2 OE cells. **(H)** By TCGA data analysis, we found that Wnt/β-catenin was enriched in HCC patients with high EPHB2 expression with a normalized ES score of 1.89 (FDR q-value of 0.14).

Fig. 6. TCF1/β-catenin is the upstream regulator of EPHB2 signaling in HCC. (A&B) Upon transfection of Δ-45β-catenin into Huh7 cells, Wnt/β-catenin activity was enhanced compared to that of wild-type β-catenin (WT), as evidenced by analysis of the expression of β-catenin downstream genes and a TOP/FOP assay (*p<0.05 and **p<0.01, t test). **(C)** Upon activation of Wnt/β-catenin activity, both the mRNA and protein levels of EPHB2 were upregulated (**p<0.01, t test). **(D)** After the addition of conditioned medium containing Wnt3a, the mRNA and protein levels of EPHB2 were similarly upregulated in Huh7 cells, when compared with CTRL (**p<0.01, t test). **(E)** Compared to injection of WT control, hydrodynamic injection of β-catenin-S33Y together with shp53 using the sleeping beauty transposon system increased EPHB2 mRNA expression in the liver (*p<0.05, t test). **(F)** *In silico* analysis identified one TCF1 putative binding site in the promoter region of EPHB2. **(G)** TCF1 expression was suppressed in shEPHB2-1 cells derived from PLC/PRF/5 or MHCC-97L cells. **(H)** Knockdown of EPHB2 expression in PLC/PRF/5 and MHCC-97L cells reduced the fold enrichment of binding to the EPHB2 promoter, as shown by ChIP assays (*p<0.05, **p<0.01, t test). **(I)** In a TCGA cohort, high EPHB2 expression was significantly associated with the presence of the CTNNB1 mutation (p<0.001).

Fig. 7. rAAV-8-shEPHB2 administration sensitized HCC cells to sorafenib treatment in NRAS/AKT driven HCC. (A) Schematic diagram of the treatment regimen with rAAV-8-NTC, rAAV-8-shEPHB2, sorafenib, or the combination of rAAV-8-shEPHB2 with sorafenib. **(B)** Representative GFP signal

images of HCC tumors derived from the four groups at the endpoint are shown. Scale bar = 2 cm. **(C)** Representative images of HCC tumors derived from the four groups at the endpoint are shown. Scale bar = 2 cm. **(D)** Graphs showing the liver/body weight ratio generated from mice in each treatment group. (*p<0.05, **p<0.01, ****p<0.0001, t test). $n = 5\text{-}6$ per group. **(E)** H&E and IHC analysis for EPHB2 expression in sections of harvested livers of mice injected with shNTC or shEPHB2 rAAV-8 particles. Scale bar = 100µm.