Oncogene (2015) **34,** 5524–5535

www.nature.com/onc

ORIGINAL ARTICLE

PROX1 promotes hepatocellular carcinoma proliferation and sorafenib resistance by enhancing β-catenin expression and nuclear translocation

Y Liu[1,2,6], X Ye[1,6], J-B Zhang[3,6], H Ouyang[1,4], Z Shen[1], Y Wu[1], W Wang[1], J Wu[1], S Tao[1], X Yang[1], K Qiao[1], J Zhang[1], J Liu[1], Q Fu[1,5] and Y Xie[1]

Aberrant activation of the Wnt/β-catenin pathway is frequent in hepatocellular carcinoma (HCC) and contributes to HCC initiation and progression. This abnormal activation may result from somatic mutations in the genes of the Wnt/β-catenin pathway and/or dysregulation of the Wnt/β-catenin pathway. The mechanism for the latter remains poorly understood. Prospero-related homeobox 1 (*PROX1*) is a downstream target of the Wnt/β-catenin pathway in human colorectal cancer and elevated *PROX1* expression promotes malignant progression. However, the Wnt/β-catenin pathway does not regulate *PROX1* expression in the liver and HCC cells. Here we report that PROX1 promotes HCC cell proliferation *in vitro* and tumor growth in HCC xenograft mice. PROX1 and β-catenin levels are positively correlated in tumor tissues as well as in cultured HCC cells. PROX1 can upregulate *β-catenin* transcription by stimulating the β-*catenin* promoter and enhance the nuclear translocation of β-catenin in HCC cells, which leads to the activation of the Wnt/β-catenin pathway. Moreover, we show that increase in *PROX1* expression renders HCC cells more resistant to sorafenib treatment, which is the standard therapy for advanced HCC. Overall, we have pinpointed PROX1 as a critical factor activating the Wnt/β-catenin pathway in HCC, which promotes HCC proliferation and sorafenib resistance.

Oncogene (2015) **34,** 5524–5535; doi:10.1038/onc.2015.7; published online 16 February 2015

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men, the seventh in women and the third leading cause of cancer-related deaths worldwide.[1] Surgical resection is often impossible for HCC patients diagnosed at advanced tumor stages. Sorafenib, an oral multikinase inhibitor of tumor cell proliferation and angiogenesis, is currently the standard of care for unresectable advanced HCC. Unfortunately, despite its beneficial effect on overall survival, response rate to sorafenib in advanced HCC patients is ~2% and most patients progress within 6 months after treatment.[2]

The Wnt/β-catenin pathway plays a key role in regulating cell proliferation, differentiation and stemness in various physiological and pathological settings. The Wnt/β-catenin pathway is activated upon the binding of certain Wnt ligands to frizzled receptors, followed by stabilization of cytosolic β-catenin through prevention of β-catenin phosphorylation by the destruction complex composed of AXIN, APC, GSK3 and CK1 and subsequent proteasomal degradation.[3] Stabilized cytosolic β-catenin then translocates into the nucleus to initiate transcription of Wnt target genes such as *c-MYC* and *cyclin D1* through interaction with T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors.

The Wnt/β-catenin pathway is heavily involved in hepatocarcinogenesis.[3,4] Aberrant activation of the Wnt/β-catenin signaling in HCC has been identified by using whole genome expression profiling.[5] Activation of the Wnt/β-catenin pathway in HCC may result from somatic mutations in the *β-catenin* and *Axin1* genes.[5–7] Further evidence also implicates dysregulation of the Wnt/β-catenin pathway through non-mutational but as yet unclear mechanisms in HCC development.[5] It is notable that *β-catenin* messenger RNA (mRNA) levels are often up-regulated in HCC,[8,9] suggesting that transcriptional dysregulation of *β-catenin* might contribute to HCC development. Several transcription factors such as AP1, TCF/LEF, NKX2-5, TRβ, ZNF191 have been shown to bind to the *β-catenin* promoter and regulate *β-catenin* transcription.[10–13] Nonetheless the mechanism of the transcriptional dysregulation of *β-catenin* in HCC is unclear. Increasing evidence also points to potential involvement of the Wnt/β-catenin pathway in sorafenib's mode of action.[14,15] Sorafenib has been shown to attenuate the Wnt/β-catenin signaling in several experimental models of liver cancer.[16]

Prospero-related homeobox 1 (PROX1) is an essential regulator in the development of multiple organs and tissues including central nervous system, heart, eye, lymphatic vessel, pancreas, and liver.[17–23] The relationship between PROX1 and cancer development is complex. PROX1 exhibits either tumor-suppressing or oncogenic activity depending on cancer types.[24] Consistent with *PROX1*'s essential role in promoting hepatocyte

[1]Key Laboratory of Medical Molecular Virology (MOE & MOH), Institute of Biomedical Sciences, Shanghai Medical College, Fudan University, Shanghai, China; [2]Department of Surgery, Huashan Hospital, Fudan University, Shanghai, China; [3]Liver Cancer Institute, Zhongshan Hospital; Key Laboratory of Carcinogenesis and Cancer Invasion (MOE), Fudan University, Shanghai, China; [4]Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China and [5]Department of Immunology, Binzhou Medical University, Yantai, China. Correspondence: Dr J Liu or Dr Y Xie, Key Laboratory of Medical Molecular Virology (MOE & MOH), Institute of Biomedical Sciences, Shanghai Medical College, Fudan University, Yixueyuan Road 138, Shanghai 200032, China or Dr Q Fu, Department of Immunology, Binzhou Medical University, Yantai 264003, China.
E-mail: liujing212@fudan.edu.cn or yhxie@fudan.edu.cn or qiangfu11@fudan.edu.cn
[6]These authors contributed equally to this work.
Received 19 May 2014; revised 19 November 2014; accepted 25 November 2014; published online 16 February 2015

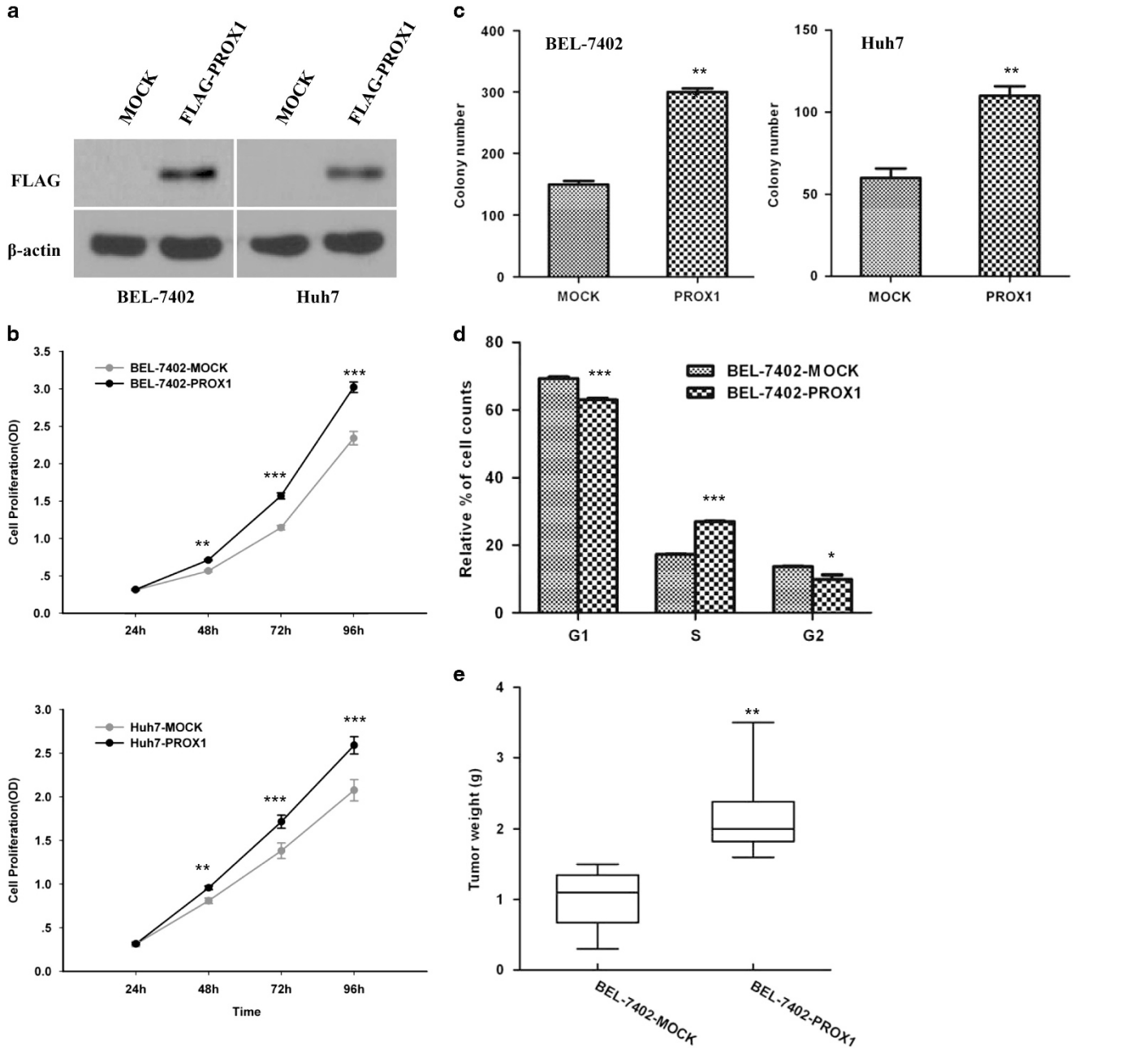


Figure 1. *PROX1* overexpression in HCC cells promotes cell proliferation *in vitro* and tumor growth *in vivo*. (**a**) Western blot verification of *PROX1* overexpression in BEL-7402 and Huh7 cells. Flag-tagged PROX1 was detected by anti-Flag mAb. (**b**) Cell proliferation and (**c**) colony formation of BEL-7402 and Huh7 cells upon *PROX1* overexpression. Means ± s.d. from three independent experiments were presented. (**d**) Cell cycle distribution of BEL-7402 cells upon *PROX1* overexpression. (**e**) *PROX1* overexpression promoted HCC growth in tumor xenograft mice. BEL-7402-MOCK and BEL-7402-PROX1 were inoculated into liver parenchyma in nude mice respectively (2.0×10^6 cells/mouse, $n = 6$). Tumor weight was measured after 8 weeks post inoculation. Significant differences were determined using Student's-*t* test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

migration in the developing liver,[23] we recently demonstrated that elevated-PROX1 protein level in HCC is an important risk factor for HCC invasion and metastasis associated with significantly worse clinical outcomes.[25] PROX1 also regulates the development of murine hepatic stem/progenitor cells and induces fetal hepatocyte proliferation.[26] It is as yet not known what kind of a role PROX1 has in HCC cell proliferation. The relationship between PROX1 and the Wnt/β-catenin pathway is also complex. In the adult mammalian hippocampus and human colorectal cancer, PROX1 has been identified as a downstream target of the Wnt/β-catenin signaling.[27,28] However, there is evidence indicating that the Wnt/β-catenin pathway does not regulate PROX1 expression in liver and HCC.[28]

In this study we investigated the relationship between PROX1 and Wnt/β-catenin pathway in HCC and its impact on HCC proliferation. We report here a crucial role of PROX1 in promoting HCC cell proliferation *in vitro* and tumor growth in HCC xenograft mice. PROX1 and β-catenin levels are positively correlated in HCC tissues and cultured HCC cells. Instead of being a downstream target of the Wnt/β-catenin pathway, we show that PROX1 acts upstream of the Wnt/β-catenin pathway in HCC. PROX1 can upregulate *β-catenin* transcription and promote nuclear

translocation of β-catenin, leading to the activation of the Wnt/β-catenin pathway and enhanced expression of Wnt target genes *c-MYC* and *cyclin D1*. Moreover, we show that increase in PROX1 expression renders HCC cells more resistant to sorafenib treatment *in vitro* and in HCC xenograft mice.

RESULTS

PROX1 promotes HCC cell proliferation *in vitro* and tumor growth in HCC xenograft mice

We previously demonstrated that PROX1 protein levels differ considerably among different HCC cell lines, ranging from being hardly detectable in BEL-7402 cells to quite abundant in Huh7 and MHCC-97H cells.[25] These cell lines are valuable tools for investigating PROX1's functions in HCC cell proliferation. Lentivirus-mediated overexpression of *PROX1* (in BEL-7402 and Huh7 cells) and knockdown of endogenous *PROX1* (in MHCC-97H and Huh7 cells) were performed (Figures 1a and 2a) and cell proliferation was analyzed using several assays. Overexpression of *PROX1* in BEL-7402 and Huh7 cells promoted cell growth (Figure 1b) and colony formation (Figure 1c). Cell cycle analysis demonstrated that there were fewer cells residing at the G1 and G2 phases and more cells at the S phase among *PROX1*-overexpressing BEL-7402 cells compared with control cells (Figure 1d). In contrast, knockdown of *PROX1* expression in MHCC-97H and Huh7 cells resulted in reduced cell growth (Figure 2b) and colony formation (Figure 2c). Percentage of G1 phase cells increased while percentage of S phase cells decreased in *PROX1*-knockdown MHCC-97H cells compared with control cells (Figure 2d).

The *in vivo* effect of PROX1 on HCC growth was further assessed in nude mice. Eight weeks after inoculation of BEL-7402 cells infected with either the *PROX1*-expressing lentivirus (BEL-7402-PROX1) or the vector lentivirus (BEL-7402-MOCK) into the liver parenchyma of nude mice, the BEL-7402-PROX1 group developed significantly larger tumors compared with the BEL-7402-MOCK group (Figure 1e). On the other hand, eight weeks after injection of MHCC-97H cells infected with the lentivirus expressing either *PROX1* si1646 precursor (MHCC-97H-si1646) or scrambled siRNA precursor control (MHCC-97H-Scr), the MHCC-97H-si1646 group developed significantly smaller tumors compared with the MHCC-97H-Scr group (Figure 2e). Taken together, these results indicate that PROX1 promotes HCC cell proliferation *in vitro* and tumor growth in HCC xenograft mice.

PROX1 and β-catenin expression levels are positively correlated in HCC

The Wnt/β-catenin pathway is often aberrantly activated during HCC development.[3–5] To assess any possible relationship between PROX1 expression and the activation of the Wnt/β-catenin pathway in HCC, we firstly examined the expression of PROX1 and β-catenin in tumor tissues from HCC patients. Immunohistochemistry (IHC) staining demonstrated that PROX1 and β-catenin expression levels were positively correlated in HCC tissues. Strong staining of β-catenin was observed in 73.5% (25/34) of HCC cases with high levels of PROX1, but only in 30.8% (8/26) of HCC cases with low levels of PROX1 (Figure 3a). The difference was significant (Fisher's exact test, $P < 0.001$). Consistent with this observation *in vivo*, the levels of β-catenin increased in BEL-7402, Huh7 (Figure 3b) and non-HCC HEK293T cells (Supplementary Figure 1) upon *PROX1* overexpression, but decreased in *PROX1*-knockdown MHCC-97H and Huh7 cells (Figure 3c).

PROX1 upregulates β-catenin transcription in HCC cells

To probe the mechanisms underlying the correlation between PROX1 and β-catenin levels in HCC, we tested the effects of PROX1 on *β-catenin* transcription, which is often upregulated in clinical HCC samples.[8,9] Indeed, *β-catenin* mRNA levels were significantly increased in BEL-7402 and Huh7 cells upon *PROX1* overexpression (Figure 4a), while they were significantly reduced after *PROX1*-knockdown in MHCC-97H and Huh7 cells (Figure 4b). Moreover, in HEK-293T that lacks endogenous *PROX1* expression, promoter luciferase reporter assays showed that exogenous PROX1 could dose-dependently activate the full-length *β-catenin* promoter (Figure 4c). To delineate the responsive region for PROX1 in the *β-catenin* promoter, serially truncated *β-catenin* promoter reporters were constructed and co-transfected, respectively, with *PROX1* expression construct into HEK-293T cells. The P(−1907/+93) construct exhibited a markedly higher PROX1-inducibility than did the P(−2692/+93) (full length) and P(−1407/+93) constructs. Further deletions toward downstream promoter as represented by the P(−907/+93) and P(−407/+93) constructs resulted in the loss of responsiveness to PROX1 stimulation (Figure 4d). The responsive region for PROX1 thus likely falls within the section between −1907 and −1407 of the *β-catenin* promoter, while the −2692/−1908 segment probably mediates negative modulation of PROX1-induced transcription upregulation. Finally, ChIP assays showed that immunoprecipitation by anti-PROX1 mAb of chromatin fragments from MHCC-97H and Huh7 cells specifically enriched *β-catenin* promoter sequences (Figure 4e), suggesting that endogenous PROX1 is associated with the *β-catenin* promoter in these HCC cells. The results collectively indicate that PROX1 upregulates *β-catenin* transcription in HCC cells, most likely through acting on its promoter.

PROX1 promotes nuclear translocation of β-catenin

One hallmark indicator of the Wnt/β-catenin pathway activation is β-catenin translocation into nucleus. In HCC IHC analyses, we noticed that 18 out of 25 (72%) of PROX1/β-catenin double high expression samples displayed stronger β-catenin signals in and around nuclei, different from mostly plasma membrane associated signals observed in other samples (Figure 3a, right panel). We went on to investigate whether PROX1 expression level might also correlate with β-catenin nuclear translocation in cultured HCC cells. Despite the marked effects on total cellular β-catenin levels imposed by PROX1 overexpression or knockdown (Figures 3b and c), there were only marginal differences in the cytoplasmic level of β-catenin between control cells and BEL-7402 and Huh7 cells overexpressing *PROX1*, or between MHCC-97H and Huh7 cells whose endogenous *PROX1* expression was knocked down (Figures 5a and b). However, protein levels of β-catenin in nuclear extracts increased significantly in *PROX1*-overexpressing BEL-7402 and Huh7 cells (Figure 5a), while they were reduced in *PROX1*-knockdown MHCC-97H and Huh7 cells (Figure 5b). Immunofluorescence also showed that overexpression of exogenous *PROX1* indeed promoted β-catenin translocation away from cytoplasmic membrane toward and into the nucleus in BEL-7402, Huh7 and HEK293T cells (Figure 5c & Supplementary Figure 1), resembling the pattern observed in PROX1/β-catenin double high-expression samples (Figure 3a, right panel). Interestingly, PROX1-overexpressing Huh7 cells displayed apparently more complete nuclear translocation of β-catenin compared with PROX1-overexpressing BEL-7402 cells (Figure 5c), which might be the result of different endogenous PROX1 levels and possibly also some other unknown differences between the two cell lines.

PROX1 activates the Wnt/β-catenin pathway in HCC cells

The PROX1-mediated upregulation of *β-catenin* expression and nuclear accumulation of β-catenin suggest that PROX1 might activate the Wnt/β-catenin pathway in HCC cells. To test this hypothesis, we investigated whether PROX1 would activate the expression of Wnt/β-catenin target genes *c-MYC* and *cyclin D1*. As shown in Figure 6a, *PROX1* overexpression in BEL-7402 and Huh7

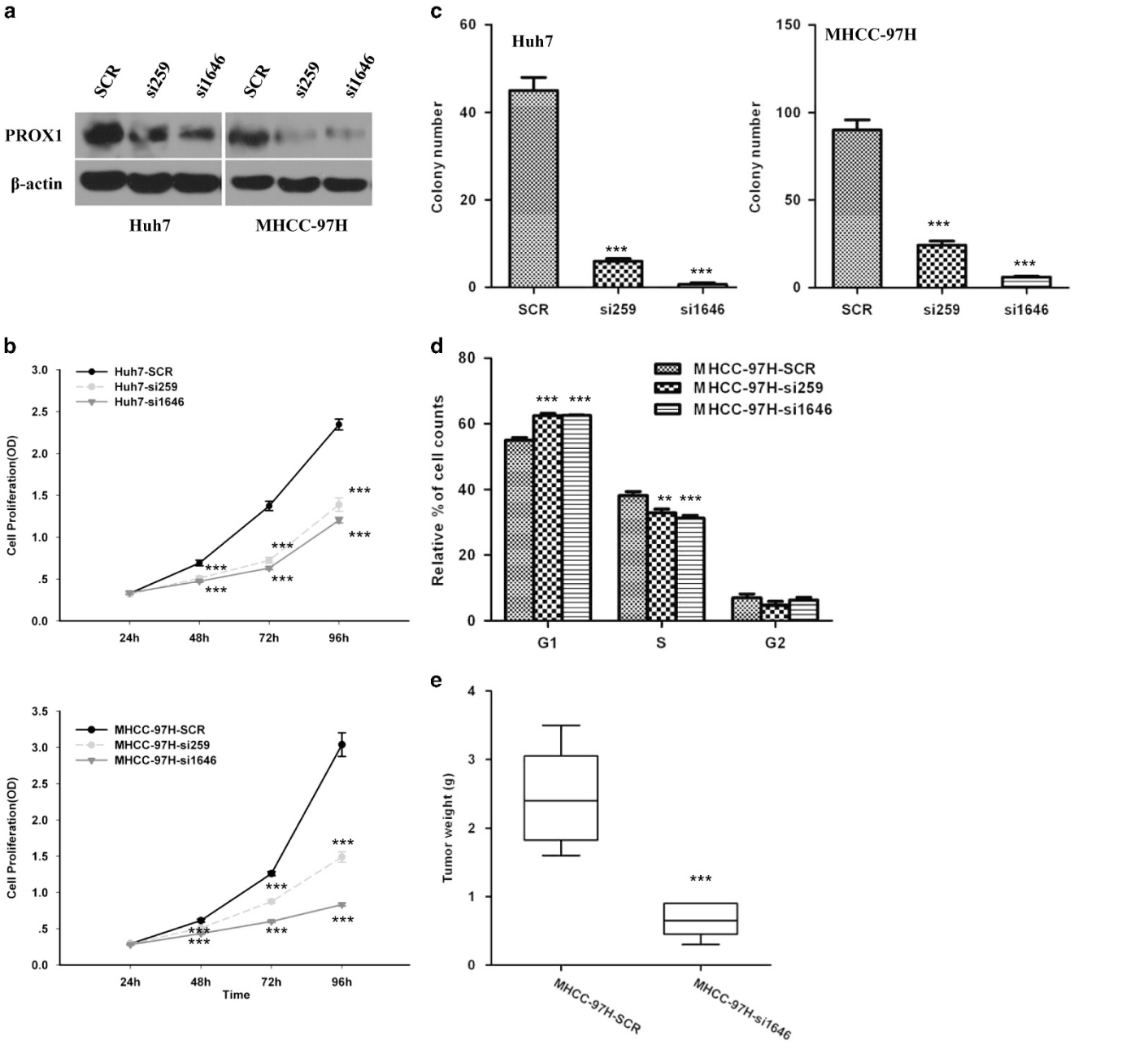


Figure 2. Knockdown of *PROX1* expression in HCC cells reduces cell proliferation *in vitro* and tumor growth *in vivo*. (**a**) Western blot verification of knockdown of endogenous *PROX1* expression in MHCC-97H and Huh7 cells. PROX1 was detected by anti-PROX1 mAb. (**b**) Cell proliferation and (**c**) colony formation of MHCC-97H and Huh7 cells upon knockdown of *PROX1* expression. Means ± s.d. from three independent experiments were presented. (**d**) Cell cycle distribution of MHCC-97H cells upon knockdown of *PROX1* expression. (**e**) Knockdown of *PROX1* expression reduced HCC growth in tumor xenograft mice. MHCC-97H-SCR and MHCC-97H-si1646 were injected subcutaneously into nude mice respectively (1.0×10^7 cell/mouse, $n = 6$). Tumor weight was measured after 8 weeks post injection. Significant differences were determined using Student's-*t* test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

cells significantly increased both the mRNA and protein levels of c-MYC and cyclin D1, whereas knockdown of *PROX1* expression in MHCC-97H and Huh7 cells had the opposite effects (Figure 6b). We further assessed whether PROX1 would activate the universal β-catenin/LEF-responsive promoter (LEF reporter) commonly used as indicator of the Wnt/β-catenin pathway activity level. Indeed, *PROX1* overexpression was able to activate the LEF reporter in BEL-7402 and Huh7 cells (Figure 6c), while knockdown of *PROX1* expression in MHCC-97H and Huh7 cells reduced the LEF reporter activation (Figure 6d). Collectively, the data indicate that PROX1 can activate the Wnt/β-catenin pathway in HCC cells.

PROX1 contributes to HCC resistance to sorafenib *in vitro* and in tumor xenograft mice

Given the opposite effects on the Wnt/β-catenin signaling by sorafenib and PROX1, we hypothesized that HCC cells with a high level of *PROX1* expression would respond differently to sorafenib treatment than HCC cells with relatively low PROX1 expression. Indeed, *PROX1* overexpression in BEL-7402 increased sorafenib resistance at all the tested sorafenib concentrations, and in Huh7 cells at drug concentrations above 5 μM (Figure 7a). On the other hand, knockdown of *PROX1* expression in MHCC-97H and Huh7 cells apparently made these cells more sensitive to

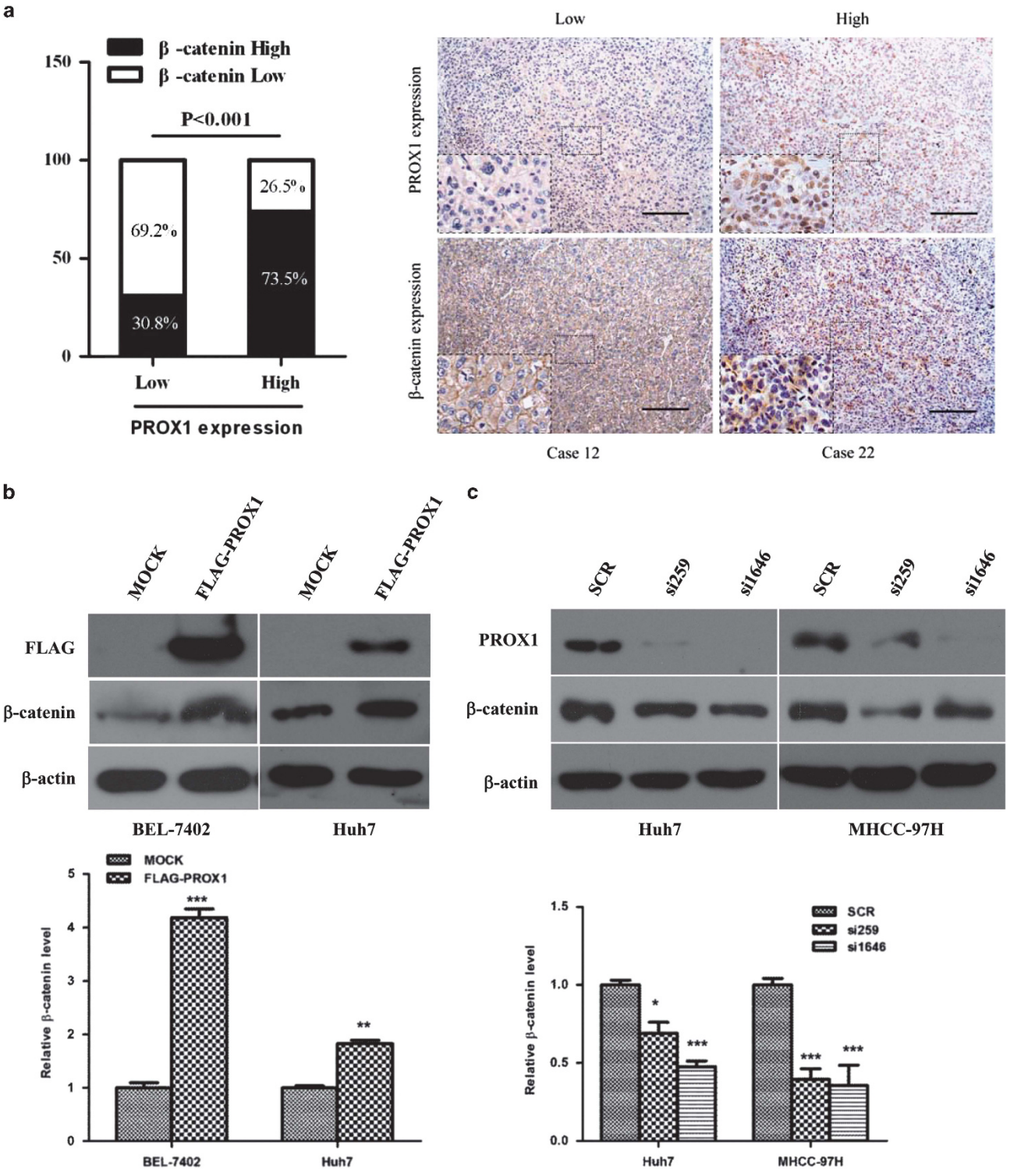


Figure 3. PROX1 and β-catenin expression levels are positively correlated in HCC. (**a**) IHC revealed that PROX1 and β-catenin expression levels were positively correlated in HCC tissues. IHC results were independently graded by two experienced pathologists. Samples with grades 0 (no staining) and 1 (weak staining) were grouped as 'low' expression, and samples with grade 2 (strong staining) were grouped as 'high' expression. Representative IHC staining images of PROX1 and β-catenin are shown on the right. Scale bars, 100 μm. (**b**) Overexpression of *PROX1* in BEL-7402 and Huh7 cells increased β-catenin expression. (**c**) Knockdown of *PROX1* expression in MHCC-97H and Huh7 cells reduced β-catenin expression. Densitometry scanning data of three independent repeat blots were plotted and presented as relative β-catenin levels against Mock or SCR. Significant differences were determined using Student's-*t* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

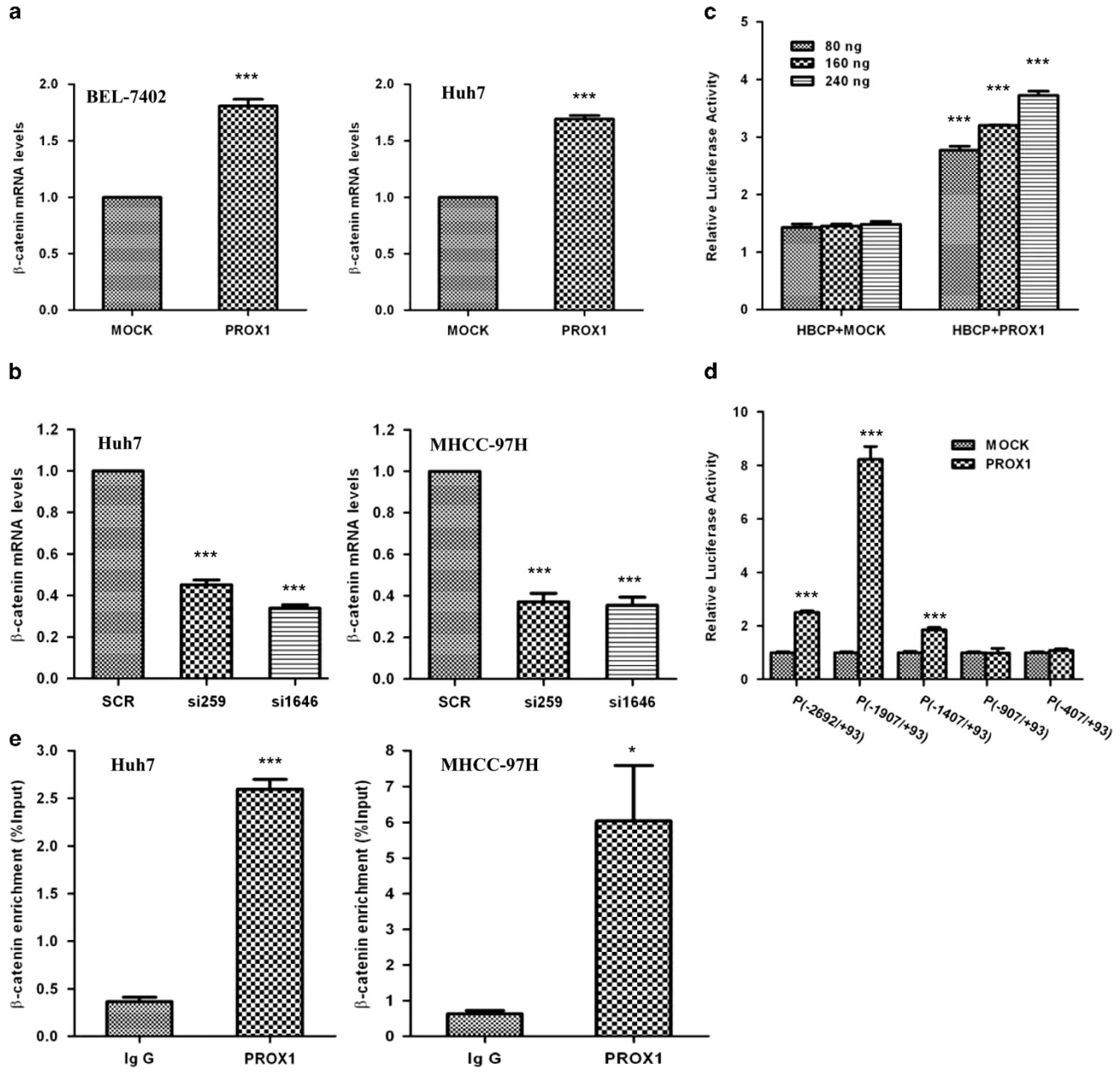


Figure 4. PROX1 activates *β-catenin* transcription. (**a**) *PROX1* overexpression in HCC cells increased *β-catenin* mRNA expression. BEL-7402 and Huh7 cells were infected with the vector lentivirus or the *PROX1*-expressing lentivirus. *β-catenin* mRNA was measured by using qrtPCR. Means ± s.d. from three independent experiments were presented as relative ratio to the control whose value was taken as 1.0. (**b**) Knockdown of *PROX1* expression in HCC cells reduced *β-catenin* mRNA expression. Huh7 and MHCC-97H cells were infected with the lentivirus either expressing *PROX1* siRNA precursor (si259 and si1646) or scrambled siRNA (SCR). Means ± s.d. from three independent experiments were presented as relative ratio to the control whose value was taken as 1.0. (**c**) PROX1 dose-dependently activated the full-length *β-catenin* promoter. HEK293T cells were co-transfected with indicated plasmids. Means ± s.d. of normalized luciferase activity from three independent experiments were presented. (**d**) Identification of the response region for PROX1 in the *β-catenin* promoter. HEK293T cells were co-transfected with various deletion constructs of the *β-catenin* promoter reporter and PROX1 expression construct. Means ± s.d. of normalized luciferase activity from three independent experiments were presented. (**e**) Endogenous PROX1 associated with the *β-catenin* promoter. ChIP-PCR was performed with sonicated chromatins immunoprecipitated from Huh7 and MHCC-97H cells by anti-PROX1 mAb or pre-immune IgG (control). *β-catenin* promoter segments were quantified by using qrtPCR against 5% input. Means ± s.d. from three independent experiments were presented. Significant differences were determined using Student's-*t* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

sorafenib at drug concentrations above 2.5 μM (Figure 7b). Consistent with this observation, *PROX1* overexpression in BEL-7402 cells counteracted sorafenib's inhibition on the LEF reporter activation, while knockdown of *PROX1* expression in MHCC-97H cells enhanced sorafenib's inhibitory effect (Figure 7c). Furthermore, in tumor xenograft mouse models, the proliferation of BEL-7402 cells with *PROX1* overexpression (BEL-7402-PROX1) was apparently less sensitive to sorafenib inhibition compared to that of the control BEL-7402 cells (BEL-7402-MOCK). These results together indicate that increase in PROX1 expression renders HCC cells more resistant to sorafenib treatment *in vitro* and *in vivo*.

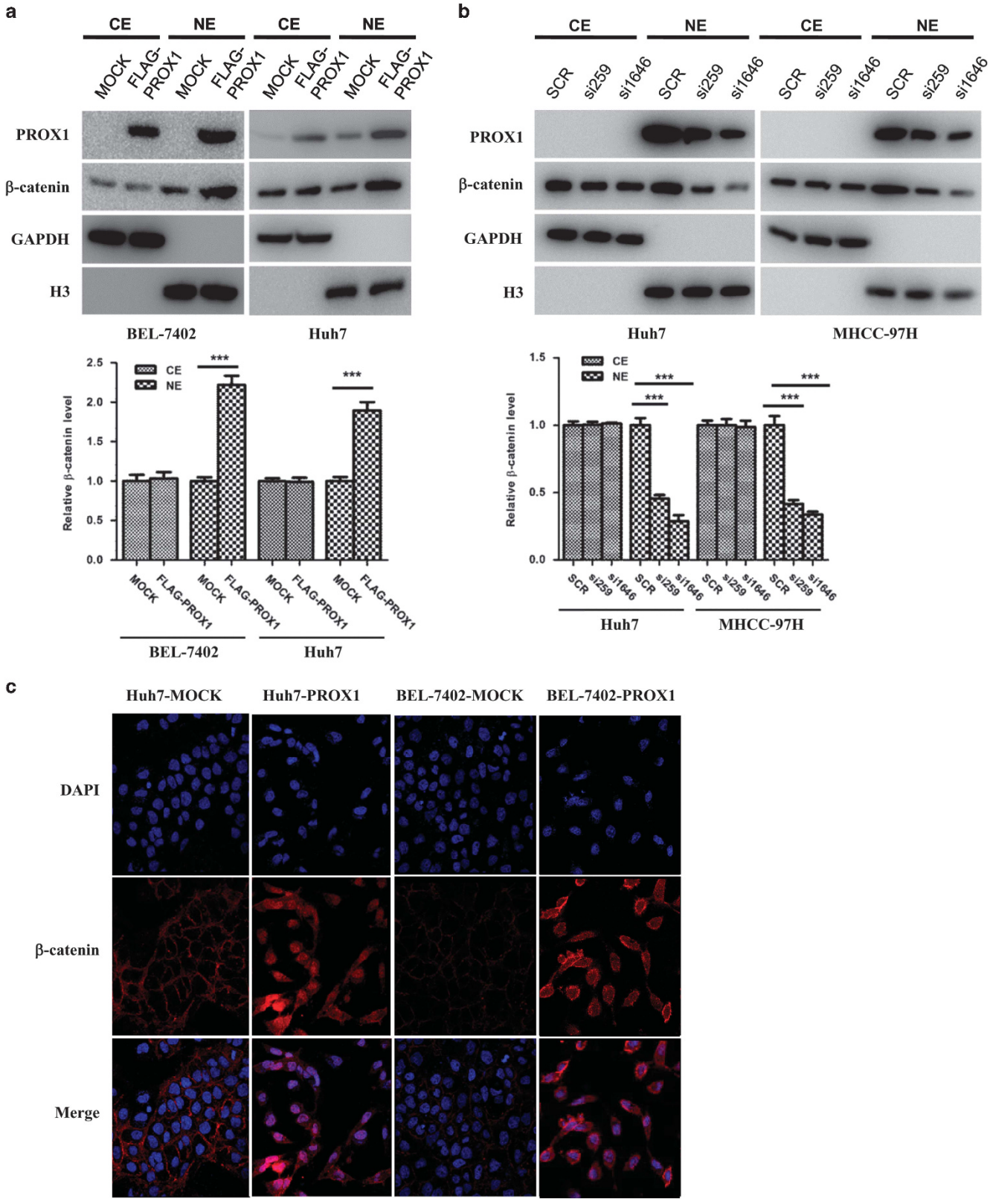


Figure 5. PROX1 promotes the nuclear translocation of β-catenin. (**a**) Cell fractionation assays revealed that overexpression of *PROX1* increased the nuclear β-catenin protein levels in BEL-7402 and Huh7 cells. (**b**) Knockdown of PROX1 expression reduced the nuclear β-catenin protein levels in MHCC-97 and Huh7 cells. Densitometry scanning data of three independent repeat blots were plotted and presented as relative β-catenin levels against Mock or SCR. Significant differences were determined using Student's-*t* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (**c**) Immunofluorescence revealed that exogenous *PROX1* expression promoted β-catenin translocation to the nucleus in BEL-7402 and Huh7 cells. CE, cytoplasmic extract; NE, nuclear extract.

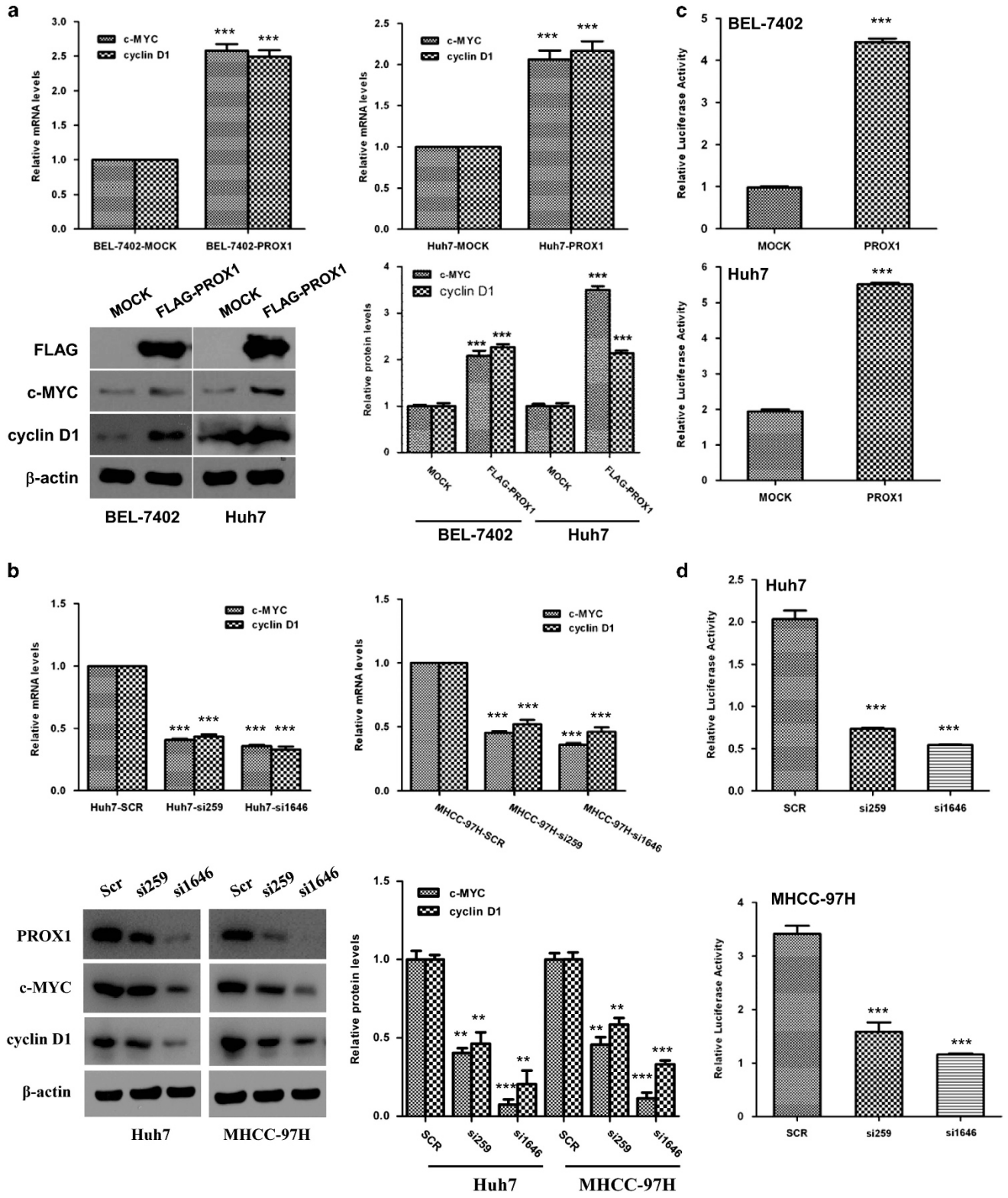
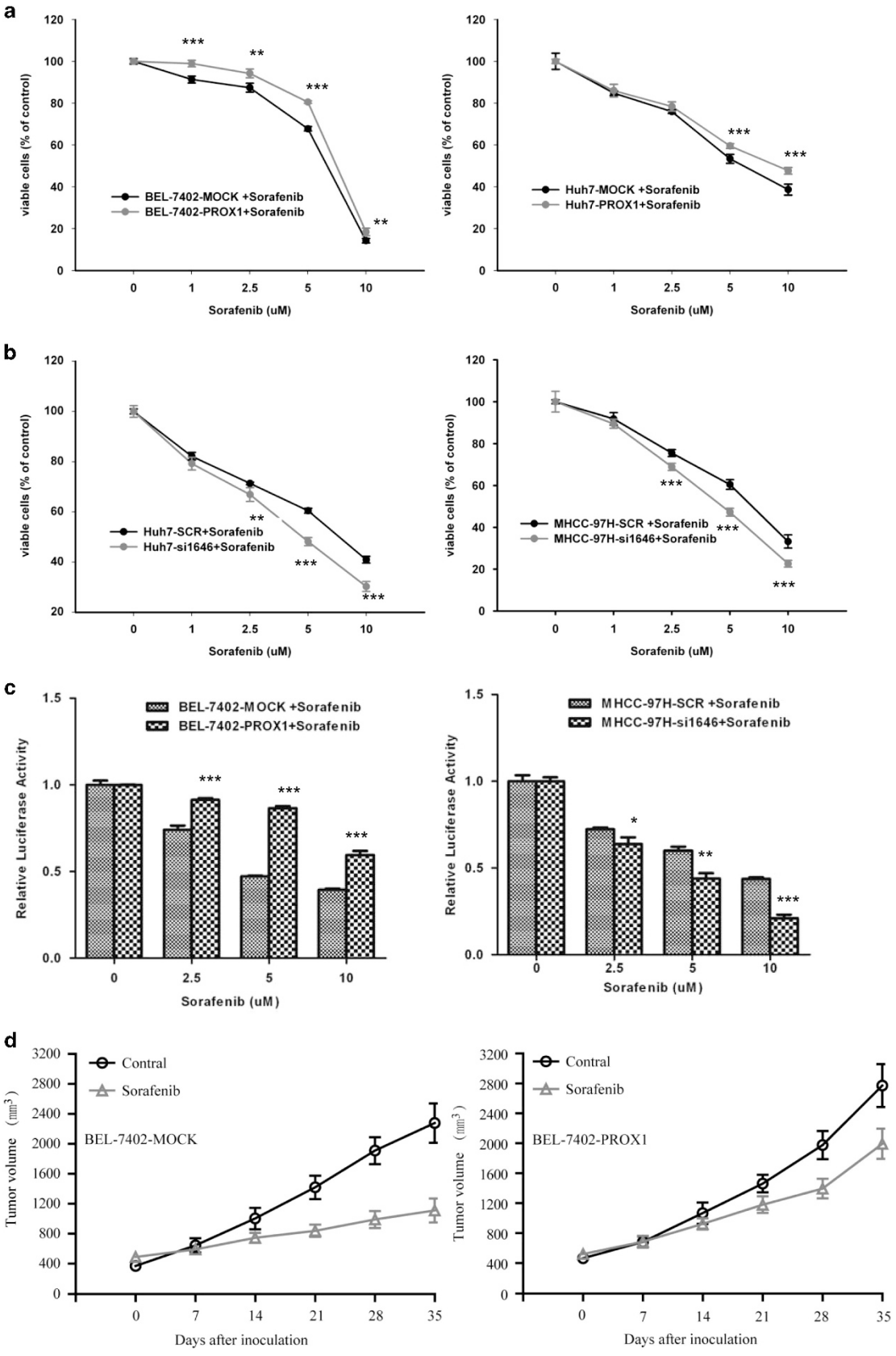


Figure 6. PROX1 activates the Wnt/β-catenin pathway. (**a**) *PROX1* overexpression in BEL-7402 and Huh7 cells upregulated *c-MYC* and *cyclin D1* mRNA expression and protein levels. Means ± s.d. from three independent experiments were presented as relative ratio to the control whose value was taken as 1.0. (**b**) Knockdown of *PROX1* expression in MHCC-97H and Huh7 cells downregulated *c-MYC* and *cyclin D1* mRNA expression and protein levels. Means ± s.d. from three independent experiments were presented as relative ratio to the control whose value was taken as 1.0. Densitometry scanning data of three independent repeat blots were plotted and presented as relative protein levels against Mock or SCR. Significant differences were determined using Student's-*t* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (**c**) Overexpression of PROX1 stimulated the LEF reporter activity in BEL-7402 and Huh7 cells. Means ± s.d. of normalized luciferase activity from three independent experiments were presented. (**d**) Knockdown of *PROX1* expression down-regulated the LEF reporter activity. Means ± s.d. of normalized luciferase activity from three independent experiments were presented. Significant differences were determined using Student's-*t* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.



DISCUSSION

The correlation between PROX1 and cancer development has been explored in several types of cancers.[24] PROX1 exhibits either tumor-suppressing or oncogenic activity depending on cancer types, reflecting the complex nature of PROX1's role in cancer development. The liver in the embryo of PROX1-knockout mouse is much smaller than that in the wild-type embryo, due to restricted migration and ensuing repopulation by hepatocytes,[23] and/or obstructed proliferation of fetal hepatocytes within the liver lobes.[26] These results point to the indispensability of PROX1 during embryonic liver development, and hint at a potential involvement of PROX1 in HCC development. We recently identified PROX1 as a crucial stimulative factor for HCC invasion and metastasis, which is consistent with PROX1's activity in promoting hepatocyte migration in the developing liver.[25] However, it was unclear whether PROX1 is also related to the more upstream process of HCC cell proliferation. This study presents the first evidence that PROX1 also has an important role in promoting HCC cell proliferation and tumor growth *in vitro* and *in vivo*, reinforcing the view that PROX1 is a critical factor in HCC development.

Aberrant activation of the canonical Wnt/β-catenin signaling pathway is often observed during the initiation and progression of HCC.[4,5] Cytoplasmic β-catenin is normally shortlived and rapidly targeted for degradation. Stabilization of cytoplasmic β-catenin leads to the accumulation of translocated β-catenin in the nucleus, and complex-formation of the latter with the TCF/LEF transcription factor in turn regulates expression of Wnt/β-catenin target genes and promotes cell proliferation.[3] The relationship between PROX1 and the Wnt/β-catenin pathway in HCC has been obscure. In the adult mammalian hippocampus and human colorectal cancer, PROX1 has been identified as a downstream target of the Wnt/β-catenin pathway, being upregulated in response to Wnt/β-catenin signaling.[27,28] However, no evidence indicates that the Wnt/β-catenin pathway regulates PROX1 expression in the liver. Petrova *et al*.[28] reported that although HepG2 cells contain a constitutively activated β-catenin mutant and a high level of *PROX1* expression, dominant negative TCF4 transfection did not affect *PROX1* expression. In addition, loss of APC in hepatocytes did not increase *PROX1* expression, despite the nuclear translocation of β-catenin. We also observed that increasing or reducing β-catenin expression in Huh7 cells had no effect on PROX1 expression (Supplementary Figure 2). These data suggest that the relationship between PROX1 and the Wnt/β-catenin pathway in HCC and normal hepatocytes differs from what has been learned in the adult mammalian hippocampus and human colorectal cancer. Instead of being a downstream target of the Wnt/β-catenin pathway, we show here that PROX1 can actually up-regulate the Wnt/β-catenin pathway in HCC cells through enhancing β-catenin transcription and promoting nuclear accumulation of β-catenin. The positive correlation between PROX1 and β-catenin expression in HCC tissues further supports this finding, albeit indirectly.

We demonstrated that PROX1 is associated with and can activate the *β-catenin* promoter in HCC cells, which might explain previous observations that β-catenin mRNA levels are frequently upregulated in HCC.[8,9] Whether PROX1 binds directly to the *β-catenin* promoter DNA remains to be clarified. In spite of possessing a DNA binding domain, PROX1 does not usually regulate transcription by directly binding to promoter DNA. Instead, it often serves as a co-regulator for transcription factors.[29,30] Nevertheless, available experimental evidence indicates that as co-regulator, PROX1 usually recruits transcriptional repression complexes, which results in inhibition of transcription.[29–31] PROX1, when involved in transcription stimulation, may recruit a different set of transcriptional complexes. The nature of these complexes remains to be identified. We have narrowed down the responsive region for PROX1 to the section between − 1907 and − 1407 in the *β-catenin* promoter. Within this region we bio-informatically predicted several binding sites for known regulators of *β-catenin* transcription such as TCF/LEF and NKX2-5.[11,13] However, the predicted TCF/LEF binding site (−1717 to − 1711) within this region is not essential for PROX1-mediated induction of *β-catenin* expression since mutation of this TCF/LEF-binding site has no marked effects on transcriptional activation by PROX1 (Supplementary Figure 3). The detailed mechanisms of PROX1-mediated stimulation of β-catenin promoter awaits elucidation. On the other hand, multiple mechanisms may potentially account for PROX1-mediated promotion of the nuclear accumulation of β-catenin. Firstly, the enhanced expression of β-catenin *per se* provides more β-catenin that could be eventually relocated to the nucleus. Secondly, we have previously shown that PROX1 is able to reduce the expression of E-cadherin in HCC cells,[25] which means less β-catenin will be trapped in membrane anchorage by means of complex formation with E-cadherin. How PROX1 promotes the nuclear accumulation of β-catenin is an important topic for further study. In addition, mechanisms underlying upregulated PROX1 expression in some HCC samples also remain to be elucidated. Research efforts along both these directions may eventually provide vital information on carcinogenesis in the liver and reveal valuable antitumor targets for the development of novel HCC therapeutics.

Currently, all efforts to develop clinically effective and safe Wnt/β-catenin pathway modulators have been complicated by multifaceted nature of the Wnt/β-catenin signaling. Toxicity of Wnt-inhibitors has been reported for several cancers[32] and is likely intolerable in cirrhotic patients with limited hepatocyte regenerative capacity. Sorafenib is well tolerated in cirrhotic patients and is the first and so far the only clinically approved drug for patients with advanced HCC. Sorafenib is an oral multikinase inhibitor which blocks tumor cell proliferation and angiogenesis by inhibiting the serine/threonine kinases Raf-1/B-Raf and the tyrosine kinases of vascular endothelial growth factor receptor (VEGFR-2/-3) and platelet derived growth factor receptor (PDGFR).[33] Although the Wnt/β-catenin pathway has not been described as a direct target of sorafenib, there is increasing evidence pointing to potentially indirect effects of sorafenib and showing its ability to antagonize the Wnt/β-catenin signaling in several experimental models of liver cancer.[16] However, resistance to sorafenib is frequently encountered in HCC treatment.[2] Our results indicate that there is a correlation between PROX1 expression and sorafenib resistance in HCC cells *in vitro* and in tumor xenograft mice. This finding suggests that PROX1 might have a role in determining sorafenib treatment outcome in HCC and points to potential strategies for improving sorafenib efficacy.

Figure 7. PROX1 contributes to sorafenib resistance of HCC *in vitro* and *in vivo*. (**a**) *PROX1* overexpression in BEL-7402 and Huh7 cells promoted resistance to sorafenib. Cell were incubated with sorafenib at various concentrations for 48 h, and then the cell viability was determined by CCK8 assay. (**b**) Knockdown of *PROX1* expression in MHCC-97H and Huh7 cells increased sensitivity to sorafenib. (**c**) Measurement of LEF reporter activity after treatment with sorafenib for 24 h in BEL-7402 and MHCC-97H cells. Left, overexpression of *PROX1* in BEL-7402 cells counteracted sorafenib's inhibition on the LEF reporter activation. Right, knockdown of PROX1 expression in MHCC-97H cells enhanced sorafenib's inhibition. Means ± s.d. of normalized luciferase activity from three independent experiments were presented. Significant differences were determined by using Student's-*t* test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (**d**) Proliferation of BEL-7402 cells with *PROX1* overexpression (BEL-7402-PROX1) was less sensitive to sorafenib inhibition (Left) compared with that of the control BEL-7402 cells (BEL-7402-MOCK) (Right).

MATERIALS AND METHODS

Clinical samples

Tumor specimens were collected during surgical resection from patients treated for HCC at Liver Cancer Institute, Zhongshan Hospital, Fudan University. The study contains 60 HCC patients assigned randomly by computer. Use of HCC specimens were approved by Zhongshan Hospital Research Ethics Committee (Permit: 2008-55). Follow-up procedures were described in our previous study.[34]

Plasmid constructs

Plasmids were generated as described previously.[25] Flag-tagged full-length *PROX1* and *β-catenin* cDNAs, full-length *LEF* cDNA were cloned in pcDNA3 (Invitrogen, Carlsbad, CA, USA) respectively. For lentivirus-mediated expression of *PROX1*, Flag-tagged *PROX1* cDNA was cloned in pWPI.1 (Addgene plasmid 12254). For lentivirus-mediated RNA interference of *PROX1* expression, DNA fragments encoding the hairpin precursors for si259 (5′-TTTCCAGGAGCAACCATAATT-3′, corresponding to nt 259–279 of the *PROX1* ORF) and si1646 (5′-GGCTCTCCTTGTCGCTCATAA-3′, corresponding to nt 1646–1666 of the *PROX1* ORF) were inserted into pLKO.1 TRC (Addgene plasmid 10879) respectively. A scrambled siRNA precursor (Scr) of similar GC-content to si259 and si1646 but no sequence identity with *PROX1* was used as a negative control.

The full length *β-catenin* promoter luciferase reporter (pGL3-HBCP) is a gift from Prof RH Dashwood of Oregon State University. Reporter plasmids containing truncated *β-catenin* promoters were kindly provided by Prof. Long Yu of Fudan University. β-catenin/LEF responsive promoter reporter was generated by inserting the *LEF* promoter into pGL2-Basic (Promega, Madison, WI, USA).

Antibodies for western blot

Antibodies used in Western blot include anti-PROX1 (polyclonal, Upstate Biotech, Lake Placid, NY, USA), anti-β-catenin (monoclonal, BD Pharmingen, San Jose, CA, USA), anti-GAPDH, anti-Histone 3 (H3), anti-c-MYC and anti-Cyclin D1 (monoclonal, Cell Signaling Inc, Danvers, MA, USA).

Cell lines, transfection and lentivirus preparation

Human HCC cell lines BEL-7402, Huh7 and embryo kidney cell line HEK293T were obtained from Cell Bank of Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences. MHCC-97H is a highly metastatic human HCC cell line, established at Liver Cancer Institute, Zhongshan Hospital, Fudan University.[35] Cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 100 U/ml penicillin G/streptomycin sulfate and 10% (v/v) fetal bovine serum (Invitrogen), and cultured at 37 °C with 5% CO_2.

The expression plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Helper plasmid pSPAX2 (Addgene plasmid 12260) or pMD2.G (Addgene plasmid 12259) were co-transfected with pLKO.1- or pWPI.1-based plasmids into HEK293T cells to package recombinant lentiviruses. Supernatants from co-transfections were used for lentiviral infection of cultured cells.

Luciferase reporter assays were performed using the Dual Luciferase Report Assay System (Promega) according to the manufacturer's instructions.

RNA isolation and quantitative real-time PCR (qrtPCR)

Total RNA was extracted with TRIzol Reagent (Invitrogen), and reverse transcription was performed using PrimeScript RT reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. For qrtPCR analysis, aliquots of cDNA were amplified by using SYBRPremix Ex Taq (TaKaRa). PCR reactions were done in triplicates with the following conditions: 95 °C/30 s, 40 cycles of 95 °C/5 s, 60 °C/15 s and 72 °C/10 s on MXP3000 cycler (Stratagene, La Jolla, CA, USA) and repeated at least three times. Relative mRNA levels were calculated with the $-\Delta\Delta$Ct method using β-actin as the control and expressed as 2^(−ΔΔCt). The following primer pairs were used: β-catenin-f/β-catenin-r: GCTATTGTAGAAGCTGGTG GAATGC/CTTCCATCCCTTCCTGTTTAGTTGC; c-MYC-f/c-MYC-r: CCAGCAGCG AC TCTGAGGAGGAAC/GCTGTGGCCTCCAGCAGAAGGTG; cyclinD1-f/cyclin D1-r: GCT CAAGTGGAACCTGGCCGCAATG/GAACTTCACATCTGTGGCACAG AGGG; β-actin-f/β-actin-r: TCCCTGGAGAAGAGCTACG/GTAGTTTCGTGGATG CCACA.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as previously described[31] and anti-PROX1 (Upstate) was used to immunoprecipitate sonicated chromatins prepared from Huh7 or MHCC-97H cells. Five percent of each post-sonication sample was saved as the input control and pre-immune IgG was used for specificity control. DNA extracted from precipitated chromatins were quantitated using qrtPCR in triplicates with primers for *β-catenin* promoter (forward/reverse, GCATATTCCTTAATTCTGCAAATGAC/G GTCACAATTCAAAAAGATTGAACCG, amplicon spanning −1710 to −1572). DNA extracted from saved input were quantitated in parallel (Ct[Input]), and results from IP by anti-PROX1 mAb or pre-immnue IgG (Ct[IP]) were then used to calculate relative specific occupancy or non-specific background using the equation: 2^(Ct[IP]-Ct[Input]) *100%.

Cell proliferation analysis

Cell proliferation was measured with a CCK-8 kit (Dojindo, Kumamoto, Japan). HCC cells infected with lentiviruses were seeded into 96-well plates in 100 μl of medium containing 10% FBS and incubated at 37 °C in 5% CO_2. After 24, 48, 72 and 96 h, the medium was replaced with 90 μl of fresh medium and 10 μl CCK-8 solution was added to each well. Cells were then incubated for 2 h at 37 °C in 5% CO_2, afterward absorbance at 450 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Each experiment was performed in triplicate and repeated in quadruplicate for each condition.

Colony formation assay

BEL-7402, Huh7 and MHCC-97H cells infected with the lentiviruses were seeded respectively in six-well plates at a density of 5×10^2 cells/well. After incubation at 37 °C for about 10–14 days, cells were washed twice with PBS, stained with Giemsa solution (AppliChem, Darmstadt, Germany), and allowed to air dry at room temperature. The number of colonies was counted. Each experiment was performed in triplicate.

Cell cycle analysis

Cell cycle distribution was determined by fluorescence-activated cell sorting (FACS) analysis. Fixed cells were incubated with RNase A and propidium iodide (PI). Flow cytometry was carried out on a FACS caliber flow cytometer (BD Biosciences, San Jose, CA, USA). Data acquisition and analysis were carried out using Cell Quest (BD Biosciences).

Immunohistochemistry

Immunohistochemistry procedures were performed as described.[34] Primary antibodies were anti-PROX1 monoclonal antibody (mAb) (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-catenin mAb (1:200, BD Pharmingen, San Diego, CA, USA). Detection was performed using the Envision-plus detection system (EnVision +/− HRP/Mo; Dako, Carpinteria, CA, USA). Signals were visualized following incubation with 3,3′-diaminobenzidine. Negative controls were treated identically except without primary antibodies. Staining was independently assessed by two experienced pathologists. The staining intensity was graded from 0–2 (0, no staining; 1, weak; 2, strong) as described previously.[25]

Tumor xenograft mouse models and sorafenib treatment

Animal care and experimental protocols were approved by the Animal Ethics Committee of Shanghai Medical College, Fudan University (Permit: 20101201-001). Male athymic BALB/c nude mice (4-weeks old, Shanghai Institute of Materia Medica, Chinese Academy of Sciences) were raised in specific pathogen-free conditions. Mice were housed six per cage with a 12-hour light/dark schedule at 25 ± 1 °C and were fed an autoclaved chow diet and water ad libitum. The mice were randomly divided into groups before injection.

The proliferation of BEL-7402-Mock or BEL-7402-PROX1 was determined following the inoculation of cells (2.0×10^6 cells/mouse suspended in 100 μl serum-free DMEM and matrigel (BD Biosciences) (1:1)) into the liver parenchyma of nude mice (6 mice/group) under anesthesia with ketamine/xylazine after opening up the abdomen. The mice were monitored every 5 days for 8 weeks, then sacrificed under anesthesia.

The proliferation of MHCC-97H-Scr or MHCC-97H-si1646 was determined following subcutaneous injection of cells (1.0×10^7 cells/mouse) into nude mice (six mice/group). Eight weeks post injection, the mice were sacrificed under anesthesia.

For sorafenib treatment of HCC-bearing mice, BEL-7402-MOCK and BEL-7402-PROX1 cells were (1.0×10^7 cells/mouse) subcutaneously inoculated into the right flanks of nude mice, respectively. Treatment was started 7 days after inoculation and mice were randomly assigned to receive a daily oral dose of either vehicle solution or 30 mg/kg sorafenib (Bayer Healthcare, Leverkusen, Germany) for 4 weeks. Tumor samples were then extracted for further analysis.

Statistical analysis

The Pearson χ^2-test or Fisher's exact test was used to compare qualitative variables, and the Student-*t*test for quantitative variables. All statistical tests were two-sided, and *P*-value < 0.05 was considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Prof RH Dashwood of Oregon State University and Prof Long Yu of Fudan University for kindly providing the *β-catenin* promoter luciferase reporters. This work is supported by the grants from the National Key Project for Infectious Diseases (2012ZX10002-006, 2012ZX10004-503, 2012ZX10002012-003), National Basic Research Program (2012CB519002), Natural Science Foundation of China (31071143, 31170148, 81472226, 81372654 and 81370730), Shanghai Municipal Health Committee (GWDTR201216), Nature Science Foundation from Shandong province (ZR2011HQ006) and Municipal Science and Technology Committee (13ZR1407100).

REFERENCES

1 Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; **127**: 2893–2917.
2 Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF *et al.* Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378–390.
3 Clevers H. Wnt/beta-catenin signaling in development and disease. *Cell* 2006. 469–480.
4 Takigawa Y, Brown AM. Wnt signaling in liver cancer. *Curr Drug Targets* 2008; **9**: 1013–1024.
5 Boyault S, Rickman DS, de Reyniès A, Balabaud C, Rebouissou S, Jeannot E *et al.* Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets. *Hepatology* 2007; **45**: 42–52.
6 de La Coste A, Romagnolo B, Billuart P, Renard CA, Buendia MA, Soubrane O *et al.* Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. *Proc Natl Acad Sci USA* 1998; **95**: 8847–8851.
7 Satoh S, Daigo Y, Furukawa Y, Kato T, Miwa N, Nishiwaki T *et al.* AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. *Nat Genet* 2000; **24**: 245–250.
8 Cui J, Zhou X, Liu Y, Tang Z. Mutation and overexpression of the beta-catenin gene may play an important role in primary hepatocellular carcinoma among Chinese people. *J Cancer Res Clin Oncol* 2001; **127**: 577–581.
9 Lai TY, Su CC, Kuo WW, Yeh YL, Kuo WH, Tsai FJ *et al.* β-catenin plays a key role in metastasis of human hepatocellular carcinoma. *Oncol Rep* 2011; **26**: 415–422.
10 Guigon CJ, Kim DW, Zhu X, Zhao L, Cheng SY. Tumor suppressor action of liganded thyroid hormone receptor beta by direct repression of beta-catenin gene expression. *Endocrinology* 2010; **151**: 5528–5536.
11 Li Q, Dashwood WM, Zhong X, Al-Fageeh M, Dashwood RH. Cloning of the rat beta-catenin gene (Ctnnb1) promoter and its functional analysis compared with the Catnb and CTNNB1 promoters. *Genomics* 2004; **83**: 231–242.
12 Liu G, Jiang S, Wang C, Jiang W, Liu Z, Liu C *et al.* Zinc finger transcription factor 191, directly binding to beta-catenin promoter, promotes cell proliferation of hepatocellular carcinoma. *Hepatology* 2012; **55**: 1830–1839.
13 Riazi AM, Takeuchi JK, Hornberger LK, Zaidi SH, Amini F, Coles J *et al.* NKX2-5 regulates the expression of beta-catenin and GATA4 in ventricular myocytes. *PLoS One* 2009; **4**: e5698.
14 Chai H, Luo AZ, Weerasinghe P, Brown RE. Sorafenib downregulates ERK/Akt and STAT3 survival pathways and induces apoptosis in a human neuroblastoma cell line. *Int J Clin Exp Pathol* 2010; **3**: 408–415.
15 Han L, Yang Y, Yue X, Huang K, Liu X, Pu P *et al.* Inactivation of PI3K/AKT signaling inhibits glioma cell growth through modulation of beta-catenin-mediated transcription. *Brain Res* 2010; **1366**: 9–17.
16 Lachenmayer A, Alsinet C, Savic R, Cabellos L, Toffanin S, Hoshida Y *et al.* Wnt-pathway activation in two molecular classes of hepatocellular carcinoma and experimental modulation by sorafenib. *Clin Cancer Res* 2012; **18**: 4997–5007.
17 Burke Z, Oliver G. Prox1 is an early specific marker for the developing liver and pancreas in the mammalian foregut endoderm. *Mech Dev* 2002; **118**: 147–155.
18 Dyer MA, Livesey FJ, Cepko CL, Oliver G. Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina. *Nat Genet* 2003; **34**: 53–58.
19 Hong YK, Harvey N, Noh YH, Schacht V, Hirakawa S, Detmar M *et al.* Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate. *Dev Dyn* 2002; **225**: 351–357.
20 Kaltezioti V, Kouroupi G, Oikonomaki M, Mantouvalou E, Stergiopoulos A, Charonis A *et al.* Prox1 regulates the notch1-mediated inhibition of neurogenesis. *PLoS Biol* 2010; **8**: e1000565.
21 Lavado A, Oliver G. Prox1 expression patterns in the developing and adult murine brain. *Dev Dyn* 2007; **236**: 518–524.
22 Risebro CA, Searles RG, Melville AA, Ehler E, Jina N, Shah S *et al.* Prox1 maintains muscle structure and growth in the developing heart. *Development* 2009; **136**: 495–505.
23 Sosa-Pineda B, Wigle JT, Oliver G. Hepatocyte migration during liver development requires Prox1. *Nat Genet* 2000; **25**: 254–255.
24 Elsir T, Smits A, Lindstrom MS, Nister M. Transcription factor PROX1: its role in development and cancer. *Cancer Metastasis Rev* 2012; **31**: 793–805.
25 Liu Y, Zhang JB, Qin Y, Wang W, Wei L, Teng Y *et al.* PROX1 promotes hepatocellular carcinoma metastasis by way of up-regulating hypoxia-inducible factor 1alpha expression and protein stability. *Hepatology* 2013; **58**: 692–705.
26 Kamiya A, Kakinuma S, Onodera M, Miyajima A, Nakauchi H. Prospero-related homeobox 1 and liver receptor homolog 1 coordinately regulate long-term proliferation of murine fetal hepatoblasts. *Hepatology* 2008; **48**: 252–264.
27 Karalay O, Doberauer K, Vadodaria KC, Knobloch M, Berti L, Miquelajauregui A *et al.* Prospero-related homeobox 1 gene (Prox1) is regulated by canonical Wnt signaling and has a stage-specific role in adult hippocampal neurogenesis. *Proc Natl Acad Sci U S A* 2011; **108**: 5807–5812.
28 Petrova TV, Nykanen A, Norrmen C, Ivanov KI, Andersson LC, Haglund C *et al.* Transcription factor PROX1 induces colon cancer progression by promoting the transition from benign to highly dysplastic phenotype. *Cancer Cell* 2008; **13**: 407–419.
29 Qin J, Gao DM, Jiang QF, Zhou Q, Kong YY, Wang Y *et al.* Prospero-related homeobox (Prox1) is a corepressor of human liver receptor homolog-1 and suppresses the transcription of the cholesterol 7-alpha-hydroxylase gene. *Mol Endocrinol* 2004; **18**: 2424–2439.
30 Song KH, Li T, Chiang JY. A Prospero-related homeodomain protein is a novel co-regulator of hepatocyte nuclear factor 4alpha that regulates the cholesterol 7alpha-hydroxylase gene. *J Biol Chem* 2006; **281**: 10081–10088.
31 Ouyang HF, Qin Y, Liu YF, Xie YH, Liu J. Prox1 directly interacts with LSD1 and recruits the LSD1/NuRD complex to epigenetically co-repress CYP7A1 transcription. *PLOS One* 2013; **8**: e62192.
32 Klaus A, Birchmeier W. Wnt signalling and its impact on development and cancer. *Nat Rev Cancer* 2008; **8**: 387–398.
33 Iyer R, Fetterly G, Lugade A, Thanavala Y. Sorafenib: a clinical and pharmacologic review. *Expert Opin Pharmacother* 2010; **11**: 1943–1955.
34 Zhu XD, Zhang JB, Zhuang PY, Zhu HG, Zhang W, Xiong YQ *et al.* High expression of macrophage colony-stimulating factor in peritumoral liver tissue is associated with poor survival after curative resection of hepatocellular carcinoma. *J Clin Oncol* 2008; **26**: 2707–2716.
35 Ye QH, Qin LX, Forgues M, He P, Kim JW, Peng AC *et al.* Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. *Nat Med* 2003; **9**: 416–423.

Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)