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Original Articles

Deacetylation of β-catenin by SIRT1 regulates self-renewal and oncogenesis of liver cancer stem cells

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ABSTRACT

Hepatocellular carcinoma (HCC) is a highly malignant liver tumor. The presence of cancer stem cells (CSCs) figures prominently in tumor invasion, therapeutic resistance and tumor recurrence resulting in poor outcome and limited therapeutic options. Wnt/β-catenin signaling is essential for cancer stem cell regulation and tumorigenesis in HCC, but its molecular mechanisms are not fully understood. Here, we demonstrate that β-catenin is overexpressed in liver CSCs, and its expression level is positively correlated with SIRT1 in HCC specimens. SIRT1 regulates the protein stability of β-catenin, thereby affecting the transcriptional activity of Wnt/β-catenin signaling in liver CSCs. Mechanistically, we show that nuclear accumulation of β-catenin results from deacetylation mediated by SIRT1. Further, nuclear β-catenin promotes the transcription of Nanog to help maintain self-renewal of liver CSCs. Taken together, our findings indicate that the deacetylation of β-catenin by SIRT1 represents a critical mechanism for regulating liver CSCs self-renewal and tumorigenesis. It provides an improved understanding of molecular mechanisms underlying β-catenin activation and tumorigenesis in HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer and is an important medical problem [1]. Recent studies have demonstrated that liver tumor initiation and progression are driven by a small subpopulation of undifferentiated cancer stem cells (CSCs) or termed tumor-initiating cells (TICs) [2]. We previously identified the homeobox protein Nanog, which is a transcription factor that is critically involved in maintenance of stem cell activity in embryonic stem cells, as a bona fide marker of liver CSCs and plays an important role in regulating their self-renewal [3]. However, it is still not clear which key components and molecular mechanisms contributing to self-renewal of liver CSCs.

Hyperactivation of Wnt/β-catenin signaling was reported to be essential for the self-renewal capacity of CSCs in a variety of human cancers, including HCC [4–7]. β-catenin is a central regulator in the Wnt signaling pathway. Regulation of β-catenin stability is one of the major mechanisms by which Wnt/β-catenin activity is tuned. It has been reported that the post-translational modification of β-catenin is an important mode of regulating of β-catenin stability and cellular location, as well as its transcriptional activity [8–10]. The function of a destruction complex in the regulation of β-catenin phosphorylation and degradation has been extensively studied. Without Wnt stimulation, β-catenin binds to the destruction complex and is sequentially phosphorylated by CK1 and GSK3, then ubiquitinated by β-TrCP, and finally undergoes proteasomal degradation [11,12]. In addition to being

Abbreviations: AFP, alpha-fetoprotein; CSCs, cancer stem cells; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FACS, Fluorescent-activated cell sorting; HCC, hepatocellular carcinoma; IF, immunofluorescence; IHC, immunohistochemistry; SEM, standard error of the mean; shRNA, short hairpin RNA; ChIP, chromatin immunoprecipitation; CHX, cycloheximide

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phosphorylated, β-catenin can also be acetylated by the histone acetyltransferase CREB/p300 at different residues [13,14]. Moreover, β-catenin can be a target for deacetylation by histone deacetylase 6 (HDAC6) [15]. Acetylation/deacetylation modification of β-catenin is involved in regulating its protein stability and transcriptional activity. However, the molecular mechanism is rarely studied.

SIRT1, sirtuin (silent mating type information regulation 2 homolog) 1, is a nicotinamide adenine dinucleotide (NAD)-dependent class III protein deacetylase. It is mainly localized to the nucleus and plays a key role in energy metabolism, maintenance of telomeres, and genomic stability by targeting a variety of non-histone proteins [16–18]. SIRT1 is found to be overexpressed in a variety of cancers, including HCC. It has been reported that SIRT1 plays an important role in maintaining self-renewal of embryonic stem cells (ESCs) and hematopoietic stem cells (HSC) [19,20].

Our previous work has shown that SIRT1 is highly expressed in liver CSCs and is essential for maintenance of the stem cells phenotype and tumorigenic potential [21]. In addition, we found that elevated levels of β-catenin were highly correlated with SIRT1 expression levels in liver CSCs. These data suggest that β-catenin may be regulated by SIRT1. However, the mechanisms whereby SIRT1 regulates β-catenin expression and transcriptional activity in liver CSCs is not known. Here, we explore whether SIRT1 is critically involved in regulating β-catenin activity in liver CSCs, and if SIRT1/β-catenin axis modulates the self-renewal and tumorigenic capacity of human liver CSCs.

2. Materials and methods

2.1. Cell lines and culture

The human HCC cell lines (Huh7, PLC/PRF/5, HepG2, SMMC-7721, 97H and 7404) and 293T were purchased from Shanghai Cell Collection (Shanghai, China). Patient-derived primary HCC cells T1115 and T1224 were obtained from patient tumor specimens with informed consent according to protocols as described previously [3]. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO) with 10% FBS at 37 °C under a 5% CO_2 atmosphere.

2.2. Clinical specimens and immunohistochemical (IHC) staining

Tissue specimens were obtained with informed consent from 148 HCC patients undergoing hepatectomy for HCC at the Institute of Hepatobiliary Surgery, Southwest Hospital, Army Medical University, China. All patients were informed with consent according to protocols approved by the Institutional Review Board of the Southwest Hospital, Army Medical University, and this study complied with by the ethical guidelines of the Helsinki Declaration. A tissue array block containing HCC samples and their corresponding noncancerous liver tissues was constructed.

Immunohistochemical (IHC) staining was performed on tissue array slides using the streptavidin biotin peroxidase complex method. Antigen retrieval was conducted by heating the tissues with 10 mmol/L EDTA (pH 8.0) of antigen retrieval solution (Dako). Then, the samples were stained with following primary antibodies, rabbit anti-β-catenin (Cell signaling) and mouse anti-SIRT1 (Cell signaling). Slides were subsequently developed using EnVision method with a DAKO kits (Dako REALTM EnVision™). Scoring for IHC staining was performed by two independent pathologists. The staining intensity was divided into 4 grades: negative, 0; weak, 1; moderate, 2; and strong, 3. The proportion scores was assigned as follow: 0, 0%; 1, 1–25%; 2, 26–50%; 3, 51–75% and 4, 76–100%. An overall protein expression score raging from 0 to 12 was calculated by combining the positivity and intensity scores. Low expression was defined as IHC score of 0–2, while high expression was defined as IHC score of 3–12. Scores were compared with overall survival, defined as the time from date of surgery to death or last known data of follow-up.

2.3. Plasmids, lentiviruses construction and cell infection

shRNA targeting β-catenin and SIRT1 were designed using “Block-iT RNAi designer” web application (ThermoFisher, https://rnaidesigner.thermofisher.com/rnaiexpress/).Oligonucleotide sequences of shRNAs as following:

Scrambled shRNA: 5′-CGTACGCGGAATACTTCGA-3’
SIRT1 shRNA#1: 5′-GCGGGAATCCAAAGGATAATT-3’
SIRT1 shRNA#2: 5′-GGGTCTTCCCTCAAAGTAAGA -3’
SIRT1 shRNA#3: 5′-GCATTAGGAACTTTAGCATGT -3’
β-catenin shRNA#1: 5′-GCTTACTGGCCATCTTTAAGT-3’
β-catenin shRNA#2: 5′-GGTGTCTGCTATTGTACGTAC-3’ shRNAs were cloned into the pLKO.1-TRC (Addgene, Plasmid 10878) according to the Addgene's pLKO.1 protocol. The efficacy of each shRNA was assessed by western blotting in Huh7 cells (Supplementary Figs. 1A and B). The shRNA2# for SIRT1 and shRNA#2 for β-catenin with the strongest knockdown efficiency were selected for all experiments in this study. The expression plasmid SIRT1 Flag (13812), Flag-SIRT1 H363Y (1792) and human beta-catenin pcDNA3.0 (16828) were obtained from Addgene.

To produce lentiviruses, HEK-293T cells were transfected with packaging plasmids pRSV-Rev, pMD2G and pMDLg/pRRE as well as transfer different lentiviral plasmids using a calcium-phosphate-precipitation-based method. Medium containing pseudotyped lentivivirus was harvested and concentrated after 48hr. Target cells were infected with 5 MOI indicated lentiviruses supplemented with 8 μg/mL polybrene (Sigma Aldrich) for 12 hr at 37 °C.

2.4. Immunoprecipitation

Protein complexes were precipitated from whole-cell lysates or different cell fractions with anti-β-catenin (Abcam) or anti-SIRT1 (Abcam) antibodies and then precipitated with protein G beads (Life technology). Immunoprecipitates were eluted by boiling the beads in loading buffer. After centrifugation, supernatants were subjected to SDS-PAGE and western blotting.

2.5. Western blot analysis

Cells were harvested and lysed in the lysis buffer for 30 min at 4 °C. Total cell extracts or immunoprecipitated protein eluates were separated in 10% SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred on PVDF membrane (Millipore). Membranes were then blocked in 5% milk for 2 hr at room temperature and blotted with specific primary antibodies overnight at 4 °C. Secondary antibodies were incubated at room temperature for 2 hr. Then the bands were visualized by chemoluminescence (Pierce) detected by Image-lab system (Bio-Rad). For quantification, signals were quantified using Image J 1.48 software.

2.6. Luciferase reporter assay

The luciferase reporter assay was performed by transfecting the reporter vector into the indicated cell lines. The pRL-SV40 vector was co-transfected in each experiment as an internal control for transfection efficiency. The luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol at 48 hr post-transfection. All experiments were carried out in triplicate.

2.7. Reverse transcription PCR analysis

Total RNA was extracted from the cells with RNAiso Plus (Takara) according to the manufacturer's protocol. For mRNA detection, reverse transcription was performed according to the protocol of PrimeScript™ RT reagent Kit with gDNA Eraser (Takara). qPCR was performed with

Table 1
Primer sequences used for real-time PCR.

Gene	Sequence	Product size
SIRT1	F: TGTGTCATAGGTTAGGTGGTGA R: AGCCAATTCTTTTTGTGTTCGTG	101 bp
β-catenin	F: CATCTACACAGTTTGATGCTGCT R: GCAGTTTTGTCAGTTCAGGGA	150 bp
Axin2	F: TACACTCCTTATTGGGCGATCA R: TTGGCTACTCGTAAAGTTTTGGT	151 bp
c-Myc	F: GTCAAGAGGCGAACACACAAC R: TTGGACGGACAGGATGTATGC	162 bp
CCND1	F: GCTGCGAAGTGGAAACCATC R: CCTCCTTCTGCACACATTTGAA	135 bp
Lgr5	F: TATGCCTTTGGAAACCTCTC R: CACCATTCAGAGTCAGTGTT	262 bp

SYBR premix Ex Taq (TaKaRa) on CFX96 Real Time PCR Detection System (Bio-Rad). GAPDH mRNA was used to normalize RNA inputs. Primers used were synthesized by Genscript Biotech and are listed in Table 1. Each reaction was carried out in triplicate, and each experiment was performed at least three independent times.

2.8. Immunofluorescence (IF)

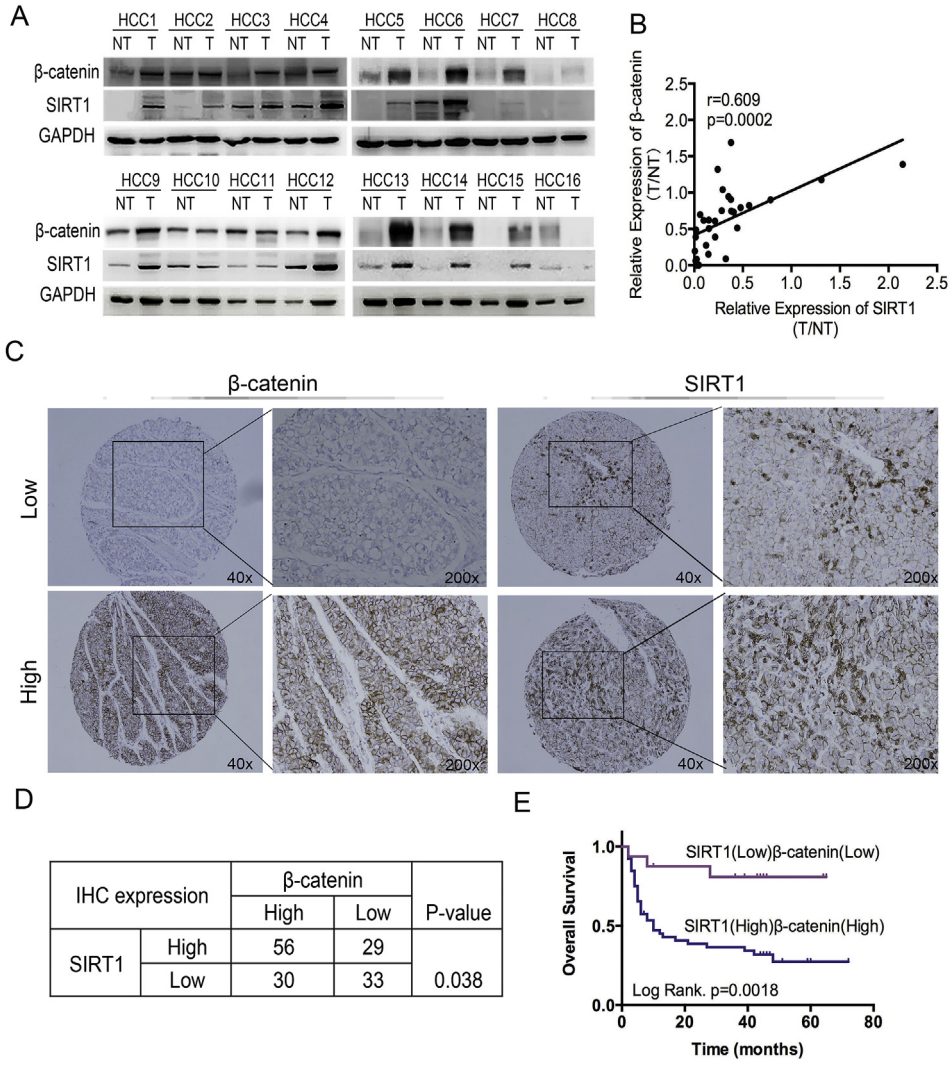
For immunofluorescence (IF), cells were cultured in 24-well on glass cover-slips and washed three times with PBS before being fixed in 4% paraformaldehyde, and permeabilized for 10 min with 0.3% Triton X-100 at room temperature. Cells were blocked with 5% goat serum (Sangon Biotech) in PBS for 1 h at room temperature, and then probed with following primary antibodies, rabbit anti-β-catenin (Cell signaling) and mouse anti-SIRT1 (Cell signaling). Secondary antibodies were goat anti-rabbit IgG-Alexa Fluor 647 (Invitrogen), or goat anti-mouse IgG-Alexa Fluor 488 (Invitrogen). Cells were further washed in PBS and the nuclei were stained with 0.5 μg/mL DAPI (Sangon Biotech) for 10 min. Cells were analyzed by using fluorescence microscopy.

2.9. Sphere formation assay

Cells were sorted and plated into Ultra Low Cluster 96-well plate (Costar). Each well was seeded with 10 cells. The cells were cultured in DMEM/F12 medium (Sigma-Aldrich) supplemented with B27 (Gibco), antibiotics, 20 ng/mL of epidermal growth factor (PeproTech), 20 ng/mL of basic fibroblast growth factor (PeproTech), and 10 ng/mL of hepatocyte growth factor (PeproTech), and 1% methyl cellulose (Sigma-Aldrich) was added to prevent cell aggregation, and individual sphere derived from a single cell was confirmed. After 4 days, equal fresh medium was added. Cells were incubated at 37 °C for 14 days, and spheres of diameter > 75 μm were counted.

2.10. Colony formation assay

For clone formation efficiency assay, 200 cells were sorted by FACS



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IHC expression		β-catenin		P-value
		High	Low	
SIRT1	High	56	29	0.038
	Low	30	33	

Fig. 1. Relationship of β-catenin/SIRT1 and HCC prognosis. (A) Western blotting analysis of β-catenin and SIRT1 expression in 16 HCC fresh tumor tissues and paired non-tumor tissues. The experiments were repeated three times. (B) Correlation between β-catenin and SIRT1 expression was analyzed by quantification of western blots in (A) using Image J software. Significance was determined using a Pearson's correlation. (C) Representative images of IHC staining for β-catenin and SIRT1 (n = 148 patients with HCC). Low expression was defined as IHC score of 0–2, while high expression was defined as IHC score of 3–12. (D) Correlation between β-catenin and SIRT1 expression in 148 HCC patients. Significance was determined using a Pearson's correlation. (E) Kaplan-Meier survival analysis was performed according to β-catenin^{High}SIRT1^{High} and β-catenin^{Low}SIRT1^{Low} expression of HCC patients. Survival (P = 0.0018 by a log-rank test) of patients who had β-catenin^{High}SIRT1^{High} expression was shorter.

Table 2
Correlation of β-catenin/SIRT1 expression and clinicopathologic parameters in HCC patients.

Variable	n	β-catenin/SIRT1				p-value
		Low/Low	High/Low	Low/High	High/High	
Age (years)						
< 50	93	12	21	27	33	
≥ 50	55	7	12	14	22	0.843
Gender						
Female	20	2	4	5	9	
Male	128	17	29	36	46	0.811
Tumor recurrence						
-	80	15	17	21	27	
+	68	4	16	20	28	0.001**
Tumor stage						
I	7	4	1	0	2	
II	69	11	19	20	19	
III	72	4	13	21	34	0.288
Tumor size (cm)#						
< 5	42	9	6	16	11	
≥ 5	99	10	26	23	40	0.61
Serum AFP level (ng/ml)#						
≤ 20	32	6	9	8	9	
21-400	45	7	14	10	14	
> 400	58	5	7	19	27	0.718
Tumor cell structure						
Trabecular	103	15	27	25	36	
Solid	45	4	6	16	19	0.705
Necrosis						
+	71	12	16	19	24	
+ +	44	2	10	13	19	
+ + +	33	5	7	9	12	0.839
Capsular invasion						
-	47	12	12	10	13	
+	60	6	11	18	25	
+ +	41	1	10	13	17	0.006**
Vascular thrombu						
-	47	14	10	10	13	
+	101	5	23	31	42	0.005**
Interstitial hyperplasia of tumor						
+	41	6	3	13	19	
+ +	67	8	22	16	21	
+ + +	40	5	8	12	15	0.072

Note: (1) *p < 0.05, **p < 0.01 significant difference. (2) x^2 test. (3) #Total number < 148 due to missing data.

and seeded per well in 24 wells plates. The cells cultured in the DMEM medium supplemented with 10% FBS for 14 days, and fresh medium was added every 3 days. The colonies were fixed with 4% formaldehyde and stained with 0.1% crystal violet (Sigma-Aldrich). Numbers of clones (> 50 cells) were counted.

2.11. Chromatin immunoprecipitation

A chromatin immunoprecipitation (ChIP) kit was purchased from Millipore, and ChIP was performed according to the manufacturer's instruction. The primer pair used for PCR to amplify Nanog promoter region was shown as following: 5′-TGGAAACGTGGTGAACCTAG-3′(F), 5′-AGTCTCACCAAGGCCATTG-3′ (R).

2.12. Tumor formation assay

NOD/SCID mice at age of 3–5 weeks, male, were maintained in pathogen-free condition at animal facility of Army Medical University. This study was approved by the Animal Research Ethics Committee of Army Medical University, and complied with the Guidelines for Animal Experiments of Laboratory Animals. The different numbers of CSCs after treatment with β-catenin shRNA and simultaneously over-expressing Nanog or only treatment with β-catenin shRNA were suspended in serum-free medium and mixed with Matrigel at the ratio of 2:1. The cells were subcutaneously injected into mice. Tumor formation was evaluated regularly after injection by palpation of injection sites.

2.13. Statistical analysis

A Student's *t*-test was used to calculate the statistical significance of the experimental data. The Kaplan-Meier survival curves and log-rank test were used for estimation of survival and difference between groups. *p < 0.05 was considered significant statistically. **p < 0.01 was considered highly significant statistically. The software tools SPSS 22.0 and GraphPad Prism 6.0 were used.

3. Results

3.1. β-catenin is positively associated with SIRT1 and poor prognosis in HCC patients

To elucidate whether β-catenin is associated with SIRT1 in HCC, we firstly measured the protein levels of β-catenin and SIRT1 in the tissue samples from 16 HCC patients. The results showed that both β-catenin and SIRT1 were highly expressed in tumor tissues compared with adjacent normal tissues (Fig. 1A). Correlation analyses showed that β-catenin was significantly correlated with SIRT1 (Fig. 1B).

Second, the expressions of β-catenin and SIRT1 were examined in a tissue microarray containing 148 HCC by IHC (Fig. 1C). The data further confirmed that β-catenin was positively associated with SIRT1 in HCC (Fig. 1D). Kaplan-Meier analysis showed that high expressions of β-catenin or SIRT1 in HCC were correlated closely with poor overall survival of the patients respectively (Supplementary Figs. 2A and B). The combined expression of double-positive for β-catenin and SIRT1 presented the prediction for the patient's worse survival outcome (Fig. 1E and Supplementary Fig. 2C).

We then moved forward to investigate the correlation between β-catenin/SIRT1 and patients' clinicopathologic features that was illustrated in Table 2. There were no statistical correlation between β-catenin/SIRT1 expression and patients' age, gender, tumor stage, tumor size, cell structure, necrosis and interstitial hyperplasia of tumor as well as AFP level in serum. But the remarkable positive correlation between β-catenin/SIRT1 expression and tumor recurrence (p = 0.001), capsular invasion (p = 0.006) and vascular thrombus (p = 0.005) were observed.

These data demonstrate that β-catenin is highly expressed in HCC. More importantly, our results reveal that β-catenin is positively associated with SIRT1, and β-catenin/SIRT1 interaction can cause poor outcome in HCC patients.

3.2. SIRT1 regulates β-catenin and activates Wnt/β-catenin in liver CSCs

Next, an obviously correlation between β-catenin and SIRT1 was detected in six HCC cell lines (Fig. 2A and B). Our previous work have proved that SIRT1 is highly expressed in liver CSCs and essential for maintaining their self-renewal and oncogenic [21]. It prompts us to investigate whether β-catenin is involved in this process. So we examined the protein level of β-catenin in liver CSCs which were isolated by our previously constructed P_{nanog}-GFP lentivirus reporter system [3]. The data showed that β-catenin was highly expressed in liver CSCs as well as SIRT1 (Fig. 2C and Supplementary Fig. 3A). Similar results were observed in cells isolated by liver CSCs markers EpCAM and CD133 (Supplementary Fig. 3B).

Further analysis revealed that β-catenin protein was significantly decreased after knockdown of SIRT1 using shRNA in liver CSCs (Fig. 2D and Supplementary Fig. 3C). To study whether SIRT1 participates in regulating the Wnt/β-catenin pathway, the pathway specific luciferase reporters was used in liver CSCs. The difference of β-catenin-stimulated TOP-Flash activities in CSCs and non-CSCs were observed firstly. It is more activation in CSCs than in non-CSCs (Supplementary Fig. 3D).

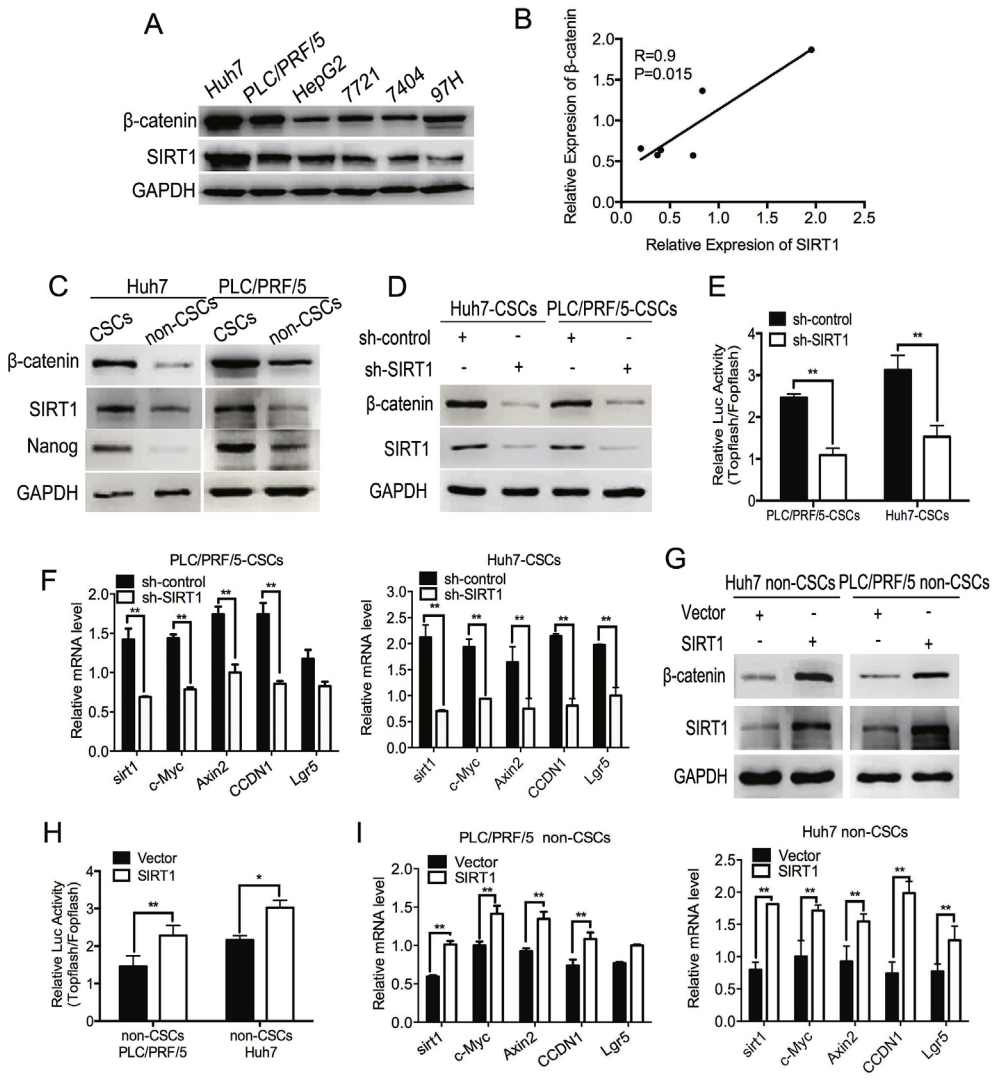

Fig. 2. β-catenin and its transcriptional activation are regulated by SIRT1 in liver CSCs. (A) Western blotting analysis of β-catenin protein levels in 6 HCC cell lines. Representative data of three independent experiments are shown. (B) Correlation between β-catenin and SIRT1 expression was analyzed by quantification of western blots in (A) using Image J software. Significance was determined using a Pearson's correlation. (C) Western blotting analysis of β-catenin and SIRT1 protein levels in liver CSCs and non-CSCs from Hun7 and PLC/PRF/5 cells. The experiments were repeated more than three times. (D) β-catenin protein levels were detected by western blotting in Huh7 and PLC/PRF/5 liver CSCs that stably express shRNA against SIRT1 (sh-SIRT1) or a control shRNA (sh-control). The experiments were repeated three times. (E) β-catenin activities were evaluated by a TOP/FOP-Flash luciferase assay in Huh7 and PLC/PRF/5 liver CSCs that stably express sh-SIRT1 or sh-control at 24hr after co-transfected with TOP/FOP-Flash reporters. The experiments were repeated three times. (F) mRNA levels of Wnt/β-catenin target genes were analyzed by RT-PCR in PLC/PRF/5 (left panel) and Huh7 (right panel) liver CSCs that stably express sh-SIRT1 or sh-control. The experiments were repeated three times. (G) Western blotting analysis of β-catenin protein levels in Huh7 (left panel) and PLC/PRF/5 (right panel) non-CSCs that transfected with SIRT1 plasmid or a control plasmid (vector). Representative data of three independent experiments are shown. (H) Huh7 and PLC/PRF/5 non-CSCs were transfected with SIRT1 plasmid or a control plasmid (vector) for 24hr, then co-transfected with TOP/FOP-Flash reporters. β-catenin activities were evaluated by TOP/FOP-Flash luciferase assay at 24hr later. The experiments were repeated three times. (I) mRNA levels of Wnt/β-catenin target genes were analyzed by RT-PCR in PLC/PRF/5 (left panel) and Huh7 (right panel) non-CSCs at 48hr after transfected with SIRT1 plasmid or a control plasmid(vector). The experiments were repeated three times. A Student's *t*-tests were used for statistical analysis in E-F and H–I. Error bars represent as mean ± s.e.m. *p < 0.05; **p < 0.01.

Knockdown of SIRT1 in CSCs abolished the TOP-Flash activity, and thus the transcriptional activity of Wnt/β-catenin (Fig. 2E). We also detected the endogenous and prototypic Wnt/β-catenin target genes, Axin2, c-Myc, CCND1 and Lgr5, whose expression levels were decreased in CSCs with knockdown of SIRT1 (Fig. 2F). In contrast, overexpression of SIRT1 resulted in an increase of β-catenin protein levels in non-CSCs (Fig. 2G and Supplementary Fig. 3E). What's more, up-regulating SIRT1 in non-CSCs strongly enhanced the activity of β-catenin-stimulated TOP-Flash (Fig. 2H), and increased Wnt/β-catenin target genes expressions (Fig. 2I).

Collectively, the above results suggest that Wnt/β-catenin signaling is activated in liver CSCs. Meanwhile, β-catenin transcriptional activity can be regulated by SIRT1 in liver CSCs.

3.3. SIRT1 deacetylates β-catenin to maintain its stability in liver CSCs

Previously, we demonstrated that SIRT1 regulated both β-catenin protein level and activated Wnt/β-catenin signaling in liver CSCs. However, the level of β-catenin transcript was not changed after knockdown SIRT1 expression in CSCs (Fig. 3A). These data suggest that SIRT1 might regulate protein turnover of β-catenin in liver CSCs. To investigate whether SIRT1 affects β-catenin protein stability, we measured β-catenin protein level in the presence of cycloheximide (CHX), an inhibitor of protein biosynthesis. As the time of CHX treatment was prolonged, the degradation of β-catenin was increased, and it was accelerated when SIRT1 was knocked down (Fig. 3B and C). Further study showed that knockdown of SIRT1 failed to induce β-catenin degradation in CSCs treated with the proteasome inhibitor MG132 (Fig. 3D). It reflected that the ubiquitination degradation pathway was involved. Consequently, the ubiquitylation of β-catenin was detected. Knockdown of SIRT1 in liver CSCs strongly enhanced the ubiquitylation level of β-catenin (Fig. 3E), indicating that SIRT1 plays an important role in regulating the protein stability of β-catenin in liver CSCs.

Protein co-immunoprecipitation (coIP) assays revealed that endogenous β-catenin and SIRT1 bound each other in Huh7 and PLC/PRF/5 cells (Supplementary Fig. 4A). However, this binding ability between β-catenin and SIRT1 was obviously decreased when liver CSCs were treated with a specific SIRT1 enzymatic activity inhibitor EX527 (Supplementary Fig. 4B). Our results have revealed that SIRT1 bound to β-catenin and regulated its ubiquitination to participate in the degradation of β-catenin. SIRT1 is an important deacetylase, and whether the deacetylase activity of SIRT1 was required to maintain the protein stability of β-catenin is unknown. Therefore, the acetylation levels of β-catenin was detected in liver CSCs with knockdown the SIRT1. Notably,

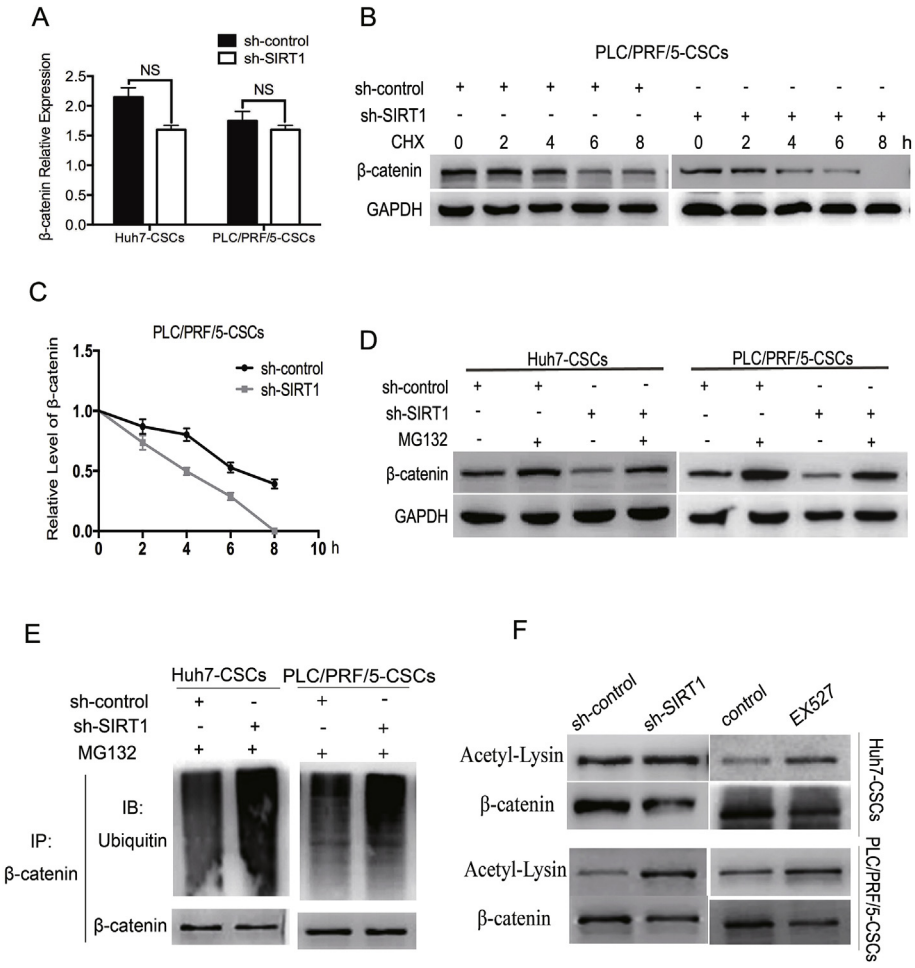

Fig. 3. Knockdown of SIRT1 induces instability of β-catenin in liver CSCs. (A) RT-PCR detection of β-catenin mRNA levels in response to transfection of sh-control or sh-SIRT1 in liver CSCs from Huh7 and PLC/PRF/5 CSCs. The graph is shown as mean ± s.e.m. Two-sided paired Student's *t*-tests were used for analysis. NS, $p > 0.05$. The experiments were repeated three times. (B) Comparison of β-catenin degradation in PCL/PRF/5 CSCs transfected with sh-control or sh-SIRT1, and then treated with control (DMSO) or 10 μg/mL CHX in a time series. CHX, cycloheximide – a protein synthesis inhibitor. The experiments were repeated three times. (C) Quantification of (B) in triplicate. Error bars represent as mean ± s.e.m. (D) Detected the degradation of β-catenin in Huh7 (left panel) and PCL/PRF/5 (right panel) CSCs after treated with sh-SIRT1 or sh-control, and inhibition of proteasome or not by MG132 (20 μM). MG132, a potent cell-permeable proteasome and calpain inhibitor. The experiments were repeated three times. (E) Ubiquitylation of β-catenin response to transfection of sh-control or sh-SIRT1 in liver CSCs from Huh7 (left panel) and PLC/PRF/5 (right panel) CSCs. Representative data of three independent experiments are shown. (F) Acetylation of β-catenin were determined by western blotting after 48hr transfected with sh-control or sh-SIRT1 or treated with 10 μM EX527 in liver CSCs from Huh7 (up) and PLC/PRF/5 (down) cells. The experiments were repeated three times.

knockdown of SIRT1 or treated with EX527 induced the acetylation of β-catenin in liver CSCs. (Fig. 3F). Moreover, inhibition of the SIRT1 enzymatic activity by EX527 resulted in decreased β-catenin protein levels (Fig. 4A) and accelerated its degradation (Fig. 4B and C) in similar fashion observed following knockdown of SIRT1 in liver CSCs. But inhibition of proteasome by MG132 inhibited the degradation of β-catenin and promoted the protein level of β-catenin to be restored in liver CSCs that treated with EX527 (Fig. 4D). It was also confirmed that the ubiquitylation of β-catenin was increased after reducing the enzymatic activity of SIRT1 by EX527 (Fig. 4E). Meanwhile, Wnt/β-catenin activity was substantially lower in liver CSCs that treated with EX527, as determined using the TOP-Flash reporter (Fig. 4F). The expression levels of Axin2, c-Myc, CCND1 and Lgr5 downstream of Wnt/β-catenin also were decreased in liver CSCs after treatment with EX527 (Fig. 4G).

Taken together, these results show that SIRT1 deacetylates β-catenin to maintain its protein stability, and regulates the activation of Wnt/β-catenin signaling in liver CSCs.

3.4. Deacetylation by SIRT1 promotes nuclear accumulation of β-catenin in liver CSCs

Nuclear accumulation of β-catenin is a critical event in many cancers, and it is essential for downstream gene expression. Therefore, nuclear β-catenin was detected in Huh7 and PLC/PRF/5 cells. The results showed that β-catenin accumulated in the nuclei in liver CSCs (Supplementary Figs. 5A and B). SIRT1 is mainly localized in the nucleus to deacetylate histones and other protein targets [18,22–24]. Whether SIRT1 plays a role in regulating the acetylation of nuclear β-catenin to maintain its nuclear localization is not known. Knockdown of SIRT1 in liver CSCs resulted in a decrease in nuclear presence and accumulation of β-catenin (Fig. 5A and B). Wnt3a is known to stabilize β-catenin and increase its nuclear accumulation, we examined the interaction of SIRT1 and β-catenin upon wnt3a stimulation in liver CSCs. The protein levels and nuclear accumulation of β-catenin increased substantially with Wnt3a treatment in a density-dependent manner in liver CSCs (Fig. 5C). However, knockdown of SIRT1 abolished Wnt3a-induced β-catenin nuclear accumulation in liver CSCs (Fig. 5B and C). Conversely, overexpression of SIRT1 maintained β-catenin protein stability and nuclear localization in non-CSCs (Fig. 5D and E). When the non-CSCs were treated with Wnt3a, nuclear β-catenin significantly increased, and its accumulation was enhanced by co-expression of SIRT1 (Fig. 5D). We have demonstrated that deacetylation activity of SIRT1 was important for maintain protein stabilization of β-catenin. So, acetylation levels of β-catenin in cytoplasm and nuclear were detected. The results showed that knockdown of SIRT1 did increase acetylation level of β-catenin, and it only induced nuclear β-catenin to acetylation (Fig. 5F). We also overexpressed a deacetylase-inactive point mutant of SIRT1 (SIRT1-H363Y) in non-CSCs. Neither total protein level of β-catenin nor nuclear β-catenin was changed by overexpression of SIRT1-H363Y (Fig. 5E and G). These results suggest that deacetylation by SIRT1 is critical for β-catenin nuclear accumulation in liver CSCs.

3.5. SIRT1/β-catenin axis participates in self-renewal of liver CSCs and tumor growth by regulating Nanog

It has been identified that SIRT1 and β-catenin were both upregulated in liver CSCs and downregulated in non-CSCs. In order to study the role of SIRT1/β-catenin axis in HCC progression, we investigated

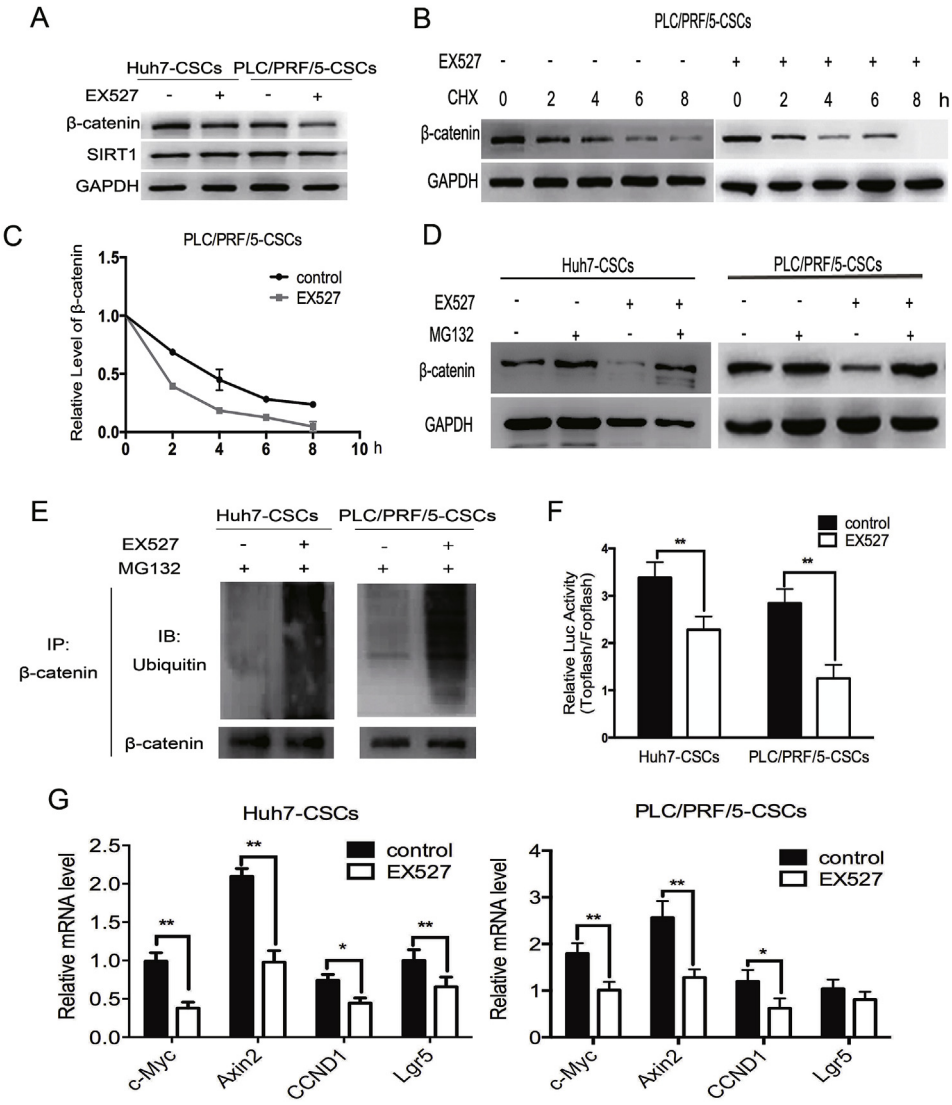

Fig. 4. Inhibition of SIRT1 deacetylase activity reduces β-catenin stabilization and transcriptional activation in liver CSCs. (A) Western blotting analysis of β-catenin protein levels in liver CSCs from Huh7 (left panel) and PLC/PRF/5 (right panel) cells at 48hr after treated with 10 μM EX527. The experiments were repeated more than three times. (B) Comparison of β-catenin degradation in PLC/PRF/5 CSCs, which were treated with CHX in a time series after pre-treated with control (DMSO) or 10 μM EX527 for 48hr. The experiments were repeated three times. (C) Quantification of (B) in triplicate. Error bars represent as ± s.e.m. (D) Detection of the effect of β-catenin degradation in liver CSCs from Huh7 (left panel) and PLC/PRF/5 (right panel) cells, which were treated with control (DMSO) or 10 μM EX527 for 48hr, and then without or with treatment of 20 μM MG132 for 2hr. The experiments were repeated three times. (E) Ubiquitylation of β-catenin response to inhibition of SIRT1 deacetylase activity by EX527 in liver CSCs from Huh7 (left panel) and PLC/PRF/5 (right panel) cells. Representative data of three independent experiments are shown. (F) Huh7 and PLC/PRF/5 CSCs were treated with control (DMSO) or 10 μM EX527, and then β-catenin transcriptional activities were determined by TOP/FOP-Flash luciferase assay. The experiments were repeated three times. Error bars represent as mean ± s.e.m. (G) mRNA levels of Wnt/β-catenin target genes in liver CSCs from Huh7 (left panel) and PLC/PRF/5 (right panel) cells were detected by RT-PCR at 48hr after treated with DMSO (control) or 10 μM EX527. The experiments repeated three times. Error bars represent as mean ± s.e.m. A Student's *t*-tests were used for statistical analysis in F an G. *p < 0.05; **p < 0.01.

whether SIRT1/β-catenin axis regulates the self-renewal of liver CSCs. Knockdown of SIRT1 or β-catenin significantly decreased the expression of Nanog in liver CSCs (Fig. 6A). Simultaneous overexpression of SIRT1 and β-catenin strongly promoted the expression of Nanog in non-CSCs (Fig. 6B). We further found that knockdown of SIRT1 or β-catenin in CSCs resulted in suppressed Nanog promoter activation (Fig. 6C, left panel), whereas promoter activation was significantly up-regulated by simultaneous overexpression of β-catenin and SIRT1 in non-CSCs (Fig. 6C, right panel). Then chromatin immunoprecipitation (CHIP) analyses showed that β-catenin bound to the promoter region of Nanog (Fig. 6D). It indicated that SIRT1/β-catenin axis might participate in self-renewal and tumorigenesis of liver CSCs by directly regulating Nanog, which is an important stemness gene in liver CSCs.

Further studies demonstrated that treatment with EX527 or knockdown of β-catenin decreased both clone formation and sphere formation abilities of liver CSCs (Fig. 6E and F). However, the inhibitory effect of sh-β-catenin on self-renewal of liver CSCs was rescued by overexpressing Nanog (Fig. 6E and F). Next, we examined the effect of β-catenin on the tumorigenesis of liver CSCs in a xenograft animal model. Compared with control, knockdown of β-catenin in liver CSCs strongly inhibited the tumorigenicity (Fig. 6H–J). Whereas up-regulating Nanog in sh-β-catenin CSCs restored both self-renewal activity and tumorigenicity (Fig. 6H–K). These results confirm that SIRT1/β-catenin axis regulates Nanog, and participates in self-renewal of liver CSCs, thereby promoting the tumor growth of HCC.

4. Discussion

In the present study, we demonstrate that β-catenin is deacetylated by SIRT1 in liver CSCs, which promotes β-catenin nuclear accumulation and activation of Wnt/β-catenin signaling, and that nuclear β-catenin participates in self-renewal and tumorigenesis via regulating Nanog in liver CSCs. Therefore, the deacetylation of β-catenin by SIRT1 represents a critical mechanism for regulating liver CSCs self-renewal and tumorigenesis.

Aberrant activation of the Wnt/β-catenin signaling pathway is vital in the development of numerous types of human cancers. Wnt/β-catenin signaling plays an essential role in the maintenance of CSC properties and is responsible for the recurrence and poor prognosis of tumor including HCC [25,26]. In this study, we confirmed that β-catenin was strongly expressed in human HCC tumor samples and liver CSCs, which caused poor prognosis. In human HCC samples and cell lines, β-catenin was highly associated with SIRT1, a class III histone deacetylase. Furthermore, inhibition of SIRT1 led to the decrease of β-catenin protein level and inhibition of Wnt/β-catenin signaling pathway activity in liver CSCs. These findings indicate that SIRT1 plays an important role in modulating β-catenin activity in liver CSCs.

β-catenin protein stability and nuclear localization are essential for

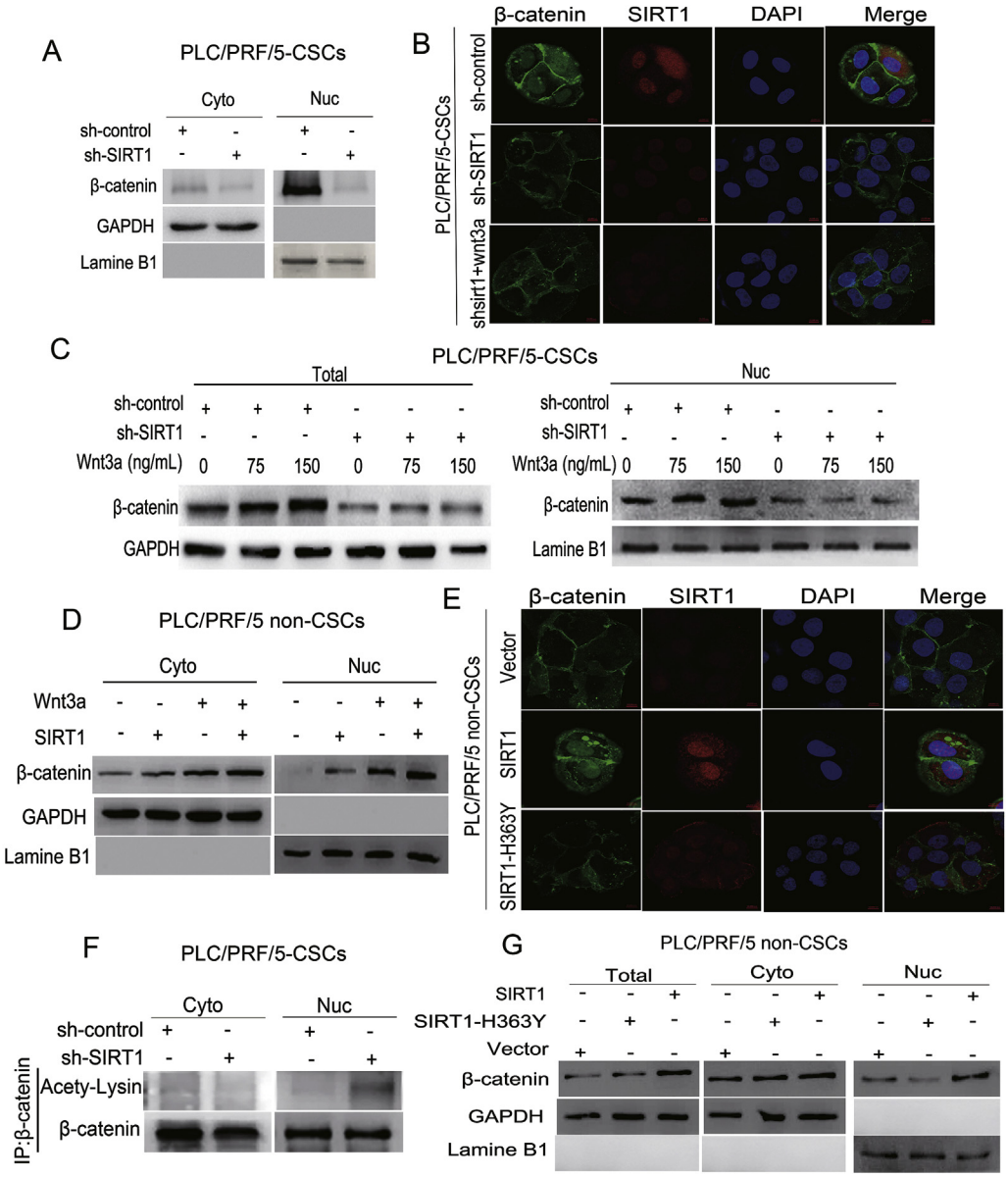

Fig. 5. β-catenin nuclear accumulation depends on SIRT1. (A) Western blotting analysis of cytoplasm (left panel) and nuclear (right panel) protein levels of β-catenin in PLC/PRF/5 CSCs after knockdown of SIRT1 by transfected with sh-SIRT1. The experiments were repeated three times. (B) IF staining of β-catenin (green), SIRT1 (red), and nuclei (DAPI, blue) in sh-control, sh-SIRT1 PLC/PRF/5 CSCs and sh-SIRT1 liver CSCs that were treated with 75 ng/mL Wnt3a for 2hr. Representative images of three independent experiments are shown. (C) Western blotting analysis of total (left panel) and nuclear (right panel) protein levels of β-catenin in sh-control and sh-SIRT1 PLC/PRF/5 CSCs, which were co-treated with 75 ng/mL or 150 ng/mL Wnt3a for 2hr. The experiments were repeated three times. (D) PLC/PRF/5 non-CSCs were transfected with SIRT1 or vector for 48hr and then treated with 75 ng/mL Wnt3a or PBS for 2hr. Cytoplasm (left panel) and nuclear (right panel) protein levels of β-catenin were determined by western blotting. The experiments were repeated three times. (E) IF staining of β-catenin (green), SIRT1 (red), and nuclei (DAPI, blue) in PLC/PRF/5 non-CSCs transfected with control vector, SIRT1, or SIRT1-H363Y mutant for 48hr. The experiments were repeated three times. (F) Acetylation of β-catenin in cytoplasm (left panel) and nuclear (right panel) were detected by western blots in liver CSCs from PLC/PRF/5 cells that stably express sh-control or sh-SIRT1. The experiments were repeated three times. (G) Total (left), cytoplasm (middle) and nuclear (right) protein levels of β-catenin were detected by western blotting in liver non-CSCs from PLC/PRF/5 cells that were transfected with vector, wild-type SIRT1 or SIRT1 deacetylase inactive H363Y mutant (SIRT1-H363Y). The experiments repeated three times.

Wnt/β-catenin pathway activation. Nuclear accumulation of β-catenin is required for transcriptional activation of Wnt signaling target genes. It is well known that β-catenin phosphorylation and subsequent degradation in the cytoplasm are required to prevent its nuclear translocation. However, the regulation of β-catenin in the nucleus has not been fully understood by now. In this study, we demonstrate that SIRT1 is important for maintaining stabilization of nucleic β-catenin. Downregulation of SIRT1 in liver CSCs accelerated the degradation of β-catenin, and caused instability of nucleic β-catenin. Previously study has reported that β-catenin can be acetylated by p300 and CERB-binding protein (CBP) acetyltransferase in epithelial cells, and be deacetylated by HDAC6. SIRT1 is well known as a histone deacetylase and it also can deacetylate numerous mammalian transcription factors important for disease. Thus, we hypothesize that SIRT1 maintains β-catenin nuclear stability through regulating β-catenin acetylation in liver CSCs. We observed that β-catenin was hyperacetylated upon SIRT1 inhibition using shRNA or treatment with the inhibitor EX527 in liver CSCs, which decreased total protein level and nuclear accumulation and transcriptional activity of β-catenin. Conversely, β-catenin nuclear localization was enhanced by overexpression of wild-type SIRT1, whereas deacetylase-inactive point mutant of SIRT1 (SIRT1-H363Y) didn't affect protein level or nuclear accumulation of β-catenin in non-CSCs. Previously, it was shown that β-catenin can be phosphorylated by destruction complex components for its subsequent degradation in both cytoplasm and nucleus [27]. Nuclear non-phosphorylated β-catenin also can be degraded regulating by kdm2a/b [8]. Therefore, hyperacetylated β-catenin, as a consequence of inhibiting SIRT1 in liver CSCs, might result in protein degradation depending on phosphorylation or non-phosphorylation status or both of them in nucleus, and it also might be relocated to cytoplasm and induced into degradation.

We identified that β-catenin and SIRT1 were both strongly expressed in liver CSCs, and β-catenin was deacetylated by SIRT1 to trigger its concentration in the nucleus and activate its transcriptional activity. Nuclear β-catenin plays an essential role in the maintenance of CSC properties, including self-renewal and special marker expression. Our data show that β-catenin is able to bind to the Nanog promoter to stimulate its expression, promote the stemness of liver CSCs and trigger oncogenesis. Additional research has demonstrated that overexpression

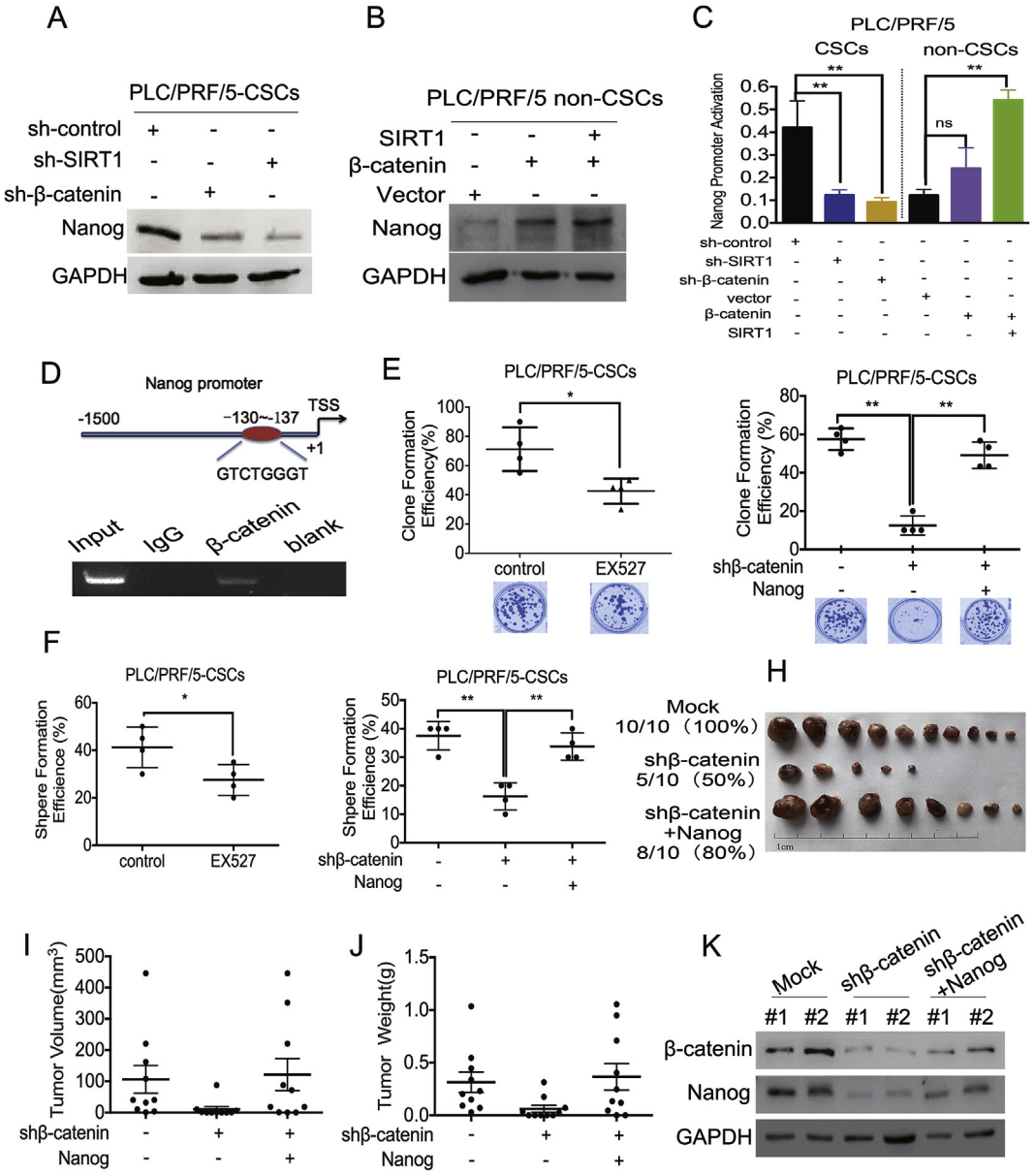

Fig. 6. SIRT1/β-catenin axis maintains the stemness of liver CSCs and promotes tumorigenesis by regulating Nanog. (A) Western blotting analysis of Nanog expression in liver CSCs from PLC/PRF/5 cells that stably express sh-β-catenin or sh-SIRT1 or sh-control. The experiments were repeated three times. (B) Western blotting analysis of Nanog in liver non-CSCs from PLC/PRF/5 cells that were transfected with vector, β-catenin or SIRT1 for 48hr. The experiments were repeated three times. (C) Knockdown of β-catenin or SIRT1 in liver CSCs (left panel) weakened the luciferase activities of Nanog promoter reporter, which were enhanced by overexpressing β-catenin and SIRT1 in liver non-CSCs (right panel). The graph is shown as mean ± s.e.m of n = 3 from one representative experiment. (D) Schematic figure shows promoter region of Nanog gene (up) and potential binding site for β-catenin (down) ChIP assay determined the binding of β-catenin to Nanog promoter regions. Representative images of three independent experiments are shown. (E and F) Clone formation efficiency (E left panel) and sphere formation efficiency (F left panel) were analyzed in PLC/PRF/5 CSCs in the presence of DMSO or 10 μM EX527. Clone formation efficiency (E right panel) and sphere formation efficiency (F right panel) of liver CSCs in PLC/PRF/5 without or with overexpressing Nanog after knockdown of β-catenin. The graph is shown as mean ± s.e.m. The experiments were repeated three times. (H–J) Live CSCs, β-catenin knockdown liver CSCs and Nanog overexpression while β-catenin knockdown liver CSCs subcutaneous injected in NOD-SCID mice, each group has 10 mice (H), Tumor volumes (I) were calculated using the formula: tumor volumes (cm^3) = 0.5 × (Width^2 × Length). We counted and weight (J) the tumors, 40 days later. (K) β-catenin and Nanog protein were detected by western blotting in tumors from mice injected with liver CSCs, β-catenin knockdown liver CSCs or Nanog overexpression while β-catenin knockdown liver CSCs. The experiments were repeated three times. A Student's *t*-tests were used for statistical analysis in C, E and F. Error bars are represented as mean ± s.e.m. *p < 0.05; **p < 0.01.

of Nanog can reverse the decrease in self-renewal and tumorigenesis of liver CSCs induced by inhibiting β-catenin. These results indicate that SIRT1/β-catenin axis participates in self-renewal and tumorigenesis via regulating Nanog in liver CSCs.

In summary, our study reveals that SIRT1 is a key regulator of β-catenin and that SIRT1 deacetylase activity contributes to nuclear stabilization of β-catenin in liver CSCs. Our findings provide an improved understanding of molecular mechanisms underlying β-catenin activation and tumorigenesis in liver CSCs.

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Author contributions

XC: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, statistical analysis; HH, CL, JS, YL and YZ: acquisition of data, technical and material support; ZZ: analysis and interpretation of data, critical revision of the manuscript; CQ: study concept, analysis and interpretation of data, critical revision of the manuscript, study supervision.

Disclosures of potential conflicts of interest

The authors disclose no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.07.021.

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