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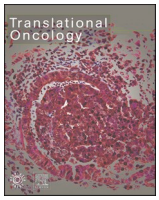

Original Research

FEN1 upregulation mediated by SUMO2 via antagonizing proteasomal degradation promotes hepatocellular carcinoma stemness

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ABSTRACT

Purpose: Metastasis of hepatocellular carcinoma (HCC) critically impacts the survival prognosis of patients, with the pivotal role of hepatocellular carcinoma stem cells in initiating invasive metastatic behaviors. The Flap Endonuclease 1 (FEN1) is delineated as a metallonuclease, quintessential for myriad cellular processes including DNA replication, DNA synthesis, DNA damage rectification, Okazaki fragment maturation, baseexcision repair, and the preservation of genomic stability. Furthermore, it has been recognized as an oncogene in a diverse range of malignancies. Our antecedent research has highlighted a pronounced overexpression of protein FEN1 in hepatocellular carcinoma, where it amplifies the invasiveness and metastatic potential of liver cancer cells. However, its precise role in liver cancer stem cells (LCSCs) remains an enigma and requires further investigation.
Methods: To rigorously evaluate the stemness attributes of LCSCs, we employed sphere formation assays and flow cytometric evaluations. Both CD133+ and CD133- cell populations were discerningly isolated utilizing immunomagnetic bead separation techniques. The expression levels of pertinent genes were assayed via real-time quantitative PCR (RT-qPCR) and western blot analyses, while the expression profiles in hepatocellular carcinoma tissues were gauged using immunohistochemistry. Subsequent immunoprecipitation, in conjunction with mass spectrometry, ascertained the concurrent binding of proteins FEN1 and Small ubiquitin-related modifier 2 (SUMO2) in HCC cells. Lastly, the impact of SUMO2 on proteasomal degradation pathway of FEN1 was validated by supplementing MG132.
Results: Our empirical findings substantiate that protein FEN1 is profusely expressed in spheroids and CD133+ cells. *In vitro* investigations demonstrate that the upregulation of protein FEN1 unequivocally augments the stemness of LCSCs. In a congruent *in vivo* context, elevation of FEN1 noticeably enhances the tumorigenic potential of LCSCs. Conversely, inhibiting protein FEN1 resulted in a marked reduction in LCSC stemness. From a mechanistic perspective, there exists a salient positive correlation between the protein expression of FEN1 and SUMO2 in liver cancer tissues. Furthermore, the level of SUMO2-mediated modification of FEN1 is pronouncedly elevated in LCSCs. Interestingly, SUMO2 has the ability to bind to FEN1, leading to a inhibition in the proteasomal degradation pathway of FEN1 and an enhancement in its protein expression. However, it is noteworthy that this interaction does not affect the mRNA level of FEN1.
Conclusion: In summation, our research elucidates that protein FEN1 is an effector in augmenting the stemness of LCSCs. Consequently, strategic attenuation of protein FEN1 might proffer a pioneering approach for the efficacious elimination of LCSCs.

Introduction

Hepatocellular carcinoma, a profoundly malignant neoplasm, ranks prominently among the principal etiologies of cancer-associated mortalities [1]. Recent scholarly endeavors have illuminated the presence of cellular subpopulations within tumors that possess the intrinsic capacity

Abbreviations: LCSCs, liver cancer stem cells; CSCs, cancer stem cells; HCC, hepatocellular carcinoma; FEN1, Flap Endonuclease1; EMT, epithelial-mesenchymal transition; SUMO2, small ubiqutin-related modifier2; PTMs, post-translational modifications.

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for self-renewal and can engender a diverse array of tumor cells. These cells are characterized by unrestrained proliferation, intrinsic self-renewal, and multi-faceted differentiation. Notably, they are implicated as central drivers behind tumor proliferation, recurrence, invasiveness, metastasis, and pharmacoresistance. These cells have been christened as Cancer Stem Cells (CSCs) [2,3].

Accumulating empirical evidence underscores the presence of a cellular subset with stem cell attributes within liver cancer tissue, termed liver cancer stem cells (LCSCs) [4]. The identification of these stem cell subpopulations has been facilitated by a gamut of immunomarker molecules. It is worth noting that the stem cell phenotype varies across CSCs originating from diverse tumors. Prominent stemness markers associated with LCSCs encompass CD90, CD44, CD133, EpCAM, Nanog, OV6, ALDH, and others [5]. Of these, CD133 and CD90 have been particularly spotlighted for their capacity to enrich LCSCs.

The underpinnings governing stemness within LCSCs are intricate, entailing a tapestry of signaling pathways such as Wnt/β-catenin, TGF-β, Hippo-YAP, PI3K/AKT/mTOR, alongside proto-oncogenes, microRNAs, and LncRNAs [6]. Existing literature emphasizes the pivotal roles of transcription factors like Oct4, Sox2, and Nanog in orchestrating CSC stemness [7]. Oct4, also christened as POU5F1, is recognized to potentiate stemness within HCC via a synergistic feedback loop with the oncogene c-Jun [8]. Nanog emerges as a linchpin, independent of the LIF-STAT3 pathway, in preserving the pluripotential differentiation capacity of pluripotent stem cells. Furthermore, it not only functions as a CSC biomarker but also sustains CSC self-renewal via the insulin-like growth factor 1 receptor signaling trajectory [9]. In LCSCs, oncogenes have also been documented to regulate stemness by modulating stemness genes [10]. As our understanding of LCSCs deepens, the targeted therapy against LCSCs is increasingly viewed as a pioneering avenue for HCC treatment. Nonetheless, the molecular mechanisms underlying LCSC stemness remain obfuscated, mandating further exploration to bolster the prevention and therapy for liver cancer metastasis.

In our antecedent investigations, we discerned a marked upregulation of FEN1 expression within hepatocellular carcinoma tissues, exhibiting a significant correlation with tumor dimensions, microvascular infiltration, and metastasis. Interestingly, protein FEN1 is implicated in fostering hepatocellular carcinoma cell invasiveness via epitomizing the epithelial-to-mesenchymal transition [11,12]. Yet, the question of whether FEN1 modulates the stemness of hepatocellular carcinoma cells remains to be addressed. Thus, anchored in our prior endeavors, we delved into the role of protein FEN1 within LCSCs.

The SUMO family, which currently consists of four major isoforms in mammals:SUMO1, SUMO2, SUMO3 and SUMO4, is a highly conserved family of proteins that are widely present in eukaryotic cells. It is noteworthy that several constituents of the SUMO chemical modification system manifest aberrant overexpression within tumors [13]. Furthermore, elevated levels of SUMO modifications are frequently observed in tumors, seemingly advancing their progression [14,15]. Guo et al. discerned an anomalous surge in SUMO1 expression within HCC, which promoted HCC proliferation [16]. Consistent with these observations, SUMO chemical alterations have been posited to play a crucial role in governing tumor cell stemness [17]. Li Du and colleagues delineated that the overexpression of SUMO E1/E2 accentuated CSC stemness, whereas the attenuation of SUMO E1/E2 expression curtailed the sustenance and self-renewal of this stemness [15]. Literature intimates that the K168 locus of FEN1 in HeLa cells can undergo modification by SUMO3, suggesting that the SUMO modification of the K168 locus of FEN1 is pivotal in modulating FEN1 expression across cellular cycles [18]. SUMO modifications is closely related to ubiquitination, potentially preventing the binding of ubiquitin to its substrate and thereby inhibiting the proteasome pathway. Previous studies have demonstrated that FEN1 can undergo degradation via the ubiquitin-proteasome pathway [19,20]. Building upon this evidence, we propose that SUMO2 protein may enhance protein levels of FEN1 in hepatocellular carcinoma cells by counteracting its proteasomal degradation pathway.

In summary, our research aimed to investigate the role of SUMO2 in regulating the upregulation of FEN1 and the impact of FEN1 on enhancing stemness in LCSCs.

Materials and methods

Cell lines and culture conditions

The Huh7 human hepatocellular carcinoma cell line was procured from the Shanghai Cell Bank of the Chinese Academy of Sciences (China), while the MHCC-97H hepatocellular carcinoma cell line was obtained from Zhongshan Hospital (Shanghai, China). Adherent cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Heclone, USA) supplemented with 10 % fetal bovine serum (FBS; Capricorn, USA). The cultures were maintained in an incubator set at 37℃ with an atmosphere of 5 % CO_2. The medium was refreshed every alternate day. LCSCs were propagated in a serum-free stem cell medium. This medium was formulated with DMEM/F12 (Gibco, USA), 2 % B27 supplement (Invitrogen, USA), 10 ng/ml basic fibroblast growth factor (bFGF; PeproTech, USA), 20 ng/ml recombinant epidermal growth factor (EGF; PeproTech, USA), 100 U/ml penicillin, and 100 g/ml streptomycin.

Isolation of CD133+ and CD133- Cells

The CD133 Microbead kit (Miltenyi Biotec) was employed to isolate CD133+ cells, adhering to the manufacturer's detailed protocol. Post enzymatic cell isolation from HCC, the cell suspension was centrifuged at 300 x g for a duration of 10 min. A volume of 300μl buffer was used to resuspend every 107 cells, followed by the addition of 20μl fluorescent receptor blocker and 10 μl of immunomagnetic beads. The mixture was subsequently incubated at 4 ℃ for 15 min in the dark. Following a buffer wash and centrifugation at 300×g for 10 min, cells were resuspended and subjected to separation using the Mini Macs® separator (Miltenyi Biotec). The efficacy of magnetic separation was assessed through flow cytometry, utilizing a phycoerythrin (PE)-labelled CD133/2 antibody (Miltenyi Biotec).

Flow cytometry analysis

For flow cytometric analysis, cells were harvested and resuspended in 80μl PBS. This suspension was treated with 20μl of FcR blocking reagent (Miltenyi Biotec, Germany) and 2μl of PE-conjugated anti-human CD133/CD90 (Miltenyi Biotec, Germany) for 10 min at 4℃ in a light-protected environment. Following a PBS wash and centrifugation at 300×g for 10 min, cells were resuspended and analyzed via flow cytometry (BD Biosciences, USA).

Real-time quantitative PCR (RT-qPCR)

All primers were custom-designed and synthesized by Shanghai Biotech (China) (Table 1). Cellular total RNA was extracted employing Trizol reagent (Invitrogen). The PrimeScript™ RT Reagent Kit (Takara, Japan) was used for reverse transcription as per manufacturer guidelines. Subsequent RT-qPCR amplification was conducted using the SYBR Premix ExTaq (Takara, Japan), with reactions read on the CFX96 Real-Time PCR Detection System (Bio-Rad).

Western blot analysis

Proteins from Huh7 and MHCC-97H cells were lysed using the RIPA (strong) lysis buffer (Beyotime, China) complemented with phosphatase and protease inhibitors (Cwbio, China). The BCA protein assay kit (Beyotime, China) facilitated protein quantification. Subsequent gel electrophoresis utilized sodium dodecyl sulphate-polyacrylamide gel, with proteins transferred onto polyvinylidene fluoride membranes

Table 1
Primer sequences for quantitative RT-qPCR.

Gene	Primer sequences
FEN1	Forward (5′−3′): CTGTGGACCTCATCCAGAAGCA
	Reverse (5′−3′): CCAGCACCTCAGGTTCCAAGA
CD90	Forward (5′−3′): GACCCGTGAGACAAAGAAGC
	Reverse (5′−3′): GCCCTCACACTTGACCAGTT
CD133	Forward (5′−3′): TGGATGCAGACCTTGACAACGT
	Reverse (5′−3′): ATACCTGCTACGACAGTCGTGGT
CD44	Forward (5′−3′): CATCCCAGACGAAGACAGTCC
	Reverse (5′−3′): TGATCAGCCATTCTGGAATTTG
Epcam	Forward (5′−3′): GCTGGTGTGTGAACACTGC
	Reverse (5′−3′): ACGCGTTGTGATCTCCTTCT
Oct4	Forward (5′−3′): CTTGCTGCAGAAGTGGGTGGAGGAA
	Reverse (5′−3′): CTGCAGTGTGGGTTTCGGGCA
Nanog	Forward (5′−3′): AATACCTCAGCCTCCAGCAGATG
	Reverse (5′−3′): TGCGTCACACCATTGCTATTCTTC
Bmi-1	Forward (5′−3′): TGGAGAAGGAATGGTCCACTTC
	Reverse (5′−3′): GTGAGGAAACTGTGGATGAGGA
Sox2	Forward (5′−3′): TGGACAGTTACGCGCACAT
	Reverse (5′−3′): CGAGTAGGACATGCTGTAGGT
GAPDH	Forward (5′−3′) : GGTGGTCTCCTCTGACTTCAACA
	Reverse (5′−3′): GTTGCTGTAGCCAAATTCGTTGT

(Millipore, USA). Post blocking with 5 % non-fat milk for 1 h, membranes were incubated with primary antibodies overnight at 4℃. Following thrice washing with TBST, membranes were exposed to secondary antibodies at room temperature for an hour. Protein bands were visualized on a ChemiDoc Imager (Bio-Rad, USA). Employed antibodies included: anti-CD133 (Immunoway, USA), anti-Nanog (Immunoway, USA), anti-Oct4 (ABmart, China), anti-Bmi1 (Immunoway, USA), anti-FEN1 (ABclonal, China), anti-SUMO2 (Genetex, America), and anti-GAPDH (used as a loading control; Cell Signaling Technology, Danvers, MA, USA).

Construction of stable cell lines

A lentiviral vector encompassing pcSLenti-CMV-B-3xFLAG-PGK-Puro-WPRE3 and pCLenti-U6-shRNA-CMV-Puro-WPRE was procured from Obio Technology Corp. , Ltd (Shanghai, China). Short-hairpin RNA (shRNA) sequences targeting the human gene SUMO2 were meticulously designed and are enumerated in Table 3. The primer sequences tailored for SUMO2overexpression vectors have been delineated in Table 2. The methodologies for transfection, screening, and the construction of FEN1 cell lines exhibiting varied expression levels were previously delineated and employed as such [11,12].

Spheroid formation assay

Cells were rendered to a homogenous suspension in serum-free stem cell medium and subsequently seeded into 6-well ultra-low attachment culture plates at a density of 2000 cells per well. After a week of incubation, spheroids surpassing a diameter of 100 μm were enumerated using an inverted microscope at a 10x magnification.

In vivo xenograft mice assay

All animal experiments were conducted in accordance with the ethical standards of animal care and under the guidance of Animal Care Committee of Chongqing Medical University. Six-week-old female nude mice were sourced from the Animal Experimentation Centre of Chongqing Medical University (China) and were housed under specific pathogen-free (SPF) conditions. CD133+/CD133- cells, post isolation using immunomagnetic beads, were subcutaneously injected into the nude mice at a concentration of 1 × 104 cells per mouse. Tumor development was monitored weekly, culminating in a comprehensive assessment at week six. Post euthanasia, the excised tumors were photographed. Segments were preserved at -80 °C for subsequent protein and RNA extractions, while others were fixed in 4 % paraformaldehyde, earmarked for further experimentation.

Table 2
Primer sequences for SUMO2 overexpressing vector.

Gene	Primer sequences
SUMO2	CMV-F CGCAAATGGGCGGTAGGCGTG
	PGK-R AAGAACGGAGCCGGTTGGCG

Table 3
The sequence of short-hairpin RNA (shRNA) targeting human SUMO2.

shRNA	Target sequence
Sh-control	TTCTCCGAACGTGTCACGT
Sh1-SUMO2	GACTGAGAACAACGATCATAT
Sh2-SUMO2	TACACCACTTAGTAAACTAAT
Sh3-SUMO2	GTGGTGCAGTTTAAGATTAAG

Co-immunoprecipitation

An optimal quantity of Protein A beads (MCE, America) was placed in a silica tube and subjected to a thrice wash cycle with PBS. The samples were centrifuged at 4 ℃ at 1000 rpm for 2 min. After discarding the supernatant, the beads were washed once with IP Lysis buffer (Beyotime, China). The pH was adjusted to lie between 7. 4–7. 5, and samples were stored at 4℃. Antibodies were added to Protein A beads in IP Lysis buffer, making up the volume to 600 μl, and then incubated under rotation at 4 °C for 3 h. Following centrifugation, the supernatant was removed. The bead-antibody complex was then combined with cell lysate and incubated overnight at 4 ℃. After a series of washes and centrifugation cycles, the appropriate volume of IP Lysis buffer was added, followed by the proportional addition of 1×SDS-PAGE protein buffer (Beyotime, China). The mixture was then boiled for 10 min. For the Western blotting experiment, the primary antibodies used were FEN1 and SUMO2. The resultant binding proteins were subjected to either LC-MS/MS or Western blot analysis, with the primary antibodies being FEN1 and SUMO2 for the latter.

Immunohistochemistry

Tissue sections were subjected to a sequential immersion in fresh xylene thrice, each for a duration of 10 min, followed by a progressive dehydration through 100 %, 95 %, and 70 % ethanol solutions, each for 5 min. Subsequent to this, the sections were washed in PBS in three cycles of 3 min each. The sections were then incubated in deionised water supplemented with 3 % H_2O_2 for a period of 10 min, post which they were extensively rinsed in PBS thrice, with each rinse lasting for 5 min. For antigen retrieval, the sections were immersed in a 0. 01 M citrate buffer (pH 6. 0) (Bioss, China), subjected to boiling for 20 min, allowed to return to ambient temperature, and subsequently washed in PBS three times, for a span of 5 min each. An optimum quantity of the diluted primary antibody was applied and the sections were incubated overnight at 4 ℃. This was followed by a re-warming phase at 37 ℃ for 45 min, after which they were washed with PBS three times, each lasting 5 min. The sections were then treated with an appropriate amount of biotin-labelled goat anti-rabbit secondary antibody (AiFang Biological, China) and allowed to incubate at room temperature for 30 min. After another series of PBS washes, a DAB (ZSGB-Bio, China) color solution, prepared according to the manufacturer's stipulation, was applied to the tissue and allowed to react for 5 min at room temperature. This was closely monitored under a microscope for cytoplasmic staining. The reaction was terminated by rinsing the tissue with water. Thereafter, a hematoxylin staining step (Beyotime, China) was performed for 60 s, followed by differentiation using hydrochloric acid-alcohol, and subsequent rinsing. Once the desired color intensity was observed under a

microscope, the sections were rehydrated through an ethanol series (70 %, 95 %, and 100 %) for 3 min each. The sections were then cleared in fresh xylene, subjected to three cycles of 5 min each, mounted, and finally visualized and captured under an upright microscope (Olympus, Japan).

Proteasome assay

Prior to harvesting, the cells were subjected to a treatment of 20 μM MG132 (MCE, America) for a duration of 4 h. Equal volume of DMSO was added to the control group.

TCGA data analysis

SUMO2 expression in HCC was compared in the ONCOMINE database (https://www.oncomine.org). SUMO2 expression and its relationship to clinical outcomes in HCC were analyzed using the GEPIA database (http://gepia.cancer-pku.cn/).

HCC specimens

The information about the 34 paired HCC and para-carcinoma tissue samples is as described earlier [11].

Subcellular localisation

The subcellular localisation of FEN1 and SUMO2 was analysed by the GeneCards database (https://www.genecards.org/).

Statistical analysis

Statistical evaluations were performed using the SPSS 23. 0 statistical software, and all values were presented as mean ± standard deviation ($X \pm SD$). The dataset comprised results from a minimum of three independent experiments. The independent samples *t*-test was employed to discern differences between two groups. A difference was deemed statistically significant at $^{*}P < 0.05$, with levels of significance denoted as follows: $^{*}P < 0.05$; $^{**}P < 0.01$.

Results

Elevated expression of FEN1 in LCSCs and its potential regulatory role in hepatocellular carcinoma cell stemness

To elucidate the potential involvement of FEN1 in modulating the stemness of LCSCs, hepatocellular carcinoma stem cells were enriched through suspension culture using a serum-free stem cell medium. Subsequent analyses via RT-qPCR and WB were employed to discern variations in the expression of FEN1 and other stemness genes between the cell spheroids and adherent cells. Notably, Huh7 cells cultivated in a

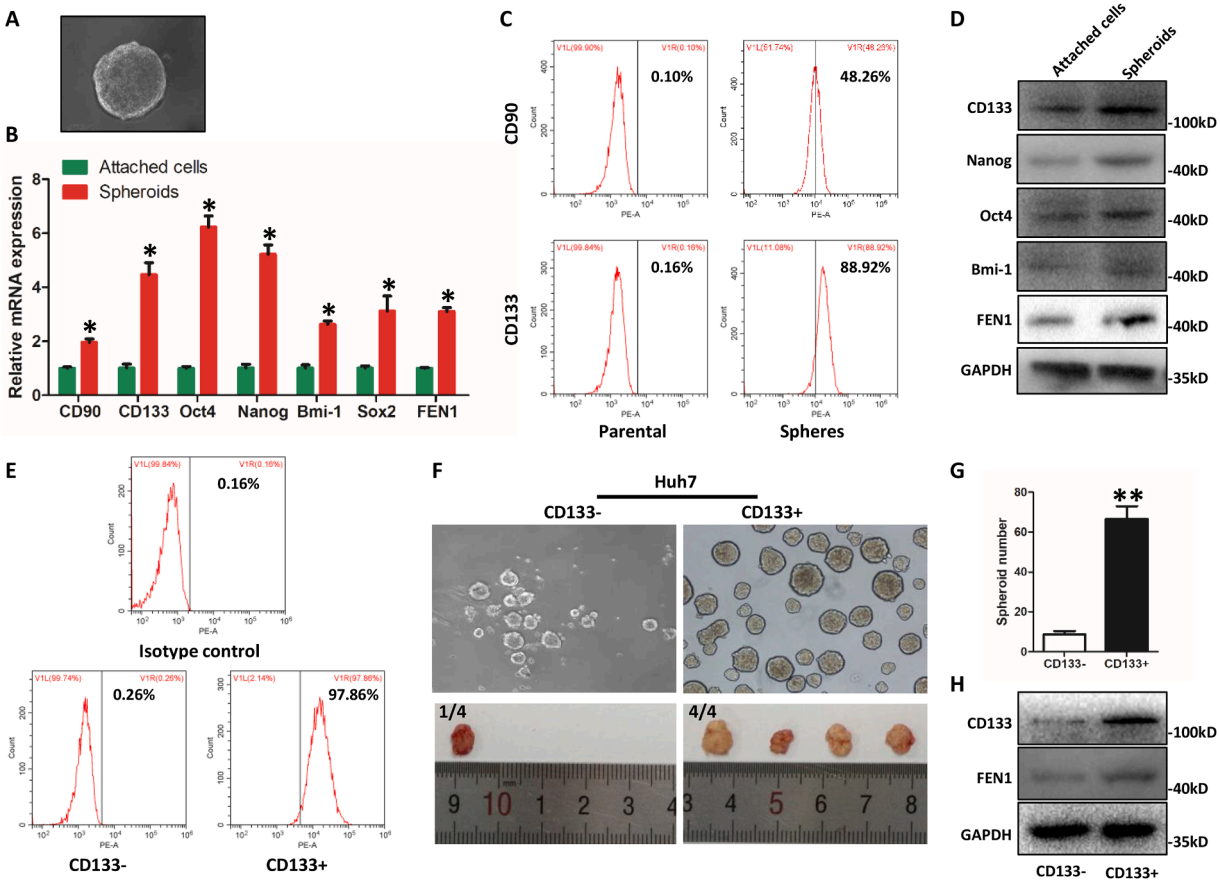

Fig. 1. Expression of FEN1 in LCSCs and its putative role in modulating stemness of hepato cellular carcinoma cells. (A)Microscopic representation of Huh7 cell spheroids. (B)RT-qPCR elucidation of A and stemness markers associated with LCSCs within Huh7 cell spheroids and adherent cells. (C)Flow cytometry profiling of CD90 and CD133 markers in Huh7 cell spheroids and adherent counterparts. (D)Western blot characterization of A and associated LCSCs stemness indicators in both Huh7 cell spheroids and adherent cells. (E)Flow cytometric quantification of the CD133+ cell fraction post-isolation. (F)Experimental depiction of cell spheroid genesis and subcutaneous tu-mor-igenicity assays in nude mice. (G)Statistical representation comparing spheroid formation effi-cacy between CD133+ and CD133- cell cohorts. (H) Western blot differentiation of FEN1 and CD133 ex-pression profiles within CD133+ spheroid entities. $^{**}P < 0.01$.

serum-free stem cell medium demonstrated spheroid formation within a span of 14 days (Fig. 1A). Flow cytometry revealed that the spheroids exhibited markedly higher expression rates of CD90 and CD133 compared to the adherent cells (Fig. 1C). Further, RT-qPCR analyses indicated a significant elevation in mRNA levels of FEN1 and notable LCSCs stemness markers such as CD90, CD133, Oct4, Nanog, Bmi-1, and Sox2 in the spheroids in contrast to the adherent cells (Fig. 1B). WB assays underscored the enhanced protein expression levels of FEN1 and LCSCs stemness markers, namely CD133, Oct4, Nanog, and Bmi-1, within the spheroids (Fig. 1D).

The molecule CD133+ is an esteemed marker for hepatocellular carcinoma stem cells, frequently employed for the sorting and identification of LCSCs. Consequently, we leveraged immunomagnetic beads to segregate CD133+/- cells from Huh7 cells. Flow cytometry ascertained that our sorted CD133+ cells constituted approximately 97. 86 % (Fig. 1E), deeming them suitable for ensuing experiments. To authenticate the properties of CD133+ cells, cell spheroid-forming and subcutaneous tumorigenic assays in nude mice were conducted. These assays revealed a superior spheroid-forming and tumorigenic capability of CD133+ cells compared to their CD133- counterparts (Fig. 1F, G). Further, WB assays emphasized a notably higher protein expression level of FEN1 in CD133+ cells (Fig. 1H). Collectively, these findings advocate the proficiency of the serum-free stem cell medium in enriching CD90+ and CD133+ LCSCs and posit FEN1 as a potential pivotal regulator in LCSCs.

Amplification of FEN1 expression enhances stemness in hepatocellular carcinoma cells

To probe deeper into the role of FEN1 in orchestrating the self-renewal of LCSCs, we initiated transfection of MHCC97H cells with an FEN1 overexpressing lentiviral vector. Subsequent RT-qPCR and WB assays highlighted that both mRNA and protein expression levels of FEN1 in the overexpressed set considerably exceeded those in the control group with an empty vector (Fig. 2A), signifying the successful establishment of a stable FEN1 overexpression cell lineage. The cell spheroid-forming capacity was discernibly higher in the FEN1 overexpression cohort (Fig. 2B, C). Western blot analyses illuminated that the LCSCs stemness markers, specifically CD133 and Nanog, in the FEN1 overexpression cohort, overshadowed those in the control group (Fig. 2D). Additionally, RT-qPCR assays revealed that the LCSCs stemness markers, including CD90, CD133, Oct4, and Nanog, in the FEN1 overexpression set, were markedly superior (Fig. 2E). Flow cytometry analyses further confirmed that the enhanced expression of FEN1 substantially bolstered the proportion of CD133+ cells (Fig. 2F, G). Moreover, *in vivo* studies in nude mice underscored that FEN1 overexpression significantly accelerated tumor proliferation in MHCC-97H CD133+ cells (Fig. 2H–K).

Down regulation of FEN1 expression curtails stemness in hepatocellular carcinoma cells

In our continued pursuit to elucidate the role of FEN1 in modulating

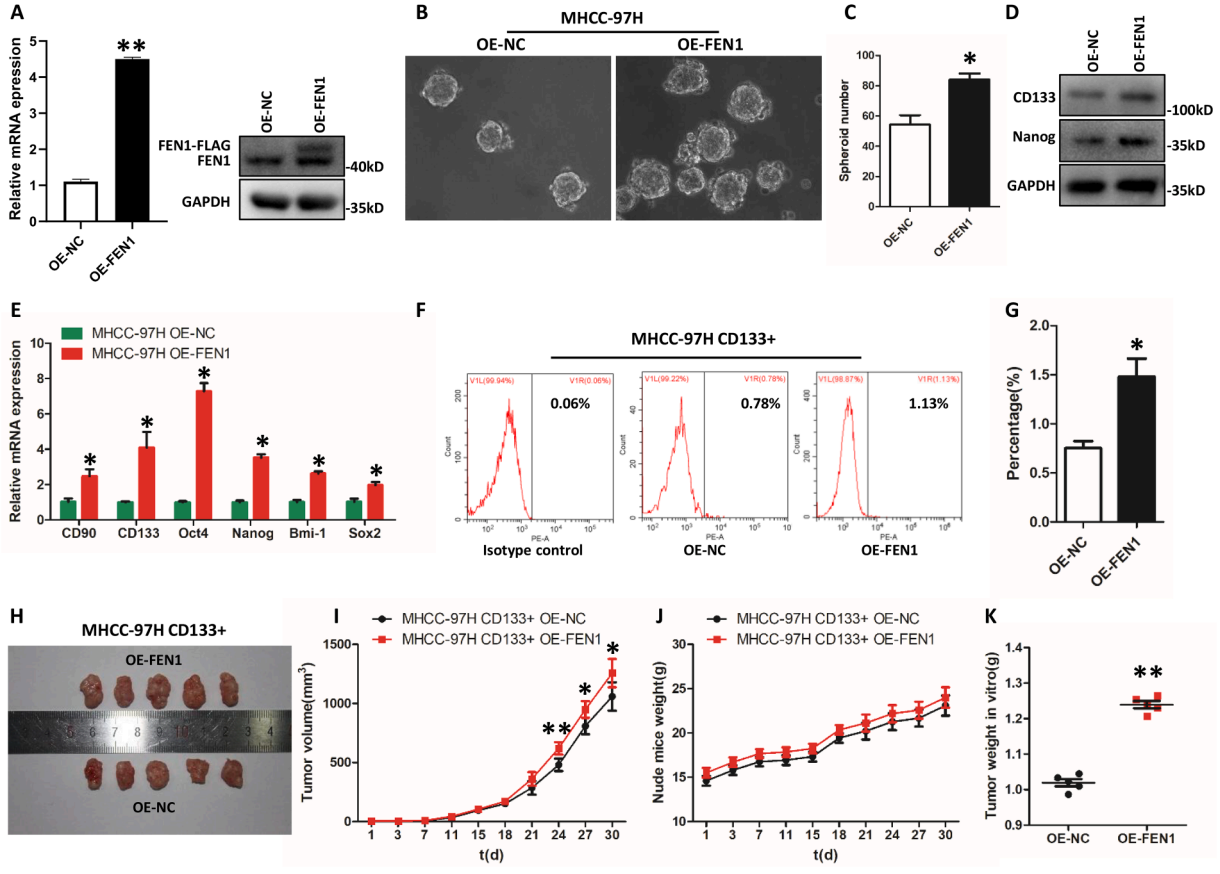

Fig. 2. Enhanced expression of FEN1 augments stemness traits in hepatocellular carcinoma cells. (A)Synchronous RT-qPCR and Western blot determinations following FEN1 overexpression in MHCC-97H cells. (B)Visual portrayal of cellular spheroids. (C)Statistical enumeration of cellular spheroid counts. (D)Western blot assessment of CD133 and Nanog subsequent to FEN1 upregu-la-tion in MHCC-97H cells. (E)RT-qPCR investigation into LCSCs stemness markers post-induction of FEN1 overexpression. (F)Flow cytometry delineation of the CD133+ cellular fraction following FEN1 upregulation. (G)Statistical visualization of the CD133+ cellular proportion exhibiting augmented FEN1 expression. (H, I)Comprehensive evaluation of subcutaneous tumor xenograft establishment and growth kinetics in nude mice. (J,K)Weight progression trajectory of nude mice and weight de-terminations of extracted subcutaneous tumors, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$.

the stemness of hepatocellular carcinoma cells, we devised three shRNA-FEN1 vectors. Following comprehensive validation via RT-qPCR and WB, an optimal interference sequence was discerned (Fig. 3A), and the subsequent transfection of Huh7 with lentiviral vectors led to the successful creation of stable FEN1 knockdown cell lines. The cell spheroid assay illuminated that the spheroidogenic propensity of the FEN1 knockdown ensemble was notably diminished in comparison to the empty vector cohort (Fig. 3B, C). The mRNA manifestation levels of the canonical LCSCs stemness markers, specifically CD90, CD133, Oct4, Nanog, Bmi-1, and Sox2, were substantially reduced in the FEN1 knockdown group vis-à-vis the empty vector assembly, as evidenced by RT-qPCR (Fig. 3D). WB assays demonstrated a conspicuous decline in the protein expression of LCSCs stemness markers CD133 and Nanog in the FEN1 knockdown group (Fig. 3E). Flow cytometry further substantiated that curtailing FEN1 expression markedly attenuated the prevalence of CD133+ cells (Fig. 3F, G). *In vivo* assays in nude mice accentuated that the suppression of FEN1 expression led to inhibited tumorigenesis in Huh7 CD133+ cells (Fig. 3H–K).

SUMO2 exhibits affinity to FEN1 in liver cancer stem cells

Protein interactions are paramount for intracellular FEN1 to operationalize its cellular functions. Keen on discerning proteins that potentially consort with FEN1 in liver cancer stem cells, we undertook immunoprecipitation of prospective binding partners from MHCC-97H and Huh7 liver cancer stem cell lines utilizing a specific FEN1 antibody (Fig. 4A). LC-MS/MS was then harnessed to analyze these protein samples. Our findings revealed that 89 proteins from MHCC-97H CD133+ cells and 46 proteins from Huh7 CD133+ cells were identified in tandem with the IP assay. Intersection of these datasets yielded 19 proteins common to both hepatocellular carcinoma cell lines, with SUMO2 featuring prominently among them (Fig. 4B). To eschew any inadvertent biases, proteins originating from both cell lines were meticulously sifted, excluding those potentially influenced by IgG-negative control antibody. Preliminary data alluded to the binding affinity between SUMO2 and FEN1 in liver cancer stem cells, a hypothesis which was later cemented by CO-IP (Fig. 4C, D). Further database mining through GeneCards unveiled that the intracellular expressions of SUMO2 and FEN1 were notably congruent (Fig. 4E).

Pronounced expression of SUMO2 in HCC and its correlation with FEN1 at the protein level

The current body of literature, both domestic and international, offers limited insights into the expression and biological function of SUMO2 in HCC. Initiating our investigation, we combed through Oncomine and GEPIA databases and unearthed that SUMO2 expression was emphatically elevated in HCC (Fig. 5A, B), and that its high expression portended an unfavorable prognosis for afflicted individuals (HR=1. 4, Logrank p = 0. 038) (Fig. 5C). Utilizing 34 paired hepatocellular carcinoma tissue samples for IHC detection and extracting cDNA from 21 of them, we discerned that both the staining intensity and IHC

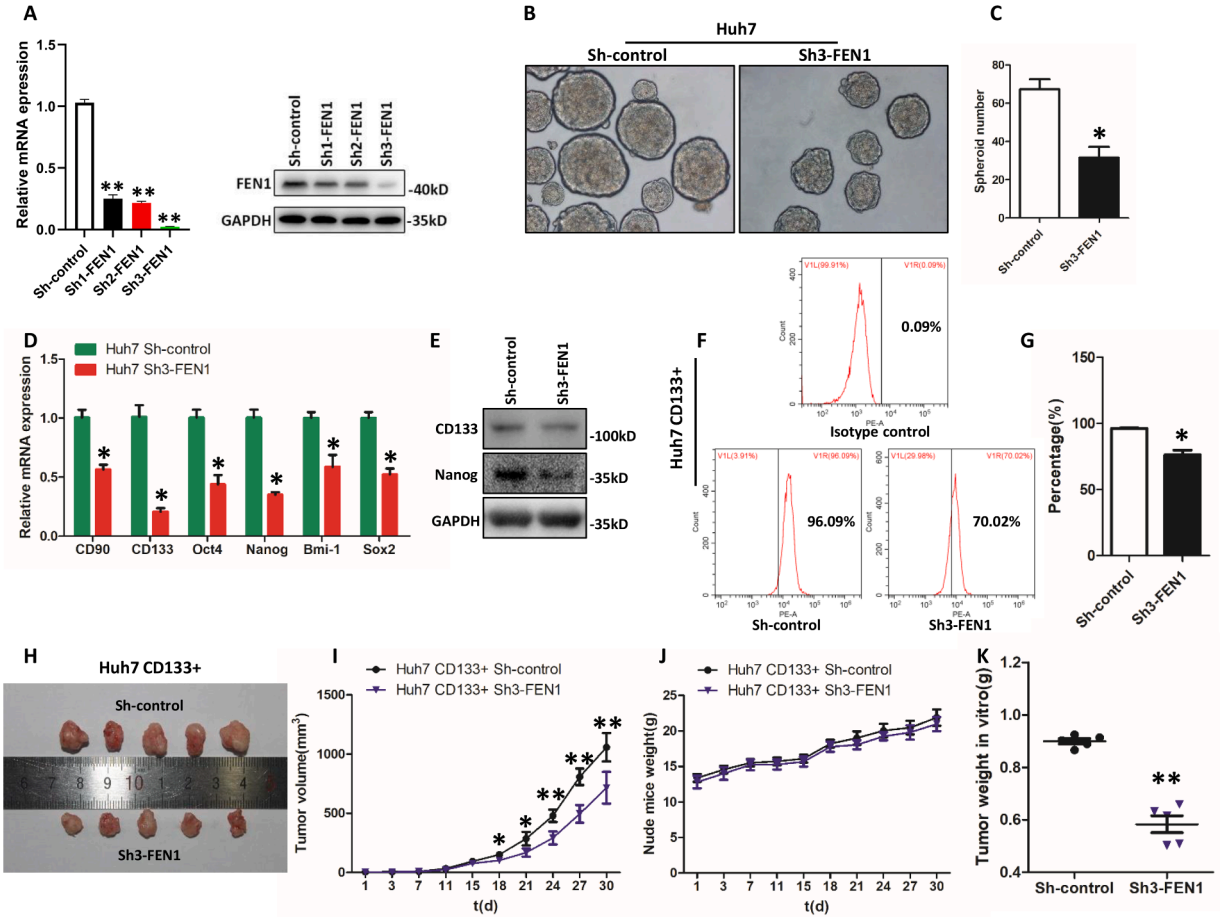

Fig. 3. Down regulation of FEN1 expression counteracts stemness characteristics in hepato cellular carcinoma cells. (A)Integrative RT-qPCR and Western blot analytics post-FEN1 gene silenc-ing in Huh7 cells. (B)Visual depiction of formed cell spheroids. (C)Statistical tabulation of cell sphe-roid frequencies. (D)RT-qPCR discernment of LCSCs stemness markers. (E)Western blot examination of CD133 and Nanog. (F)Flow cytometric analysis spotlighting the CD133+ cell subset. (G) Statistical delineation highlighting the fraction of CD133+ cells manifesting reduced FEN1 ex-pres-sion. (H, I)Empirical assessment of subcutaneous tumor xenograft initiation and growth pat-terns in nude mice. (J,K)Weight trajectory of nude mice and quantification of isolated subcutaneous tumor masses. *$P < 0.05$, **$P < 0.01$.

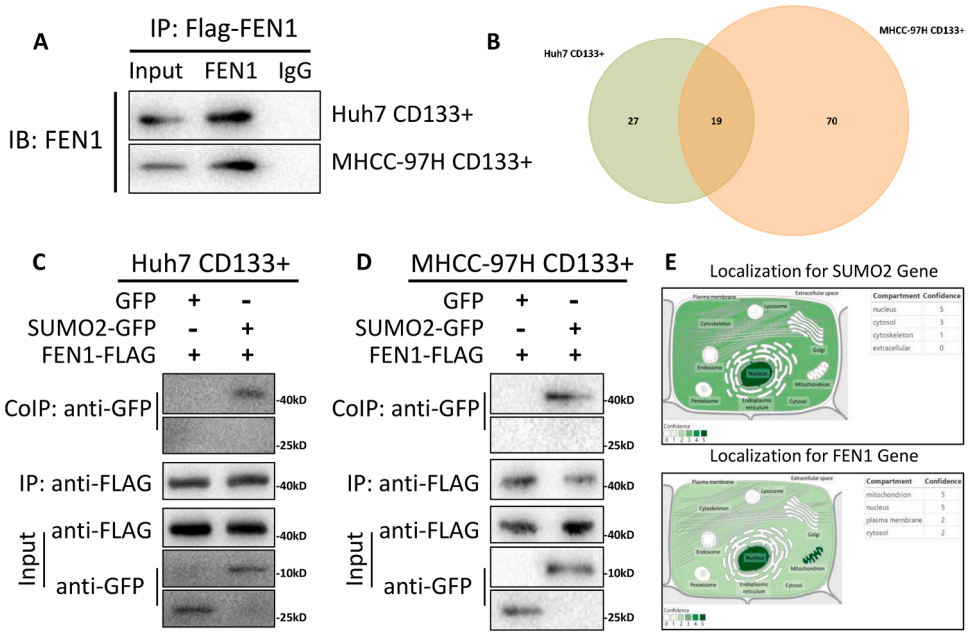

Fig. 4. Interaction between SUMO2 and FEN1 proteins in liver cancer stem cells. (A)Immunoprecipitation (IP) assay executed to isolate potential protein candidates binding to FEN1 in MHCC-97H and Huh7-derived hepatocellular carcinoma stem cells. (B)Liquid Chroma-tog-raphy-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) enabled identification, with a Venn dia-grammatic representation of proteins sourced from the two distinct liver cancer cell lines. (C, D) Co-immunoprecipitation (CO-IP) assays reaffirmed the molecular interaction between SUMO2 and FEN1 in liver cancer stem cells. (E)GeneCards database analyses highlighted intracellular locali-za-tion patterns of SUMO2 and FEN1 proteins.

score of SUMO2 were significantly heightened in hepatocellular carcinoma tissues as opposed to paracarcinoma samples (Fig. 5D, G). RT-qPCR analyses further accentuated the superior mRNA expression of SUMO2 in hepatocellular carcinoma specimens compared to paracarcinoma samples (Fig. 5E). While Pearson's correlation coefficient analysis did not unveil a statistically significant association between the mRNA expressions of SUMO2 and FEN1 ($r = 0.414$, $p = 0.062$) in hepatocellular carcinoma tissues (Fig. 5F), a robust positive correlation was observed in terms of their IHC scores ($r = 0.671$, $p < 0.01$) (Fig. 5H). This underscores a compelling positive interrelation between SUMO2 and FEN1 at the protein level within hepatocellular carcinoma.

The SUMO2 modification level of FEN1 in LCSCs was significantly increased, and SUMO2 could up-regulate the protein level of FEN1

To delve deeper into the SUMOylation dynamics of FEN1 within LCSCs, we embarked on an analytical foray, encompassing normal liver cell LO2, Huh7 adherent cells, and Huh7 stem cell spheroids. Our observations unveiled a marked upregulation of FEN1 in Huh7 stem cell spheroids when juxtaposed with the other two groups (Fig. 6A). Advancing further, we employed magnetic bead sorting to isolate CD133+ cells, wherein we discerned that the SUMO2 modification extent of FEN1 within these CD133+ cells was appreciably amplified (Fig. 6B, C).

Intrigued by the potential regulatory interplay between SUMO2 and the expression of FEN1, we fashioned three shRNA-SUMO2 vectors, subsequently introducing them into MHCC-97H. Post rigorous validations via RT-qPCR and Western blot, we pinpointed the two most efficacious interference sequences, affirming the successful establishment of SUMO2 stable knockdown hepatoma cell lines. Probing into FEN1 expression with RT-qPCR and Western blot revealed an intriguing observation: while the knockdown of SUMO2 appeared to leave the mRNA levels of FEN1 largely unperturbed, it did manifestly curtail the protein expression of FEN1 (Fig. 6D, E). To further buttress our understanding of SUMO2s regulatory influence on FEN1, we induced overexpression of SUMO2 in Huh7 cells employing lentiviral vector transfection. Consistent with our anticipations, both RT-qPCR and Western blot analyses indicated that while the mRNA and protein expressions of SUMO2 eclipsed those in the empty vector group (thereby confirming the success of our overexpression endeavor), the overexpression of SUMO2 remained largely neutral to the mRNA levels of FEN1. Remarkably, however, it did potentiate the protein expression of FEN1 (Fig. 6F, G).

SUMO2 can up-regulate FEN1 protein expression by counteracting the proteasomal degradation pathway

Studies have indicated that FEN1 can be degraded by the proteasome pathway and that SUMOylation can counteract this process. To investigate how SUMO2 regulates FEN1, we utilized the proteasome inhibitor MG132 in both SUMO2-stable knockdown and overexpression cells-MHCC97H and HUH7. Our results showed that MG132 was able to restore the inhibitory effect of SUMO2 down-regulation on FEN1 expression (Fig. 7A, B). Furthermore, adding MG132 could enhance the up-regulation of FEN1 expression that is induced by SUMO2 overexpression (Fig. 7C, D). Finally, according to our evidence, the protein expression of FEN1 was almost unchanged when MG132 was added to the control group and the stable knockdown or overexpression group. These findings rigorously demonstrate that SUMO2 has the capability to up-regulate the protein expression of FEN1 by antagonizing its proteasomal degradation pathway.

Discussion

Mirroring the inherent properties of conventional stem cells, cancer stem cells (CSCs) possess a distinctive capacity for self-renewal and engendering a diverse repertoire of tumor cells. This quintessential trait underscores their pivotal role in orchestrating events such as tumorigenesis, recurrence, metastasis, and notable resistance to chemotherapy. Notably, within the landscape of hepatocellular carcinoma, subsets of cells echoing CSC properties, denominated as LCSCs, have been identified [3]. A corpus of literature has underscored the prominence of

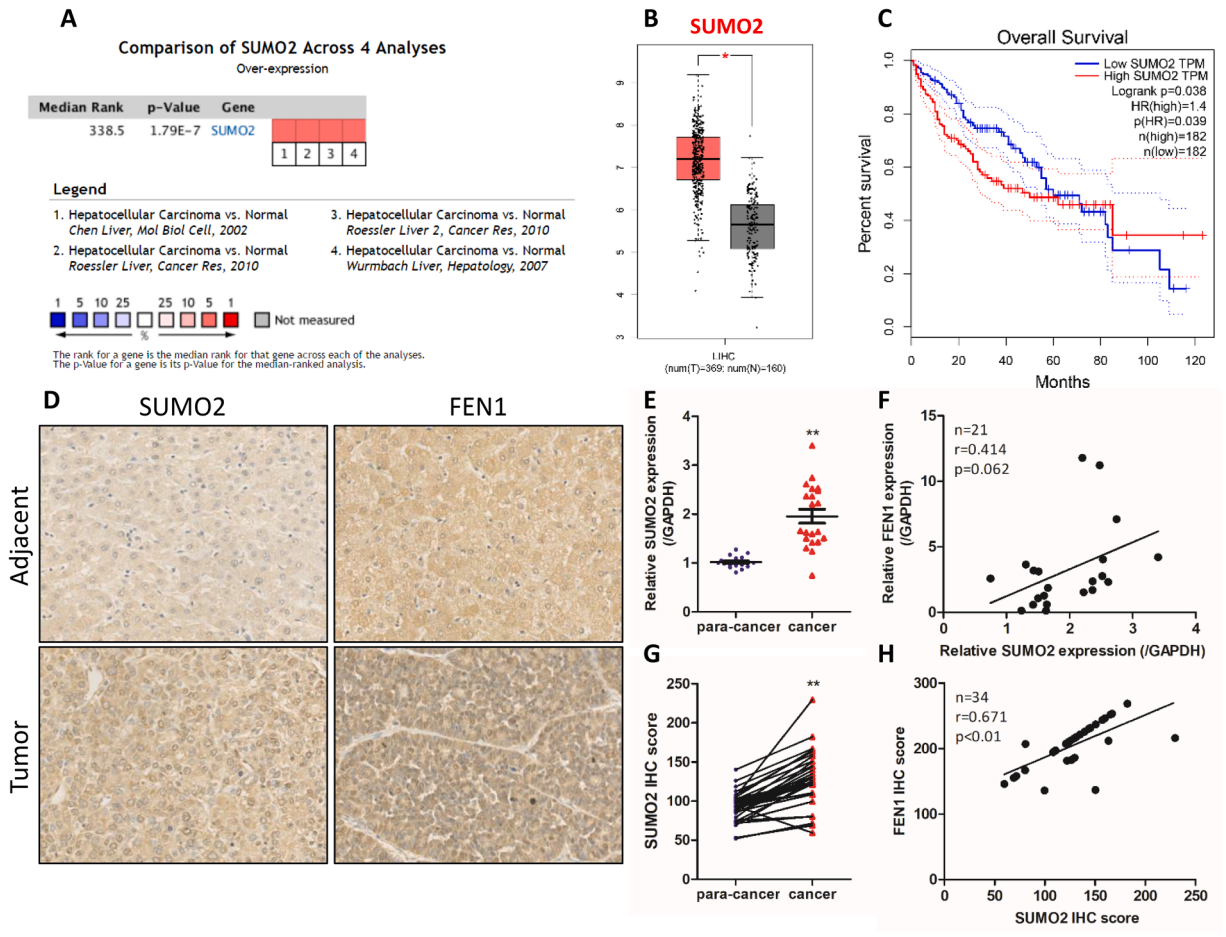

Fig. 5. Elevated expression of SUMO2 in HCC and its positive association with FEN1 tissue expression. (A)Utilization of the Oncomine database facilitated the survey of SUMO2 expression across four disparate liver cancer gene chips. (B)Genome-wide analysis using the GEPIA database discerned the expression profiles of SUMO2 within TCGA hepatocellular carcinoma datasets. (C) GEPIA database-driven analyses explored the prognostic correlation between SUMO2 expression and the overall survival of hepatocellular carcinoma patients. (D)Immunohistochemical (IHC) de-tec-tion underlined the expression dynamics of SUMO2 and FEN1 within hepatocellular carcinoma and adjacent paracarcinoma tissues. (E)RT-qPCR assays determined the transcriptional levels of SUMO2 mRNA in liver cancer specimens. (F) Quantitative correlations between FEN1 mRNA and SUMO2 mRNA expressions were established employing Pearson's correlation coefficient. (G) Evalu-ation of IHC-derived scores representing SUMO2 expression within liver cancer samples. (H) Statis-tical-ly-derived correlation between IHC scores of FEN1 and SUMO2. Note: Comprehensive data on FEN1 mRNA levels and IHC scoring within HCC has been documented in prior publications and hence is omitted in the present context.

CD133 and CD90 as pivotal markers delineating LCSCs [4,21,22]. Concurrently, central transcriptional regulators like Oct4, Sox2, and Nanog have been pinpointed as instrumental in the orchestration of stemness [7,8]. Yet, a thorough understanding demands further meticulous probing into the intricate molecular and biological machinations governing LCSCs.

The multifaceted molecule FEN1 has been heralded for its prowess in safeguarding genomic stability [23,24]. Its sterling role in tumor progression, coupled with its standing as a salient biomarker for a myriad of malignancies, has been widely recognized [25–29]. The association of FEN1 with pivotal oncogenic processes, such as cellular proliferation, migratory behaviors, drug resistance, and tumorigenesis, has been well-documented [11,30–33]. For instance, it has been elucidated that FEN1 bolsters proliferation in non-small-cell lung cancers, while its inhibition amplifies the therapeutic impact of cisplatin [34]. Study reports Aidi injection may exert anti-hepatocellular carcinoma effects by inhibiting molecules such as FEN1 [35]. A confluence of investigations reaffirm the heightened expression of FEN1 in HCC [11,33]. Certain groundbreaking works have illuminated the modus operandi of FEN1, revealing its self-maintenance and promotion of hepatocellular carcinoma proliferation via an m6A-dependent paradigm [36]. Furthermore, there exists empirical evidence suggesting FEN1s potential to modulate hepatocellular carcinoma trajectories by liaising with ubiquitin-specific proteases [33]. Complementing this, our prior endeavors substantiated that the TGFβ1- miR-140-5p axis exerts regulatory influence over FEN1, culminating in the promotion of the epithelial-mesenchymal transition (EMT) within HCC [11]. Collectively, this wealth of evidence resonates with the overarching hypothesis underscoring FEN1s seminal involvement in HCC's ontogeny.

The intricate nexus between tumor stem cells and the EMT process has been the focal point of a plethora of studies. Within certain malignancies, distinct cellular subpopulations, enriched in CSCs, conspicuously undergo EMT [37–40]. Augmenting this, several research endeavors have postulated that EMT induction catalyzes tumor initiation [37,40–42]. It has been claimed that MSCs can induce EMT transition to produce a CSC-like state [43]. Notably, a striking congruence exists between the molecular footprints governing EMT and stem cell origination; signaling cascades like TGF-β, Wnt/β-catenin, Hedgehog, and Notch stand implicated in the inception of both paradigms [44,45]. Drawing inspiration from this intimate liaison between EMT and CSCs, and buttressed by our prior observations of FEN1 potently fostering EMT in HCC, we postulated a significant regulatory role of FEN1 within LCSCs. Our methodical approach, harnessing serum-free stem cell medium to enrich LCSCs, revealed that hallmarks of LCSCs, namely CD133, CD90, Nanog, and Oct4, exhibited heightened levels in spheroids relative to adherent cells. Complementing this, our flow cytometry analyses

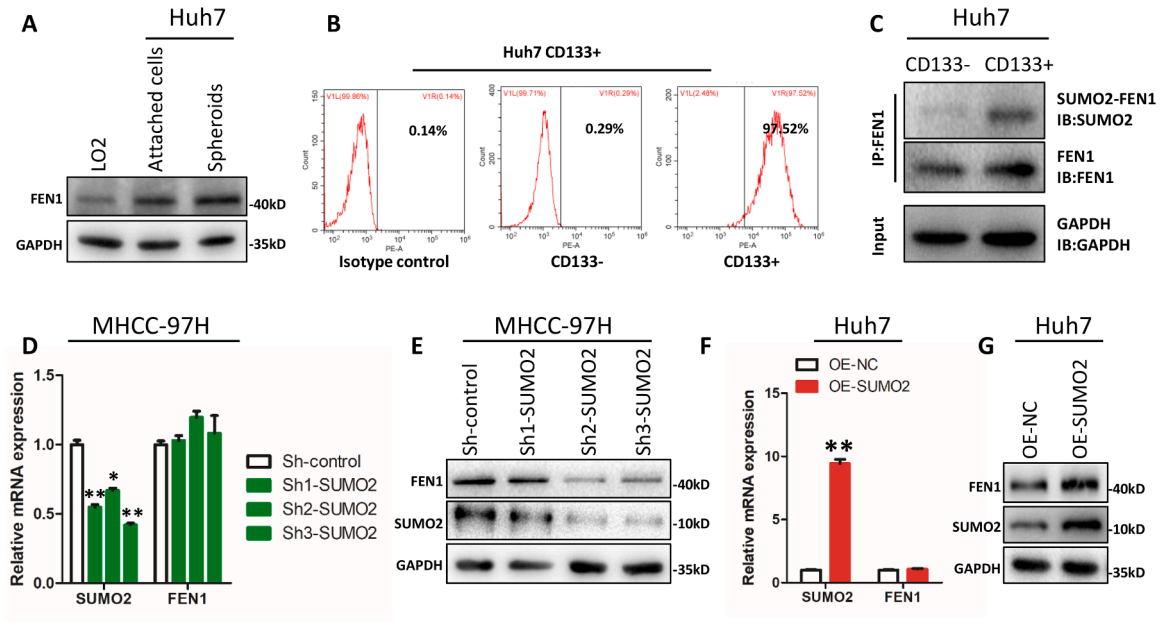

Fig. 6. Modulation of FEN1 by SUMO2 in HCC: emphasis on the ascendant regulation at the protein scale. (A)Western blot methodologies discerned FEN1 protein expression profiles across normal liver cells, liver cancer adherent cells, and liver cancer stem cell spheroids. (B)Flow cytomet-ric ap-proaches quantified the fraction of CD133+ cells within the analyzed sam-ples. (C)Immunoprecipitation assays were instrumental in gauging the modification extent of FEN1 by SUMO2 within Liver Cancer Stem Cells (LCSCs). (D, F)RT-qPCR analyses scrutinized the tran-scrip-tional influence exerted by SUMO2 on FEN1 mRNA levels. (E, G)Western blot techniques delved into the modulation of FEN1protein expression by SUMO2.

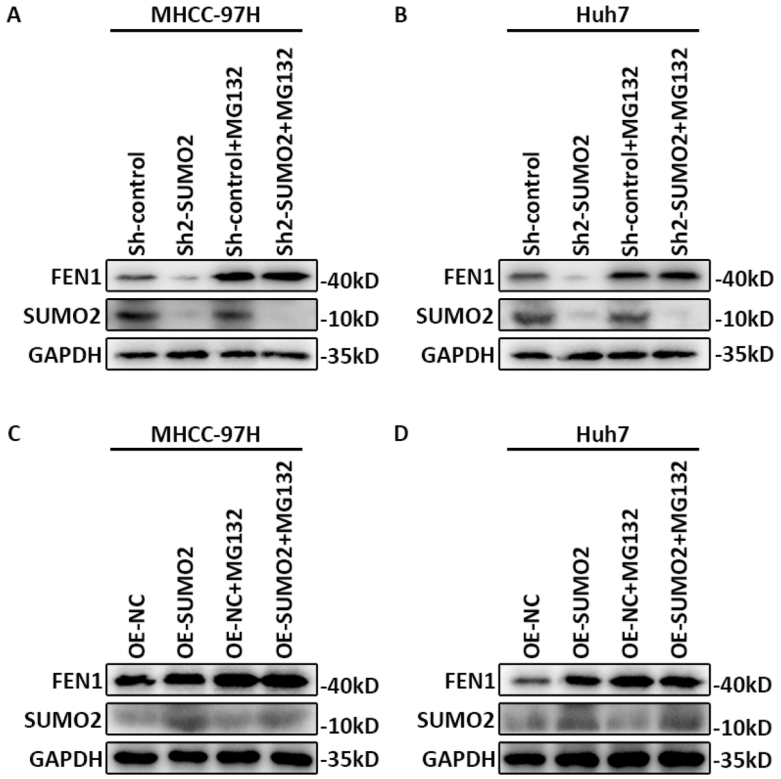

Fig. 7. SUMO2 up-regulates the protein expression of FEN1 by antagonising its proteasomal degradation pathway. (A, B)Western blot analyses of FEN1, SUMO2 and GAPDH expression levels in SUMO2-stable knockdown cells after treating with MG132 for 4 h. (C, D)Western blot analyses of FEN1, SUMO2 and GAPDH expression levels in MHCC97H and HUH7 cells with stable overexpression of SUMO2.

illuminated a pronounced upregulation of both CD133 and CD90 within these spheroids, advocating for their utility as representative LCSC models. As our investigation unfolded, we discerned that FEN1 enjoys pronounced expression within these spheroids and CD133+ cells. Remarkably, a surge in FEN1 levels was observed to robustly amplify stemness attributes within LCSCs, whilst its attenuation manifested an inverse effect. This compendium of findings unambiguously accentuates the central role FEN1 assumes in the narrative of LCSC development.

Metastasis, an intricate and often lethal phenomenon within the cancer trajectory, is responsible for an overwhelming majority of cancer-attributed mortalities, accounting for more than 90 % [46,47]. A compelling link exists between tumor stem cells and the machinations of metastasis. Remarkably, it has been demonstrated that a scant subset of tumor cells, endowed with the intrinsic ability for self-renewal, possesses the potential to reestablish the primary tumor in distant anatomical locations [48]. Several investigative endeavors have intimated that metastatic propagation of colon cancer might be singularly orchestrated by cellular subgroups bearing tumorigenic initiation capacity [49]. Scholars have reported that cancer stem cells in breast cancer can reprogram the tumor microenvironment to promote tumor metastasis [50]. Furthermore, a spectrum of genes pivotal in steering stemness—including, but not limited to, Nanog, Sox2, KLF4, Snai2, and Oct4—have been identified as promulgators of both stemness and metastasis across diverse tumor types [51]. Within the purview of our study, we discerned that modulations in the expression of FEN1 elicited concomitant fluctuations in the stemness marker CD133, as well as the quintessential transcriptional effector, Nanog. On this evidentiary basis, we postulate that FEN1 might amplify tumor metastasis through a nuanced regulation of the stemness attributes inherent to HCC cells. Drawing upon the entirety of our empirical data, we posit that FEN1 stands as a promising candidate with potential diagnostic and therapeutic ramifications in the context of HCC.

An evolving body of literature underscores the significance of sumoylation—a ubiquitous post-translational modification—in modulating myriad cellular processes, ranging from cell cycle dynamics and DNA damage repair to drug resistance and tumorigenesis [52]. It has been revealed that SUMO-1 modification is instrumental in calibrating the function of FEN1 during critical phases of DNA replication and repair [53,54]. SUMO2, in its own right, has been implicated in the intricate tapestry of tumor evolution. For instance, SUMO2/3 has been identified as a catalyst for gastric cancer progression, acting through the modulation of NSUN2 stability and its nuclear translocation [55]. Additionally, literature cites SUMO2/3 as a pivotal agent driving colorectal cancer progression and oxaliplatin resistance by orchestrating the sumoylation modification of the DNA assembly constituent, Ku80 [56]. Recent revelations align with our experimental evidence, advocating that SUMO2 exerts a positive regulatory influence on hepatocellular carcinoma progression [57,58]. Concurrently, sumoylation modifications have been firmly established as regulators of CSC stemness [15,17]. Leveraging immunoprecipitation coupled with LC-MS/MS, our results unveiled an interaction matrix between FEN1 and SUMO2. Subsequent co-immunoprecipitation assays validated the binding dynamics between FEN1 and SUMO2 within HCC cells, and a concomitant positive correlation was discerned at their protein expression levels in hepatic malignancies. A thorough literary exploration further revealed sumoylation sites within FEN1 [18,53]. Expanding on this, our co-immunoprecipitation assays evinced an augmented level of SUMO2-induced sumoylation of FEN1 in LCSCs. Thus, we forward the hypothesis that SUMO2 might modulate the expression amplitude of FEN1 through sumoylation-mediated mechanisms. This hypothesis gained further empirical traction as our Western blot and RT-qPCR analyses delineated that both knockdown and overexpression of SUMO2 predominantly influenced the protein expression spectrum of FEN1, leaving its mRNA transcriptional landscape unperturbed.

The intricate world of post-translational modifications (PTMs) showcases SUMO proteins as pivotal players, possessing the capability to subtly influence the functionality of target proteins. This is achieved by modulating a myriad of other PTMs, encompassing ubiquitination, acetylation, and phosphorylation, to name but a few. Both the molecular architectures of SUMO and ubiquitin-binding substrates prominently feature lysine residues at their interaction sites, suggesting a potential competitive interplay between the two. This competition may ultimately inhibit the degradation role played by the proteasome pathway. It has been previously delineated that FEN1 is susceptible to degradation through the ubiquitin-proteasome cascade [34,36]. Melding this knowledge with our empirical data, we found that SUMO2 latches onto the SUMO modification site of FEN1, facilitating its modification. This act attenuated the proteasomal degradation pathway of FEN1, thereby anchoring the expression and subcellular localization of FEN1 and, consequentially, augmenting the stemness of hepatocellular carcinoma cells. Intriguingly, specific case studies highlight SUMO3's potential to expedite the ubiquitination and subsequent degradation of FEN1. Contrarily, SUMO2, despite bearing a sequence highly homologous to SUMO3, did not manifest a similar modulatory function [18], a finding that resonates with our observation of SUMO2 acting to elevate FEN1 protein levels. This disparity may well be rooted in the intricate crosstalk between PTMs. As elucidated in existing literature, PTMs do not operate in isolation [59]. Instead, they frequently engage in a multifaceted interplay, encompassing processes like phosphorylation, SUMOylation, and ubiquitination [18]. These interactions can either amplify or counteract one another, offering a roadmap for our continued exploration into the regulatory dynamics between SUMO2 and FEN1. Ultimately, delving deep into the intricate functionalities and interrelationships of PTM-regulated proteins is instrumental in advancing our understanding of tumorigenesis.

In summary, our investigative endeavors unequivocally attest to the elevated expression of FEN1 within LCSCs for the first time. Furthermore, FEN1 has demonstrated firstly its prowess in amplifying the proportion of CD133+ cells, enhancing the expression of the stemness key transcription factor, Nanog, bolstering cellular spheroid formation capacity, and propelling *in vivo* tumor growth. Of paramount significance, FEN1s influential role in modulating LCSC stemness was first found to be intricately intertwined with SUMO2. Consequently, FEN1 emerges as a promising molecular target, not only for obliterating LCSCs but also as a therapeutic avenue for HCC treatment.

Ethical approval

34 pairs of human HCC samples were obtained by surgical resection from HCC patients in the Second Hospital of Chongqing Medical University. Informed consent was provided by all patients. The protocols were reviewed and approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University, Chongqing, China. All animal experiments were conducted following the ethical standards of animal care and approved by the Animal Care Committee of Chongqing Medical University.

Declaration of competing interest

The authors declare that they have no competing interests.

CRediT authorship contribution statement

Zhenxiang Peng: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Shuling Wang:** Investigation. **Diguang Wen:** Writing – review & editing, Methodology, Formal analysis, Investigation, Data curation, Conceptualization. **Zhechuan Mei** and **Hao Zhang** proofread and revised the manuscript of the article and participated in the statistical analysis of the data. **Shengtao Liao:** Writing – review & editing, Formal analysis, Supervision, Investigation, Conceptualization. **Lin Lv:** Writing – review & editing, Supervision, Formal analysis, Project administration, Investigation, Methodology. **Chuanfei Li:** Writing – review & editing, Validation, Supervision, Investigation, Software, Resources, Conceptualization, Project administration, Methodology, Funding acquisition.

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