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Inhibition of EZH2 Attenuates Sorafenib Resistance by Targeting NOTCH1 Activation-Dependent Liver Cancer Stem Cells *via* NOTCH1-Related MicroRNAs in Hepatocellular Carcinoma ☆,☆☆

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ABSTRACT

Acquired resistance and intrinsic to sorafenib therapy represents a major hurdle in improving the management of advanced hepatocellular carcinoma (HCC), which has been recently shown to be associated with the emergence of liver cancer stem cells (CSCs). However, it remains largely unknown whether and how histone posttranslational modifications, especially H3K27me3, are causally linked to the maintenance of self-renewal ability in sorafenib-resistant HCC. Here, we found that NOTCH1 signaling was activated in sorafenib-resistant HCC cells and NOTCH1 activation conferred hepatoma cells sorafenib resistance through enhanced self-renewal and tumorigenecity. Besides, the overexpression of EZH2 was required for the emergence of cancer stem cells following prolonged sorafenib treatment. As such, modulating EZH2 expression or activity suppressed activation of NOTCH1 pathway by elevating the expression of NOTCH1-related microRNAs, hsa-miR-21-5p and has-miR-26a-1-5p, *via* H3K27me3, and consequently weakened self-renewal ability and tumorigenecity and restored the anti-tumor effects of sorafenib. Overall, our results highlight the role of EZH2/NICD1 axis, and also suggest that EZH2 and NOTCH1 pathway are rational targets for therapeutic intervention in sorafenib-resistant HCC.

Introduction

Receptor tyrosine kinase inhibitor (RTKi) sorafenib is presently used as a standard of care in patients with recurrent metastatic hepatocellular carcinoma (HCC) but durable responses are not common [1]. However, therapy resistance and tumor recurrence are common and represent major obstacles to the improvement of patient survival in HCC [2]. Thus, elucidation of the mechanisms underlying recurrence and therapy resistance is fundamental for the development of new therapeutic treatments for HCC.

Therapeutic resistance and relapse in HCC relates to the extensive intratumoral genetic and phenotypic heterogeneity characteristic of these tumors [3]. Evidence indicates that a subpopulation of stem-like cells, termed cancer stem cells (CSCs) [4, 5]. Accumulating data shows that liver CSCs accumulate after long-term RTKi treatments and are likely to contribute to their failure and subsequent disease progression [2, 5–7]. The development of CSCs and maintenance of their "stemness" are associated with aberrations of several molecular cascades involving signaling triggered by Notch and Wnt/beta-catenin [4, 8–10]. Notch signaling regulates cell-fate determination throughout development in many organ systems, including liver [11]. There are four Notch receptors in mammals (Notch1–4) and five ligands (Delta-like 1 (DLL1), DLL3, DLL4, JAG1, and JAG2) [12]. Notch activation requires Notch receptors to bind to a ligand located in the adjacent cells [12]. Upon ligand binding, the intracellular domain of Notch1 (NICD) is cleaved, after which it translocates to the nucleus to regulate downstream target gene transcription, such as the HES (hairy enhancer of split) and Herp/Hey families of basic helix–loop–helix transcriptional repressors [12]. In HCC, higher expression of Notch 1, 3, 4 and Jagged 1 correlated with aggressive phenotype, while Notch 2 had the opposite result [13–15]. Recently, some

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studies showed that Notch1 could promote HCC cell growth, metastasis and stemness *via* activation of Stat3 signaling pathway, and Notch3 could promote liver CSCs self-renewal of tumor cells *via* LSD1 activation by inducing deacetylation, indicating activation of Notch signaling pathway promotes self-renewal of liver CSCs [16, 17]. However, direct evaluation of Notch signaling as drivers of sorafenib-resistant HCC remain unclear.

Epigenetic modifications have been implicated in cancer progression and are potential drivers of drug resistance [18, 19]. The overexpression of EZH2 has been reported in numerous cancer types including advanced hepatocellular carcinoma, suggesting its role in modulating several cellular processes involved in cell survival and drug resistance, and inhibition of EZH2 has resulted in the attenuation of drug resistance in tumor and stem cells [19–25]. However, direct demonstration about the role of EZH2 in acquired resistance to sorafenib of HCC is lacking, and the responsible mechanisms need further investigation.

EZH2 plays a key role in the regulation of the Notch signaling pathway [26–29]. In some tumors, EZH2 can epigenetically silence microRNAs, such as miRNA34a, or JAG1 or NOTCH1 to regulate the NOTCH1 pathway [26, 28, 29]. However, in other tumors, independent of its catalytic histone H3 lysine 27 methyltransferase activity and of the Polycomb Repressive Complex 2 but instead to transcriptional activation marks, EZH2 increases NOTCH1 expression by directly binding to the NOTCH1 promoter and further promotes CSC properties or expands CSCs [26, 28, 29]. However, the effect and mechanism of EZH2 inhibition on NOTCH pathway in acquired resistance to sorafenib of HCC is unknown.

Here, we found that NOTCH1 signaling is activated, and EZH2 is overexpressed in sorafenib-resistant *in vivo* and *in vitro* models, and active EZH2/NICD1 axis confers hepatoma cells sorafenib resistance through enhanced stem-cell properties. Furthermore, molecular and pharmacological inhibition of EZH2 attenuated NOTCH1 activation by increasing the expression of NOTCH1-related microRNAs, hsa-miR-21 and has-miR-26a, and consequently weakened self-renewal ability and tumorigenecity and reestablished sensitivity to sorafenib. Taken together, these results suggest that pharmacological targeting of EZH2 and NOTCH1 pathway are promising strategies to overcome sorafenib resistance in HCC.

Materials and Methods

Cell Culture

Human hepatoma cell lines Huh7 and SMMC-7721 were obtained directly from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. All cultures were maintained in a humidified incubator at 37 ˚C and 5% CO_2. The cell lines have been characterized at the cell bank by DNA fingerprinting analysis using short tandem repeat markers. All cell lines were placed under cryostage after they were obtained from the cell bank and used within 6 months of thawing fresh vials, as described previously [30].

Construction of Plasmids, RNA Interference (RNAi), shRNAs, and Transfection

Expression plasmids encoding wild-type EZH2 was constructed as described previously [30]. pcDNA3-intracellular domain of NOTCH1 (NICD1) plasmid was kindly provided by Dr. Jon C. Aster (Brigham and Women's Hospital, Boston, MA, USA). Luciferase reporter plasmid containing the 3′-flanking region (3'-UTR) of JAG1 were amplified from cDNA library of Huh7 cells and constructed in pGL3-Basic vector as described previously [30]. All plasmid constructs were confirmed by DNA sequencing. Plasmid transfections were performed using Lipofectamine 2000 (Invitrogen, USA). The shRNA against *EZH2* gene shEZH2s and corresponding scrambled shRNA (Sigma, USA) were used for RNA interference as described previously [30]. Control, NOTCH1, JAG1 siRNAs (Santa Cruz Biotechnology, USA), miR-21-5p mimic, miR-26a-1-5p mimic, negative control miRNA (miR-NC), anti-miR-21-5p, anti-miR-26a-1-5p, and anti-miR-NC (Thermo fisher, USA) transfections were conducted with X-tremeGENE (Roche Applied Science, Germany). Gene silencing effect was confirmed by immunoblotting and real-time PCR at 48 hours posttransfection. The sequences are listed in Supplementary Table S1.

Treatment of Cells with Inhibitors in Vitro and Establishment of Sorafenib-Resistant Cells In Vitro

Different amounts of inhibitor(s) were dissolved in dimethyl sulfoxide. Cells were plated in 6-well plates. When cells reached 60% confluence, they were treated with the appropriate dose of inhibitor at different times. After treatment, cells were collected for further experiments and analyses. Chemical reagents are listed in Supplementary Table S3.

Huh7 and SMMC-7721 cells were exposed to sorafenib from low dose (2.5 μM) and when cells stably grew then started to change to the higher dosage of sorafenib (5, 7.5, and then 10 μM). Medium containing sorafenib was replaced every 2 days for 3 months. In the ends, the cells could grow slowly in medium containing 10 μM sorafenib (a clinically relevant dose).

Immunobloting

Immunobloting assay was used to analyze protein expression as described previously [30]. In brief, after extraction, proteins in cell lysates were first resolved by SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membrane and subsequently incubated with the primary antibody. After incubation with secondary antibodies, the signals were visualized by Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, USA) according to the manufacturer's instructions. Antibody list used is shown in Supplementary Table S2.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, NY) and reverse transcription was performed with 500 ng of RNA using PrimeScript™ RT Master Mix (Takara, Japan) or All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia, USA), according to the manufacturer's instructions. qPCR was performed with FastStart Universal SYBR® Green Master (Roche, USA) on an Applied Biosystems 7500 Real Time PCR System supplied with analytical software (Applied Biosystems, USA). The average of the technical replicated was normalized to GAPDH or U6 levels using the comparative CT method ($2^{-\triangle\triangle CT}$). Average and standard deviations of at least 3 experiments are shown in the figures. The primer pairs were listed in Supplementary Table S1.

Annexin V/Propidium Iodide Staining and Flow Cytometry

Annexin V/propidium iodide (PI) staining was carried out using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, USA) according to the manufacturers' instructions.

To identify the liver CSC population, the cells were labeled with antibodies against NANOG-PE (Miltenyi Biotec, USA), and the corresponding isotype antibody as a control to exclude nonspecific background staining. The stained cells were then subjected to flow cytometry analysis using FACS Calibur (Backman Coulter DxFLEX, USA).

Colony Formation Assay

Two days after transfection, 3×10^3 tumor cells per well were resuspended in DMEM containing 0.3% agarose (Promega, USA). This suspension was laid over DMEM containing 0.6% noble agarose in six-well plates and further overlaid with DMEM. The plates were then incubated for 14 days in a 5% CO_2 incubator at 37 ˚C, with replenishment of medium every other day. Colonies were imaged using Olympus IX71 and macroscopically visible colonies in three randomly chosen fields per well were counted for quantification.

Sphere Formation Assay

Spheres were generated in DMEM:F12 (Life Technologies, USA) supplemented with 2% B-27 (Life Technologies, USA), EGF, bFGF (PeproTech, USA), 100 IU/mL penicillin, and 100 mg/mL streptomycin on ultralow attachment plates for 14 days. Spheres were imaged using Olympus IX71 and macroscopically visible colonies in three randomly chosen fields per well were counted for quantification.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was carried out with the ChIP Assay Kit (Millipore, USA) according to the manufacturer's instructions as described previously [30]. The percentage of the bound DNA was quantified against the original DNA input by PCR analysis. The PCR primer sets used are shown in Supplementary Table S1.

Luciferase Activity Assay

The pGL3-JAG1–3′-UTR plasmid containing 3′-UTR of JAG1 mRNA and pGL3-Basic control vector were used for assessing the effect of microRNAs on JAG1 expression, the primer set used is shown in Supplementary Table S1. Luciferase activity assay was performed as described previously [30].

Animal Models, Histology, Immunohistochemistry Analysis, and Evaluation

All animal experiments were approved by the Institutional Animal Care and Use Committee of Zhejiang Chinese Medical University (Zhejiang, China) and carried out in accordance with the approved guideline“code of practice: animal experiments in cancer research” (Netherlands Inspectorate for Health Protection, Commodities and Veterinary Public Health, 1999).

To generate sorafenib-resistant subcutaneous tumors, 1×10^7 Huh7 cells in 200 μl phosphate buffered saline were injected subcutaneously in 3–4 week-old male nude mice. After 2 weeks, mice were randomly allocated into groups and treated with sorafenib (60 mg/kg/intraperitoneal injection (i.p.), once every other day) for 2 months. On day 60 after the start of treatment, tumors were removed.

To establish *in vivo* tumor formation model, 100 to 10,000 sorafenib-resistant cells from Huh7 or 7721 cell, were mixed with Matrigel, and were injected subcutaneously into the flanks of 3-to 4-week-old male nude mice. Tumors grew for approximately 4 weeks and were removed.

Male nude mice 3 to 4 weeks old were injected subcutaneously with stable scrambled shRNA or shEZH2-#1 clones from Huh7-Res or 7721-Res cell. Tumors grew for approximately 4 weeks and were removed.

Tumors were measured twice weekly and volumes. Tumor size was measured with digital calipers and calculated based on the following formula: $\text{length} \times (\text{width})^2 \times \pi/6$. Tumors sections from subcutaneous tumor xenografted male nude mice were H&E stained, immunohistochemically analyzed, and evaluated as described previously [31].

Statistical Analysis

Experimental data were presented as mean ± standard error of mean of at least 3 independent replicates through analyzing with GraphPad Prism 5 (GraphPad Software, La Jolla, CA) and assessing comparisons between different groups by the Student t test, one-way ANOVA, and two-way ANOVA analysis of variance.

Results

Tumors Exhibit Relapse and Distant Metastases After Long-Term Exposure to Sorafenib

To study acquired resistance to sorafenib, we firstly established an *in vivo* sorafenib-resistant xenograft model using Huh7 cell with a high dose of sorafenib treatment (Figure 1*A*). Mice were orally administered sorafenib at a dose of 60 mg/kg/day on the 14th day after post-implant for 2 months. In agreement with the previous data, we found dramatic reductions of tumor weights and volumes without significant loss of body weight after sorafenib treatment, suggesting sorafenib suppressed tumor growth (Figure 1*B*) [32]. The tumor growth slope of the acquired resistant tumor 42 days before the development of resistance showed a slow increase in tumor volume, but showed a sharp increase after this period (Figure 1*B*). In addition, we detected lung, liver, and spleen metastasis in all nude mice in both the DMSO-treated and sorafenib-treated groups (Figure 1*B*). Tumor secondary growth and multiple organ metastasis indicated tumor recurrence and tumor cell resistance formation.

Long-Term Sorafenib Treatment Confers Hepatoma Cells Enhanced Self-Renewal Ability and Tumorigenecity

We next established sorafenib-resistant Huh7 (Huh7-Res) and SMMC-7721 (7721-Res) cells *in vitro*, which were generated by prolonged exposure to increasing doses of sorafenib (Figure 1*C*). To confirm successful establishment of sorafenib-resistant cells, we firstly evaluate the effects of sorafenib on cell apoptosis by Annexin V staining, sorafenib-resistant cells were found less sensitive to sorafenib-induced apoptosis than mock-treated cells (Figure 1*D*). We next evaluated the tumorigeneic and self-renewal ability in sorafenib-resistant cells by clone and sphere formation assays, and observed significantly more hepatospheres in both cells than parental cells (Figure 1*E*). Besides, Immunoblotting was performed to check the expression of cell stem cells (CSCs) markers, and showed that the expressions of NANOG, SOX2, and OCT4 significantly increased in sorafenib-resistant cells compared to parental cells (Figure 1*F*). We also observed sorafenib-resistant cells with higher percentage of CSCs marker NANOG in cell surface than parental cells by flow cytometry analysis (Figure 1*F*).

NOTCH1 Signaling is Activated in Sorafenib-Resistant in Vivo and In Vitro Models.

Sorafenib cannot target but enrich liver CSCs as evidenced by frequent tumor relapse and resistance after long-term therapy [7, 32, 33]. NOTCH1 activation promotes self-renewal of liver CSCs [10, 11, 16, 33]. Therefore, we hypothesized that the enrichment of liver CSCs in sorafenib-resistant cells is regulated by NOTCH signaling. Firstly, we should evaluate whether NOTCH signaling is active in sorafenib-resistant cells. We measured the expression of four receptors and six ligands in sorafenib-resistant *in vivo* and *in vitro* models. We found that both *JAG1* gene and *NOTCH1* gene are significantly increased in the sorafenib-resistant *in vivo* and *in vitro* models (Figure 2, *A* and *B*). Due to the differences *in vivo* and *in vitro* models, sorafenib-resistant tumors derived from Huh7 cell exhibited significant up-regulation of two receptors (*NOTCH2* and-*3*) compared with control tumors, while Huh7-Res cells exhibited no significant change of two genes. Due to the heterogeneity between different cell lines, Huh7-Res cells showed significant up-regulation of two ligands (*DLL1* and-*3*), while 7721-Res cells showed significant increase of a receptor (*NOTCH3*) (Figure 2, *A* and *B*). Collectively, these results demonstrated that several NOTCH receptors or ligands known to be necessary for NOTCH1 activation were up-regulated in sorafenib-resistant *in vivo* and *in vitro* models.

Upon ligand binding, active NICD1 translocates to the nucleus to regulate downstream target gene transcription [12]. Therefore, we performed immunoblotting analysis to check the protein levels of NICD1 and JAG1, and performed real-time PCR to the expressions of active NOTCH1 signaling genes (*HES1*, *HEY1*, and *HEY2* genes). Consistent with the changes from real-time PCR assay, tumors with acquired sorafenib resistance and SMMC-7721-Res/Huh7-Res cells expressed significantly higher levels of NICD1 and JAG1 than controls (Figure 2, *C* and *D*). Besides, the expression of *HES1*, *HEY1*, and *HEY2* genes also significantly increased in sorafenib-resistant tumors and cells than controls (Figure 2, *E* and *F*). Collectively, these findings demonstrate that NOTCH1 signaling is activated in sorafenib-resistant *in vivo* and *in vitro* models.

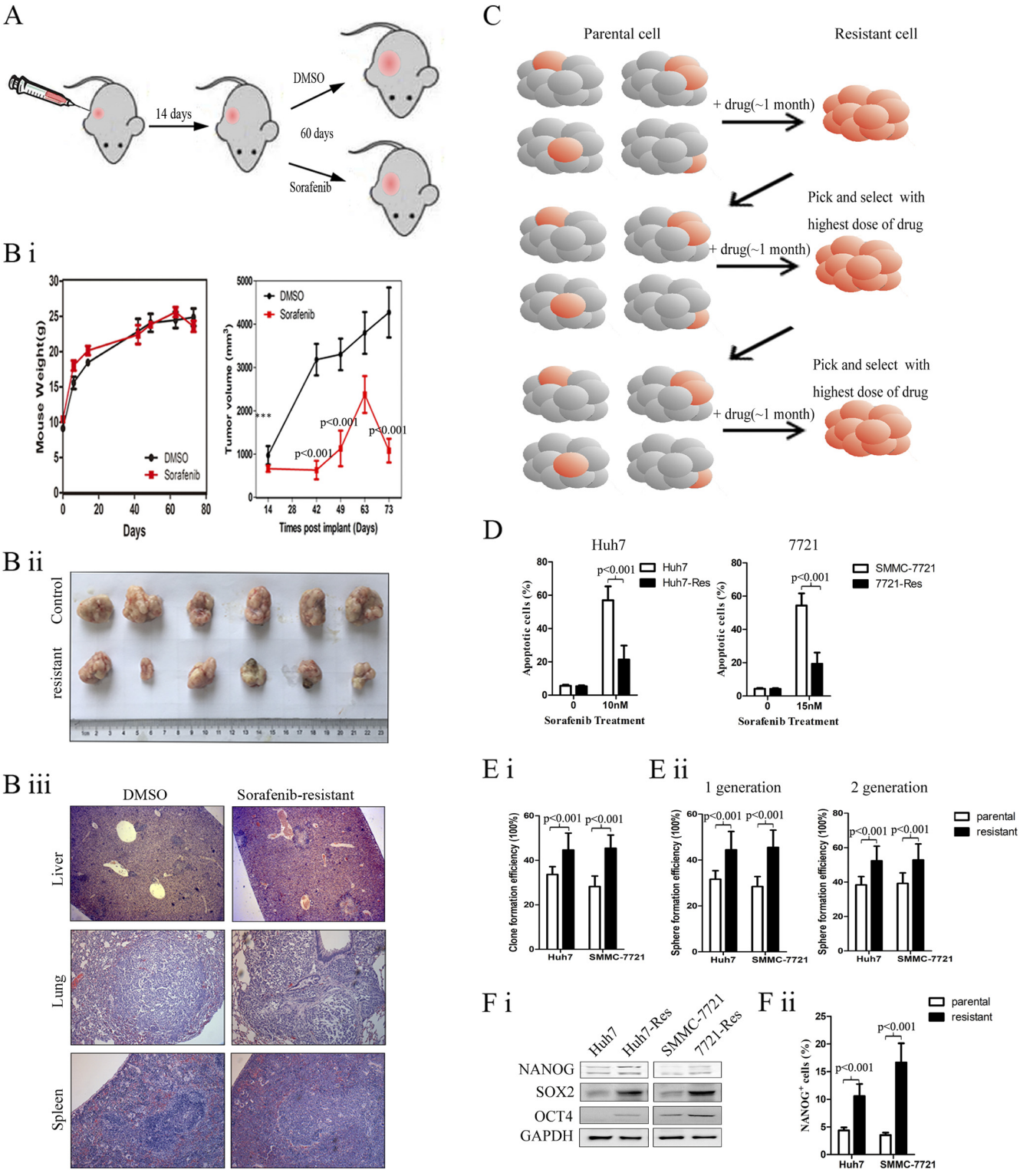


Figure 1. The establishment and identification of sorafenib-resistant cells *in vivo* and *in vitro*. (A) Schedule for the establishment of sorafenib-resistant tumors derived from Huh7 cells *in vivo*. (B) Sorafenib treatment inhibits tumor growth *in vivo*, but long-term treatment did not inhibit metastasis. i) Left: mouse weight, Right: tumor volume. ii) Final tumors images are represented. iii) Representative pictures of liver, lung and spleen with metastatic tumors stained for H&E. n = 6/group. (C) Schedule for constructing sorafenib-resistant cell lines derived from Huh7, and SMMC-7721 (marked with Huh7-Res and 7721-Res) *in vitro*. (D) Flow cytometric assay to detect apoptosis cells (Annexin V^+ population) in parental and sorafenib-resistant cells. The percentage (%) of Annexin V^+ population was calculated. Cells were treated with indicated doses of sorafenib for 48 h. Clone formation efficiency, and sphere formation efficiency, in the first and second generations, assays in parental and sorafenib-resistant cells. (E) i) Immunoblotting analysis of CSC markers (NANOG, SOX2, and OCT4) in parental and sorafenib-resistant cells. ii) Flow cytometric assays to detect $NANOG^+$ population in parental and sorafenib-resistant cells. The percentage (%) of $NANOG^+$ cells was calculated.

NOTCH1 Activation Confers Hepatoma Cells Sorafenib Resistance Through Enhanced Self-Renewal and Tumorigenecity

Secondly, we should evaluate whether active *NOTCH1* signaling is required for liver CSCs of sorafenib-resistant cells. We used a NOTCH1 inhibitor LY3039478, and a small molecular inhibitor (compound E) of γ-secretase, a protease necessary for NOTCH1 activation [34–36]. We performed clone and sphere formation assay to check tumorigenicity and self-renewal ability. Treatment with LY3039478 or compound E effectively reduced clone and sphere formation frequencies of sorafenib-resistant cells than controls (Figure 3*A*). Silencing *NOTCH1* or *JAG1* gene with siRNAs also significantly decreased clone and sphere formation frequencies than control siRNA (siCtl) in sorafenib-resistant cells (Figure 3, *B* and *C*). Conversely, NICD1 overexpression or NOTCH1 activation by JAG1 was sufficient to confer hepatoma cells increased clone and sphere formation frequencies in parental cells (Figure 3, *D* and *E*). These data suggest that NOTCH1 activation is required for self-renewal ability and tumorigenecity of sorafenib-resistant cells.

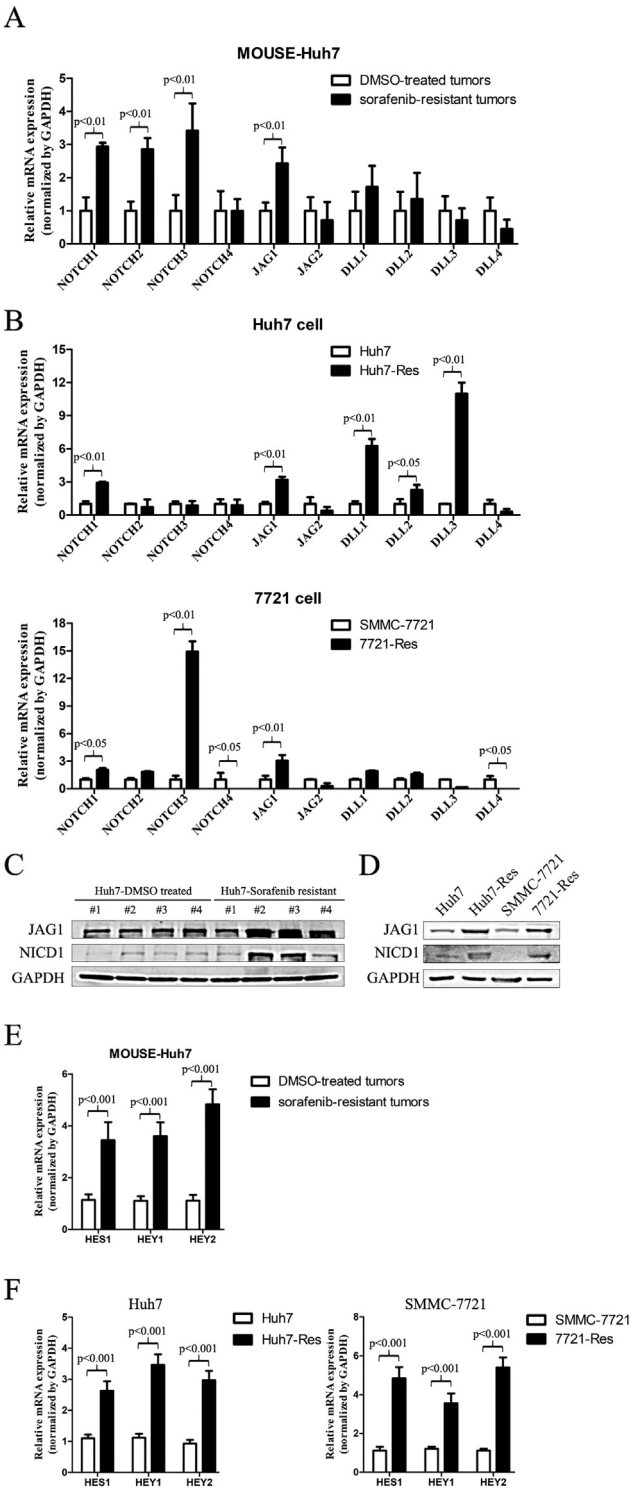


Figure 2. Notch signaling is activated in sorafenib-resistant tumors and cells. (A-B) Real-time PCR analysis of notch receptors and ligands in DMSO-treated and Sorafenib-resistant tumors (A), and in parental and sorafenib-resistant cells (B). (C-D) Immunoblotting analysis of JAG1 and NICD1 in DMSO-treated and Sorafenib-resistant tumors (C), and in parental and sorafenib-resistant cells (D). (E-F) Real-time PCR analysis of active Notch signaling genes (*HES1*, *HEY1*, and *HEY2*) in DMSO-treated and Sorafenib-resistant tumors (E), and in parental and sorafenib-resistant cells (F).

Genetic and Pharmacological Inhibition of EZH2 Inhibits Activation of NOTCH1 Signaling in HMT-Dependent Manner

Many developmental and Notch pathway genes are established targets of polycomb repressors and the associated histone mark H3K27me3, which restrain their activity in non-expressing cell types [37]. EZH2 is a member of the polycomb repressive complex 2 (PRC2), and has been characterized as general self-renewal regulators of hepatic stem cells [38]. Interesting, we did not observe that the H3K27 methyltransferase EZH2 is down-regulated but up-regulated in sorafenib-resistant tumors and cells than controls by immunoblotting (Figure 4*A*). EZH2 has been reported to serve as a transcriptional repressor though H3K27me3 in HMT-dependent manner, but there is recent evidence supporting the activating role of EZH2 in an HMT activity-independent manner [21, 22, 25–28, 38–40]. We hypothesized that EZH2 altered the NOTCH1 signaling pathway and in turn affected self-renewal ability and tumorigenicity of sorafenib-resistant cells. We conducted loss-of-function analysis of EZH2 *in vitro*, and achieved the stable knockdown of EZH2 in Huh7-Res and 7721-Res cells with lentivirus-mediated shRNA against EZH2 (shEZH2). Two different shEZH2s, shEZH2-#1 and shEZH2-#2, both markedly repressed EZH2 protein expression (Figure 4*B*). Genetic silencing of EZH2 with shEZH2 or pharmacological inhibition of EZH2 by DZNep or GSK343 decreased the amount of JAG1 and NICD1 in sorafenib-resistant cells by immunoblotting (Figure 4*B*). We next performed real-time PCR assays to detect expression of the NOTCH1 and *JAG1* genes at the transcriptional level. Except that silencing EZH2 in Huh7-Res reduced *NOTCH1* expression but was no significant, EZH2 depletion significantly inhibited the expression of *JAG1* and *NOTCH1* genes in sorafenib-resistant cells (Figure 4, *C* and *D*). What's more, genetic and pharmacological inhibition of EZH2 suppressed NOTCH1 activation as shown by significantly reduced *HES1*, *HEY1*, and *HEY2* expression in sorafenib-resistant cells by real-time PCR assay (Figure 4, *E* and *F*). These findings indicate that EZH2 depletion inhibits *JAG1* and *NOTCH1* expression and activation of NOTCH1 signaling in HMT-dependent manner.

Genetic and Pharmacological Inhibition of EZH2 Suppresses Self-Renewal Ability and Tumorigenicity through Activation of NOTCH1 Signaling.

Liver CSCs are highly dependent on EZH2 for their self-renewal capability and tumorigenic activity, and EZH2 depletion contributes to the eradication of liver CSCs [38, 41]. Because knockdown of EZH2 was more prominent with shEZH2-#1 than shEZH2-#2, we used shEZH2-#1 for most of the subsequent experiments (Figure 4*B*). Regarding the relative self-renewal potential of sorafenib-resistant cells, EZH2 knockdown significantly decreased clone and sphere formation frequencies than controls (Figure 5*A*). In keeping with these findings, limiting dilution analysis showed that sorafenib-resistant cells with EZH2 knockdown required more cells and a longer incubation time to generate tumors as compared with those with scrambled shRNA, indicating that sorafenib-resistant cells with shEZH2 were significantly less enriched in CSCs, compared with that in their scrambled counterparts (Figure 5*B*). Next, we established a xenograft model to examine the role of EZH2 in tumor

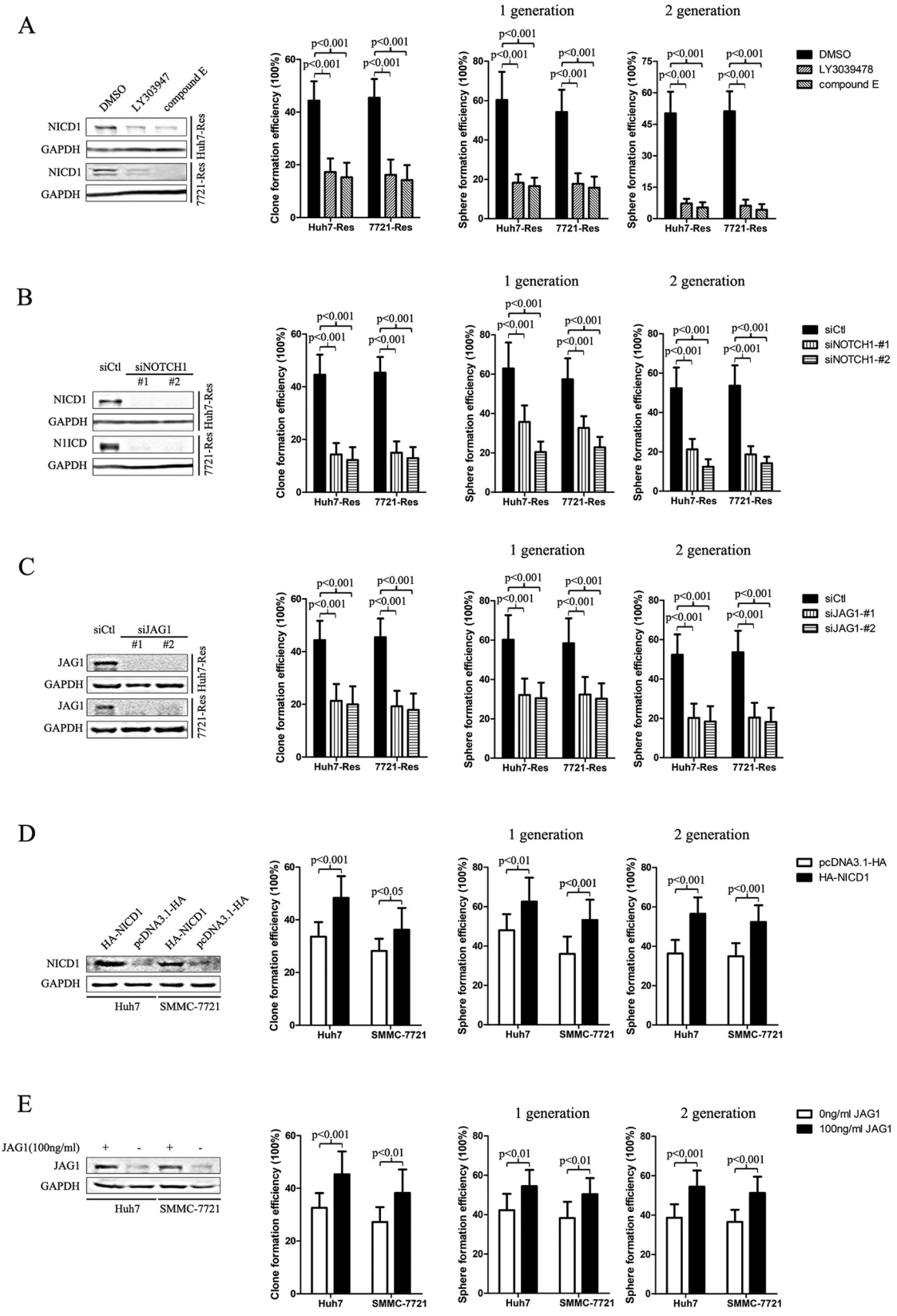


Figure 3. Activation of Notch1 signaling is required for the self-renewal and tumorigenic for sorafenib-resistant cells. (A) Left: Immunoblotting analysis of NICD1. Right: Effect of Notch1 inhibitor LY303947 and γ-secretase inhibitor compound E on clone formation and sphere formation of sorafenib-resistant cells. Sorafenib-resistant cells were treated with 10 μM LY303947 or 5 μM compound E for 48 h. (B) Left: Immunoblotting analysis of NICD1. Right: Effect of NOTCH1 siRNAs (siNOTCH1) and control siRNA (siCtl) on clone formation and sphere formation of sorafenib-resistant cells. (C) Left: Immunoblotting analysis of JAG1. Right: Effect of JAG1 siRNAs (siJAG1) and siCtl on clone formation and sphere formation of sorafenib-resistant cells. (D) Left: Immunoblotting analysis of NICD1. Right: Effect of NICD1 overexpression on clone formation and sphere formation of HCC cells. (E) Left: Immunoblotting analysis of JAG1. Right: Effect of adding JAG1 protein on clone formation and sphere formation of HCC cells. Clone formation and sphere formation assays were performed after 2 weeks. N = 10/group.

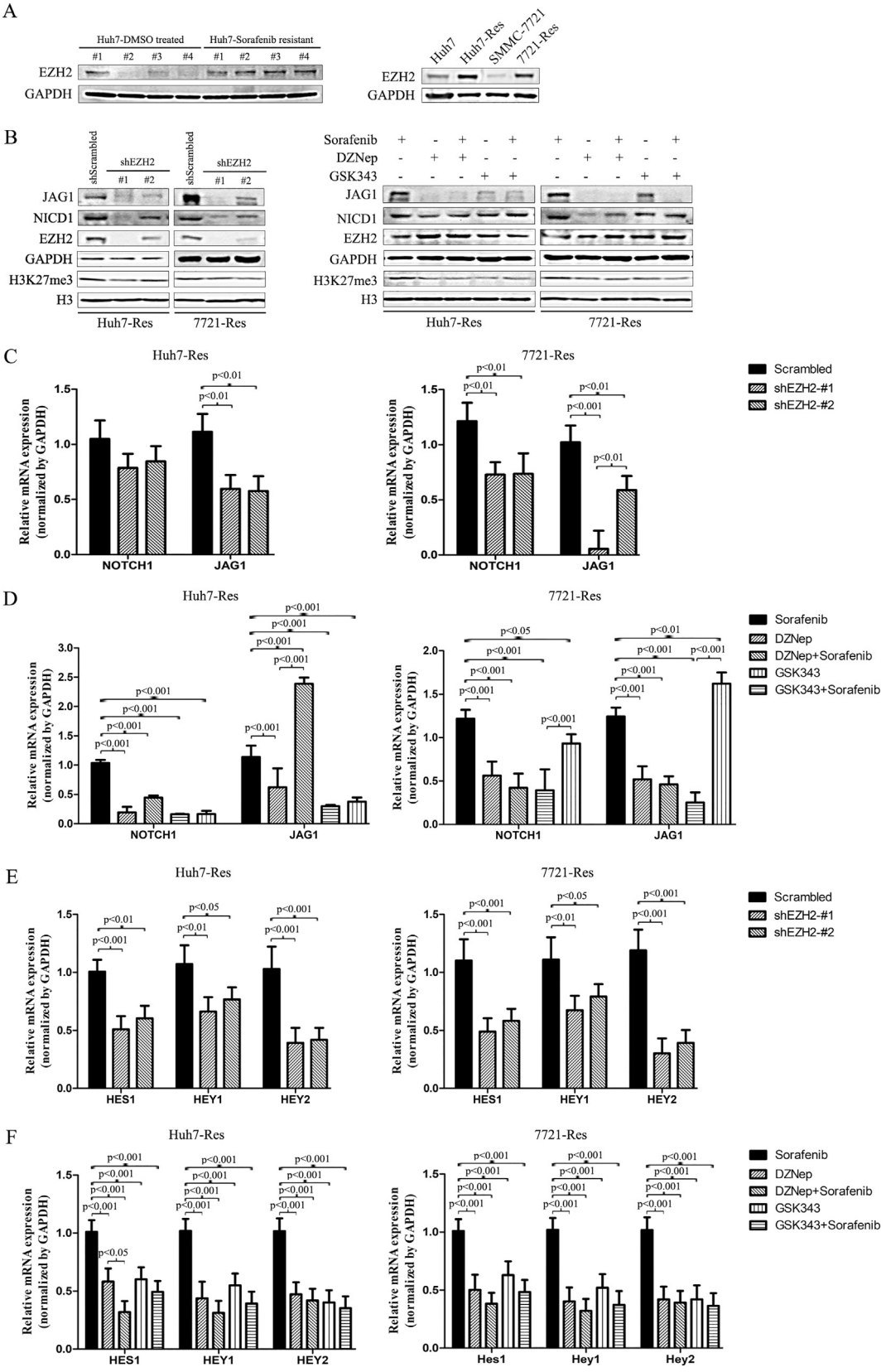
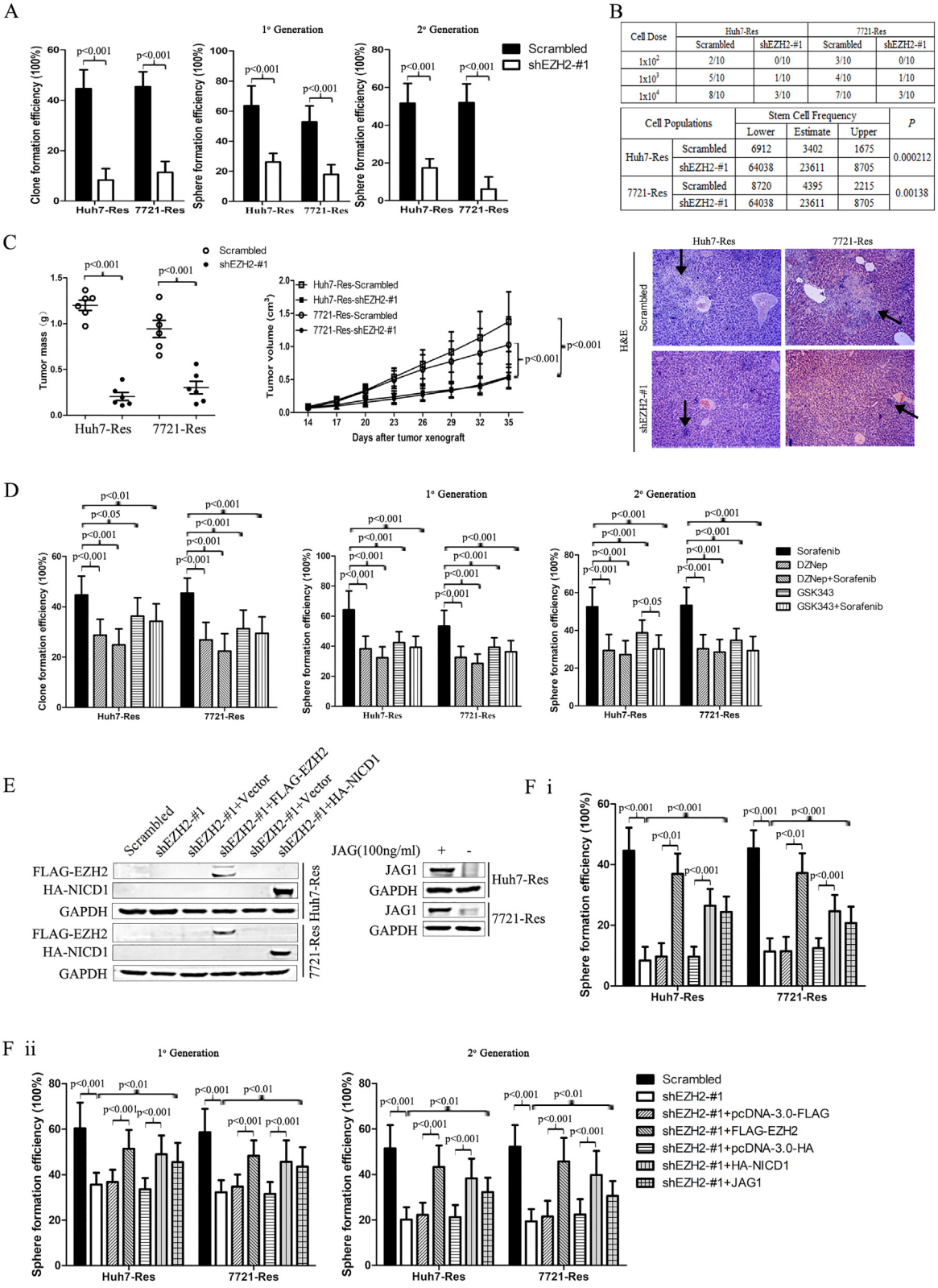


Figure 4. Genetic and pharmacological inhibition of EZH2 suppresses the activation of Notch signaling in sorafenib-resistant cells. (A) Immunoblotting analysis of EZH2 in DMSO-treated and Sorafenib-resistant tumors derived from Huh7 cells, and Huh7, Huh7-Res, SMMC-7721, and 7721-Res. (B) Left: Sorafenib-resistant cells expressing scrambled shRNA (scrambled) and two different EZH2 shRNAs (shEZH2, #1 and #2) were analyzed for the indicated proteins by immunoblotting. Right: Sorafenib-resistant cells were treated with EZH2 inhibitors DZNep and GSK343, and then were analyzed for the indicated proteins by immunoblotting. (C-F) Effects of shEZH2s and scrambled (C, E) and EZH2 inhibitors DZNep and GSK343 (D, F) on *NOTCH1* and *JAG1* gene (C-D) or active Notch signaling genes (*HES1*, *HEY1*, and *HEY* gene) (E-F) by real-time PCR. Sorafenib-resistant cells were treated with 10 μM Sorafenib, 5 μM DZNep, 10 μM Sorafenib + 5 μM DZNep, 3 μM GSK343, 10 μM Sorafenib + 3 μM GSK343 for 48 h. Results are expressed as the relative expression (mean ± SD). N = 3, each in triplicate.



Cell Dose	Huh7-Res		7721-Res	
	Scrambled	shEZH2-#1	Scrambled	shEZH2-#1
$1x10^2$	2/10	0/10	3/10	0/10
$1x10^3$	5/10	1/10	4/10	1/10
$1x10^4$	8/10	3/10	7/10	3/10

Cell Populations		Stem Cell Frequency			P
		Lower	Estimate	Upper	
Huh7-Res	Scrambled	6912	3402	1675	0.000212
	shEZH2-#1	64038	23611	8705	
7721-Res	Scrambled	8720	4395	2215	0.00138
	shEZH2-#1	64038	23611	8705	

Figure 5. EZH2 is essential for the maintenance of liver CSC self-renewal and tumorigenecity in sorafenib-resistant cells. (A) Sphere formation efficiency in the first and second generations, and clone formation efficiency assays with shEZH2 or scrambled in sorafenib-resistant cells were performed after 2 weeks. (B) Effect of EZH2 knockdown on the tumor-forming frequency of sorafenib-resistant cells, as determined by limiting dilution assays. CSC frequency was calculated using the ELDA (http://bioinf.wehi.edu.au/software/elda). (C) Sorafenib-resistant cells stably infected with shEZH2 and scrambled vectors were analyzed for tumorigenic potential; Final tumor masses are represented, and tumor volume was monitored every 3 days with indicated (mean ± SD, n = 6/group). Representative pictures of liver with metastatic tumors stained for H&E. (D) Effect of EZH2 inhibitors DZNep and GSK343 on Sphere formation and clone formation. Sorafenib-resistant cells were treated with 10 μM Sorafenib, 5 μM DZNep, 10 μM Sorafenib + 5 μM DZNep, 3 μM GSK343, 10 μM Sorafenib + 3 μM GSK343. (E) Effect of overexpressing EZH2 or NICD1, or adding JAG1 on EZH2-mediated self-renewal and tumorigenecity. Sorafenib-resistant cells were stably infected with scrambled and shEZH2 vectors, and then transfected with EZH2 or NICD1 vector, or added JAG1 protein for 24 h. Sphere formation efficiency in the first and second generations, and clone formation efficiency assays were performed after 2 weeks.

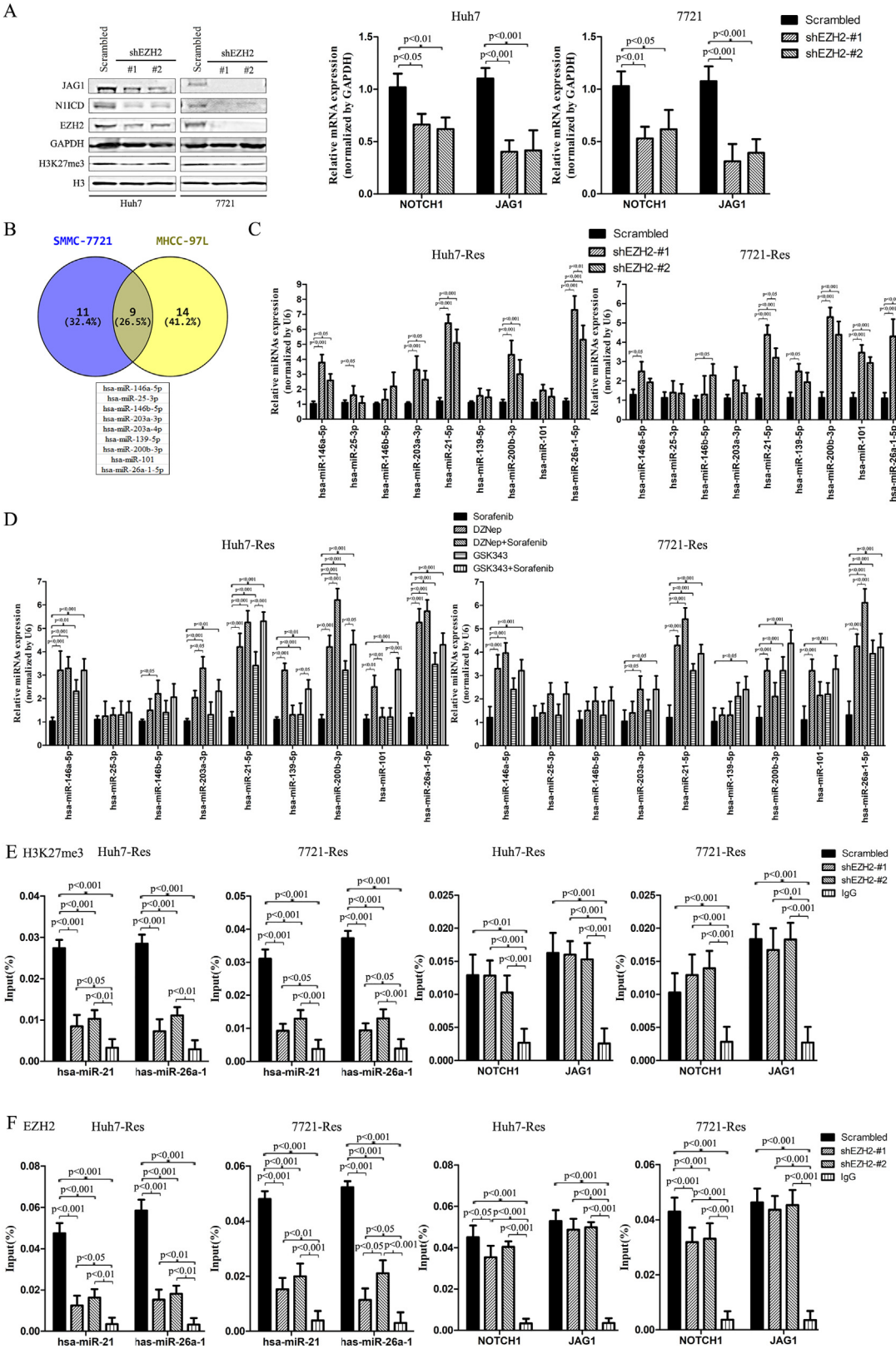


Figure 6. Genetic and pharmacological inhibition of EZH2 results in the up-regulation of Notch-related miRNAs by decreasing H3K27me3 modification in sorafenib-resistant cells. (A) Effect of EZH2 knockdown on the protein level of JAG1 and NICD1 by immunoblotting or the expression of *NOTCH1* and *JAG1* gene by real-time PCR in Huh7 and SMMC-7721 cells. (B) A Venn diagram illustrates the distribution of up-regulated miRNA upon EZH2 knockdown in SMMC-7721 and MHCC97L-luc (Up), the table lists a set of 9 miRNAs that were universally up-regulated across two cell lines (Down). Two-fold increases in miRNA expression were defined as up-regulation. (C-D) Effects of EZH2 knockdown (C) or EZH2 inhibition by DZNep or GSK343 (D) on the expression of 9 Notch-related miRNAs by real-time PCR in Huh7-Res and 7721-Res cells. E-F) Effects of the recruitment of EZH2 (E) and the enrichment of H3K27me3 mark (F) on hsa-miR-21 and has-miR-26a-1 loci upon EZH2 knockdown by ChIP-qPCR analysis in Huh7-Res and 7721-Res cells. Sorafenib-resistant cells were treated with 10 μM Sorafenib, 5 μM DZNep, 10 μM Sorafenib +5 μM DZNep, 3 μM GSK343, 10 μM Sorafenib + 3 μM GSK343 for 48 h. Results are expressed as the relative expression (mean ± SD). N = 3, each in triplicate.

development and metastasis in nude mice, and observed that with EZH2 knockdown, the tumorigenic potential, growth and liver metastasis of sorafenib-resistant cells were inhibited (Figure 5*C*). Besides, EZH2 inhibition by DZNep or GSK343 also significantly decreased clone and sphere formation frequencies than sorafenib treatment, and combined with sorafenib treatment did not always have significant results (Figure 5*D*). These findings indicate that EZH2 depletion abrogates liver CSCs stemness *in vivo* and *in vitro* in HMT-dependent manner in sorafenib-resistant cells.

To further determine whether EZH2 can restore self-renewal through activation of NOTCH1 signaling in sorafenib-resistant cells, we overexpressed EZH2 or NICD1, or added JAG1 protein to rescue the effect of EZH2 knockdown on self-renewal ability and tumorigenicity in sorafenib-resistant cells (Figure 5*E*). We observed overexpressed EZH2 significantly restored clone and sphere formation frequencies of sorafenib-resistant cells (Figure 5*F*). What's more, overexpression of NICD1 or NOTCH1 activation by JAG1 can partly restore self-renewal potential and tumorigenicity (Figure 5*F*). Collectively, overexpression of EZH2 promotes the self-renewal of liver CSCs through activation of NOTCH1 signaling.

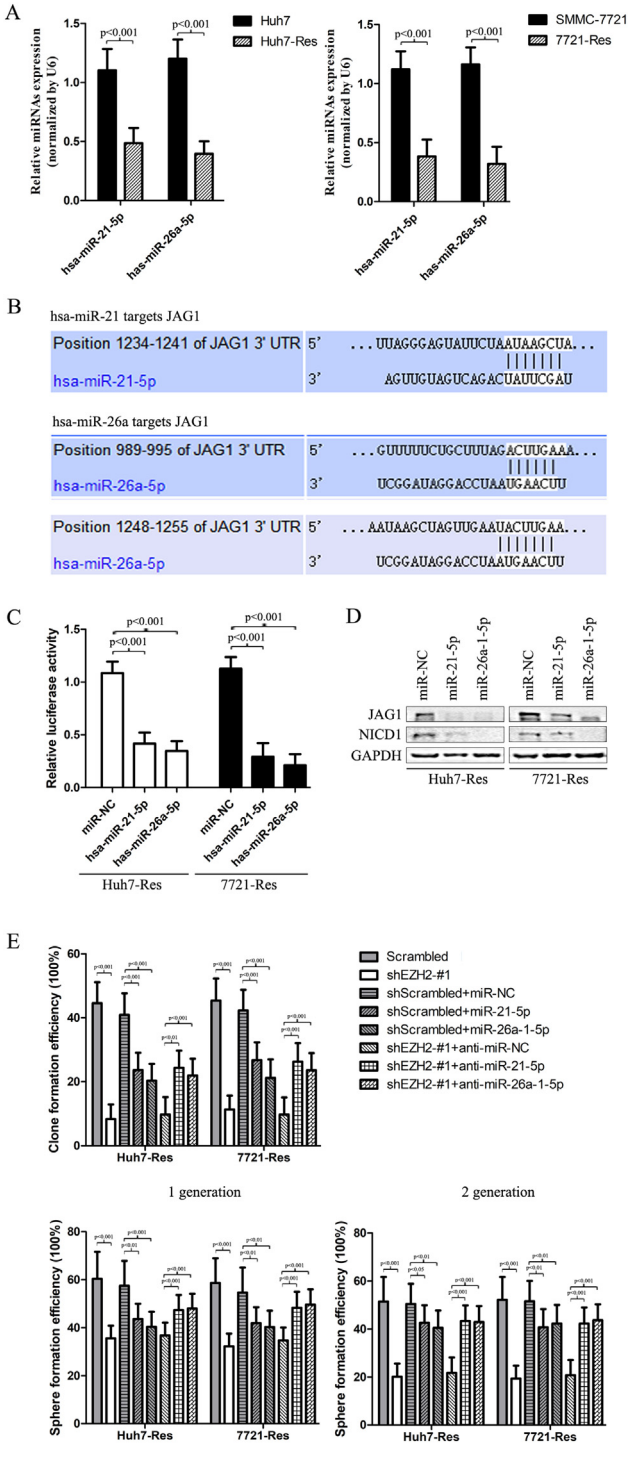


Figure 7. MiR-21-5p and miR-26a-1-5p directly target *JAG1* gene and weaken stem-cell properties *via* Notch1 signaling. (A) Real-time PCR analysis of miR-21-5p and miR-26a-1-5p in Huh7, Huh7-Res, SMMC-7721, and 7721-Res. (B) Schematic representation of the miR-21-5p or miR-26a-1-5p and their binding sequences in the 3'-UTR of the JAG1 mRNA. (C) Analysis of luciferase activity in sorafenib-resistant cells for 48 h after co-transfection with the control Renilla luciferase expression construct pGL3-Basic and a luciferase reporter plasmid containing the 3'-UTR of JAG1. Luciferase activity in each sample was normalized to the Renilla and luciferase activity of the control cells. Each experiment was performed in triplicate. (D) Immunoblotting analysis of JAG1 and NICD1 protein in sorafenib-resistant cells, which were transfected with miR-21-5p mimic, miR-26a-1-5p mimic or negative control miRNA (miR-NC) mimic for 48 h. (E) Effect of miR-21-5p and miR-26a-1-5p on EZH2-mediated self-renewal and tumorigenecity. Sorafenib-resistant cells with scrambled or shEZH2 were transfected with either miR-21-5p mimic, miR-26a-1-5p mimic or miR-NC mimic, or anti-miR-21-5p, anti-miR-26a-1-5p or anti-miR-NC for 24 h. Clone formation efficiency, and Sphere formation efficiency in the first and second generations assays were performed after 2 weeks.

EZH2 Epigenetically Silences NOTCH1-Related MicroRNAs and Promotes NOTCH1 Activation

Next we explored how EZH2 promotes NOTCH1 signaling activation in HMT-dependent manner in sorafenib-resistant cells. Notch suppressors participates in the splicing process of Notch active form through protein–protein interaction, and consequently decreased the amount of NICD1 at the protein level, while microRNAs target *JAG1* and *NOTCH1* genes, and consequently decreased *JAG1* and *NOTCH1* expression at the transcriptional level [42, 43]. EZH2 epigenetically silences multiple tumor suppressor microRNAs to promote liver cancer metastasis, *via* H3K27me3 [44]. Therefore, we hypothesized that EZH2 targeted and repressed microRNAs and consequently reduced *JAG1* and *NOTCH1* expression and activated NOTCH1 pathway. First, we need to determine which miRNAs are likely to be involved in the regulation of NOTCH1 signaling. We searched published articles and the TargetScan website (http://www.targetscan.org/vert_72/), and found that 81 miRNAs are involved in the regulation of NOTCH1 signaling, mainly in *JAG1* and *NOTCH1*, defined as NOTCH1-related-miRNAs (Supplementary Table. S4–6). Next, we need to determine which NOTCH1-related-miRNAs may be subject to epigenetic regulation of EZH2 in sorafenib-resistant cell lines. There is no literature study on the epigenetic regulation of miRNA expression by EZH2 in sorafenib-resistant cell lines. However, Sandy Leung-Kuen Au et al. performed qPT-PCR-based TaqMan miRNA expression arrays in SMMC-7721, MHCC97L-luc, and HepG2 cell lines, and compared the miRNA expression profile of cells upon EZH2 knockdown [44]. What's more, lentiviral-mediated shEZH2 stably knockdown EZH2 in Huh7 and 7721 cells significantly inhibited EZH2 and JAG1 protein expression and decreased NICD1 protein and histone H3K27me3 modification by immunoblotting, which was consistent with sorafenib-resistant cells (Figure 6*A*). Besides, EZH2 knockdown significantly suppressed the expression of *JAG1* and *NOTCH1* genes in Huh7 and 7721 cells by real-time PCR (Figure 6*A*). These data implicate a similar mechanism for regulation in resistant and non-resistant cell lines. Due to the characteristics of sorafenib-resistant cell, we selected the results in SMMC-7721 and MHCC97L-luc cells for preliminary analysis, and found that simultaneously up-regulated in both cell lines upon EZH2 depletion was observed for nine miRNAs, namely hsa-miR-146a-5p, hsa-

miR-25-3p, hsa-miR-146b-5p, hsa-miR-203a-3p, hsa-miR-21-5p, hsa-miR-139-5p, hsa-miR-200b-3p, hsa-miR-101, and hsa-miR-26a-1-5p (Supplementary Table.S7, Figure 6*B*) [44].

In support of our hypothesis, blockade of EZH2 expression or inhibition of EZH2 activity by DZNep and GSK343 in sorafenib-resistant cells led to elevated expression of multiple NOTCH1-related microRNAs (Figure 6, *C* and *D*). Hsa-miR-21-5p and hsa-miR-26a-1-5p were further validated candidate miRNAs that were simultaneously upregulated in both cell lines upon EZH2 depletion (Figure 6, *C* and *D*). EZH2 exerts transcriptional repression through H3K27me3 in HMT-dependent manner [38]. We performed chromatin immunoprecipitation (ChIP)-qPCR assays to measure the recruitment of EZH2 and the enrichment of H3K27me3 mark on miR-21 and miR-26a-1 loci in Huh7-Res and 7721-Res cells. The results showed significant enrichments of EZH2 and H3K27me3 on miR-21 and miR-26a-1 loci in Huh7-Res and 7721-Res cells, whereas genetic knockdown of EZH2 abrogated these occupancies, but did not affect the occupancies at the JAG1 promoter (Figure 6, *E* and *F*). Interestingly, although EZH2 knockdown did not reduce the enrichment of the H3K27me3 marker, but the recruitment of EZH2 was reduced on the NOTCH1 promoter, suggesting that EZH2 can be independent of the activity of histone methyltransferase by direct binding to the promoter, acting as an activator of the NOTCH1 gene, just as EZH2 regulates NOTCH1 signaling in breast cancer (Figure 6, *E* and *F*) [38]. The data suggests EZH2 targets NOTCH1-related miRNAs and promotes NOTCH1 activation in HMT-dependent manner.

MiR-21-5p and miR-26a-1-5p Suppress NOTCH1 Signaling and Stem-Cell Properties by Targeting JAG1

To evaluate the role of hsa-miR-21-5p and hsa-miR-26a-1-5p in sorafenib-resistant cells, we performed real-time PCR to examine the expression levels of miR-21-5p and miR-26a-1-5p, and found that miR-21-5p and miR-26a-1-5p are significantly down-regulated in sorafenib-resistant cells than controls (Figure 7*A*). TargetScan (www.targetscan.org) was used in an attempt to identify the targets of miR-21-5p and miR-26a-1-5p, and found that JAG1 was identified as a potential target gene of miR-21-5p and miR-26a-1-5p (Figure 7*B*). A luciferase assay was performed in sorafenib-resistant cells, which revealed that overexpression of the miR-21-5p mimic and miR-26a-1-5p mimic, but not the negative control miRNAs (miR-NC) mimic, significantly inhibited the activity of luciferase fused with the 3′-UTR of JAG1 (Figure 7*C*). To further verify whether JAG1 is a functional target gene of miR-21-5p and miR-26a-1-5p in sorafenib-resistant cells, the cells were transfected with either miR-21-5p mimic, miR-26a-1-5p mimic or miR-NC mimic, and the protein level of JAG1 and NICD1 were determined by immunoblotting. Overexpression of miR-21-5p mimic and miR-26a-1-5p mimic significantly inhibited JAG1 expression and NICD1 protein in Huh7-Res and 7721-Res cells than miR-NC mimic (Figure 7*D*). To evaluate the effects of miR-21-5p or miR-26a-1-5p overexpression on EZH2-mediated stem-cell properties, the resistant cells with scrambled were transfected with either miR-21-5p mimic, miR-26a-1-5p mimic or miR-NC mimic, and the resistant cells with shEZH2 were transfected with either anti-miR-21-5p, anti-miR-26a-1-5p or anti-miR-NC, we next performed clone and sphere formation assays, and found that overexpression of miR-21-5p mimic and miR-26a-1-5p mimic significantly inhibited clone and sphere formation frequencies of sorafenib-resistant cells, similar to the effect of EZH2 knockdown, but that overexpression of anti- miR-21-5p and anti-miR-26a-1-5p significantly rescued the decreased clone and sphere formation frequencies of sorafenib-resistant cells due to EZH2 knockdown (Figure 7*E*). These data suggest miR-21-5p and miR-26a-1-5p target *JAG1* gene and suppress NOTCH1 signaling to weaken stem-cell properties in sorafenib-resistant cells.

Discussion

Liver CSCs, like normal stem cells, display the stem-cell properties of self-renewal and the ability to give rise to new tumors [4]. Drug-resistant cells have more CSCs, resulting in cancer relapse and metastasis [4, 32]. In our study, we found that sorafenib-resistant tumors exhibit relapse and distant metastasis, and that sorafenib-resistant cells have enhanced self-renewal ability and tumorigenecity and increased CSC markers.

CSCs may have core regulatory genes and developmental signaling pathways, similar to those of normal-tissue stem cells [4, 10, 11]. Activation of Notch signaling was found to promote liver CSC self-renewal through the transcriptional regulation of downstream target genes that participate in cell-fate determination [11, 13, 14, 16, 17]. In HCC, Notch signaling is activated and plays an oncogenic role [13, 14, 16]. Herein, we further determined that NOTCH1 signaling is activated in sorafenib-resistant cells from *in vitro* and *in vivo* models, and consequently promoted liver CSCs self-renewal and conferred hepatoma cells sorafenib resistance. As such, inhibition of NOTCH1 activation by inhibitors or silencing *NOTCH1* or *JAG1* gene weakened self-renewal ability and tumorigenecity in sorafenib-resistant cells. Conversely, NICD1 overexpression and NOTCH1 activation by JAG1 enhanced self-renewal ability and tumorigenecity in parental cells. Our data suggest that NOTCH1 signaling acts as an essential mediator of the resistance to sorafenib in HCC though enhanced self-renewal ability and tumorigenecity. Further studies are required to clarify whether NOTCH1 signaling plays a central role in development and progression of acquired sorafenib resistance in HCC and the relationship of NOTCH1 signaling with other commonly altered pathways in sorafenib-resistant HCC.

Poised epigenetic states drive cancer cells to adapt to different pressures through adaptive chromatin remodeling, including conventional and targeted therapy [45]. Enrichment of CSCs is a common way for cancer cells to adapt to drug stress [7, 45]. Epigenetic regulation machinery involves multiple proteins with distinct functions [33, 45]. In our study, we found that EZH2 was overexpressed following prolonged sorafenib treatment, and EZH2 depletion weakened stem-cell properties of sorafenib-resistant cells and reversed this resistance. In term of mechanism, alterations of epigenetic modifiers can affect HCC epigenome in multiple aspects and determine HCC outcome [19]. Herein, we found that EZH2 depletion increased the expression of multiple NOTCH1-related miRNAs, and indirectly suppressed NOTCH1 activation in sorafenib-resistant cells. Conversely, EZH2 overexpression in sorafenib-resistant cells epigenetically silenced the expression of NOTCH1h-related miRNAs, which in turn increased the expression of NOTCH1 and JAG1, indirectly activating NOTCH1 signaling. However, like other solid cancers, other mechanisms modified by EZH2 may also be involved in controlling acquired sorafenib resistance in HCC, for example, transcriptional activation of NOTCH1, kinase group reprogramming, and immune escape of cancer cells [21, 27, 46]. Therefore, we identified EZH2 served as an important molecular switch controlling acquired sorafenib resistance in HCC.

MicroRNAs (miRNAs or miRs) are small non-coding RNAs involved in the posttranscriptional control of hundreds of target genes, and are recognized as key regulators in numerous cellular processes including pluripotency [47]. Aberrant expression of miRNAs considerably contributes to regulation of CSC and drug resistance [19, 48]. Herein, we found multiple miRNAs, including miR-21-5p and miR-26a-1-5p, were up-regulated upon EZH2 depletion in sorafenib-resistant cells. Besides, miR-21-5p and miR-26a-1-5p targeted JAG1 and inhibited NOTCH1 activation, thereby indirectly eradicating CSCs.

In conclusion, our results indicate that sorafenib resistance in HCC may be due in part to a compromised direct anti-tumor effect which appears to be regulated by EZH2. Active NOTCH1 signaling by resistance to sorafenib may be associated with epigenetic regulators, which down-regulate NOTCH1-associated microRNAs as a mechanism to escape sorafenib stress. We anticipate that our findings will have direct clinical implications as EZH2 inhibitors are already in advanced clinical development to overcome/delay HCC resistance.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2020.01.002.

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