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Overexpression of PER3 Inhibits Self-Renewal Capability and Chemoresistance of Colorectal Cancer Stem-Like Cells via Inhibition of Notch and β-Catenin Signaling

Feng Zhang,* Hong Sun,† Sai Zhang,† Xin Yang,‡ Guogang Zhang,* and Tao Su†

*Department of Cardiovascular Medicine, Xiangya Hospital, Central South University, Changsha, P.R. China
†The Medical Research Center of Xiangya Hospital, Central South University, Changsha, Hunan, P.R. China
‡Department of General Surgery, The Third Hospital of Changsha, Changsha, P.R. China

PER3, a circadian clock gene, plays an important role in colorectal cancer, but its action and underlying mechanism in colorectal cancer stem-like cells (CSCs) remain unclear. In this study, the colorectal CSCs were enriched in colorectal HCT-116 sphere-forming cells, expressing lower levels of stem cell markers CD133, CD44, LGR5, and SOX2 compared with HCT-116 cells. A drug-resistant strain from HCT-116 was established. Western blot and qRT-PCR analysis showed that PER3 was downregulated in colorectal CSCs and drug-resistant HCT-116. Overexpression of PER3 could strengthen 5-FU-induced inhibitory effects on colorectal CSCs, but knockdown of PER3 decreased its inhibition of colorectal CSCs. In addition, overexpression of PER3 in colorectal CSCs resulted in reduced colony formation efficiency in a soft agar medium and self-renewal efficiency. Inversely, knockdown of PER3 enhanced self-renewal of colorectal CSCs. Overexpression of PER3 decreased stemness markers and Notch1, Jagged1, β-catenin, c-Myc, and LGR5 in colorectal CSCs. When Notch or β-catenin signaling was inhibited, the chemoresistance and self-renewal capability of colorectal CSCs were decreased. It was confirmed that PER3 can reduce chemoresistance and self-renewal capability of colorectal CSCs via inhibition of Notch and β-catenin signaling. Our results reveal that PER3 plays a critical role in maintaining the stemness of colorectal CSCs and may be a promising target for elimination of CSCs.

Key words: Colorectal cancer stem-like cells (CSCs); Circadian clock; PER3; Self-renewal; Chemoresistance

INTRODUCTION

Colorectal cancer is the third leading cause of cancer deaths among American men and women[1]. Despite increased screening and advances in treatment for colorectal cancer over the past 20 years, the prognosis for patients with metastatic disease remains poor[2]. Thus, to develop novel therapeutic strategies for colorectal cancer patients with poor response to conventional chemotherapy, it is necessary to search for new targets that play an important role in chemoresistance, recurrence, and metastasis.

It has been shown that disruption of the circadian rhythm is correlated with cancer development and progression in humans including breast, endometrial, prostate, and colon cancer[3]. In particular, PER3 is significantly downregulated in colorectal cancer tissue compared to adjacent normal mucosa, and lower PER3 expression is associated with poorer survival rates[4,5]. These findings imply that PER3 possibly acts as a tumor suppressor, and its downregulation is implicated in colorectal cancer development and progression. However, its underlying mechanisms in colorectal cancer chemoresistance and recurrence are still obscure.

Cancer stem-like cells (CSCs) have self-renewal capacity and differentiation potential. The CSC theory presumes that tumor maintenance, chemoresistance, and recurrence are due to the existence of a small subpopulation of CSCs. CSCs have been sorted from colorectal cancer cells and can be characterized by markers CD133, CD44, LGR5, SOX2[6–8], etc. Studies confirm that the spheroid cultures of colorectal cancer HCT-116 cells can enrich colorectal CSCs[9], which provides an optional approach for establishing a colorectal CSC model. The clock genes have been shown to have various effects on c-Myc/p21 and Wnt/β-catenin pathways,

Address correspondence to Guogang Zhang, Department of Cardiovascular Medicine, Xiangya Hospital, Central South University, Xiangya Road 87, Changsha 410008, Hunan, P.R. China. Tel: +86-731-84327695; Fax: +86-731-84327695; E-mail: xyzgg2006@sina.com *or* Tao Su, The Medical Research Center of Xiangya Hospital, Central South University, Xiangya Road 87, Changsha 410008, Hunan, P.R. China. Tel: 86-0731-84327628; Fax: +86-731-84327332; E-mail: csusutao@sina.com

which are involved in the regulation of CSC stemness[3,10]. Elimination of colorectal CSCs represents a promising strategy for curing colorectal cancer[11]. However, whether PER3 plays a critical role in colorectal CSCs remains unclear.

In the present study, colorectal CSCs were enriched in HCT-116 sphere-forming cells, and a drug-resistant strain from HCT-116 was established. The expression profile of PER3 in those models was detected. The effects of PER3 on chemoresistance and self-renewal of colorectal CSCs were investigated. Our results show novel action mechanisms for PER3 in colorectal cancer as well as a new target for treatment of this malignant disease.

MATERIALS AND METHODS

Cell Culture

The HCT-116 human colorectal cancer cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and antibiotics at 37°C in a 5% CO_2 atmosphere. The 5-FU-resistant line HCT-116/R was established as previously described[12] by exposing its parental cell line to the IC_{50} of 5-FU for 3 months, followed by further exposure to a 10-fold higher dose for another 6 months under the same cultivation conditions as the parental cell line.

Sphere-Forming Cell Culture

To obtain sphere cultures, monolayer cells were enzymatically and manually dissociated into a single-cell suspension, plated at a density of 5×10^3 cells/well in six-well ultralow plates (Corning, Acton, MA, USA) containing serum-free medium DMEM/F12 (Gibco, Carlsbad, CA, USA), supplemented with commercial hormone mix B27 (Gibco), 20 ng/ml EGF (PeproTech, Rocky Hill, NJ, USA), 10 ng/ml bFGF (PeproTech), 0.4% bovine serum albumin (Gibco), 4 mg/ml insulin (Gibco), 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C. After being cultured for 6 days, the colorectal cancer spheres were collected, dissociated into single-cell suspensions, and resuspended in fresh medium for serial subcultivation every 6 days. To gain a PER3 overexpression model, the HCT-116 sphere-forming cells were infected with lentivirus packaged pGMLV-PE1-per3 (Spheres-Lv-per3). The HCT-116 sphere-forming cells were then infected with lentivirus packaged per3-siRNA (Spheres-per3-siRNA) to establish a PER3 knockdown model.

Quantitative RT-PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and then the RNA was reverse transcribed using the PrimeScript RT Master Mix Perfect Real Time kit (TaKaRa, Dalian, P.R. China) to obtain the cDNA. Using the cDNA as the template, a real-time PCR assay was performed using the following pairs of primers: PER3, 5′-gcaggtctatgccagtgtga-3′ (forward) and CTR2, 5′-tgccttgtggttctgtttgt-3′ (reverse); CD33, 5′-tcaaggacttgcg aactctc-3′ (forward) and 5′-gtctccttgatcgctgttg-3′ (reverse); CD44, 5′-agcagcacttcaggaggttac-3′ (forward) and 5′-ccatg tgagtgtctggtagca-3′ (reverse); LGR5, 5′-aatttgcgaagccttc aatc-3′ (forward) and 5′-gggatttctgttaacgcattg-3′ (reverse); Sox2, 5′-ccatccacactcacgcaaaa-3′ (forward) and 5′-tatac aaggtccattcccccg-3′ (reverse); and β-actin, 5′-aggggccgga ctcgtcatact-3′ (forward) and 5′-ggcggcaccaccatgtaccct-3′ (reverse). The 20-μl real-time PCR reaction included 0.5 μl of cDNA template, 0.25 μl of primer F, 0.25 μl of primer R, 10 μl of RNase-free dH_2O, and 8 μl of 2.5× Real Master Mix (SYBR Green I). The reaction conditions included a predenaturation step at 95°C for 10 s, and 40 cycles of 95°C for 15 s and 60°C for 60 s. After the reaction, the data were subjected to statistical analysis.

Soft Agar Colony Formation Assay

Cells were seeded at a density of 1,000 cells per well in six-well plates and allowed to grow for 10 days. Clones were fixed with 4% methanol and stained with Giemsa dye (Sigma-Aldrich, St. Louis, MO, USA), and clone numbers were counted microscopically. The colony formation efficiency = (clone number/inoculated cell number) × 100%.

Self-Renewal Assay

To investigate self-renewal capacity, single-cell suspensions prepared from parental cells or tumor spheres of HCT-116 cells were diluted to 1,000 cells/ml. One microliter of the single-cell suspension was plated in 96-well ultralow plates containing 150 ml of serum-free medium per well. Wells containing no cells or more than one cell were excluded, and those with one cell were marked and monitored daily under a microscope (Nikon Eclipse TE2000-S; Nikon, Japan) for 6 days, and the colonies were counted. The self-renewal efficiency = (clone number/inoculated cell number) × 100%.

Drug Sensitivity Assay

HCT-116, HCT-116/R, or HCT-116 sphere-forming cells or its PER3 overexpression and knockdown cells, as indicated, were seeded in a 96-well plate and cultured at 37°C in a humidified 5% CO_2 atmosphere for 24 h. 5-FU at various concentrations (0, 10, 20, 30, 40, and 50 μg/ml) was then administrated to cell cultures. An MTT assay was applied to examine cell viability after 48 h of incubation. The IC_{50} values of each drug were estimated using the relative survival curve at a concentration that inhibited

50% cell survival. 5-FU at a concentration of 20 μg/ml was administrated to HCT-116 spheres and HCT-116 Spheres-Lv-per3 cell cultures. An MTT assay was applied to examine cell viability after 0, 12, 24, 36, and 48 h of incubation. The optical density at 570 nm (OD570) of each well was measured with an ELISA reader (ELX-800 type; Bio-Tek Instruments, Winooski, VT, USA). The relative inhibition rate = (OD value at 0 h − OD value at 12, 24, 36, or 48 h)/(OD value at 0 h). Three independent experiments were performed in triplicate.

Tumorigenicity Assay

The 1×10^4 cells were injected subcutaneously into the back of 4-week-old BALB/C nude mice (supplied by the Shanghai Experimental Animal Center, Chinese Academy of Sciences, Shanghai, P.R. China). The mice were reared for 1 month, and tumor growth was examined visually. Tumor volumes were calculated in accordance with the formula: V (transplanted tumor volume, mm^3) = L (longest diameter, mm) × W (minimum diameter, $mm)^2 \times 0.5$. At the end of the experiment, mice were sacrificed under deep anesthesia with pentobarbital. The tumors were then dissected and captured.

Western Blot

Cells were lysed in cell lysate and then centrifuged at 12,000 × g for 20 min at 4°C. The supernatant was collected and denatured. Proteins were separated in 10% SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was treated with TBST containing 50 g/L skim milk at room temperature for 4 h, followed by incubation with the primary antibodies: anti-CD133 (1:500; Proteintech, Chicago, IL, USA), anti-CD44 (1:1,000; Proteintech), anti-LGR5 (1:1,000; Proteintech), anti-Sox2 (1:5,000; Proteintech), anti-PER3 (1:500; Abcam, Cambridge, MA, USA), and anti-β-actin (1:1,000; Cell Signaling Technology, Danvers, MA, USA), respectively, at 37°C for 1 h. Membranes were rinsed and incubated for 1 h with the corresponding peroxidase-conjugated secondary antibodies. Chemiluminescence detection was performed with the ECL kit (Pierce Chemical, Rockford, IL, USA). The amount of the protein of interest, expressed as arbitrary densitometric units, was normalized to the densitometric units of β-actin.

Statistical Analysis

All data are presented as mean ± standard deviation. The means of groups were compared with one-way analysis of variance, and after checking for equal variance, comparisons between two means were performed using the least significant difference (LSD) method. Student's t-test was used for comparison of two groups. In all cases, $p < 0.05$ was considered statistically significant. All statistical tests were performed using SPSS, v. 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

PER3 Is Downregulated in Drug-Resistant HCT-116 Cells

It is known that CSCs are implicated in tumor chemoresistance. To determine whether PER3 is associated with drug resistance, a HCT-116 cell model with resistance to 5-FU (HCT-116/R) was established. The relative cell viability was higher in HCT-116/R than in HCT-116 cells when treated with different concentrations of 5-FU (Fig. 1A). The IC_{50} of 5-FU in HCT-116/R cells was 38.10 μg/ml, higher than that (24.07 μg/ml) in HCT-116 cells. The stemness marker miR-21 was highly expressed in HCT-116/R cells compared with control (Fig. 1B). However, the levels of PER3 mRNAs and protein were significantly reduced in HCT-116/R cells compared with HCT-116 cells (Fig. 1C and D). These results confirm that downregulation of PER3 is possibly implicated in colorectal cancer cells acquiring chemoresistance and stemness.

Colorectal Cancer Sphere-Forming Cells Have Stem-Like Features

In order to identify the stem-like features of colorectal cancer sphere-forming cells, the expression levels of stemness markers in colorectal cancer cell line HCT-116 cells and its sphere-forming cells were detected using qRT-PCR and Western blot analysis. The expression of CD133, CD44, LGR5, and SOX2 mRNA was upregulated in HCT-116 sphere-forming cells compared with HCT-116 cells (Fig. 2A–D). Moreover, the production of those tumor stem cell markers reached peaks in HCT-116 sphere-forming cells of passage 3 (P3). Thus, the HCT-116 sphere-forming cells of P3 were used in the following studies. Western blot analysis further demonstrated that CD133, CD44, LGR5, and SOX2 protein increased in HCT-116 sphere-forming cells (Fig. 2E). The tumor stem cell markers were highly expressed in colorectal cancer sphere-forming cells, suggesting that colorectal CSCs were enriched in the HCT-116 sphere-forming cells.

PER3 Is Decreased in Colorectal Cancer Sphere-Forming Cells

To examine whether an aberrant PER3 expression exists in colorectal CSCs, the levels of PER3 mRNAs and protein in primary human colon epithelial cells (HCEpic), HCT-116, and its sphere-forming cells were compared using qRT-PCR and Western blot analysis.

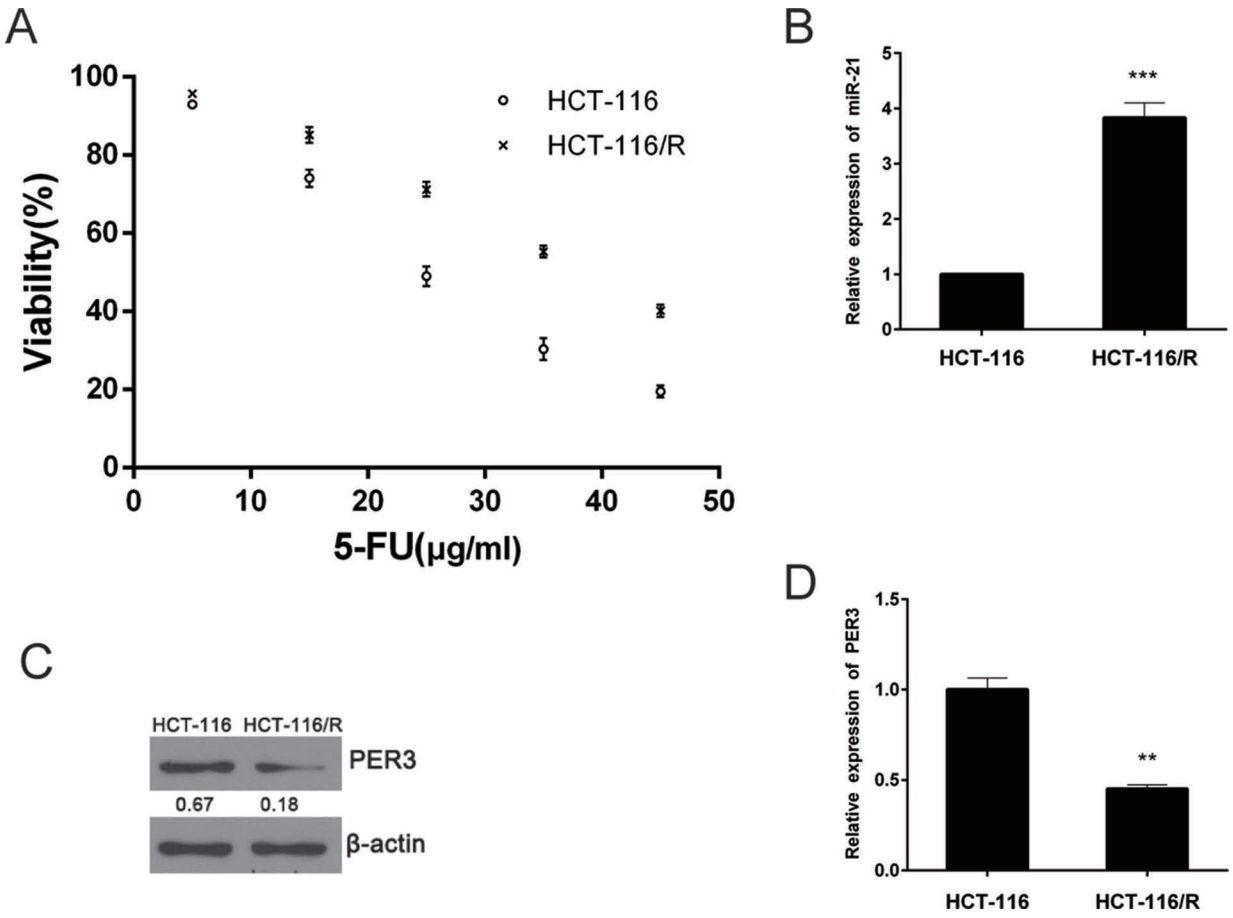

Figure 1. The expression of PER3 in drug-resistant HCT-116. (A) The relative viability of HCT-116 and HCT-116 with resistance to 5-FU (HCT-116/R) was determined by MTT. (B) The relative expression of miR-21 in HCT-116 and HCT-116/R was detected using qRT-PCR. The protein (C) and relative mRNAs (D) in both cellular models were analyzed using Western blot and qRT-PCR respectively. $^{**}p<0.01$ versus HCT-116 group; $^{***}p<0.001$ versus HCT-116 group.

The results showed that levels decreased gradually and occurred least in sphere-forming cells derived from HCT-116 (Fig. 2F and G). This suggests that the dysregulation of PER3 is possibly correlated with the function of colorectal CSCs.

PER3 Enhances Chemosensitivity of Colorectal CSCs

To validate the role of PER3 in colorectal CSCs, HCT-116 sphere-forming cells were infected with lentivirus packaged pGMLV-PE1-per3 (Spheres-Lv-per3). PER3 was significantly increased in infected HCT-116 tumor sphere-forming cells (Fig. 3A and B). The relative inhibition rate in HCT-116 sphere-forming cells exposed to 20 μg/ml 5-FU increased in a time-dependent manner (Fig. 3C). Cell viability in HCT-116 sphere-forming cells was suppressed by 5-FU in a concentration-dependent manner (Fig. 3D). Overexpression of PER3 could strengthen the inhibitory effect of 5-FU on HCT-116 sphere-forming cells (Fig. 3C and D). Conversely, knockdown of PER3 by its siRNA resulted in enhanced cell viability in HCT-116 sphere-forming cells treated with different concentrations of 5-FU (Fig. 3E and F). This confirms that PER3 can enhance the sensitivity of HCT-116 sphere-forming cells to 5-FU.

PER3 Reduces Self-Renewal of Colorectal CSCs

To validate whether PER3 affects stem-like behavior of colorectal CSCs, the stemness markers (CD133, CD44, LGR5, and SOX2), soft agar colony formation, and self-renewal assays were performed. The results showed that overexpression of PER3 in HCT-116 sphere-forming cells resulted in decreased stemness marker expression (Fig. 4A), colony formation efficiency in soft agar medium (Fig. 4B and C), self-renewal efficiency (Fig. 4D), a fewer number, and a smaller size of nonadherent spheres (Fig. 4E). More importantly, the nude mouse transplantation tumor experiment displayed that the oncogenicity of HCT-116 sphere-forming cells

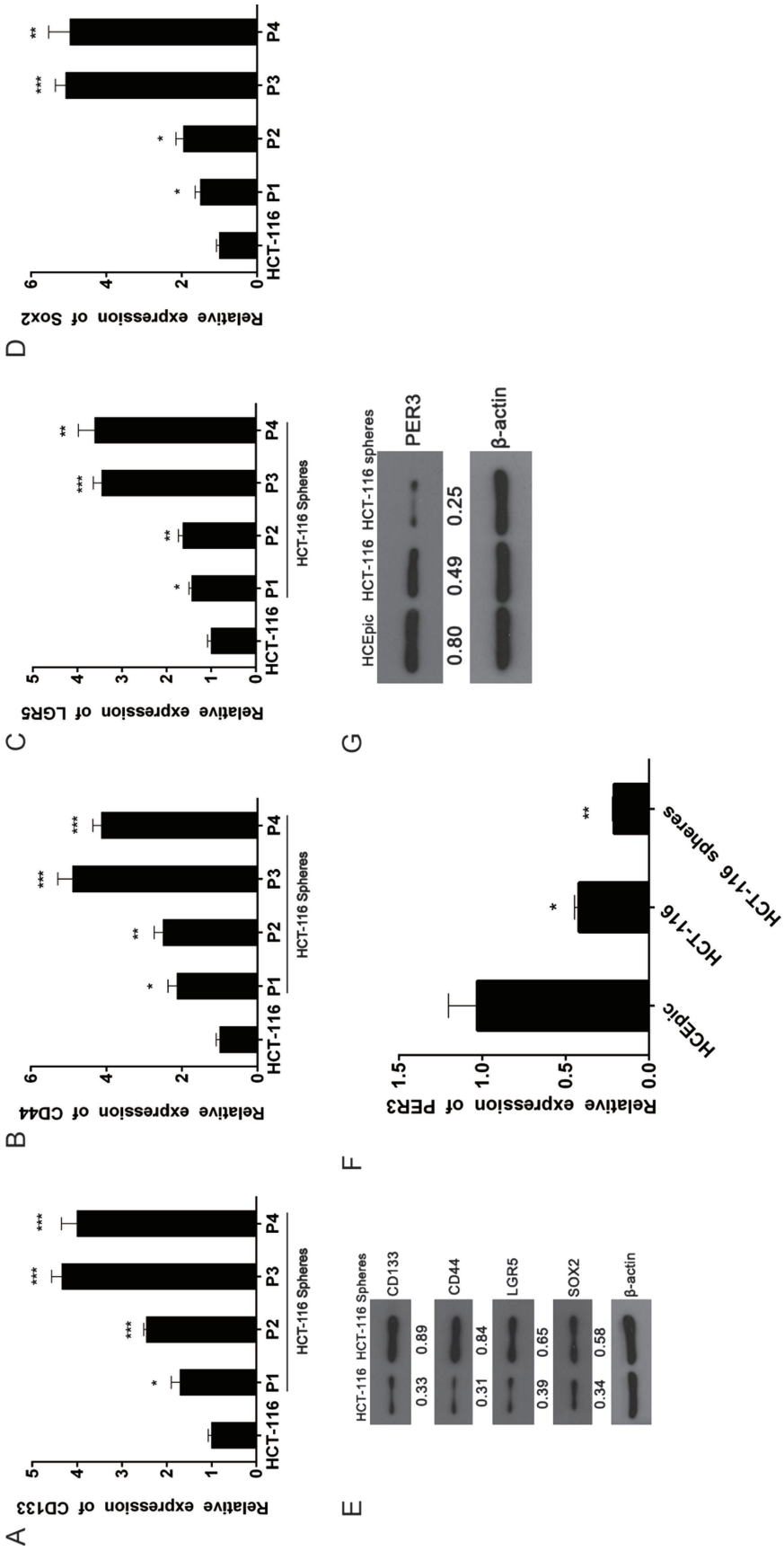

Figure 2. The expression of PER3 in the HCT-116 sphere-forming cells. The expressions of CD133 (A), CD44 (B), LGR5 (C), Sox2 (D) mRNA, and protein (E) increased in HCT-116 sphere-forming cells of passage 1 (P1), P2, P3, and P4. The PER3 mRNA (F) and (G) protein decreased in HCT-116 sphere-forming cells. $^{*}p<0.05$ versus HCT-116 group or HCEpic group in (F); $^{**}p<0.01$ versus HCT-116 group or HCEpic group in (F); $^{***}p<0.001$ versus HCT-116 group.

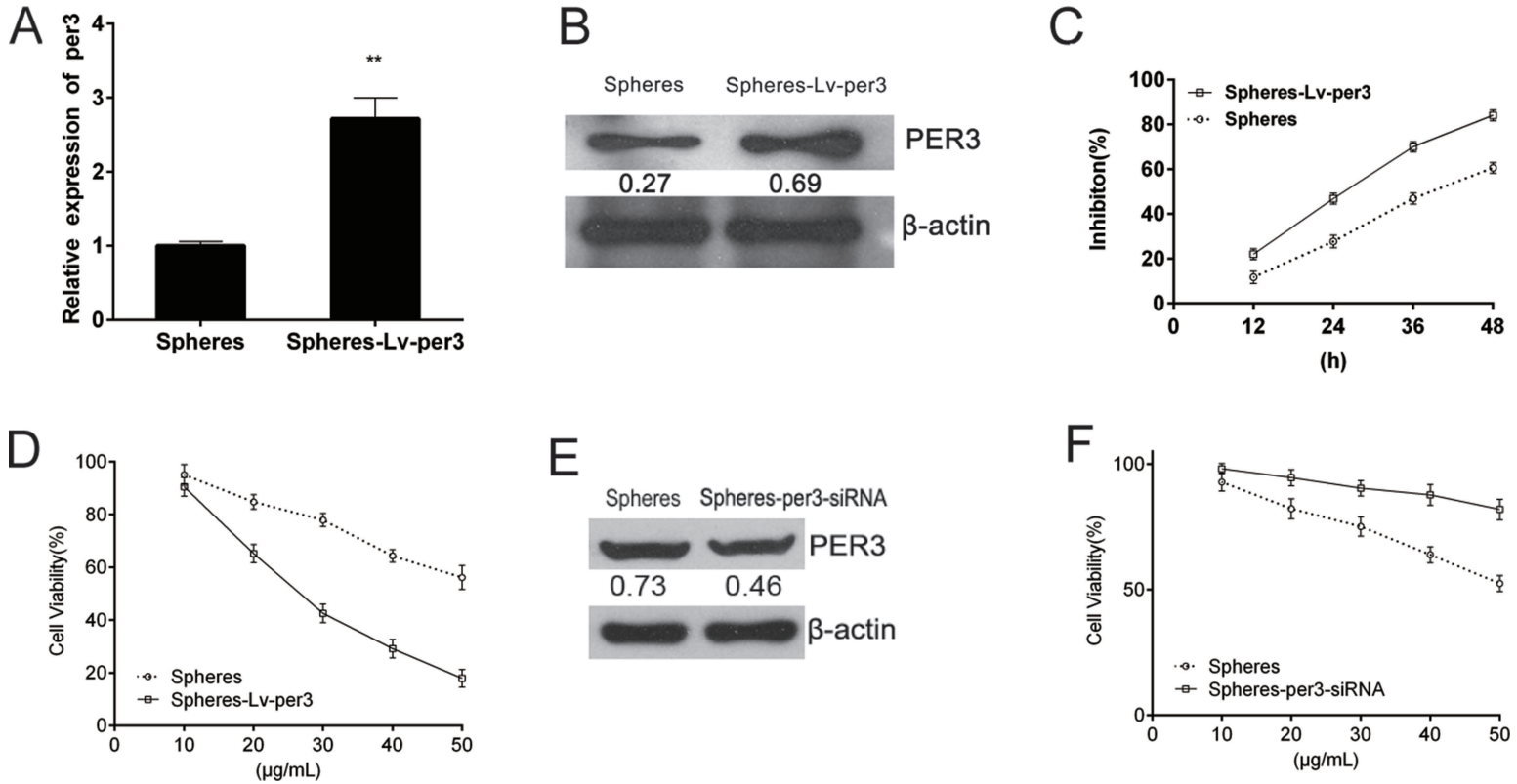

Figure 3. PER3 affects chemoresistance of HCT-116 sphere-forming cells. (A, B) PER3 was overexpressed in HCT-116 sphere-forming cells (Spheres) transfected with lentivirus packaged pGMLV-PE1-per3 vector (Spheres-Lv-per3). Overexpression of PER3 enhanced 5-FU inhibition of HCT-116 sphere-forming cells in a time-dependent (C) and concentration-dependent (D) manner. Knockdown of PER3 (E) in HCT-116 sphere-forming cells (Spheres-per3-siRNA) reduced 5-FU suppression of its cell viability (F). $^{**}p < 0.01$ versus control.

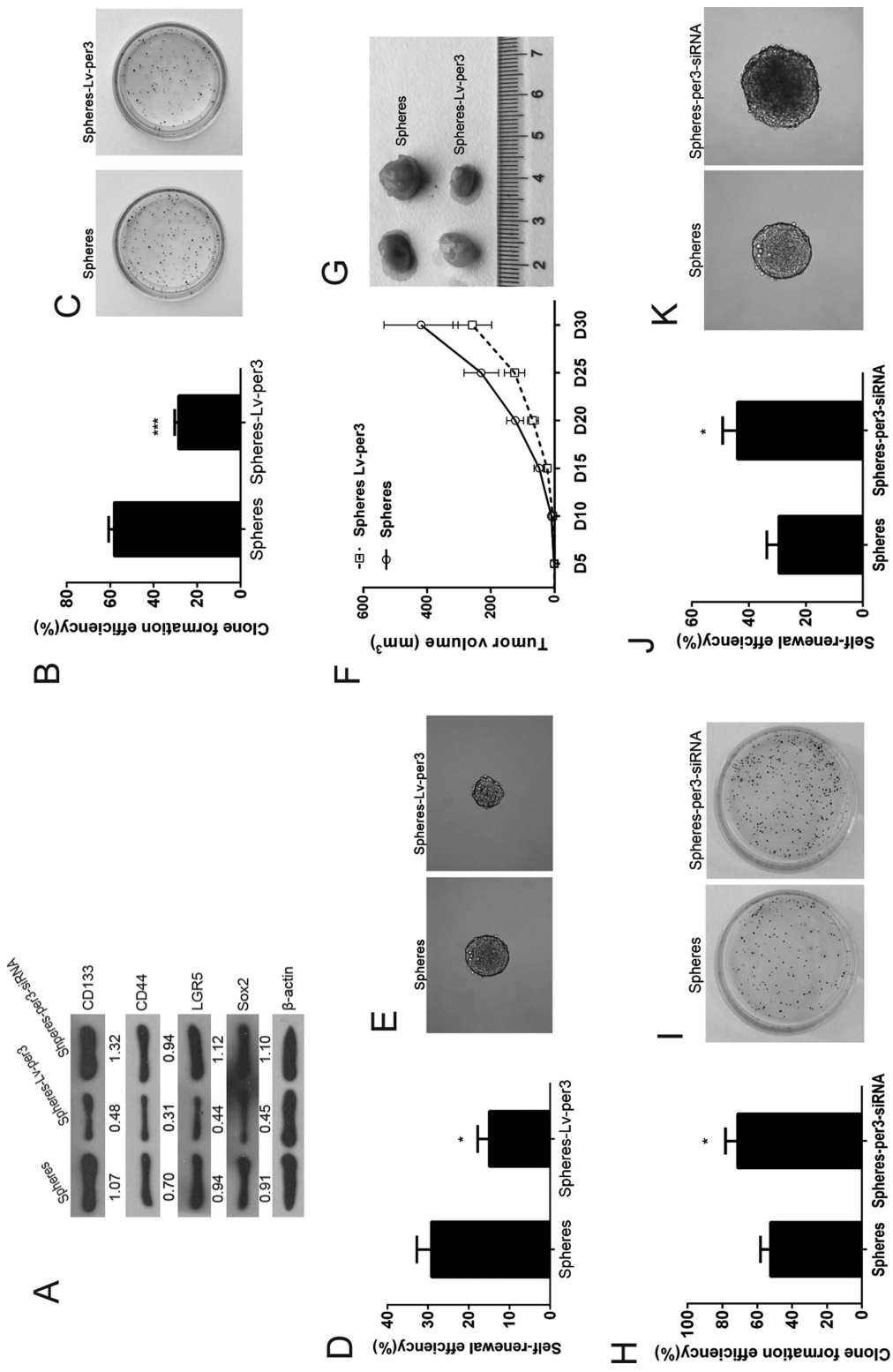

Figure 4. PER3 affects self-renewal of HCT-116 sphere-forming cells. (A) Overexpression of PER3 reduced the stemness marker expression in HCT-116 sphere-forming cells, but knockdown of PER3 enhanced that expression. Overexpression of PER3 suppressed clone formation efficiency (B, C) in soft agar culture and self-renewal efficiency (D, E) in single-cell suspension culture. Cells (1×10^4) were transplanted into nude mouse. (F) The volume of tumor transplantation from day 5 to day 30 was measured. (G) The representative images of HCT-116 sphere-forming cell tumor tissue in nude mouse. Knockdown of PER3 increased clone formation efficiency (H, I) in soft agar culture and self-renewal efficiency (J, K) in single-cell suspension culture. $*p<0.05$ versus control; $***p<0.001$ versus control.

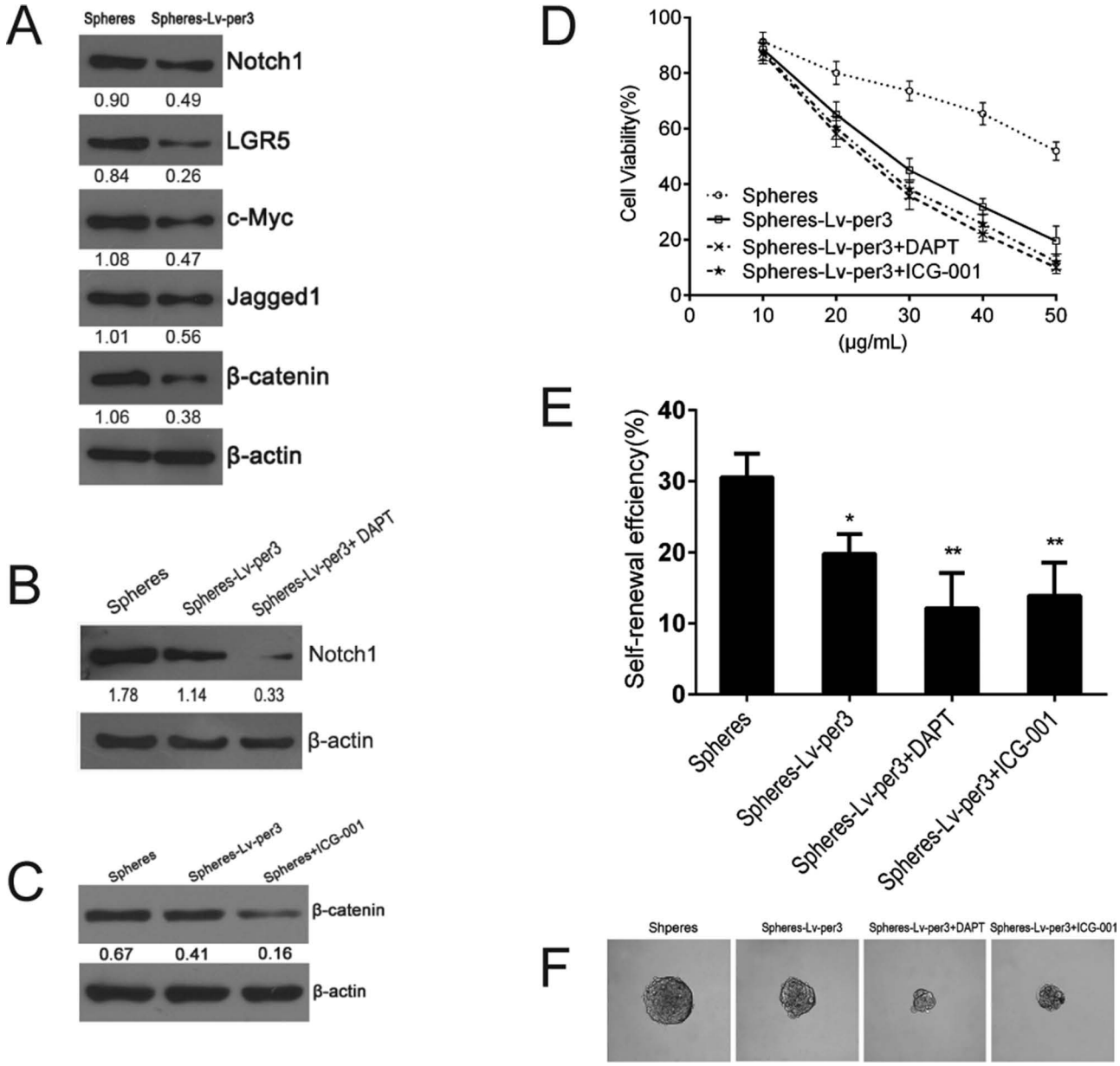

Figure 5. Notch and β-catenin involved in PER3 functioning in colorectal CSCs. (A) The representative image of Western blot analysis of the effects of overexpression of PER3 on the expression of Notch1, LGR5, c-Myc, Jagged1, and β-catenin in HCT-116 sphere-forming cells. Notch inhibitor DAPT and β-catenin inhibitor ICG-001 decreased Notch1 (B) and β-catenin (C) expression in Spheres-Lv-per3. DAPT and ICG-001 inhibited cell viability (D) of Spheres-Lv-per3 and reduced self-renewal (E, F) in HCT-116 sphere-forming cells. *$p < 0.05$ versus control; **$p < 0.01$ versus control.

was higher than that of HCT-116 sphere-forming cells infected with lentivirus packaged pGMLV-PE1-per3 vector (Fig. 4F and G). Inversely, knockdown of PER3 led to increased stemness marker expression (Fig. 4A), colony formation efficiency in soft agar medium (Fig. 4H and I), self-renewal efficiency (Fig. 4J), and a greater number and larger-size nonadherent spheres (Fig. 4K). These results demonstrate that PER3 may function as a suppressor in colorectal CSCs maintaining self-renewal.

Notch and β-Catenin Signaling Are Involved in PER3 Controlling Stem-Like Features

Notch and β-catenin signaling play an important role in CSC maintenance. To confirm whether Notch and β-catenin signaling are involved in PER3 functioning in colorectal CSCs, the expression of Notch1, Jagged1, β-catenin, c-Myc, and LGR5 in HCT-116 sphere-forming cells was detected using Western blot analysis. Overexpression of PER3 led to reduced Notch1, Jagged1, β-catenin, c-Myc,

and LGR5 in HCT-116 sphere-forming cells (Fig. 5A). Notch inhibitor DAPT and β-catenin inhibitor ICG-001 decreased Notch1 and β-catenin expression in HCT-116 sphere-forming cells overexpressing PER3, respectively (Fig. 5B and C), and resulted in lower cell viability of those cells (Fig. 5D) and reduced self-renewal (Fig. 5E and F) in HCT-116 sphere-forming cells treated with different concentrations of 5-FU. These data suggest that PER3 suppresses chemoresistance and the self-renewal capability of colorectal CSCs, at least partly, via inhibition of Notch and β-catenin signaling.

DISCUSSION

Conventional treatments for colorectal cancer include surgery, radiotherapy, and chemotherapy. To some extent, chemotherapy can effectively alleviate tumor-related symptoms and prolong survival. However, the effectiveness of chemotherapy will gradually decline with the occurrence of resistance to conventional chemotherapeutic agents such as 5-FU[13]. Tumor chemoresistance is closely correlated with CSCs. It is reported that the drug-resistant colorectal cancer HCT-116 cells exhibit enhanced stem-like features[14].

In the present study, a 5-FU-resistant HCT-116 cell model (HCT-116/R) was successfully established. Consistent with a previous report, our results showed that miR-21, which can induce stem-like features in colorectal cancer cells[15], was significantly increased in HCT-116/R compared with HCT-116 cells. Moreover, we found that the expression of PER3 was downregulated in drug-resistant HCT-116 cells. These data suggest that downregulation of PER3 is possibly implicated in colorectal cancer cells acquiring chemoresistance and stemness.

Thus, in order to reveal the effects of PER3 on functions and features of CSCs, colorectal CSCs were enriched by the spheroid cultures of HCT-116 cells[9]. Our results confirmed that the stemness markers CD133, CD44, LGR5, and SOX2 were upregulated in HCT-116 sphere-forming cells compared with HCT-116 cells, suggesting that HCT-116 sphere-forming cells can be used as a model for colorectal CSCs. We then found that the expression of PER3 was downregulated in HCT-116 sphere-forming cells. This further highlights the potential correlation between PER3 deregulation and colorectal cancer cells acquiring stem-like features or colorectal CSCs maintaining stemness.

To elucidate this problem, PER3 was overexpressed or knocked down in colorectal CSCs. It was demonstrated that overexpression of PER3 resulted in increased drug sensitivity, decreased self-renewal, and tumorigenesis in colorectal CSCs, but knockdown of PER3 caused increased drug sensitivity and decreased self-renewal. Overexpression of PER3 decreased stemness markers and Notch1, Jagged1, β-catenin, c-Myc, and LGR5 in colorectal CSCs. When Notch or β-catenin signaling was inhibited by its corresponding inhibitor, the chemoresistance and self-renewal capability of colorectal CSCs were decreased. It suggests that PER3 may play a suppressor role in colorectal CSCs maintaining self-renewal and chemoresistance. The underlying mechanisms are involved in the inhibition of Notch1, Jagged1, β-catenin, c-Myc, and LGR5.

The Notch pathway includes four receptors (Notch 1–4) and five ligands (DLL-1, DLL-3, DLL-4, Jagged1, and Jagged2) in adjacent cells[16]. Aberrant activation of the notch signaling pathway may contribute to the development of tumors, including colon carcinoma. Notch signaling plays an important role in the regulation of self-renewal, differentiation, chemosensitivity, invasion, and migration of CSCs[17,18]. Therefore, downregulation of Notch1 and Jagged1 may contribute to colorectal CSCs losing self-renewal capability and chemoresistance[19–21].

The aberrant activation of Wnt/β-catenin signaling plays an important role in the initial development of colorectal cancer. β-Catenin is a downstream signal of the Wnt/β-catenin pathway, which contributes to stem cell self-renewal and cancer chemoresistance[10,22]. c-Myc is a critical regulator of the cell cycle through downregulation of p21 and activation of cyclin D1[23,24]. The increased nuclear accumulation of β-catenin induced by Wnt may promote c-Myc and cyclin D1 expression, resulting in enhanced proliferative capability in colorectal CSCs[25,26]. Thus, overexpression of PER3-induced inhibition of β-catenin will facilitate colorectal CSC death[27,28].

LGR5 is an adult intestinal stem cell marker frequently detected in human colorectal cancers[29]. A high level of LGR5 indicates a poor prognosis in colorectal cancer patients[30]. Overexpression of LGR5 will augment resistance to 5-FU-based chemotherapy in colorectal cancer[31]. Downregulation of LGR5 may suppress a stem-like phenotype by inactivation of Wnt/β-catenin signaling[32], induce cell apoptosis by modulating Bcl-2/Bcl-xL/Bax, and enhance chemosensitivity in colorectal CSCs[33].

In conclusion, our data illustrate that PER3 is downregulated in colorectal CSCs and 5-FU-resistant HCT-116. PER3 can also reduce the chemoresistance and self-renewal capability of colorectal CSCs, and the underlying action mechanisms are involved in the inhibition of Notch and β-catenin signaling. Our results provide novel action mechanisms for PER3 in the regulation of colorectal CSCs as well as offer a new target for treatment of this malignant disease.

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