Knockdown of hnRNPAB reduces the stem cell properties and enhances the chemosensitivity of human colorectal cancer stem cells

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Abstract. Heterogeneous ribonucleoprotein AB (hnRNPAB) is one of the main members of the nuclear heterogeneous ribonucleoprotein family and plays a crucial role in the occurrence and development of tumours. A previous study by the authors demonstrated that hnRNPAB was highly expressed in colorectal cancer tissues and was closely associated with a poor prognosis of patients. However, the contribution of hnRNPAB to the tumorigenesis and drug resistance of colorectal cancer (CRC) stem cells (CSCs) remains elusive. The aim of the present study was thus to examine whether hnRNPAB can enhance the characteristics of colorectal CSCs and chemotherapeutic drug resistance by altering the cell cycle and the apoptosis of colorectal CSCs. The results revealed that the expression of hnRNPAB in colorectal CSCs was increased compared with that in their parental cells. The knockdown of hnRNPAB reduced the sphere formation of and the levels of CSC markers in colorectal CSCs, enhanced sensitivity to 5-fluorouracil and oxaliplatin chemotherapy and increased apoptosis. Taken together, these data indicate the role of hnRNPAB in maintaining CSC properties and provide a novel therapeutic target for the treatment of CRC and particularly, drug resistance.

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Introduction

Colorectal cancer (CRC) is one of the most common malignant tumours in humans and is the third most common type of cancer and the second leading cause of cancer-related mortality worldwide (1). Chemotherapy is the main treatment modality for CRC, particularly for patients with stage IV CRC and patients with tumour progression following surgery, who have a high mortality rate. 5-Fluorouracil (5-FU) and oxaliplatin are the first-line chemotherapy agents (2). 5-FU-based therapies, such as FOLFOX (5-FU, folinic acid and oxaliplatin) have been used as standard therapies for advanced-stage CRC (3).

However, chemoresistance significantly affects the effectiveness of CRC therapy, resulting in tumour recurrence accompanied by metastasis. Cancer stem cells (CSCs) are responsible for tumour initiation, tumour growth, metastasis and resistance to chemotherapy (4,5). Therefore, the elucidation of the mechanisms that maintain colorectal CSCs is essential for the development of novel therapeutic treatment strategies for CRC.

Heterogeneous ribonucleoprotein AB (hnRNPAB) is a main member of the widely expressed RNA-binding protein hnRNP family, which is mainly involved in the process of mRNA selective splicing, mRNA stabilization and gene transcription regulation (6). Research has indicated that the inhibition of hnRNPAB interaction with the p53 family member, p63α, may be responsible for craniofacial diseases, such as Hay-Wells syndrome (7).

In addition to craniofacial and neurodegenerative diseases (7,8), hnRNPAB is closely related to tumours and functions as a transcriptional repressor or activator, as it binds DNA and leads to the enhancement or inhibition of the expression of downstream genes, which are involved in the progression and metastasis of a series of tumours, including lung (9), prostate (10) and liver cancers (11). Hua *et al* (10) found that hnRNPAB interacted with lncRNA PCAT19 to activate a series of cell cycle-related genes, and promoted the growth and metastasis of prostate cancer. Zhou *et al* (12)

found that the overexpression of hnRNPAB induced the epithelial-mesenchymal transition process and the metastasis of liver cancer. Another study confirmed that hnRNPAB promoted the progression of liver cancer by inhibiting the expression of lnc-ELF209 (11).

A previous study by the authors confirmed that hnRNPAB was highly expressed in CRC tissues and was closely associated with the poor prognosis of patients (13). However, the role of hnRNPAB in the stemness characteristics and drug resistance of colorectal CSCs is not yet not fully understood. In the present study, the properties and drug resistance of hnRNPAB in colorectal CSCs were investigated. It was found that hnRNPAB expression was increased in colorectal CSCs compared with their parental cells, and stem cell properties and drug resistance were increased. The knockdown of the *hnRNPAB* gene in colorectal CSCs reduced the characteristics of human colorectal CSCs and enhanced their sensitivity to drugs by changing the cell cycle and increasing apoptosis. These results suggest that hnRNPAB may be involved in the regulation of colorectal CSCs and may function as a regulator of the drug resistance process in CRC.

Materials and methods

Cell lines and colorectal CSC culture. The human CRC cell lines, SW480 (cat. no. TCHu172)and HT29 (cat. no. TCHu103), were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. The SW480 and HT29 cells were cultured in Leibovitz's L-15 or McCoy's 5A medium (HyClone; Cytiva) supplemented with 10% foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2. The SW480 and HT29 cells with good cell growth and 80% confluency were washed with PBS and grown in serum-free Leibovitz's L-15 or McCoy's 5A medium supplemented with stem cell culture medium [20 ng/ml epidermal growth factor (EGF); Invitrogen; Thermo Fisher Scientific, Inc.] B27 (1:50; Gibco; Thermo Fisher Scientific, Inc.) and 10 ng/ml basic fibroblast growth factor (bFGF; Invitrogen; Thermo Fisher Scientific, Inc.). At 72 h after the replacement of the stem cell culture medium, SW480CSCs and HT29CSCs were obtained by culturing the cells in stem cell medium for 14 consecutive days, which has been confirmed in previous experiments (14), and harvested for subsequent experiments as needed.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated using RNAiso Plus (cat. no. 9108; Takara Biotechnology Co., Ltd.), and cDNA was synthesized using a PrimeScript™ RT Reagent kit (cat. no. RR037A; Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. mRNA expression levels were analysed using qPCR with SYBR Premix Ex Taq™ (cat. no. RR820; Takara Biotechnology Co., Ltd.). The forward and reverse primers were designed and synthesized by Dalian Bao Biotech Co., Ltd. The thermocycling conditions were as follows: Denaturation at 95˚C for 5 min, followed by 35 cycles of denaturation at 95˚C for 15 sec and annealing/elongation at 60˚C for 30 sec. GAPDH was used as an internal control. The sequences were as follows: hnRNPAB forward, 5'-AAGAAGTCTATCAGC AGCAGCAGTATG-3' and reverse, 5'-CTCCACCTCCAC CACCACCTC-3'; GAPDH forward, 5'-ATGACATCAAGA AGGTGGTGAAGCAGG-3' and reverse, 5'-GCGTCAAAG GTGGAGGAGTGGG-3'. The samples were normalized to GAPDH. Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (15).

Western blot analysis. Total protein was extracted from the cells using the Protein Extraction kit (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd), and the protein concentration was quantified using a BCA Protein Assay kit (cat. no. PC0020; Beijing Solarbio Science & Technology Co., Ltd.). Equal amounts (40 μg per lane) of protein were separated using 10% SDS-polyacrylamide gels. The protein was transferred onto a nitrocellulose membrane at 4˚C under a constant flow of 200 mA for 1 h using a dry transfer system. The membrane was blocked in 5% skimmed milk for 1 h at room temperature and incubated overnight at 4˚C with anti-hnRNPAB (1:1,000; cat. no. ab199724; Abcam), anti-octamer-binding transcription factor 4 (OCT4; 1:1,000; cat. no. HRP-60242; Proteintech Group, Inc.), anti-SOX2 (1:1,000; cat. no. 11064-1-AP; Proteintech Group, Inc.), anti-Bcl-2 (1:1,000; cat. no. ab32124; Abcam), anti-Bax (1:1,000; cat. no. ab32503; Abcam), anti-caspase-3 (1:1,000; cat. no. ab32351; Abcam) and anti-GAPDH (1:2,000; cat. no. ab8245; Abcam) primary antibodies. Subsequently, the membrane was washed and incubated in an HRP-conjugated antibody solution (1:5,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 1 h at room temperature. The membrane was washed and visualized using Super ECL plus supersensitive luminescent solution (Bio-Rad Laboratories, Inc.) and exposed using X-ray film. Quantity One software v4.6.6 (Bio-Rad Laboratories, Inc.) was used to quantify the band intensities.

Cell transfection. Lentiviral constructs expressing hnRNPAB shRNA (HBLV-sh-hnRNPAB-PURO) and negative control (HBLV-PURO) were purchased from Hanbio Biotechnology Co, Ltd. The sequences for hnRNPAB shRNA-1, hnRNPAB shRNA-2 and negative control shRNA were as follows: 5'-GGUAGUACAAACUACGGCATT-3', 5'-GGAGAG GTCGTTGACTGTACAATAA-3' and 5'-UUCUCCGAA CGUGUCACGUTT-3', respectively. A total of $1x10^5$ cells from each of the HT29CSCs and SW480CSCs were plated in a low-adherence six-well plate and one well was taken as an example. Subsequently, 120 μl HBLV-PURO (NC control virus), HBLV-sh-hnRNPAB-1-PURO and HBLV-sh-hnRNPAB-2-PURO virus (both viral titers were $1x10^8$ TU/ml; multiplicity of infection, 100) was added, and 5 μl Polybrene (2 mg/ml) were added to bring the medium to 2 ml. After an additional 72 h, the cells were collected, and the effects of gene silencing were verified by RT-qPCR and western blot analysis after 48-72 h.

Cell Counting Kit-8 (CCK-8) assay. The cells were infected with the lentivirus according to the aforementioned steps. The transfected cells were counted, and $1x10^4$ cells were spread in a 96-well plate. 5-FU (0.025, 1, 10 and 100 μmol/l; cat. no. 51-21-8; MilliporeSigma) and oxaliplatin (0, 0.02, 0.1, 1, 1 and 10 μmol/l; cat. no. 61825-94-3; MilliporeSigma) were added followed by incubation at 37˚C in a cell incubator for 48 h. Subsequently, 10 μl CCK-8 detection solution (cat. no. CK04; Dojindo Laboratories, Inc.) were added to each

well, incubated at 37˚C in a cell incubator for 2 h, and the absorbance was measured at 450 nm using a microplate reader at 37˚C. The cell growth inhibition rate was calculated as follows: Inhibition rate (IR)=(absorbance of the control group NC-absorbance of the experimental group)/absorbance of the control group. The concentration at which each drug produced 50% inhibition of growth (IC_{50}) was estimated using the IR.

Sphere formation assay. For the sphere formation assay, a total of 800 cells were suspended in serum-free medium and plated into an attachment plate. The cells were then cultured in Leibovitz's L-15 or McCoy's 5A medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 20 ng/ml EGF (Invitrogen; Thermo Fisher Scientific, Inc.), B27 (1:50; Gibco; Thermo Fisher Scientific, Inc.) and 10 ng/ml bFGF (Invitrogen; Thermo Fisher Scientific, Inc.). For serial passaging, the primary spheres were collected and resuspended in Leibovitz's L-15 or McCoy's 5A medium with the aforementioned supplements following trypsin dissociation. Finally, the number of spheres was counted under a microscope (IX53; Olympus Corporation), and the size of spheres was estimated using ImageJ v6.0 software (National Institutes of Health), as previously described (16).

Flow cytometry. For the flow cytometric analysis of CSC markers, the cells were digested into single-cell suspensions and washed with PBS. A total of $1x10^6$ cells were then resuspended in 100 μl PBS containing 0.5% BSA and 10 μl fluorophore-conjugated primary antibodies against CD133 (1:100; cat. no. ab305371; Abcam) and CD44 (1:100; cat. no. ab284640; Abcam) for 10 min in the dark at 4˚C. Subsequently, the tubes were removed by centrifugation (500 x g, 5 min at 37˚C) and washed twice with 500 μl PBS buffer. The cells were then suspended in 200 μl PBS each and analysed using a FACS Vantage SE (BD Biosciences). For the detection of apoptosis, the HT29CSCs and SW480CSCs were infected with the lentivirus and treated with 5-FU (10 μmol/l) or oxaliplatin (1 μmol/l) for 48 h. The cells were resuspended in 100 μl 1X conjugated buffer, mixed with 5 μl of Annexin V/FITC, and incubated at room temperature in the dark for 5 min. Subsequently, 10 μl of 20 μg/ml propidium iodide solution (PI) were added. The cells were then suspended in 400 μl PBS and analysed using a fluorescence-activated cell sorting (FACS) Vantage SE (BD Biosciences).

Statistical analysis. Data are expressed as the mean ± standard deviation (SD). Comparisons between two groups were performed using a Student's unpaired t-test. For testing among multiple groups, one-way analysis of variance (ANOVA) with the SNK-q test or Tukey's post hoc test was conducted. Statistical analyses were performed using SPSS software (version 19.0; IBM Corp.). Each experiment was performed three times, and P<0.05 was considered to indicate a statistically significant difference.

Results

hnRNPAB is highly expressed in colorectal CSCs and may be related to the stemness of CSCs. The SW480CSCs and HT29CSCs were obtained from a suspension culture of the human CRC cell lines, SW480 and HT29, respectively, grown in suspension (Fig. 1A), which was confirmed by a previous study by the authors (14). The present study detected the mRNA and protein expression of hnRNPAB in the SW480CSCs, HT29CSCs and their parental SW480 cells and HT29 cells using RT-qPCR and western blot analysis, respectively. The relative mRNA expression of hnRNPAB in the SW480, SW480CSC, HT29 and HT29CSC groups was 1.06±0.03, 3.15±0.03, 0.73±0.05 and 2.59±0.07, respectively, as detected using RT-qPCR. Compared with those in the SW480 and HT29 groups, the mRNA expression levels of hnRNPAB in the SW480CSC and HT29CSC groups were increased (P<0.01; Fig. 1B). Furthermore, the same trend was observed for the hnRNPAB protein levels detected using western blot analysis (P<0.01; Fig. 1C and D).

The present study also detected the protein expression of OCT4 and SOX2, which are characteristic markers of embryonic stem cells. The results of western blot analysis revealed that the protein expression levels of OCT4 in the SW480, SW480CSC, HT29 and HT29CSC groups were 0.26±0.02, 0.44±0.02, 0.22±0.01 and 0.48±0.02, respectively; the protein expression levels of SOX2 in the SW480, SW480CSC, HT29, and HT29CSC groups were 0.32±0.01, 0.54±0.02, 0.19±0.01 and 0.28±0.01, respectively. The protein expression levels of OCT4 and SOX2 in the SW480CSCs and HT29CSCs were significantly higher than those in their parental SW480 cells and HT29 cells, consistent with the same trend observed for hnRNPAB expression (P<0.05; Fig. 1C-F). These results suggested that hnRNPAB may be related to the stemness of CSCs.

OCT4 and SOX2 expression levels decrease in colorectal CSCs following the knockdown of the hnRNPAB gene. To investigate the effects of hnRNPAB on the stemness of colorectal CSCs, stable hnRNPAB-silenced colorectal CSCs were established using two independent shRNAs (sh-hnRNPAB-1 and sh-hnRNPAB-2), with shRNA-NC (sh-NC) as a negative control. Compared with that in the sh-NC group, the mRNA level of hnRNPAB in the experimental groups (sh-hnRNPAB-1 and sh-hnRNPAB-2) of SW480CSCs and HT29CSCs was significantly decreased, as measured using RT-qPCR (P<0.01; Fig. 2A). Furthermore, the same trend was observed for the hnRNPAB protein levels detected using western blot analysis (P<0.01; Fig. 2B-E). These results suggested that the *hnRNPAB* gene in SW480CSCs and SW620CSCs was successfully silenced.

To examine the effects of EC cell properties, the levels of two EC cell biomarkers, OCT4 and SOX2, were detected using western blot analysis. The protein expression of OCT4 in the SW480CSC sh-NC, sh-hnRNPAB-1 and sh-hnRNPAB-2 groups was 0.24±0.01, 0.14±0.01 and 0.19±0.01, respectively, and the protein expression of SOX2 in the SW480CSC sh-NC, sh-hnRNPAB-1 and sh-hnRNPAB-2 groups was 0.48±0.02, 0.19±0.01 and 0.20±0.01, respectively. The results revealed that the protein expression of OCT4 and SOX2 in the sh-hnRNPAB-1 and sh-hnRNPAB-2 groups of SW480CSCs decreased significantly compared with that in the sh-NC group of SW480CSCs (P<0.05; Fig. 2B and C). In addition, the protein expression of OCT4 and SOX2 in the sh-hnRNPAB-1 and sh-hnRNPAB-2 groups of HT29CSCs decreased significantly compared with that in the sh-NC group of HT29CSCs (P<0.01; Fig. 2D and E).

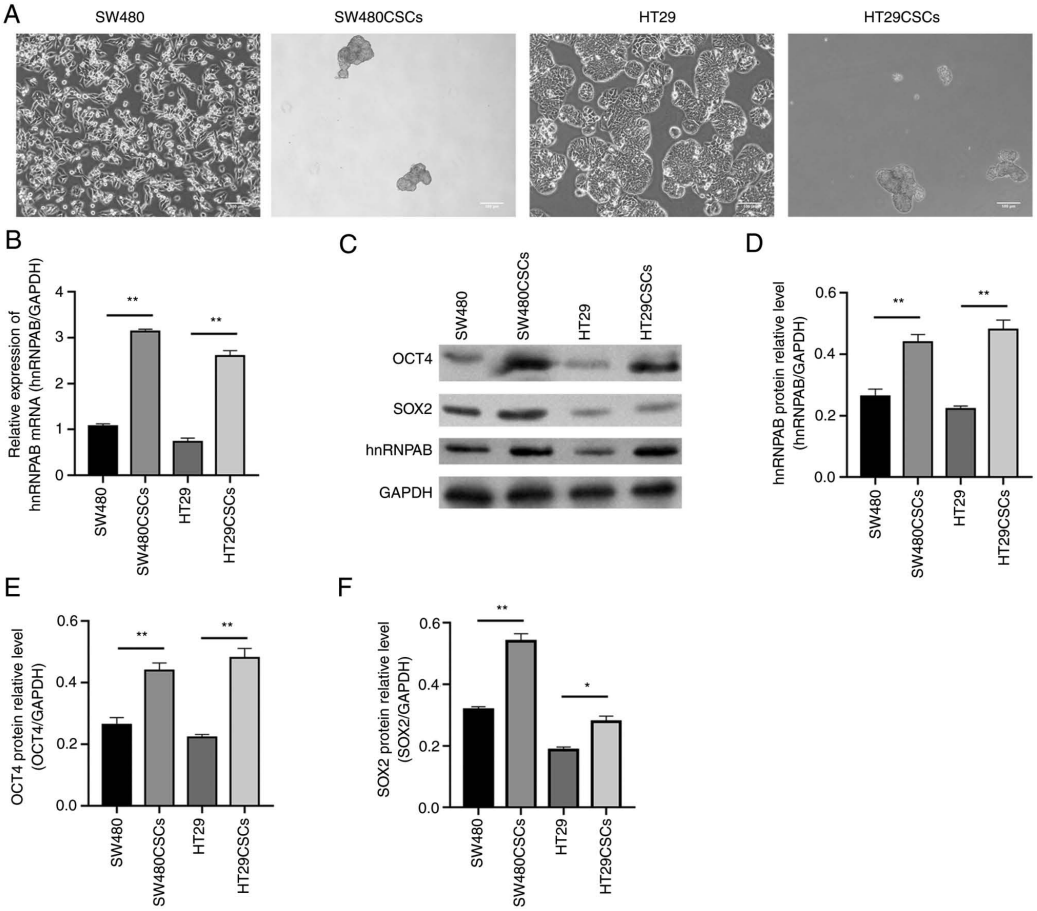

Figure 1. hnRNPAB is highly expressed in SW480CSCs and HT29CSCs and may be related to the stemness of CSCs. (A) Morphology of SW480, SW480CSCs, HT29, HT29CSCs, SW480CSCs and HT29CSCs aggregated to form three-dimensional spheres. (B) The mRNA expression levels of hnRNPAB in SW480, SW480CSCs, HT29 and HT29CSCs were measured using reverse transcription-quantitative PCR. (C-F) The protein expression levels of OCT4, SOX2 and hnRNPAB in SW480, SW480CSCs, HT29 and HT29CSCs were examined using (C) western blot analysis and (D-F) the protein levels were quantified. The expression levels of (D) hnRNPAB, (E) OCT4, and (F) SOX2 increased in colorectal CSCs. Each bar represents the mean ± SD of three independent experiments. *P<0.05 and **P<0.01. hnRNPAB, heterogeneous ribonucleoprotein AB; CSCs, cancer stem cells; OCT4, octamer-binding transcription factor 4.

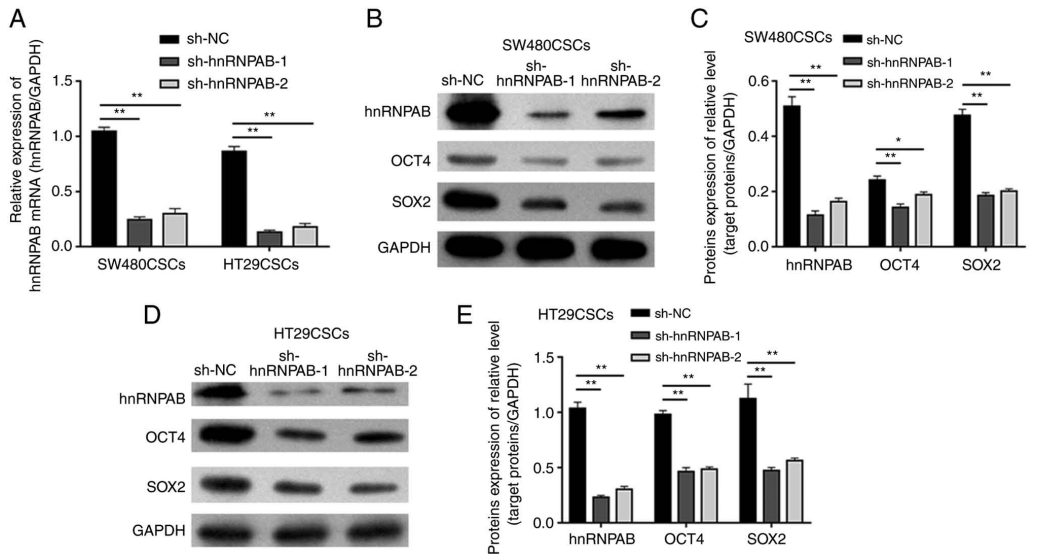

Figure 2. Expression of hnRNPAB, OCT4 and SOX2 in colorectal CSCs in which the *hnRNPAB* gene was silenced. (A) The mRNA expression levels of hnRNPAB in SW480CSCs and HT29CSCs transfected with sh-hnRNPAB-1, sh-hnRNPAB-2 and sh-NC were analysed using reverse transcription-quantitative PCR. (B and C) The protein expression levels of hnRNPAB, OCT4, and SOX2 in SW480CSCs transfected with sh-hnRNPAB-1, sh-hnRNPAB-2 and sh-NC were examined using (B) western blot analysis and (C) the protein levels were quantified. (D and E) The protein expression levels of hnRNPAB, OCT4, and SOX2 in HT29CSCs transfected with sh-hnRNPAB-1, sh-hnRNPAB-2 and sh-NC were examined using (D) western blot analysis and (E) the protein levels were quantified. Each bar represents the mean ± SD of three independent experiments. *P<0.05 and **P<0.01. hnRNPAB, heterogeneous ribonucleoprotein AB; CSCs, cancer stem cells; OCT4, octamer-binding transcription factor 4.

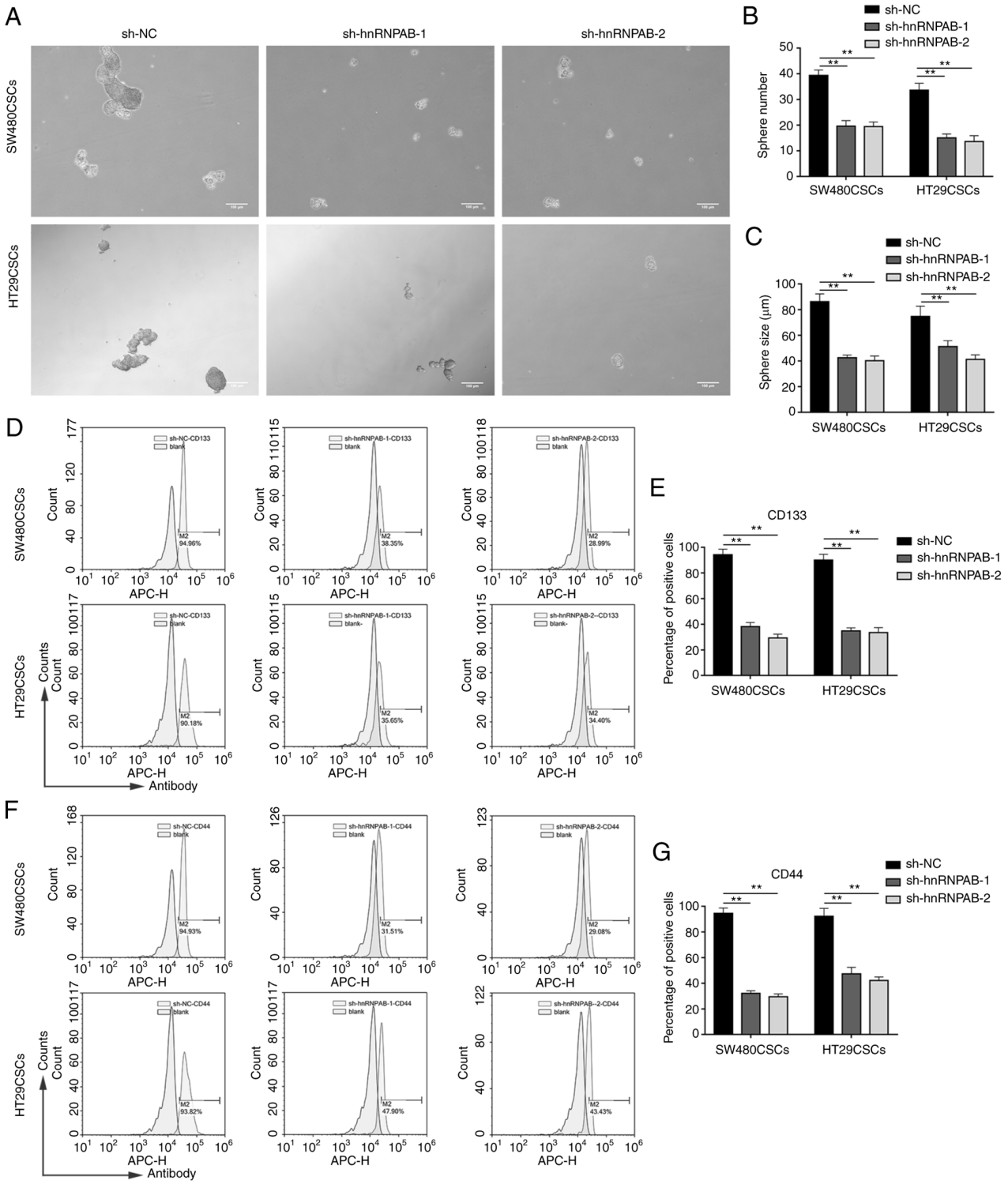

Figure 3. Reduction in stemness of colorectal CSCs after the silencing of the *hnRNPAB* gene. (A) Spheres derived from SW480CSCs or HT29CSCs transfected with sh-hnRNPAB-1, sh-hnRNPAB-2 and sh-NC, (B) the summary of sphere number, and (C) the average sphere size; n=5). (D and E) The expression level of CD133 was significantly decreased in the sh-hnRNPAB-1 and sh-hnRNPAB-2 groups compared with the sh-NC group in SW480CSCs and HT29CSCs. (F and G) The expression level of CD44 was significantly decreased in the sh-hnRNPAB-1 and sh-hnRNPAB-2 groups compared with the sh-NC group in SW480CSCs and HT29CSCs. Each bar represents the mean ± SD of three independent experiments. **P<0.01. hnRNPAB, heterogeneous ribonucleoprotein AB; CSCs, cancer stem cells.

Stemness is reduced in colorectal CSCs after the silencing of the hnRNPAB gene. To verify the effects of hnRNPAB on the stemness of CSCs, spheroid formation assays were employed. The number and average diameter of the spheres derived from the colorectal CSCs in which hnRNPAB was knocked down were less than those derived from the sh-NC group (P<0.01; Fig. 3A-C), confirming that the stemness of the colorectal CSCs decreased after the silencing of the *hnRNPAB* gene.

Subsequently, the levels of two CSC markers, CD133 and CD44, were detected. Flow cytometric analysis revealed

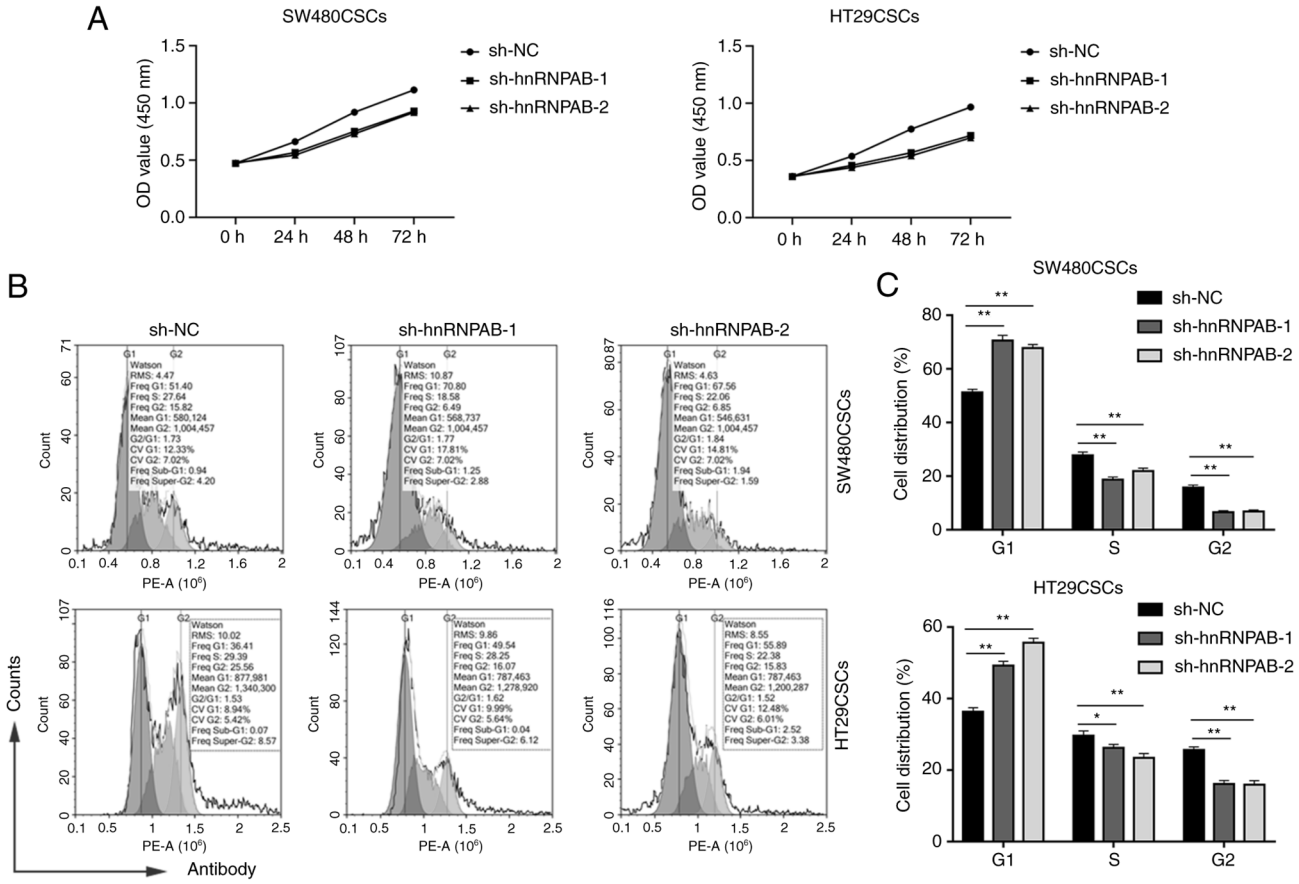

Figure 4. Silencing of hnRNPAB inhibits tumour stem cell proliferation and prevents cell G1/S transition *in vitro*. (A) The effect of hnRNPAB on cell viability was assessed using CCK-8 assays at 24, 48 and 72 h (n=6). (B and C) Flow cytometry results illustrating the cell phase distribution in SW480CSCs or HT29CSCs transfected with sh-hnRNPAB-1, sh-hnRNPAB-2 and sh-NC. Each bar represents the mean ± SD of three independent experiments. *P<0.05 and **P<0.01. hnRNPAB, heterogeneous ribonucleoprotein AB; CSCs, cancer stem cells.

that CD133 was expressed in 94.23±4.31% of sh-NC cells, 38.13±3.27% of sh-hnRNPAB-1 cells and in 29.34±2.91% of sh-hnRNPAB-2 cells in the SW480CSCs; it was also expressed in 90.03±4.56% of sh-NC cells, 34.85±2.39% of sh-hnRNPAB-1 cells and in 33.47±3.93% of sh-hnRNPAB-2 cells in the HT29CSCs (P<0.01; Fig. 3D and E). In addition, flow cytometric analysis revealed that CD44 was expressed in 94.56±4.16% of sh-NC cells, 32.13±1.98% of sh-hnRNPAB-1 cells and in 29.53±2.15% of sh-hnRNPAB-2 cells in the SW480CSCs; it was also expressed in 93.23±6.25% of sh-NC cells, 47.37±5.10% of sh-hnRNPAB-1 cells and in 42.23±2.72% of sh-hnRNPAB-2 cells in the HT29CSCs (P<0.01; Fig. 3F and G). These results demonstrated that the expression levels of CD133 and CD44 were significantly decreased in the sh-hnRNPAB-1 and sh-hnRNPAB-2 groups compared with their sh-NC group in colorectal CSCs.

Silencing of hnRNPAB inhibits colorectal CSC proliferation and prevents cell G1/S transition in vitro. A CCK-8 assay was then performed to examine the effects of hnRNPAB on the growth of colorectal CSCs. The results revealed that hnRNPAB silencing markedly attenuated the viability of the SW480CSCs and HT29CSCs in the sh-hnRNPAB-1 and sh-hnRNPAB-2 groups compared with the sh-NC group (Fig. 4A).

To explore the mechanisms through which hnRNPAB accelerated cell proliferation, the effects of hnRNPAB on cell cycle progression were detected using flow cytometry. As presented in Fig. 4B and C, compared with the sh-NC group, the sh-hnRNPAB-1 and sh-hnRNPAB-2 groups of SW480CSCs and HT29CSCs exhibited a significantly increased percentage of cells in the G1 phase (51.32±1.09% vs. 70.56%±2.01 and 67.79±1.37%; and 36.32±1.13% vs. 49.24±1.21 and 55.67±1.26%, respectively), a decreased percentage of cells in the S phase (27.82±1.21% vs. 18.76±0.92 and 21.87±1.15%; and 29.67±1.29% vs. 26.21±1.03 and 23.48±1.21%, respectively), as well as a corresponding decrease in the proportion of cells in the G2 phase (15.86±0.87% vs. 6.61±0.54 and 6.83±0.63%; and 25.62±0.92% vs. 16.12±0.98 and 15.92±1.17%, respectively) (P<0.05; Fig. 4B and C). Compared with the sh-NC group, the percentage of G1 phase cells in the sh-hnRNPAB group increased significantly, indicating that hnRNPAB silencing induced G1 phase arrest. These results suggested that hnRNPAB accelerated colorectal CSC cell cycle progression by facilitating the G1/S transition to promote cell proliferation.

Silencing of hnRNPAB enhances the sensitivity of colorectal CSCs to the chemotherapeutic drugs, 5-FU and oxaliplatin. To investigate the role of hnRNPAB in sensitivity to the chemotherapeutic drugs, 5-FU and oxaliplatin, a cytotoxicity assay was performed using the hnRNPAB knockdown groups (sh-hnRNPAB-1 and sh-hnRNPAB-2 groups) in the SW480CSCs and HT29CSCs. As shown in Fig. 5A, compared

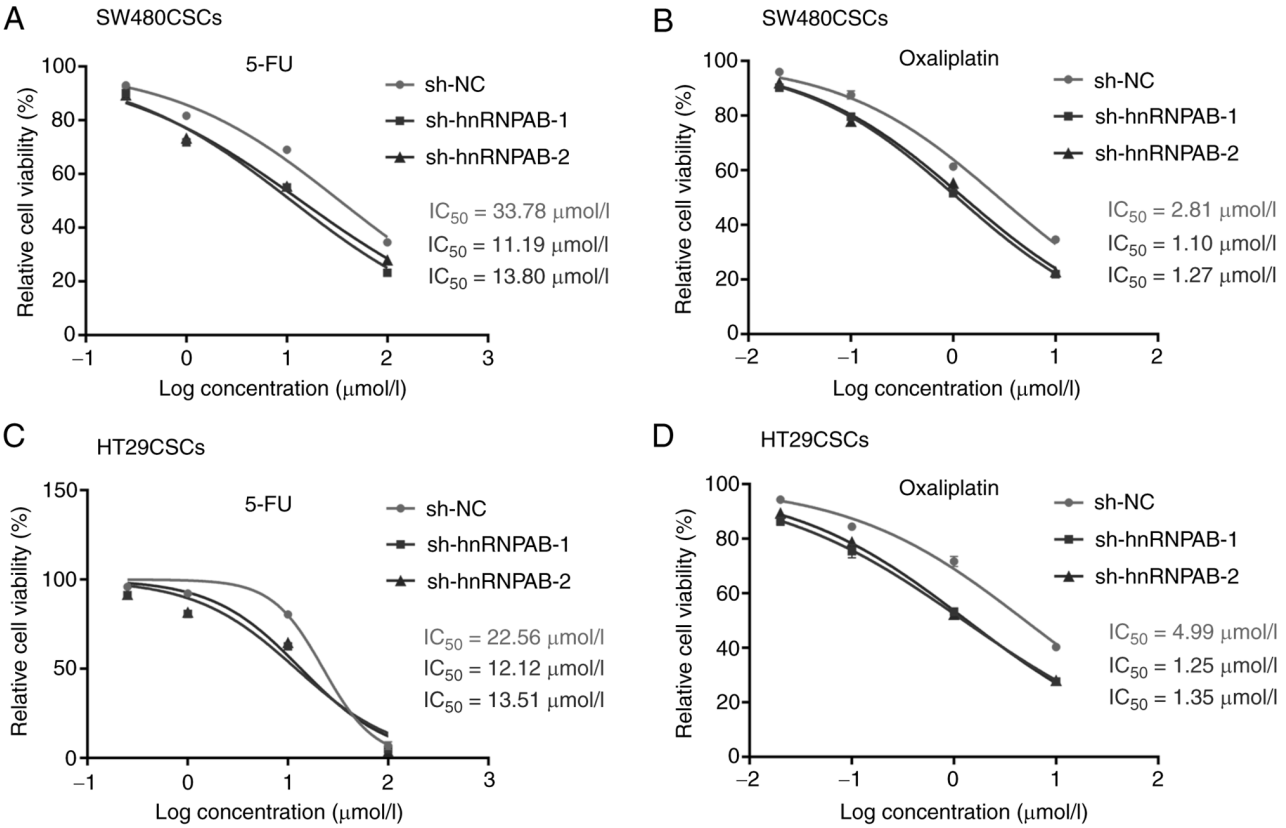

Figure 5. Silencing of hnRNPAB enhances the sensitivity of SW480CSCs and HT29CSCs to the chemotherapeutic drugs, 5-FU and oxaliplatin. (A and B) SW480CSCs transfected with sh-hnRNPAB-1, sh-hnRNPAB-2 and sh-NC were treated with various concentrations of (A) 5-FU or (B) oxaliplatin. (C and D) HT29CSCs transfected with sh-hnRNPAB-1, sh-hnRNPAB-2 and sh-NC were treated with various concentrations of (C) 5-FU or (D) oxaliplatin. Cell viability was measured using CCK-8 assays to determine whether hnRNPAB sensitizes cells to anticancer drugs. hnRNPAB, heterogeneous ribonucleoprotein AB; CSCs, cancer stem cells; 5-FU, 5-fluorouracil.

with the sh-NC group, the viability of the cells in the sh-hnRNPAB-1 and sh-hnRNPAB-2 groups decreased markedly following treatment with 5-FU in the SW480CSCs. The amount of 5-FU required to achieve the same level of cell death as the sh-NC group in the SW480CSCs was much lower in the sh-hnRNPAB-1 and sh-hnRNPAB-2 groups. The respective IC_{50} values for 5-FU were 11.19 μmol/l (sh-hnRNPAB-1), 13.80 μmol/l (sh-hnRNPAB-2) and 33.78 μmol/l (sh-NC) in the SW480CSCs (Fig. 5A). As shown in Fig. 5B, compared with the sh-NC group, the viability of the cells in the sh-hnRNPAB-1 and sh-hnRNPAB-2 groups decreased significantly following treatment with oxaliplatin in the SW480CSCs. Similar results were obtained in the sh-hnRNPAB-1 and sh-hnRNPAB-2 groups of HT29CSCs (Fig. 5C and D).

The results revealed that the viability of the cells in the sh-hnRNPAB group and the sh-NC group decreased significantly with the increasing drug concentrations, regardless of whether 5-FU or oxaliplatin were added. However, the sh-hnRNPAB groups exhibited are more significant decrease than the sh-NC group.

Knockdown of hnRNPAB increases the apoptotic rate of colorectal CSCs treated with the chemotherapeutic drugs, 5-FU and oxaliplatin. The present study then examined whether hnRNPAB exerts any effect on colorectal CSC apoptosis following treatment with the chemotherapeutic drugs, 5-FU and oxaliplatin. The sh-hnRNPAB-1 and sh-hnRNPAB-2 groups, compared with the sh-NC group, exhibited a higher percentage of apoptotic SW480CSCs treated with DMSO (6.95±0.53 and 7.27±0.44% vs. 1.42±0.39%, respectively), 5-FU (25.43±0.67 and 22.12±0.56% vs. 13.58±0.42%, respectively) and oxaliplatin (21.83±1.03 and 19.68±0.92% vs. 8.46±0.52%, respectively), as determined using flow cytometry ($P<0.01$; Fig. 6A and B). In addition, the sh-hnRNPAB-1 and sh-hnRNPAB-2 groups, compared with the sh-NC group, exhibited a higher percentage of apoptotic cells in the HT29CSCs treated with DMSO (6.45±0.34 and 8.53±0.38% vs. 0.87±0.26%, respectively), 5-FU (27.28±0.69 and 24.87±0.73% vs. 9.83±0.65%, respectively) and oxaliplatin (22.75±0.82 and 22.40±0.56% vs. 9.62±0.51%, respectively), as determined using flow cytometry ($P<0.01$; Fig. 6C and D). In the two types of colorectal CSCs, the apoptotic rate of the sh-hnRNPAB groups and sh-NC group was increased following treatment with 5-FU and oxaliplatin. Compared with the sh-NC group, the knockdown of hnRNPAB in the SW480CSCs and HT29CSCs resulted in a higher apoptotic rate in response to the chemotherapeutic drugs, 5-FU and oxaliplatin.

In addition, the levels of apoptosis-related indicators (Bax, Bcl-2 and cleaved caspase-3) were examined using western blot analysis. The results revealed that compared with the sh-NC group, in the sh-hnRNPAB-1 and sh-hnRNPAB-2 groups of SW480CSCs treated with 5-FU or oxaliplatin, the expression of the pro-apoptotic proteins, Bax and cleaved caspase-3, was

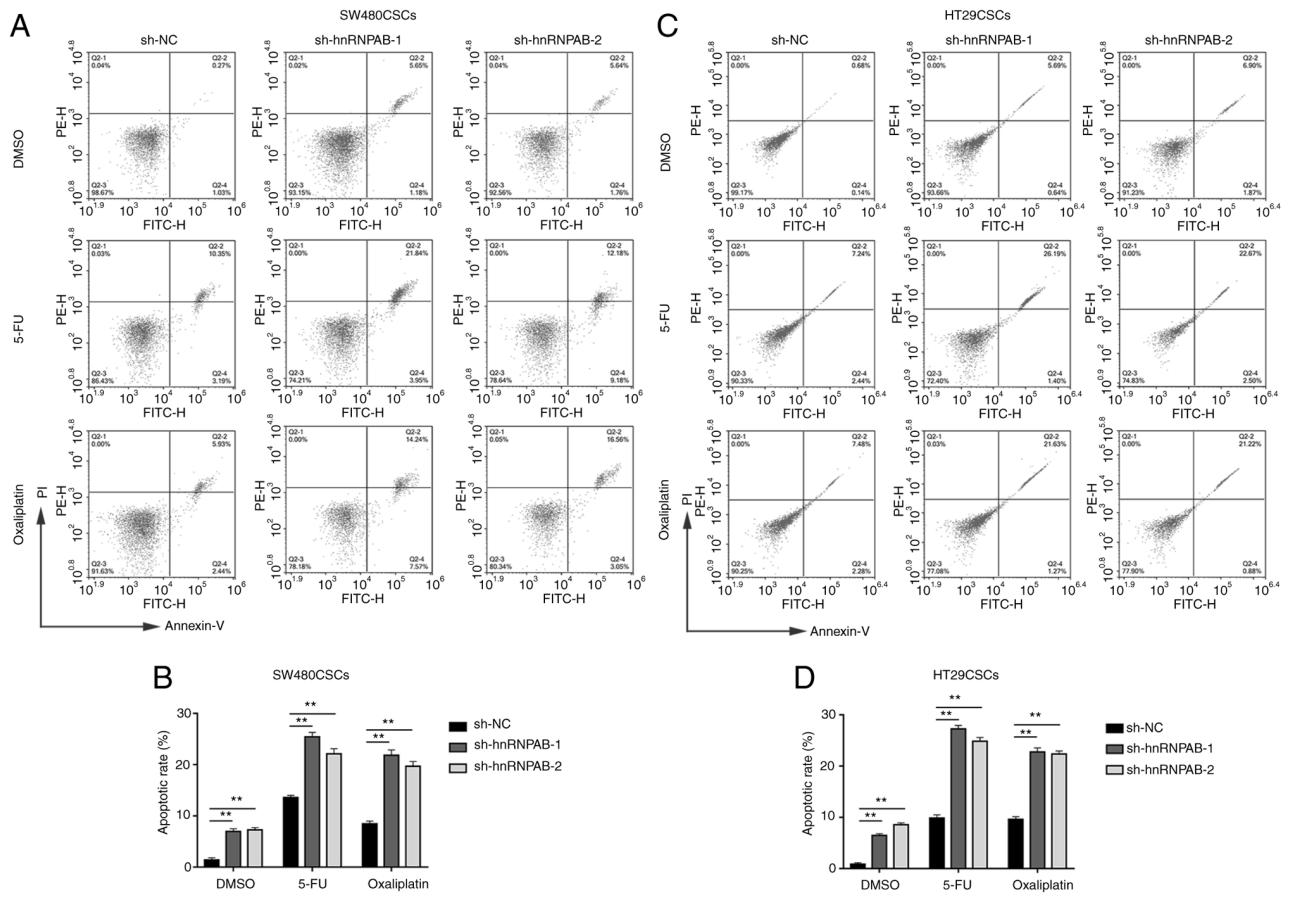

Figure 6. Knockdown of hnRNPAB increases the apoptotic rate of SW480CSCs and HT29CSCs treated with the chemotherapeutic drugs, 5-FU and oxaliplatin. (A and B) Comparison of apoptosis between the sh-NC group and the sh-hnRNPAB-1 or sh-hnRNPAB-2 groups in SW480CSCs in response to 5-FU or oxaliplatin treatment examined using flow cytometry. (C and D) Comparison of apoptosis between the sh-NC group and the sh-hnRNPAB-1 or sh-hnRNPAB-2 groups in HT29CSCs in response to 5-FU or oxaliplatin treatment examined using flow cytometry. Each bar represents the percentage of apoptotic cells with mean ± SD of three independent experiments. **P<0.01. hnRNPAB, heterogeneous ribonucleoprotein AB; CSCs, cancer stem cells; 5-FU, 5-fluorouracil.

increased, while the expression of the anti-apoptotic protein, Bcl-2, was decreased; the expression of apoptosis-related proteins in the sh-hnRNPAB groups increased or decreased more significantly (P<0.01; Fig. 7A-D). In addition, the same trend was observed for the expression of Bax, Bcl-2 and cleaved caspase-3 in the HT29CSCs with hnRNPAB silencing treated with 5-FU or oxaliplatin (P<0.01; Fig. 7E-H). The above results suggested that silencing hnRNPAB increased the sensitivity of SW480CSCs and HT29CSCs to chemotherapeutic drugs by promoting cell apoptosis.

Discussion

hnRNPs are a large class of RNA-binding proteins with important roles in multiple aspects of nucleic acid metabolism, including the packaging of nascent transcripts, mRNA alternative splicing and gene translational regulation. Studies have found that the abnormal expression of hnRNPs plays a crucial role in the occurrence and development of lung, liver, colorectal, breast, pancreatic cancer and other malignant tumours (17). As an early classification of the hnRNP family, hnRNPAB was first purified from 40S granules of human HeLa cells, and it can be divided into four subtypes: hnRNPA1, hnRNPA2/B1, hnRNPA3 and hnRNPA0 (18). Previous studies have found that hnRNPAB is highly expressed in CRC and is closely related to various prognoses of CRC. In the present study, it was found that the stemness characteristics of colorectal CSCs were increased compared with their parent cells, the resistance to chemotherapeutic drugs was increased, and the expression of hnRNPAB was increased. The knockdown of hnRNPAB expression in colorectal CSCs decreased the stemness of CSCs, which were sensitive to chemotherapeutic drugs and cell apoptosis increased.

CSCs are considered subsets of tumour cells with high tumorigenicity, multilineage differentiation potential, and self-renewal capacity (19). Several preclinical studies have demonstrated the presence of CSCs in human CRC and have demonstrated significant contributions of CRC CSCs to clinical tumour progression, chemoresistance, and treatment failure (20). CSCs can express specific surface markers. CRCs that recur following chemotherapy failure are enriched in cells expressing tumour stem cell markers, i.e., enriched in CSCs (21). For CRC, the CSC markers are OCT4, SOX2, CD44, CD133, CD29, Nanog and Lgr5 (22-24). It has been have found that the increase in identifiable stemness-related biomarkers in tumour cells is associated with treatment resistance and cancer recurrence (25).

Determining the function of hnRNPAB in CSCs is critical for understanding the molecular mechanisms of tumour occurrence and development. According to previous research

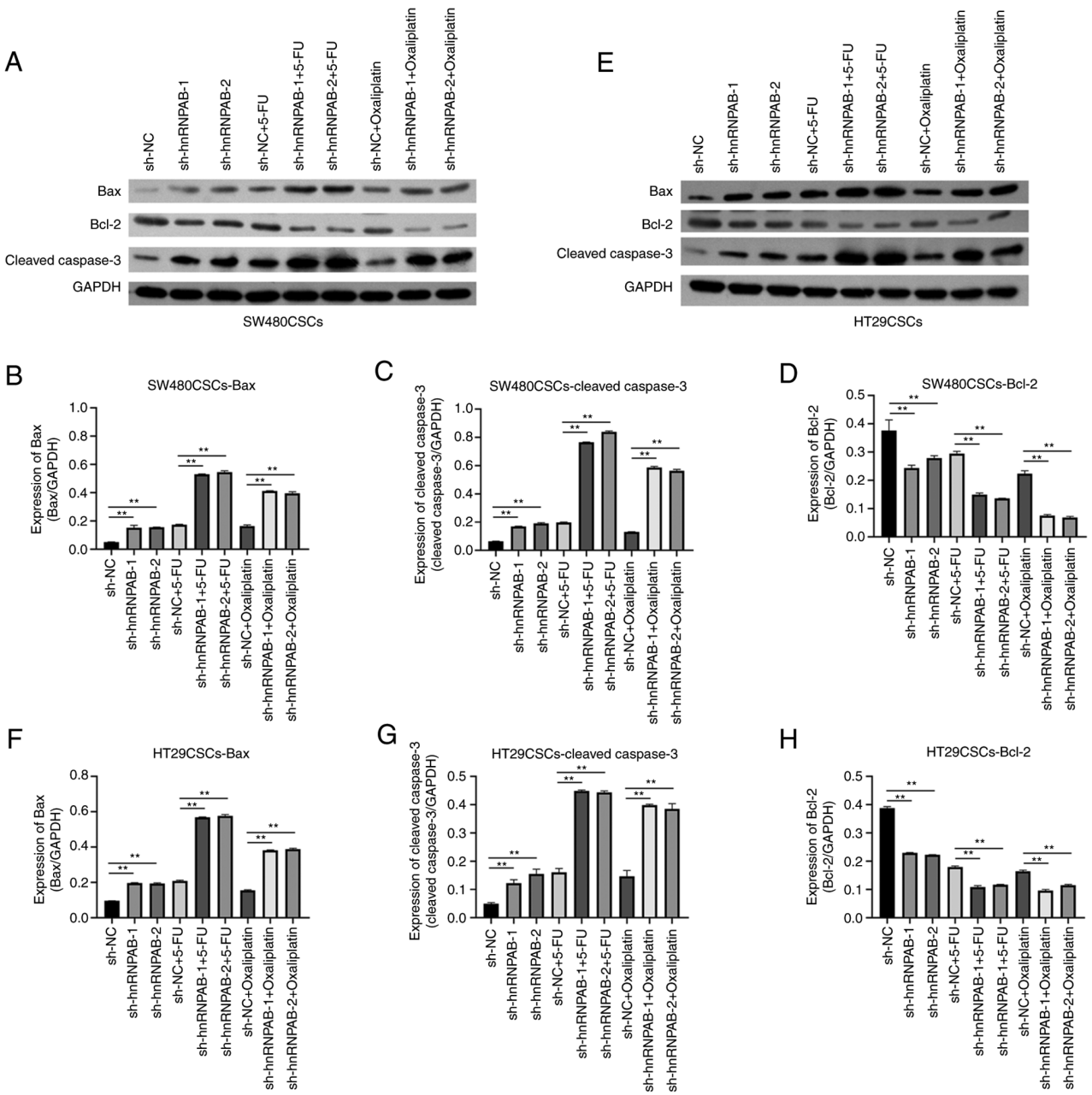

Figure 7. The expression of the apoptosis-related proteins, Bax, Bcl-2 and cleaved caspase-3, in SW480CSCs and HT29CSCs after the silencing of hnRNPAB and treatment with 5-FU or oxaliplatin was examined using western blot analysis. (A-D) The expression of Bax, Bcl-2 and cleaved caspase-3 in SW480CSCs after the silencing of hnRNPAB and treatment with 5-FU or oxaliplatin was detected using (A) western blot analysis and (B-D) the protein levels were quantified. (E-H) The expression of Bax, Bcl-2, and cleaved caspase-3 in HT29CSCs after the silencing of hnRNPAB and treatment with 5-FU or oxaliplatin was examined using (E) western blot analysis and (F-H) the protein levels were quantified. Each bar represents the mean ± SD of three independent experiments. **P<0.01. hnRNPAB, heterogeneous ribonucleoprotein AB; CSCs, cancer stem cells; 5-FU, 5-fluorouracil.

methods (14), the authors cultured colorectal cancer CSCs, termed SW480CSCs and HT29CSCs, and found that the expression of hnRNPAB increased in colorectal CSCs, as shown by RT-qPCR and western blot analysis. To further elucidate the role of hnRNPAB in CSCs, shRNPAB in SW480CSCs and HT29CSCs was silenced using two independent shRNAs. Spheroid formation experiments have been used to verify the proliferation, self-renewal and differentiation abilities of different cell populations of CSCs (26). In the present study, the spheroid colony formation ability of colorectal CSCs with hnRNPAB silencing was lower than that of the normal control group (the number and size of cells were decreased).

Accumulating evidence has indicated that hnRNPAB can regulate stem cell proliferation, the cell cycle and apoptosis (27,28). Choi *et al* (27) found that hnRNPA2/B1 knockout reduced the expression of the embryonic stem cell markers, OCT4, NANAG and SOX2, inhibited the proliferation of human embryonic stem cells and induced cell arrest in the G0/G1 phase, thus regulating the self-renewal and multipotential of human embryonic stem cells. Chen *et al* (28) reported that miRNA-8064 targeted hnRNPAB to inhibit the self-renewal of colorectal CSCs through the Wnt/β-catenin pathway. The stemness capability of colorectal CSCs was previously assessed using flow cytometry to examine the

percentage of cells expressing tumour stem cell markers, such as OCT4, SOX2, CD44 and CD133 *in vitro* (29). In the present study, the levels of the colorectal CSC markers, CD44, CD133, OCT4 and SOX2, were reduced in colorectal CSCs after hnRNPAB silencing, suggesting a reduction in stemness.

Konishi *et al* (30) found that hnRNPA0 was highly expressed in CRC cells. The knockdown of hnRNPA0 in the HCT116 cells reduced the number of cells in the G1 phase, and increased that in the S and G2/M phase; it also increased the expression of cleaved caspase-3 and promoted apoptosis. These results indicate that hnRNPA0 inhibits the apoptosis of CRC cells by maintaining the promotion of the G2/M phase. Liu *et al* (31) found that the silencing hnRNPA2/B1 reduced the total clone number of CRC cells with cetuximab treatment and significantly prevented cell migration and invasion, and the MIR100HG/hnRNPA2B1/TCF7L2 regulatory loop, regulated cetuximab resistance and tumour metastasis. In the present study, it was found that the knockdown of hnRNPAB reduced cell proliferation by blocking the cell transition from the G1/S to the S/G2 phase. It was hypothesized that hnRNPAB may be one of the kinases that promotes the transformation of cells from the G1 to the S phase. Thus, hnRNPAB promoted the growth of cancer cells by regulating CSC properties, as well as alterations in the cell cycle.

In addition, the present study examined the effects of hnRNPAB in colorectal CSCs treated with the chemotherapeutic drugs, oxaliplatin and 5-FU. The results revealed that colorectal CSCs with a high hnRNPAB expression were more resistant to the chemotherapeutic drugs, oxaliplatin and 5-FU. The knockdown of hnRNPAB enhanced the sensitivity of colorectal CSCs to the chemotherapeutic drugs and promoted cell apoptosis.

In conclusion, the present study demonstrated that the knockdown of hnRNPAB reduced the growth of colorectal CSCs, enhanced the sensitivity to the chemotherapeutic drugs, 5-FU and oxaliplatin, and promoted cell apoptosis. Therefore, the inhibition of hnRNPAB may be considered a novel molecular therapeutic strategy for CRC and drug-resistant patients. However, the underlying mechanisms require further investigation.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JZ, SC, JD and JLi conceived and designed the experiments. JZ, JLiu and SC performed the experiments and the statistical analysis. JZ, JD and JLi participated in the discussion and interpretation of the data. JZ wrote the manuscript. JD and JLi supervised all the experimental work. JD and JLi confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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