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Baicalin reduces the stemness potential of hepatoblastoma rather than hepatocellular carcinoma and improves its chemo-sensitivity

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Abstract: Introduction: Hepatoblastoma (HB) is the most common malignant liver tumor in children. Although surgery and chemotherapy have greatly improved the survival rate of children, the emergence of chemotherapy resistance still threatens the survival of most patients. Baicalin（Ba）is a kind of flavonoid bioactive substance with strong anti-tumor effect, but the effect of Ba on HB remains to be explored. Therefore, studying the effect and mechanism of Ba on HB may provide new opportunities for improving chemotherapy resistance. **Objective：** To explore the effect and mechanism of Ba on the anti-tumor effect and cisplatin sensitivity of HB cell line Hep G2 and hepatocellular carcinoma cell line Hep 3B. **Methods:** The anti-tumor effects of Ba on Hep G2 and Hep 3B cells and its influence on cisplatin sensitivity were evaluated by phenotypic experiments；Western blotting and colony formation assay are used to detect the influence of Ba on the stemness potential of two cell lines；Finally, the effect of Ba on the sensitivity of Hep G2 tumor-bearing mice to cisplatin was further verified in vivo. **Results:** Ba can significantly inhibit the malignant phenotype and stemness potential of Hep G2 in vitro, and increase its sensitivity to cisplatin, but it has no effect on Hep 3B. Further mechanism studies have shown that Ba can inhibit the clone ball formation of Hep G2 cells and down-regulate the expression of Oct4 and Sox2 proteins, but this effect has not been found in Hep 3B cell lines. Further in vivo verification showed that Ba increases the sensitivity of Hep G2 tumor-bearing mice to cisplatin. **Conclusion:** Our study confirms the important role of Ba in HB chemotherapy resistance. Ba increases its chemotherapeutic sensitivity to cisplatin by reducing the stemness of HB cells, and this effect is selective for tumor types and has no effect on hepatocellular carcinoma.
Key words: baicalin, hepatoblastoma, hepatocellular carcinoma, cisplatin, chemotherapy sensitivity, Oct4, Sox2.

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***Abbreviations*:** HB, Hepatoblastoma; Ba, Baicalin; PLC, Primary liver cancer; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; cHCC-ICC, intrahepatic cholangiocarcinoma; CSCs, cancer stem cells.

***Authors' Contributions*:** All authors read and approved the final manuscript.

***Competing interests*:** The author declare that they have no conflicts of interest.

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Introduction

Primary liver cancer (PLC) is one of the most common malignant tumors worldwide [1, 2], which is mainly divided into hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC), hepatoblastoma (HB), and the combination of hepatocellular carcinoma and intrahepatic cholangiocarcinoma (cHCC-ICC) [3]. Among them, HB is the most common hepatic malignancy in children [4-6], and accounts for more than 90% of primary hepatic malignancies in children under 5 years of age. On the other hand, HCC is one of the most common solid malignancies and is the leading cause of cancer-related deaths worldwide [7, 8]. Surgery and chemotherapy are currently the main treatments for HB and HCC [9, 10]. In particular, the advent of platinum-based drugs has greatly improved the prognosis of the disease, but the development of tumor recurrence and multidrug resistance still poses a serious threat to patient survival [11-13]. Even survivors may be at risk of permanent hearing loss due to cisplatin in the later stages of chemotherapy, which not only affects the quality of life of patients, but also has a serious impact on children's speech and language development. Therefore, overcoming drug resistance and increasing chemotherapy sensitivity remain important measures to improve patient prognosis.

Scutellaria baicalensis, an herbal medicine, has been recorded in ancient texts thousands of years ago and is widely used to treat a variety of respiratory and digestive diseases, especially liver and bile-related diseases. Baicalin (Ba) is the most important bioactive flavonoid in Scutellaria baicalensis. It has antioxidant [14], antiviral [15], anti-inflammatory [16], and neuroprotective [17] and other more effects. In recent years, it has been found that BA exerts antitumor effects through various pathways such as induction of apoptosis [18], autophagy [19], senescence [20], and cell cycle blockade [21, 22]. However, the study of baicalin on hepatoblastoma and Hep 3B hepatocellular carcinoma is still a gap, and its role in regulating the tumor stemness of hepatoblastoma remains to be explored.

It is well known that the stemness potential of cancer cells is closely related to tumor recurrence and multidrug resistance [23-25]. A population of cancer stem cells (CSCs) with stem cell-like biological properties such as self-renewal, tumorigenic ability, and chemoresistance exists in cancer cells [26]. CSCs are not sensitive to conventional radiotherapy; on the contrary, with the alteration of the tumor microenvironment by radiotherapy, CSCs are enriched, their ability to proliferate and invade and metastasize is further enhanced, and they show greater resistance to chemotherapeutic drugs, leading to tumor recurrence [27]. Therefore, increasing chemotherapy sensitivity by reducing tumor stemness potential is a current research hotspot in cancer treatment.

Here, our study found that baicalin significantly inhibited the malignant phenotype of HB cell line Hep G2 in vitro and increased its sensitivity to cisplatin, but had no effect on HCC cell line Hep 3B. Further experiments revealed that Ba inhibited clone ball formation in HB and decreased the expression of Oct4 and Sox2 proteins at the protein level, but did not have this effect on Hep 3B. Further validation in vivo similarly demonstrated that BA increased the sensitivity of hepatoblastoma to cisplatin. This suggests that baicalein increases the chemosensitivity of hepatoblastoma to cisplatin by decreasing the tumor stemness of hepatoblastoma, and that this effect is selective for tumor type and does not affect hepatocellular carcinoma. Our study confirms the important role of BA in HB chemoresistance, which brings hope for clinical increase of cisplatin sensitivity, reduction of dosage, and decrease of its ototoxicity and nephrotoxicity.

Methods and Materials

Materials

Ba (purity ≥98%) was purchased from Beijing Solebao Technology Company, and prepared into a 60 solution with dimethyl sulfolide (DMSO, Sigma, St. Louis, USA), which was stored at -20℃.

Cell Lines

HB cell line Hep G2 and HCC cell line Hep 3B all purchased from the American Type Culture Collection (ATCC, Maryland, USA) and cultured in a DMEM medium (High glucose) containing 10% FBS, 100 U/mL penicillin and 100 g/ml Streptomycin (these reagents were obtained from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, US), and in a humid environment of 37℃, the concentration of CO_2 was set at 5%. Negative control group (normal culture), positive control group (cisplatin 1ug/ml) [28], experimental group (Ba 20uM) and combined treatment group (cisplatin 1ug/ml + Ba 20uM) were set for the experiment, and each group was treated with drugs for 24 h.

Cell Proliferation Assay

Hep G2 and Hep 3B cells were digested and resuspended. The cells were diluted to 1×10^4/mL and seeded on a 96-well culture plate with 3 replicate wells in each group. For the dose assessment test, the medium was then replaced with a fresh medium containing a range of Ba concentrations (0, 10, 15, 20, 40 and 60), and the cells were then cultured for another 24 hours. To determine the change in cell proliferation, each well was treated with 5 mg/mL MTT solution (MTT; Yeasen, Shanghai, China) 20uL, incubate at 37℃ for 4h. Discard the supernatant, and finally add

100uL DMSO solution to each well and mix well. Use a microplate reader (Bio-Rad, Hercules, CA, USA) to detect the optical density D (λ) at 490nm.

Invasion Assay

The digested cell pellet was resuspended in serum-free DMEM, diluted to 5×10^6/mL, Add 200ul cell suspension to the upper chamber after being precoated with a matrigel (BD Biosciences) for 30 minutes at 37℃. Then 600 ul of medium containing 10% fetal bovine serum was added to the lower chamber; After the intervention of each group, remove the upper chamber and add 4% paraformaldehyde to fix the cells for 30 s; Add crystal violet staining solution and stain at room temperature for 20 minutes; after natural drying, observe the cell invasion under a microscope and determine the number of cells penetrating the membrane.

Wound Healing Assay

Cells were seeded on a 6-well culture plate and cultured normally until a cell monolayer was formed. A pipette tip was used to make a mark along the bottom of the culture plate. After the cell debris was removed by PBS, each group was intervened with drugs for 24 hours and then the medium was changed. After 48 h, the wound area was observed under an inverted microscope, and the cell mobility was calculated.

ELISA

The cells were inoculated with 96-well culture plates, with 3 replicates in each group, and the groups were treated with drugs for 24 h. The LDH content in the cell culture medium of each group was detected by the enzyme-linked immunosorbent assay (ELISA)-based LDH Activity Assay Kit (Yuanmu Biotechnology Co., Ltd., Shanghai, China). The experiment process was carried out in strict accordance with the ELISA kit instructions, and the microplate reader reads the results and performs statistical analysis.

Flow Cytometry Analysis

After group intervention, cells in each group were harvested by free EDTA-trypsin digestion, and then stained according to the instructions of the Annexin V Apoptosis Detection Kit (BD Biosciences). The labeled cells are then analyzed by flow cytometry to detect early and late apoptotic cells.

Colony Formation Assay

The cells in each group were adjusted to 5000 cells /mL and inoculated into the 96-well plate with the cell density of 1000 cells/well. The cells were dispersed evenly by gentle shaking, and the corresponding treatments were carried out in different experimental groups. Changed the serum-free culture medium every 3 days, and took out the culture plate after incubating for 2 weeks. Washed 3 times with PBS, fixed with 4% paraformaldehyde for 20 minutes, discard the fixative, and add an appropriate amount of 0.02% crystal violet staining solution to stain for 30 minutes. Observe cell colonies under an optical microscope.

Western Blotting

Each group of cells added an appropriate amount of pre-cooled lysis buffer (containing 10% PMSF) to extract whole protein. After protein quantification by BCA method, the protein is denatured by boiling water. Load the same amount of protein (50 ug) onto SDS-PAGE gel (10% separation gel), electrophoresis (80 V, 40 mA, 2.5 h), transfer membrane (110 V, 200 mA, 100 min). After blocking with 7% skimmed milk for 1 hour. Antibodies Oct4, Sox2 and β-actin (all purchased from Cell Signaling Technology, MA, USA) were incubated with the membrane to label the target protein, and then exposed and developed using a scanner. All the pictures are analyzed and processed by the biological image analysis system (ImageJ version 1.48; National Institutes of Health, Bethesda, MD, USA).

Construction of Tumor-Bearing Mouse Model

Collect the logarithmic phase cells and adjust the cells to 5×10^7 cells/mL after washing twice with PBS. Inject 0.2 mL of cell suspension into the armpits of the forelegs of nude mice, and then continue to raise them in a clean environment. When the tumor volume reached 100 mm^3, the mice were randomly divided into three groups (10 mice/group), Intraperitoneal injections of saline, Ba (50 mg/kg), and cisplatin (15 mg/kg) were given every other day [29]. The body weight and tumor volume of nude mice were observed every 4 days. Nude mice were sacrificed 15 days later, and tumor tissues were obtained by dissection. This experiment was performed according to the regulations and guidelines approved by the Animal Ethics Committee of the Fifth Central Hospital of Tianjin (Tianjin, China).

Apoptosis Assays

The tumors were resected and apoptosis was detected by TUNEL assay kit (Roche Diagnostics) according to the manufacturer's instructions. The nuclei were stained with DAPI (Zhongshan Goldenbridge, Beijing, China). The cell staining was then analyzed using confocal fluorescence microscopy (Carl Zeiss AG, Germany). The green fluorescence, which indicates the apoptosis, was observed at 520nm. The blue fluorescence, which indicates the nuclei, was observed at 460 nm.

Statistical Analysis

All data are presented as means ± SD and were analyzed using GraphPad Prism 6 software (San Diego, CA). One-way analysis of variance (ANOVA) or the

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unpaired Student's *t*-test was used to evaluate the significance of differences among treatment groups, as appropriate. A value of $P < 0.05$ was considered to be statistically significant.

Results

Ba Inhibits Malignant Phenotypes of HB cells, But Has no Effect on HCC cells

It is well known that high proliferative ability, invasiveness and migration ability are important characteristics of malignant phenotypes of tumor cells. Preliminary experiments showed that BA had a certain inhibitory effect on Hepatoma cell line. Based on this, we investigated the effect of Ba on the malignant phenotype of Hep G2 and Hep 3B, MTT, wound healing assay and invasion assay were conducted, respectively. As shown in Fig 1C, When the concentration of Ba reached 20, it started to have a dose-dependent inhibitory effect on Hep G2 cell viability, but all concentrations did not have an inhibitory effect on Hep 3B. Fig 1A, B showed that Ba (20uM) significantly reduced the migration and invasion capacity of Hep G2 compared with the control group, while in Hep 3B, there was no significant difference between the control group and the Ba group. So, it was indicated that Ba had no effect on the malignant phenotype of Hep 3B cells.

Ba Increases the Chemotherapy Sensitivity of HB, but has no Effect on HCC

Chemotherapy is one of the important measures for the treatment of malignant tumors, but due to the differences in the biological characteristics of tumors and the heterogeneity of cancer cells, the efficacy of chemotherapy drugs on different cancer cells is different. Chemosensitivity is an important indicator for evaluating the effectiveness of chemotherapy drugs on a certain tumor cell. The higher the sensitivity, the easier the tumor cells will be killed. Here, in order to study whether Ba can increase the sensitivity of Hep G2 and Hep 3B to cisplatin, we conducted verification in vitro. The results in Hep G2 showed that compared with the normal group, the LDH content of

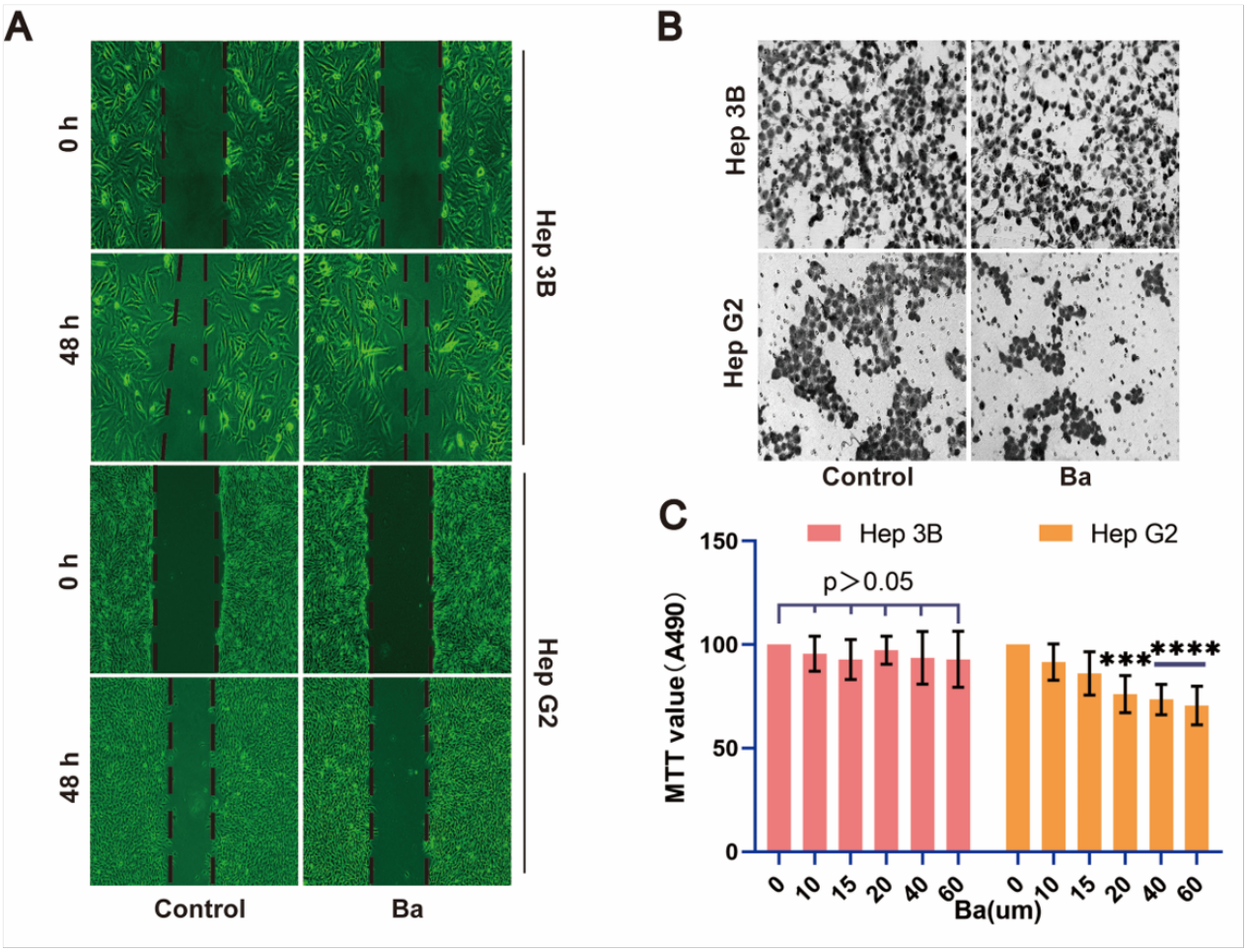

FIGURE 1 | Ba inhibits malignant phenotypes of HB, but has no effect on HCC. **(A)** Hep 3B and Hep G2 cell migration under Ba and normal conditions was analyzed over 48 h using a wound healing assay (scale bar, 50 um); **(B)** Hep 3B and Hep G2 cell invasion was examined by transwell assay (scale bar, 50 um); **(C)** MTT assay was used to evaluate the viability of both types of cells treated with different concentrations of Ba. Data are shown as means ± SD (n = 3); ***$P < 0.001$ and ****$P < 0.0001$ when compared with the control (Student' s t test).

the drug treatment group was significantly increased, and the combination of Ba and cisplatin group had a more significant increase in LDH compared with the individual treatment groups (Fig 2A). This indicated that Ba combined with cisplatin increased the sensitivity of Hep G2 cells to cisplatin, and flow cytometry results also showed that the apoptosis of Hep G2 cells in the combined group was significantly increased compared with other groups (Fig 2B). Although the Hep 3B cell experiment also showed that Ba or cisplatin alone increased the apoptosis of the cells, there was no significant difference between the single drug and the combined group, indicating that Ba could not increase the sensitivity of Hep 3B cells to cisplatin.

Ba Reduces the Stemness Maintenance Potential of HB, But Has No Effect On HCC

As stem cells in tumor cell population play a decisive role in the occurrence and development of tumors, the characteristics of stem cells with unlimited proliferation and self-renewal ability can be used to try to culture and reproduce tumor cells under certain conditions, so as to determine the chemotherapy-drug sensitive response of the cells. Colony formation assay is an important experimental method to detect the stem potential of cells and their sensitivity to killing factors. The clone ball formation rate reflects two important traits of cell population dependence and proliferation ability. In recent years, Oct4 and Sox2 have been shown to play a key role in regulating the proliferation of tissue cells, determining the direction of cell differentiation, and maintaining the pluripotency of cells. Therefore, it is not clear whether the effect of BA on HB and HCC is related to cells stemness potential. To this end, We studied the effect of Ba on the stem potential of Hep G2 and Hep 3B˄ Fig 3A, B showed that Ba significantly inhibited the clone ball formation of Hep G2 cells , and the Oct4 and Sox2 protein expressions were also significantly decreased compared with the normal group. However, there was no difference between Hep 3B cells and the normal group, indicating that Ba had no effect on the stem potential of Hep 3B cells.

Ba Increased the Sensitivity of HB to Cisplatin In Vivo, But Had No Effect On HCC

In order to verify the above possible mechanism of action, we carried out the following in vivo subcutaneous tumor formation experiments in nude mice. First, we established Hep G2 tumor-bearing mouse models by injecting cell suspension into the foreleg armpits of nude mice. When the tumor body grew to 100mm^3, all mice were randomly divided into 4 groups, each with 8 mice, and were given (1) blank normal saline;(2) Ba; (3) Cisplatin ;(4) Combined (Ba+ Cisplatin). The administration was continued for 28 days, and the tumor volume was observed every 4 days. After the administration, the rats in each group were anesthetized with isoflurane, the tumor mass was stripped off, and weighed. Appropriate amounts of tumors in each group were taken, fixed with 4% paraformaldehyde, dehydrated, embedded, and sectioned to make paraffin sections. In situ apoptosis kits were used to detect the apoptosis of tumor cells in each group. As shown in the Fig 4A, B, in Hep G2 tumor-bearing mice, tumor volume and mass were observed in other groups compared with control. Similarly, the reduction in Cisplatin group was more significant. It is worth noting that the combination of Ba and Cisplatin showed more significant tumor inhibition than the single-drug group. In addition, tumor tissue apoptosis detection also showed that the combination group was superior to the individual treatment groups (Fig 4C).

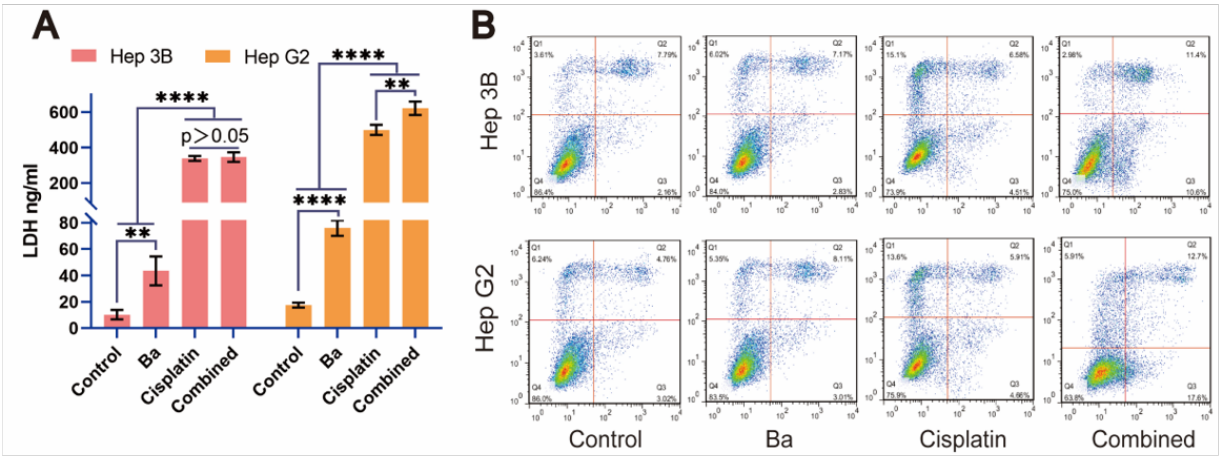

FIGURE 2 | Ba increases the chemotherapy sensitivity of HB, but has no effect on HCC. **(A)** Lactate dehydrogenase (LDH) expression in Hep3B and Hep G2 cells was measured by enzyme-linked immunosorbent assay (ELISA) following exposure of cells to the indicated treatments. **(B)** Cell apoptosis was examined by flow cytometry. Data are shown as means ± SD (n = 3); $^{**}P < 0.01$ and $^{****}P < 0.0001$ when compared with another group (Student's t test).

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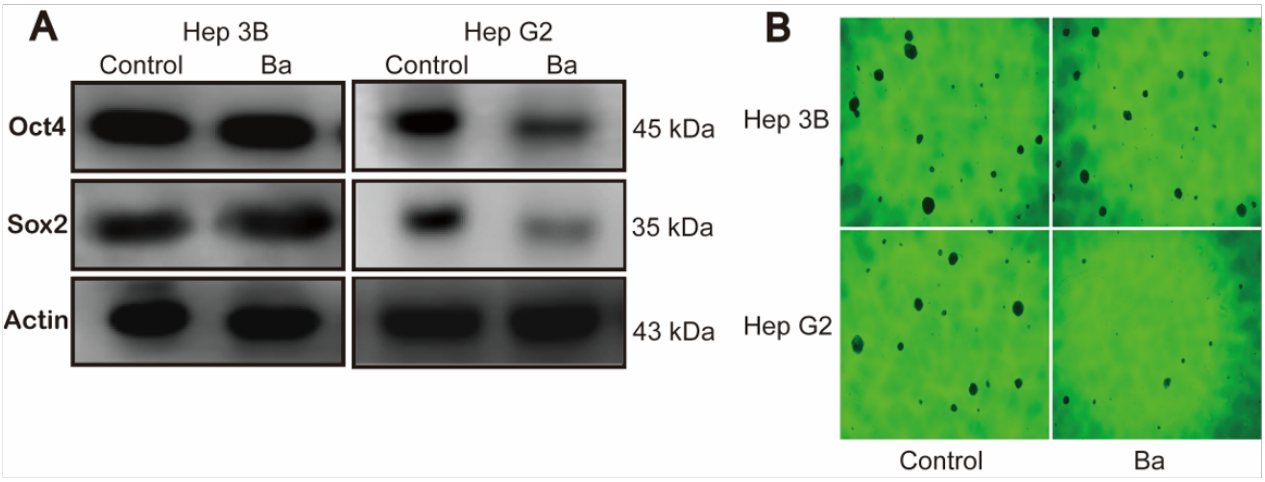

FIGURE 3 | (A) Ba reduces the stemness maintenance potential of HB, but has no effect on HCC. **(B)** Colony formation assay was used to detect the proliferation of Hep3B and Hep G2 cells. The expression of OCT4 and SOX2 proteins were examined by western blotting.

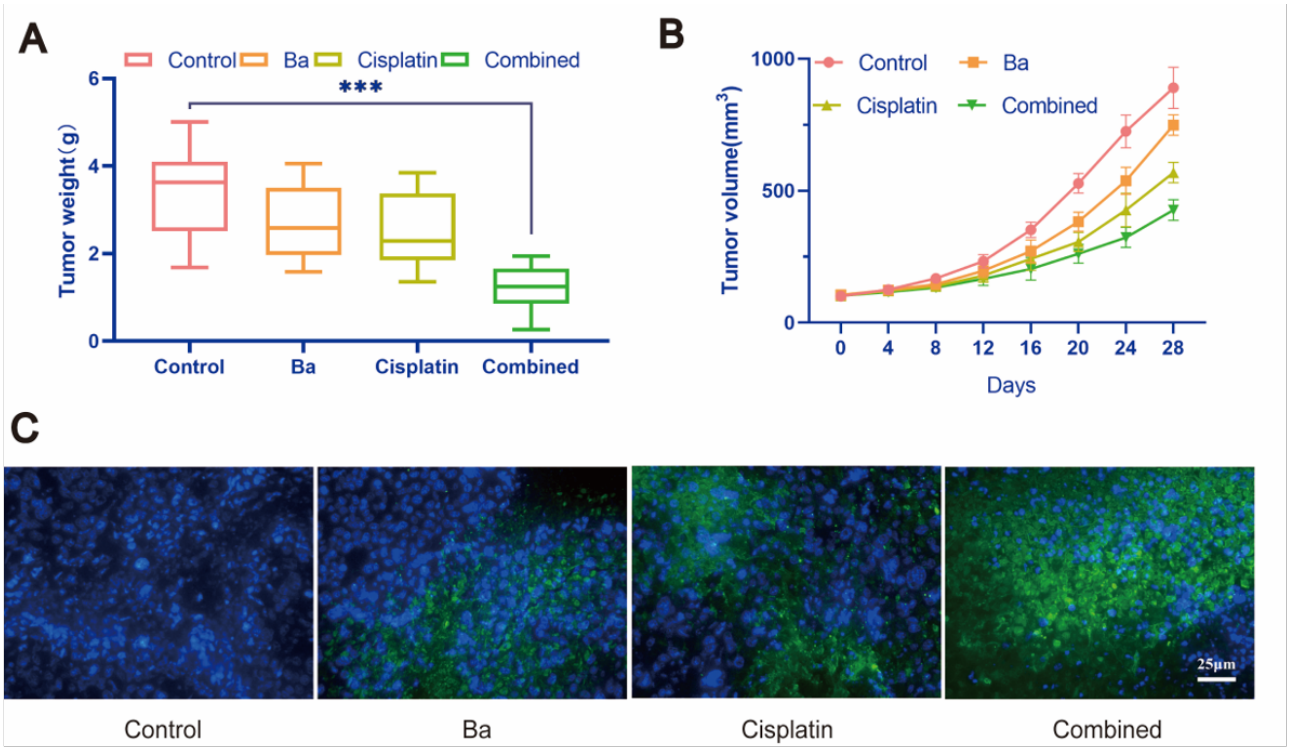

FIGURE 4 | Ba inhibits tumor growth and increases the sensitivity of Hep G2 cells to Cisplatin in vivo. BALB/c nude mice were inoculated with Hep G2 cells and subsequently treated with vehicle, Ba, Cisplatin, or a combination of Ba and Cisplatin. **(A)** Tumor were weighted immediately after dissection from nude mice. **(B)** Tumor volumes were measured every 4 days over a 28-day period. **(C)** Apoptosis in tumor xenograft sections was detected by TUNEL staining (scale bar, 25uM) was examined by flow cytometry. Data are shown as means ± SD (n = 3); $^{**}P < 0.01$ and $^{****}P < 0.0001$ when compared with another group (Student's t test).

Discussion

Although surgical treatment is an important method for liver cancer [10], due to the low rate of early detection, more than half of patients have unresectable primary tumors or distant metastases. Even if they have received liver resection or other local treatments, the recurrence rate of patients is still as high as 70% [30]. To date, chemotherapy is still the most promising method for the treatment of tumors. Recent clinical studies of multiple chemotherapeutic drugs have shown that adjuvant or neoadjuvant systemic therapy is effective for advanced liver cancer [31-33], and surgery combined with chemotherapy has significantly improved patients survival rate [34-36].

Cisplatin is the most commonly used first-line drug in HB chemotherapy. It can be interlinked with and within the DNA strand to form CDDP-DNA complex, which ultimately interferes with DNA replication and

synthesis. In the past few years, with the continuous improvement of chemotherapy regimens, Cisplatin based combination chemosensitizer significantly increased the overall survival rate of HB patients, reaching 70-80% of the total 5-year survival rate [37]. Unfortunately, clinical applications 80% of HB patients develop resistance after four cycles of chemotherapy [38,39], which severely limits the efficacy of chemotherapy and is an important cause of treatment failure. Therefore, the search for drugs that improve the sensitivity of Cisplatin chemotherapy is important to improve the prognosis of hepatoblastoma.

Baicalin, a flavonoid bioactive substance, has been shown to have antitumor effects in human HCC cell lines MHCC-97H, HCC-LM3, and SMMC-7721 [40-43]. However, the antitumor effect of Ba on Hep G2 and Hep 3B cells, and its effect on the chemotherapeutic sensitivity of tumor cells remain to be further explored. Inspired by this, we investigated the effect of Ba on Hep G2 and Hep 3B cells in vitro. Encouragingly, we found that Ba inhibited the malignant phenotype (invasion, migration and proliferation ability) of hepatoblastoma Hep G2 cells and increased the chemosensitivity of Hep G2 cells to Cisplatin in vitro and in vivo. Unfortunately, Ba did not have similar effects on hepatocellular carcinoma Hep 3B cells, suggesting that the antitumor effects of Ba are somewhat selective.

Further, chemotherapy sensitivity has always been the focus of tumor chemotherapy treatment, and the existence of tumor stem cells is an important reason for chemotherapy resistance. Meanwhile, previous studies have identified many mechanisms of Cisplatin resistance, such as reduced drug accumulation, increased DNA repair, resistance to Cisplatin toxicity, involvement of long-stranded non-coding RNAs, and epithelial-mesenchymal transition [44, 45]. Notably, a recent study found that Cisplatin induces stemness characteristics in tumor cells, leading to Cisplatin resistance [46, 47], while inhibition of tumor stem cells significantly increases Cisplatin sensitivity [48, 49]. Tumor stem cells are usually hidden in cancer nests, in a quiescent state, with inactive DNA replication, able to evade the effect of DNA damage induced by chemotherapeutic drugs such as Cisplatin, and have an enhanced ability to repair DNA damage, which are one of the main causes of tumor recurrence and drug resistance [50]. Like stem cells, tumor stem cells are able to initiate tumorigenesis and have the ability to self-renew and differentiate, which is closely related to their high expression of stemness genes such as Oct4 and Sox2. Sox2 and Oct4 regulate the proliferation of tissue cells, determine the direction of cell differentiation and maintain stem cell totipotency. Together with NANOG, they work in an interdependent transcriptional network in which Sox2 can regulate Oct4 expression in a direct or indirect manner, thereby affecting the multipotency of stem cells. This network stimulates not only the transcription of its own genes, but also the expression of other key genes, including FGF4 and Zfp42/Rex1 [51]. Recently, several studies have also reported aberrant expression of Oct4 and Sox2 in various malignant tissues or cell lines [52-55]. High levels of Oct4 increased malignant potential with a more primitive and aggressive tumor phenotype, while low Oct4 expression resulted in a degraded malignant phenotype with a more differentiated morphology [56]. Yuan [57] found that Sox2 and Oct4 were expressed in both hepatocellular carcinoma tissues and cell lines, and that clone formation and invasiveness of cancer cells decreased after downregulating them. Sox2 and Oct4play key roles in HCC [58], and several hepatocellular carcinoma characteristic genes are controlled by Oct4 [59], which is important for the self-renewal of hepatocellular carcinoma stem cells and tumor metastasis [60-62]. In addition, high levels of Sox2 and Oct4 were found to be associated with poor prognosis in HCC patients [63,64]. It is important to note that Oct4 and Sox2 are involved in tumorigenicity and drug resistance as key transcription factors [65-67]. Sox2 and Oct4 overexpression reduces chemotherapy sensitivity [68] and is an important factor in tumor resistance to Cisplatin. For example, in studies on Cisplatin resistance, it was found that lung cancer cells transfected with Oct4 increased their resistance to Cisplatin [69], as well as in liver cancer cells, by mechanisms that may be related to activation of TCL1, AKT and ABCG2 [70]. In addition, increased expression of Sox2 similarly increased Cisplatin resistance in advanced hepatocellular carcinoma [71]. Cisplatin resistance was also found in the hepatoma cell line MHCC97-L overexpressing Oct4 plus NANOG, while melanoma cells overexpressing Oct4 had not only higher Cisplatin resistance but also stronger proliferative capacity [72]. In contrast, Oct4 knockdown increased the sensitivity of tumors to Cisplatin [69, 73].

So, is the mechanism by which Ba increases Cisplatin sensitivity also related to its inhibition of tumor stemness in HB? For this reason, we further investigated the effect of Ba on the stemness of tumor cells. Our study found that Ba reduced the rate of clone ball formation in hepatoblastoma and significantly inhibited Sox2 and Oct4 protein expression at the protein level. Again, these effects were not observed in hepatocellular carcinoma Hep 3B cells. This suggests that Ba increases the chemosensitivity of hepatoblastoma to Cisplatin by decreasing the tumor stemness of hepatoblastoma, and that this effect is selective for tumor type and does not affect hepatocellular carcinoma.

Finally, although we have shown that Ba inhibits the malignant phenotype of hepatoblastoma and increases the chemosensitivity of Cisplatin, this may be related to the inhibition of Sox2 and Oct4 protein expression

and reduction of their stemness potential. However, Ba did not show the same effect in Hep 3B cells, and the exact mechanism on Hep G2 cells is not yet clear, and we need to investigate further in the future.

References

1. McGlynn KA, Petrick JL, El-Serag HB. Epidemiology of Hepatocellular Carcinoma. Hepatology. 2021 Jan;73 Suppl 1(Suppl 1):4-13.
2. Satriano L, Lewinska M, Rodrigues PM, Banales JM, Andersen JB. Metabolic rearrangements in primary liver cancers: cause and consequences. Nat Rev Gastroenterol Hepatol. 2019 Dec;16(12):748-766.
3. Wang G, Wang Q, Liang N, et al. Oncogenic driver genes and tumor microenvironment determine the type of liver cancer. Cell Death Dis. 2020 May 4;11(5):313.
4. Ferrari A, Brecht IB, Gatta G, et al. Defining and listing very rare cancers of paediatric age: consensus of the Joint Action on Rare Cancers in cooperation with the European Cooperative Study Group for Pediatric Rare Tumors. Eur J Cancer. 2019 Mar; 110:120-126.
5. Lupo PJ, Schraw JM, Desrosiers TA, Nembhard WN, Plon SE. Association Between Birth Defects and Cancer Risk Among Children and Adolescents in a Population-Based Assessment of 10 million Live Births. JAMA Oncol. 2019 Aug 1;5(8):1150-1158.
6. Feng J, Polychronidis G, Heger U, et al. Incidence trends and survival prediction of hepatoblastoma in children: a population-based study. Cancer Commun (Lond). 2019 Oct 24;39(1):62.
7. Chen Z, Xie H, Hu M, et al. Recent progress in treatment of hepatocellular carcinoma. Am J Cancer Res. 2020 Sep 1;10(9):2993-3036.
8. Wei W, Zeng H, Zheng R, et al. Cancer registration in China and its role in cancer prevention and control. Lancet Oncol. 2020 Jul;21(7): e342-e349.
9. Brock PR, Maibach R, Childs M, Rajput K, Neuwelt EA. Sodium Thiosulfate for Protection from Cisplatin-Induced Hearing Loss. N Engl J Med. 2018 Jun 21;378(25):2376-2385.
10. Yang JD, Heimbach JK. New advances in the diagnosis and management of hepatocellular carcinoma. BMJ. 2020 Oct 26;371:m3544.
11. Gabr A, Kulik L, Mouli S, et al. Liver Transplantation Following Yttrium-90 Radioembolization: 15-Year Experience in 207-Patient Cohort. Hepatology. 2021 Mar;73(3):998-1010.
12. Kim N, Cheng J, Jung I, et al. Stereotactic body radiation therapy vs. radiofrequency ablation in Asian patients with hepatocellular carcinoma. J Hepatol. 2020 Jul;73(1):121-129.
13. Pisa R, Kapoor TM. Chemical strategies to overcome resistance against targeted anticancer therapeutics. Nat Chem Biol. 2020 Aug;16(8):817-825.
14. Lei K, Shen Y, He Y, et al. Baicalin Represses C/EBPbeta via Its Antioxidative Effect in Parkinson's Disease. Oxid Med Cell Longev. 2020 May 21:2020:8951907.
15. Su HX, Yao S, Zhao WF, et al. Anti-SARS-CoV-2 activities in vitro of Shuanghuanglian preparations and bioactive ingredients. Acta Pharmacol Sin. 2020 Sep;41(9):1167-1177.
16. Paudel KR, Kim DW. Microparticles-Mediated Vascular Inflammation and its Amelioration by Antioxidant Activity of Baicalin. Antioxidants (Basel). 2020 Sep 20;9(9):890.
17. Song X, Gong Z, Liu K, Kou J, Liu K. Baicalin combats glutamate excitotoxicity via protecting glutamine synthetase from ROS-induced 20S proteasomal degradation. Redox Biol. 2020 Jul: 34:101559.
18. Huang L, Peng B, Nayak Y, et al. Baicalein and Baicalin Promote Melanoma Apoptosis and Senescence via Metabolic Inhibition. Front Cell Dev Biol. 2020 Aug 25:8:836.
19. Tan HY, Wang N, Man K, et al. Autophagy-induced RelB/p52 activation mediates tumour-associated macrophage repolarisation and suppression of hepatocellular carcinoma by natural compound baicalin. Cell Death Dis. 2015 Oct 22;6(10): e1942.
20. Wang Z, Ma L, Su M, et al. Baicalin induces cellular senescence in human colon cancer cells via upregulation of DEPP and the activation of Ras/Raf/MEK/ERK signaling. Cell Death Dis. 2018 Feb 13;9(2):217.
21. Li-Weber M. New therapeutic aspects of flavones: the anticancer properties of Scutellaria and its main active constituents Wogonin, Baicalein and Baicalin. Cancer Treat Rev. 2009 Feb;35(1):57-68.
22. Himeji M, Ohtsuki T, Fukazawa H, et al. Difference of growth-inhibitory effect of Scutellaria baicalensis-producing flavonoid wogonin among human cancer cells and normal diploid cell. Cancer Lett. 2007 Jan 8;245(1-2):269-74.
23. López de Andrés J, Griñán-Lisón C, Jiménez G, Marchal JA. Cancer stem cell secretome in the tumor microenvironment: a key point for an effective personalized cancer treatment. J Hematol Oncol. 2020 Oct 15;13(1):136.
24. Liu YC, Yeh CT, Lin KH. Cancer Stem Cell Functions in Hepatocellular Carcinoma and Comprehensive Therapeutic Strategies. Cells. 2020 May 26;9(6):1331.

25. Wang N, Wang S, Li MY, et al. Cancer stem cells in hepatocellular carcinoma: an overview and promising therapeutic strategies. Ther Adv Med Oncol. 2018 Dec 21:10:433535969.
26. Sell S, Leffert HL. Liver cancer stem cells. J Clin Oncol. 2008 Jun 10;26(17):2800-5.
27. Saygin C, Matei D, Majeti R, Reizes O, Lathia JD. Targeting Cancer Stemness in the Clinic: From Hype to Hope. Cell Stem Cell. 2019 Jan 3;24(1):25-40.
28. Shi L, Lin H, Li G, et al. Cisplatin enhances NK cells immunotherapy efficacy to suppress HCC progression via altering the androgen receptor (AR)-ULBP2 signals. Cancer Lett. 2016 Apr 1;373(1):45-56.
29. Tan HY, Wang N, Man K, et al. Autophagy-induced RelB/p52 activation mediates tumour-associated macrophage repolarisation and suppression of hepatocellular carcinoma by natural compound baicalin. Cell Death Dis. 2015 Oct 22;6(10):e1942.
30. Zhu XD, Li KS, Sun HC. Adjuvant therapies after curative treatments for hepatocellular carcinoma: Current status and prospects. Genes Dis. 2020 Feb 29;7(3):359-369.
31. Jackson R, Psarelli EE, Berhane S, Khan H, Johnson P. Impact of Viral Status on Survival in Patients Receiving Sorafenib for Advanced Hepatocellular Cancer: A Meta-Analysis of Randomized Phase III Trials. J Clin Oncol. 2017 Feb 20;35(6):622-628.
32. Kudo M, Finn RS, Qin S, et al. Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. Lancet. 2018 Mar 24;391(10126):1163-1173.
33. Abou-Alfa GK, Meyer T, Cheng AL, et al. Cabozantinib in Patients with Advanced and Progressing Hepatocellular Carcinoma. N Engl J Med. 2018 Jul 5;379(1):54-63.
34. Zsiros J, Brugieres L, Brock P, et al. Dose-dense cisplatin-based chemotherapy and surgery for children with high-risk hepatoblastoma (SIOPEL-4): a prospective, single-arm, feasibility study. Lancet Oncol. 2013 Aug;14(9):834-42.
35. Pritchard J, Brown J, Shafford E, et al. Cisplatin, doxorubicin, and delayed surgery for childhood hepatoblastoma: a successful approach--results of the first prospective study of the International Society of Pediatric Oncology. J Clin Oncol. 2000 Nov 15;18(22):3819-28.
36. Zsiros J, Maibach R, Shafford E, et al. Successful treatment of childhood high-risk hepatoblastoma with dose-intensive multiagent chemotherapy and surgery: final results of the SIOPEL-3HR study. J Clin Oncol. 2010 May 20;28(15):2584-90.
37. Czauderna P, Haeberle B, Hiyama E, et al. The Children's Hepatic tumor International Collaboration (CHIC): Novel global rare tumor database yields new prognostic factors in hepatoblastoma and becomes a research model. Eur J Cancer. 2016 Jan; 52:92-101.
38. Marin JJG, Cives-Losada C, Asensio M, et al. Mechanisms of Anticancer Drug Resistance in Hepatoblastoma. Cancers (Basel). 2019 Mar 22;11(3):407.
39. von Schweinitz D, Hecker H, Schmidt-von-Arndt G, Harms D. Prognostic factors and staging systems in childhood hepatoblastoma. Int J Cancer. 1997 Dec 19;74(6):593-9.
40. Ke M, Zhang Z, Xu B, et al. Baicalein and baicalin promote antitumor immunity by suppressing PD-L1 expression in hepatocellular carcinoma cells. Int Immunopharmacol. 2019 Oct; 75:105824.
41. Wang Y, Yin S, Zhou Y, et al. Dual-function of Baicalin in nsPEFs-treated Hepatocytes and Hepatocellular Carcinoma cells for Different Death Pathway and Mitochondrial Response. Int J Med Sci. 2019 Sep 7;16(9):1271-1282.
42. Tan HY, Wang N, Man K, et al. Autophagy-induced RelB/p52 activation mediates tumour-associated macrophage repolarisation and suppression of hepatocellular carcinoma by natural compound baicalin. Cell Death Dis. 2015 Oct 22;6(10): e1942.
43. Zhang X, Tang X, Liu H, et al. Autophagy induced by baicalin involves downregulation of CD147 in SMMC-7721 cells in vitro. Oncol Rep. 2012 Apr;27(4):1128-34.
44. Shen DW, Pouliot LM, Hall MD, Gottesman MM. Cisplatin resistance: a cellular self-defense mechanism resulting from multiple epigenetic and genetic changes. Pharmacol Rev. 2012 Jul;64(3):706-21.
45. Galluzzi L, Senovilla L, Vitale I, et al. Molecular mechanisms of cisplatin resistance. Oncogene. 2012 Apr 12;31(15):1869-83.
46. Weng CH, Wu CS, Wu JC, et al. Cisplatin-Induced Giant Cells Formation Is Involved in Chemoresistance of Melanoma Cells. Int J Mol Sci. 2020 Oct 24;21(21):7892.
47. Naik PP, Mukhopadhyay S, Panda PK, et al. Autophagy regulates cisplatin-induced stemness and chemoresistance via the upregulation of CD44, ABCB1 and ADAM17 in oral squamous cell carcinoma. Cell Prolif. 2018 Feb;51(1): e12411.
48. Zhao M, Wang T, Hui Z. Aspirin overcomes cisplatin resistance in lung cancer by inhibiting cancer cell stemness. Thorac Cancer. 2020 Nov;11(11):3117-3125.
49. Schmidtova S, Dorssers L, Kalavska K, et al. Napabucasin overcomes cisplatin resistance in

ovarian germ cell tumor-derived cell line by inhibiting cancer stemness. Cancer Cell Int. 2020 Aug 3:20:364.
50. Marin JJG, Herraez E, Lozano E, Macias RIR, Briz O. Models for Understanding Resistance to Chemotherapy in Liver Cancer. Cancers (Basel). 2019 Oct 29;11(11):1677.
51. Kashyap V, Rezende NC, Scotland KB, et al. Regulation of stem cell pluripotency and differentiation involves a mutual regulatory circuit of the NANOG, OCT4, and SOX2 pluripotency transcription factors with polycomb repressive complexes and stem cell microRNAs. Stem Cells Dev. 2009 Sep;18(7):1093-108.
52. Chang KJ, Yin JZ, Huang H, Li B, Yang MH. Arsenic trioxide inhibits the growth of cancer stem cells derived from small cell lung cancer by downregulating stem cell-maintenance factors and inducing apoptosis via the Hedgehog signaling blockade. Transl Lung Cancer Res. 2020 Aug;9(4):1379-1396.
53. Sedlic F, Seiwerth F, Sepac A, et al. Mitochondrial ROS Induce Partial Dedifferentiation of Human Mesothelioma via Upregulation of NANOG. Antioxidants (Basel). 2020 Jul 10;9(7):606.
54. Congras A, Hoareau-Aveilla C, Caillet N, et al. ALK-transformed mature T lymphocytes restore early thymus progenitor features. J Clin Invest. 2020 Dec 1;130(12):6395-6408.
55. Pedregal-Mallo D, Hermida-Prado F, Granda-Diaz R, et al. Prognostic Significance of the Pluripotency Factors NANOG, SOX2, and OCT4 in Head and Neck Squamous Cell Carcinomas. Cancers (Basel). 2020 Jul 4;12(7):1794.
56. Gidekel S, Pizov G, Bergman Y, Pikarsky E. Oct-3/4 is a dose-dependent oncogenic fate determinant. Cancer Cell. 2003 Nov;4(5):361-70.
57. Yuan F, Zhou W, Zou C, et al. Expression of Oct4 in HCC and modulation to wnt/beta-catenin and TGF-beta signal pathways. Mol Cell Biochem. 2010 Oct;343(1-2):155-62.
58. Wu G, Wilson G, Zhou G, et al. Oct4 is a reliable marker of liver tumor propagating cells in hepatocellular carcinoma. Discov Med. 2015 Oct;20(110):219-29.
59. Ye C, Zhang X, Chen X, et al. Multiple novel hepatocellular carcinoma signature genes are commonly controlled by the master pluripotency factor OCT4. Cell Oncol (Dordr). 2020 Apr;43(2):279-295.
60. Liu L, Liu C, Zhang Q, et al. SIRT1-mediated transcriptional regulation of SOX2 is important for self-renewal of liver cancer stem cells. Hepatology. 2016 Sep;64(3):814-27.
61. Zhang C, Huang S, Zhuang H, et al. YTHDF2 promotes the liver cancer stem cell phenotype and cancer metastasis by regulating OCT4 expression via m6A RNA methylation. Oncogene. 2020 Jun;39(23):4507-4518.
62. Wen W, Han T, Chen C, et al. Cyclin G1 expands liver tumor-initiating cells by Sox2 induction via Akt/mTOR signaling. Mol Cancer Ther. 2013 Sep;12(9):1796-804.
63. Wang L, Guo ZY, Zhang R, et al. Pseudogene OCT4-pg4 functions as a natural microRNA sponge to regulate OCT4 expression by competing for miR-145 in hepatocellular carcinoma. Carcinogenesis. 2013 Aug;34(8):1773-81.
64. Du XM, Wang LH, Chen XW, et al. Prognostic value of Sox2 expression in digestive tract cancers: A meta-analysis. J Huazhong Univ Sci Technolog Med Sci. 2016 Jun;36(3):305-312.
65. Lai SC, Su YT, Chi CC, et al. DNMT3b/OCT4 expression confers sorafenib resistance and poor prognosis of hepatocellular carcinoma through IL-6/STAT3 regulation. J Exp Clin Cancer Res. 2019 Nov 26;38(1):474.
66. Tang J, Sui CJ, Wang DF, et al. Targeted sequencing reveals the mutational landscape responsible for sorafenib therapy in advanced hepatocellular carcinoma. Theranostics. 2020 Apr 6;10(12):5384-5397.
67. Chang TS, Wu YC, Chi CC, et al. Activation of IL6/IGFIR confers poor prognosis of HBV-related hepatocellular carcinoma through induction of OCT4/NANOG expression. Clin Cancer Res. 2015 Jan 1;21(1):201-10.
68. Lim YC, Kang HJ, Moon JH. C-Met pathway promotes self-renewal and tumorigenecity of head and neck squamous cell carcinoma stem-like cell. Oral Oncol. 2014 Jul;50(7):633-9.
69. Chen YC, Hsu HS, Chen YW, et al. Oct-4 expression maintained cancer stem-like properties in lung cancer-derived CD133-positive cells. PLoS One. 2008 Jul 9;3(7): e2637.
70. Wang XQ, Ongkeko WM, Chen L, et al. Octamer 4 (Oct4) mediates chemotherapeutic drug resistance in liver cancer cells through a potential Oct4-AKT-ATP-binding cassette G2 pathway. Hepatology. 2010 Aug;52(2):528-39.
71. Lee JH, Suh JH, Kang HJ, et al. Tonicity-responsive enhancer-binding protein promotes stemness of liver cancer and cisplatin resistance. EBioMedicine. 2020 Aug; 58:102926.
72. Yin X, Zhang BH, Zheng SS, et al. Coexpression of gene Oct4 and Nanog initiates stem cell characteristics in hepatocellular carcinoma and promotes epithelial-mesenchymal transition through activation of Stat3/Snail signaling. J Hematol Oncol. 2015 Mar 11:8:23.
73. Samardzija C, Luwor RB, Volchek M, et al. A

critical role of Oct4A in mediating metastasis and disease-free survival in a mouse model of ovarian cancer. Mol Cancer. 2015 Aug 11:14:152.