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COX-2 Promotes Migration and Invasion by the Side Population of Cancer Stem Cell-Like Hepatocellular Carcinoma Cells

Zhe Guo, MD, PhD, Jing-Hang Jiang, MD, Jun Zhang, MD, Hao-Jie Yang, MD, Fu-Quan Yang, MD, Ya-Peng Qi, MD, Yan-Ping Zhong, MD, Jie Su, MD, Ri-Rong Yang, MD, PhD, Le-Qun Li, MD, PhD, and Bang-De Xiang, MD, PhD

Abstract: Cancer stem cells (CSCs) are thought to be responsible for tumor relapse and metastasis due to their abilities to self-renew, differentiate, and give rise to new tumors. Cyclooxygenase-2 (COX-2) is highly expressed in several kinds of CSCs, and it helps promote stem cell renewal, proliferation, and radioresistance. Whether and how COX-2 contributes to CSC migration and invasion is unclear. In this study, COX-2 was overexpressed in the CSC-like side population (SP) of the human hepatocellular carcinoma (HCC) cell line HCCLM3. COX-2 overexpression significantly enhanced migration and invasion of SP cells, while reducing expression of metastasis-related proteins PDCD4 and PTEN. Treating SP cells with the selective COX-2 inhibitor celecoxib down-regulated COX-2 and caused a dose-dependent reduction in cell migration and invasion, which was associated with up-regulation of PDCD4 and PTEN. These results suggest that COX-2 exerts pro-metastatic effects on SP cells, and that these effects are mediated at least partly through regulation of PDCD4 and PTEN expression. These results further suggest that celecoxib may be a promising anti-metastatic agent to reduce migration and invasion by hepatic CSCs.

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Abbreviations: 5-Fu = 5-fluorouracil, bFGF = basic fibroblast growth factor, CCK8 = Cell Counting Kit 8, CDDP = cisplatin, COX-2 = cyclooxygenase-2, CSCs = cancer stem cells, EGF = epidermal growth factor, FACS = fluorescence-activated cell sorting, FBS = fetal bovine serum, GAPDH = glyceraldehyde-3-phosphate dehydrogenase, HCC = hepatocellular carcinoma, MP = main population, PDCD4 = programmed cell death protein 4, PGE2 = prostaglandin E2, PTEN = phosphatase and tensin homolog deleted on chromosome ten, PTGS2 = prostaglandin-endoperoxide synthase 2, SP = side population.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies and the third most common cause of cancer-related death worldwide.[1] Surgical resection is considered the first-line treatment for patients with early-stage HCC,[2] and combining resection with adjuvant therapy can significantly prolong survival.[3] However, intrahepatic and distant metastasis after surgery remains common.[4] This highlights the need for better understanding of the molecular processes behind HCC invasion and metastasis in order to develop novel therapeutic strategies.

Evidence suggests that in many malignancies, including HCC, low-abundance cancer stem cells (CSCs) are responsible for tumor recurrence and metastasis. CSCs initiate and sustain tumor growth, translocating from the primary tumor to distant tissues, where they give rise to new tumors.[5,6] In previous work, our group identified several surface markers of hepatic CSCs (CD133, CD90, EpCAM) and showed that they may be linked to HCC tumor onset and/or progression.[7] We found EpCAM expression to be associated with shorter survival time, and CD90 expression to be associated with early HCC recurrence. These findings suggest that treatments specifically targeting CSCs may be useful and necessary for effectively treating HCC.

Research into CSCs in HCC and other cancers is hampered by the lack of standard markers for identifying and isolating CSCs. Studies have focused on side population (SP) cells as most likely CSC candidates.[8,9] A reproducible method for isolating SP cells based on Hoechst 33342 efflux has been described using fluorescence-activated cell sorting (FACS). Applying this approach to the HCC cell lines Huh7 and PLC/PRF/5 showed that the SP fraction makes up <1% of the total cell population.[10] These low-abundance SP cells

showed cancer stem-like properties both in culture and in vivo in transplant experiments.

Cyclooxygenase-2 (COX-2), also called prostaglandin-endoperoxide synthase 2 (PTGS2), is up-regulated in several kinds of CSCs,[11–16] and it may play an essential role in promoting stem cell renewal, proliferation, and radioresistance.[15–18] Given the documented influence of COX-2 on stem cell-like properties, which are now recognized as critical for tumor metastasis and recurrence, we wanted to examine in molecular detail whether and how COX-2 regulates invasion and metastasis by hepatic CSCs.

To do this, we up-regulated COX-2 expression in the HCC cell line HCCLM3 and examined the effects on migration and invasion by SP cells. We repeated the experiments in the presence of the COX-2 inhibitor celecoxib. This work provides the first molecular insights into how COX-2 may help drive SP migration and invasion.

MATERIALS AND METHODS

The study protocol was approved by the institutional review board of the Tumor Hospital of Guangxi Medical University, Nanning, China.

HCC Cell Lines and Cell Culture

The human HCC cell lines HCCLM3 and Huh7 were purchased from the Liver Cancer Institute of Zhongshan Hospital, Fudan University (Shanghai, China). Cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM; Gibco, California, USA) containing 10% (v/v) fetal bovine serum (FBS; Gibco) and $1\times$ penicillin/streptomycin (Gibco). Cultures were incubated at $37^\circ C$ in a humidified atmosphere containing 5% CO_2.

SP Cell Analysis

To identify and isolate SP fractions and main population (MP) fractions, cells were adjusted to a concentration of 1×10^6 cells/mL in high-glucose DMEM supplemented with 5% FBS, then incubated at $37^\circ C$ for 90 min with 6 µg/mL Hoechst 33342 dye (Sigma, Missouri, USA) alone or in the presence of 50 µM verapamil (Sigma). Cell suspensions were washed with cold phosphate-buffered saline (PBS), then centrifuged and resuspended in high-glucose DMEM supplemented with 5% FBS and 7-amino-actinomycin D (1 mg/L; Invitrogen, California, USA) in order to label dead cells. Cells were filtered through a 40-µm cell strainer (BD Falcon, califorina, USA) to obtain single-cell suspensions, maintained at $4^\circ C$ before analysis, then sorted by a FACSComp flow cytometer (BD). All experiments were done in triplicate.

Spheroid Formation Assay

Isolated SP and MP cells were suspended in serum-free DMEM/F12 medium containing B27 supplement (1:50; Invitrogen), 20 ng/mL epidermal growth factor (Invitrogen), and 20 ng/mL basic fibroblast growth factor (Invitrogen). Cells were cultured for 2 weeks at a density of 2000 cells/well in ultra-low-attachment 6-well plates (Corning, Massachusetts, USA). Spheroids were counted and photographed under a microscope (Nikon, Tokyo, Japan). All experiments were performed in triplicate.

Cell Viability Assay

To determine cell viability, the Cell Counting Kit 8 (CCK8; Dojindo, Kumamoto, Japan) was used according to the

manufacturer's instructions. Briefly, 5×10^3 cells/well were seeded into 96-well plates and incubated overnight. Drugs were applied at different concentrations and left on cells for 24 or 48 hr. Then CCK8 reagent was added to the cultures, they were incubated for 4 hr, and the plates were analyzed on a microplate reader (Thermo, Massachusetts, USA).

Tumorigenicity Assay

Varying numbers of SP and MP cells (5×10^3 to 5×10^5) were suspended in a 1:1 (v/v) mixture of 200 µL PBS and Matrigel (BD, USA). The suspensions were injected subcutaneously into 6-week-old male nude mice under anesthesia: each mouse received an injection of SP cells on the left side of the back, and an injection of MP cells on the right side of the back. Tumor growth was monitored weekly for 4 weeks.

Analysis of Putative Stem Cell Markers by Flow Cytometry

We analyzed SP and MP cells for expression of the putative CSC markers ABCG2, CD133, CD90, EpCAM, CD13, and CD44. Cells were first incubated with Hoechst 33342. Excess dye was removed by resuspending 1×10^6 cells/mL in high-glucose DMEM with 2% FBS. Cells were incubated in the dark at $4^\circ C$ for 30 min with fluorescence-conjugated monoclonal antibodies, including mouse anti-human ABCG2-PE (Miltenyi, Bergisch Gladbach, Germany), CD133-PE (Miltenyi), EpCAM-PE (Miltenyi), CD13-PE (BD), CD90-APC (BD), and CD44-FITC (BD). Isotype-matched mouse antibodies served as controls. Samples were analyzed using a FACSComp flow cytometer (BD) and Flowjo software (Treestar, California, USA). Representative data from 3 independent experiments are shown.

Migration and Invasion Assays

Cell migration was analyzed using Transwell cell culture chambers (8 µm pore size; Corning), and cell invasion was analyzed using the same Transwell chambers precoated with Matrigel (Corning). Briefly, cells (5×10^4 cells/chamber) were resuspended in FBS-free medium and added to the upper chamber of the Transwell plates. Medium containing 10% FBS was added to the well, and cultures were incubated for 24 hr. Cells that migrated and invaded to the lower surface were fixed with methanol for 5 min at room temperature, stained for 10 min with Giemsa solution (ZSGQ-BIO, Beijing, China), and counted at $200\times$ magnification from 5 fields of each filter to obtain an average. All experiments were done in triplicate.

Creation of a Stable HCCLM3 Cell Line Overexpressing COX-2

A lentiviral vector expressing green fluorescent protein (LV-GFP) was purchased from Shanghai Cancer Institute (Shanghai, China) and used to construct a lentiviral vector constitutively overexpressing COX-2 (LV-COX-2-GFP). This expression construct or the empty vector LV-GFP was transfected into HCCLM3 cells, which were then cultured for more than 2 weeks in the presence of 500 mg/mL G418 (Invitrogen). A stable cell line overexpressing COX-2 was obtained and named HCCLM3-COX-2. The corresponding negative control cell line was named HCCLM3-NC.

Quantitative Real-Time PCR

Total RNA was extracted from cells using TRIzol (Invitrogen) according to the manufacturer's instructions and used to

synthesize cDNA with the Prime Script RT Reagent Kit with gDNA Eraser (TaKaRa, Shiga, Japan). Then the cDNA was used as template in quantitative real-time PCR (qRT-PCR) with the SYBR Green/ROX Master Mix in a 7300 RT-PCR System (Applied Biosystems, California, USA). Expression of target genes was determined using the $2^{-\Delta\Delta Ct}$ method and normalized to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers to amplify specific gene sequences are shown in Table 1. All experiments were performed in triplicate.

Western Blotting

Cells were lysed with Radio Immunoprecipitation Assay lysis buffer containing 1% Phenylmethanesulfonyl fluoride and proteins were harvested. Protein concentrations were measured using the bicinchoninic acid protein assay (Beyotime, Shanghai, China). All proteins were resolved on 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked in 5% nonfat milk overnight at 4°C and incubated overnight at 4°C with antibodies against COX-2 (1:500; Abcam, Cambridge, UK), phosphatase and tensin homolog deleted on chromosome ten (PTEN) (1:1000; Abcam) or programmed cell death protein 4 (PDCD4) (1:1000; Cell Signaling Technology, Massachusetts, USA). Membranes were then incubated for 1 hr with the appropriate horseradish peroxidase-conjugated secondary antibody. After washing with Tris-Buffered Saline and Tween 20, proteins were detected by enhanced chemiluminescence (Thermo) and quantified using ImageLab 5.0 (BioRad, California, USA). Protein levels were normalized to the amount of GAPDH detected on the same blot. Representative data from 3 independent experiments are shown.

Quantification of Prostaglandin E2

Prostaglandin E2 (PGE2) concentration in culture medium was determined using an enzyme-linked immunosorbent assay kit following the manufacturer's instructions (Cayman Chemical, Michigan, USA). Briefly, 5×10^3 cells/well were seeded into 24-well plates and incubated for 24 hr with celecoxib at 12.5, 25, or 50 μM.

Statistical Analysis

All analyses were performed using SPSS 19.0 (IBM, New York, USA). Data were reported as mean ± standard deviation. Differences between means were assessed for significance using Student t test or 1-way analysis of variance. The threshold for significance was defined as a 2-sided $P < 0.05$.

RESULTS

Higher Proportion of SP Cells and COX-2 Expression in HCCLM3 Cells Than in Huh7 Cells

We used flow cytometry to examine the relative proportions of HCCLM3 and Huh7 cells that pumped out Hoechst 33342 dye. The SP gate was defined as the region of the FACS plot from which cells disappeared when cultures were exposed to the transport inhibitor verapamil. Based on this criterion, the proportion of SP cells was 16.9 ± 1.8% in HCCLM3 cultures and 1.0 ± 0.3% in Huh7 cultures ($P < 0.01$, Fig. 1A and B).

The HCCLM3 and Huh7 cell lines were then compared in terms of COX-2 expression in order to determine whether expression correlated with stem cell-like phenotype. COX-2 mRNA was 8.24-fold more abundant in HCCLM3 cells than in Huh7 cells ($P < 0.01$, Figure 1C) and immunoblotting showed significantly more COX-2 protein in HCCLM3 cells ($P < 0.01$, Figure 1D). This correlates with the previous observation that HCCLM3 cells display a much stronger stem cell-like phenotype than Huh7 cells.[19] In order to ensure sufficient numbers of SP cells for subsequent detailed analysis, we prepared SP and MP cells from the HCCLM3 cell line.

SP Subpopulation of HCCLM3 Cells Exhibits CSC-Like Properties

Self-renewal is a defining characteristic of CSCs, so we evaluated the self-renewal properties of HCCLM3 SP cells. SP cells formed larger, more compact and more numerous spheroids than MP cells (Figure 2A).

Resistance to conventional chemotherapeutics is another defining characteristic of CSCs, so we measured the resistance of SP and MP cells to 2 structurally and functionally unrelated drugs, 5-fluorouracil (5-Fu) and cisplatin (CDDP). SP cells were consistently more resistant to 5-Fu and CDDP than MP cells (Figure 2B).

To compare differentiation ability, we cultured sorted SP and MP cells separately for 1 week under the same conditions before staining with Hoechst 33342 and analyzing. SP cells differentiated into both SP and MP cells with an SP proportion of 28.5 ± 2.5%, whereas MP cells produced predominantly MP cells (Figure 2C).

To compare their tumorigenicity in vivo, SP and MP cells were injected into opposite sides of the back of nude mice, and tumorigenesis was monitored. Injections of as few as 5×10^3 SP cells initiated tumors in 1 of 5 mice, while 5×10^5 MP cells were required to cause tumors in 2 of 5 mice (Table 2). Tumor

TABLE 1. Primer Sequences to Measure Expression of Cancer Stem Cell-Associated Genes Using Quantitative Real-Time PCR

Gene	Accession No.	Sequence (5′→3′)
COX-2	NM_000963	F: CCAGCACTTCACGCATCAG
		R: GCTGTCTAGCCAGAGTTTCACC
PDCD4	NM_145341	F: GAGCGTGCAGATAATGACAAGGAAT
		R: GGATTTGACGGCTCCTCTACTGTTT
PTEN	NM_000314	F: TTAGCACTGGATACTCCTAGAGCAC
		R: AGCCTGCACACAATCTACAGTTC
GAPDH	NM_002046.3	F: CTGGGCTACACTGAGCACC
		R: AAGTGGTCGTTGAGGGCAATG

F = forward; R = reverse.
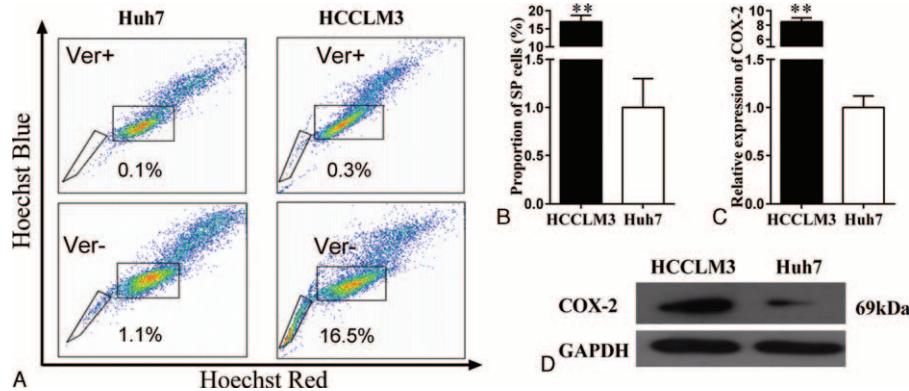
FIGURE 1. Side population (SP) cell fraction and cyclooxygenase-2 (COX-2) expression in the HCC cell lines Huh7 and HCCLM3. (A) Identification of SP and main population (MP) cells by flow cytometry. (B) The proportion of SP cells differed significantly between HCCLM3 and Huh7 lines. (C) Quantitative real-time PCR (qRT-PCR) showed a significantly higher level of COX-2 mRNA in HCCLM3 cells than in Huh7 cells. Gene expression was normalized to that of GAPDH. Data are shown as mean ± standard deviation. (D) Western blotting showed a higher level of COX-2 protein in HCCLM3 cells than in Huh7 cells. GAPDH served as a loading control. HCC = hepatocellular carcinoma. $^{**}P < 0.01$.

pathology was confirmed by histopathology. These results indicate that SP cells are much more tumorigenic, consistent with our recently accepted manuscript ''Side population in hepatocellular carcinoma HCCLM3 cells is enriched with stem-like cancer cells,'' to be published in Oncology Letters.

Next we examined the expression of various putative CSC markers in SP and MP cells (Figure 2D). Both cell types were nearly 100% positive for CD44 and did not express detectable amounts of CD133 or EpCAM. SP cells expressed significantly higher levels of the CSC marker ABCG2 than MP cells did ($35.8 \pm 4.1\%$ vs $11.2 \pm 2.7\%$), and the same was true for the markers CD90 ($7.0 \pm 0.8\%$ vs $3.5 \pm 1.0\%$) and CD13 ($81.9 \pm 1.6\%$ vs $63.3 \pm 0.9\%$) (all $P < 0.05$).

SP Cells Show Greater Migration and Invasion Ability Than MP Cells

Using a Transwell-based migration and invasion assay, we found SP cells to possess higher migration and invasion ability than MP cells (Figure 3).

COX-2 Is Highly Expressed in SP Cells

As expected, COX-2 mRNA was 17.49-fold more abundant in SP cells than in MP cells ($P < 0.01$, Figure 4A), and immunoblotting showed a similar result for COX-2 protein ($P < 0.01$, Figure 4B).

COX-2 Overexpression Promotes Chemoresistance, Migration, and Invasion of SP Cells

HCCLM3-COX-2 cultures contained a higher percentage of SP cells than HCCLM3-NC cultures ($19.9 \pm 1.4\%$ vs $17.3 \pm 0.6\%$, $P < 0.05$, Figure 5A). COX-2 mRNA was 2.4-fold more abundant in SP cells from HCCLM3-COX-2 than in SP cells from HCCLM3-NC, and similar results were observed at the protein level (both $P < 0.05$, Figure 5C and D).

SP cells from HCCLM3-COX-2 cultures were consistently more resistant to 5-Fu and CDDP than SP cells from HCCLM3-NC cultures (Figure 5B), and they contained significantly greater numbers of migrating and invading cells (both $P < 0.001$, Figure 5E and F).

## COX-2 Overexpression Down-Regulates PDCD4 or PTEN to Promote SP Migration and Invasion

Migration and invasion processes are complex and associated with alterations in metastasis-related proteins, including PDCD4[20] and PTEN.[21] We applied qRT-PCR and Western blotting to SP cells from HCCLM3-COX-2 and HCCLM3-NC cultures to examine whether COX-2 expression influences migration and invasion by affecting endogenous PDCD4 or PTEN expression. Higher COX-2 expression was associated with lower levels of PDCD4 and PTEN mRNA and protein (Figure 5C and D). These results suggest that changes in PDCD4 and PTEN expression help mediate the pro-metastatic effects of COX-2 on SP cells.

COX-2 Inhibitor Celecoxib Inhibits SP Cell Migration and Invasion

To confirm that COX-2 overexpression drives SP migration and invasion in our experimental system, we repeated the experiments in the presence of the COX-2 inhibitor celecoxib. Using a CCK8 assay, we found that celecoxib significantly inhibited SP cell proliferation in a dose- and time-dependent manner ($P < 0.01$, Figure 6A), and that concentrations of 12.5 and 25 μM had no obvious effect on cell proliferation. Using these concentrations in migration and invasion assays, we found that celecoxib significantly repressed both activities in a dose-dependent manner (Figure 6C and D).

COX-2 Inhibitor Celecoxib Inhibits SP Cell Migration and Invasion in Part by Reducing COX-2 Expression

Analyses using qRT-PCR and Western blotting to determine COX-2 expression in SP cells in the presence or absence of celecoxib showed that treatment with 50 μM celecoxib reduced COX-2 mRNA levels to $74.0 \pm 18.6\%$ of the level in the absence of inhibitor, and that treatment with 100 μM celecoxib reduced levels to $33.1 \pm 4.5\%$ of controls (Figure 6E). Similar dose-dependent decreases in COX-2 protein were observed by Western blotting (Figure 6F). These results suggest that celecoxib suppresses SP cell migration and invasion in part by inhibiting COX-2 expression.
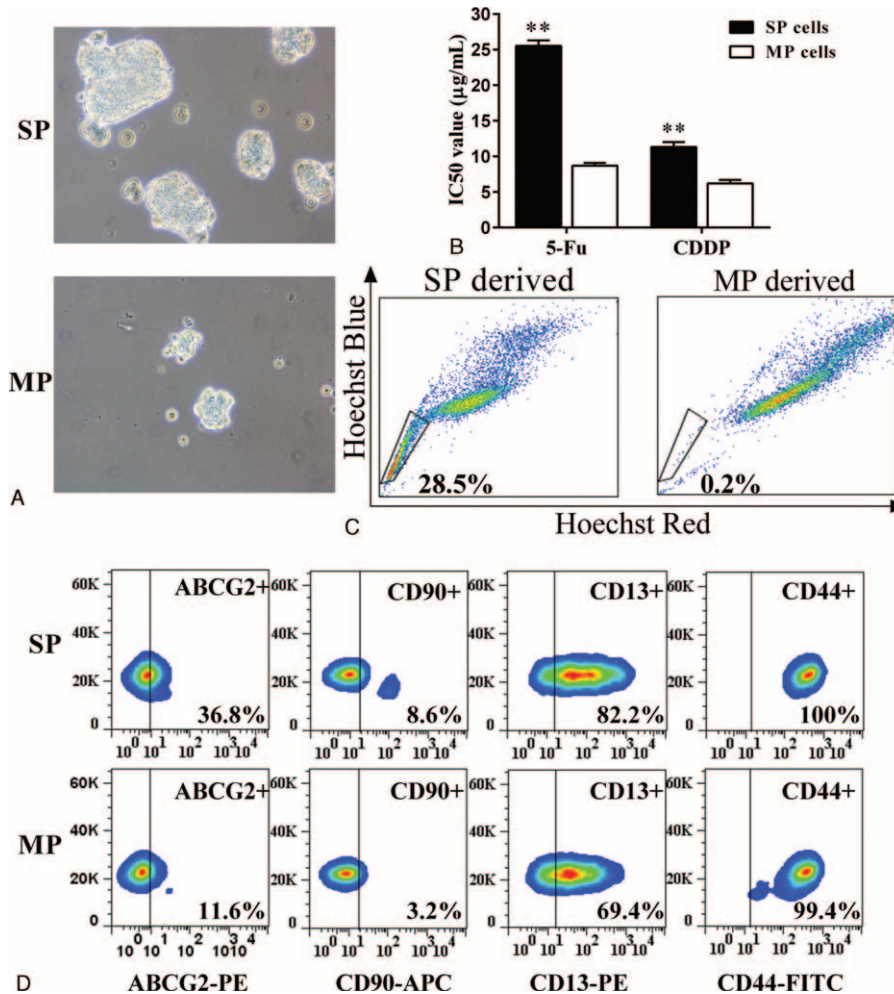
FIGURE 2. SP cells from the HCCLM3 cell line are enriched for cancer stem cell-like cells. (A) Spheroid formation by SP and MP cells after 2-week culture in modified medium was visualized by microscopy (magnification, ×200). SP cells formed larger and more compact spheroids than MP cells. (B) IC$_{50}$ values of SP and MP cells for 5-fluorouracil (5-Fu) or cisplatin (CDDP) were measured using the CCK8 assay. SP cells were consistently more resistant to 5-Fu and CDDP than MP cells. (C) Differentiation ability was compared between sorted SP and MP cells using Hoechst 33342 staining after 1 week in culture. SP cultures still contained 28.5% Hoechst 33342-negative cells, while MP cultures contained only 0.2% of such cells. (D) SP and MP cells were stained with fluorochrome-conjugated monoclonal antibodies or isotype antibodies and analyzed by flow cytometry. Nearly all SP and MP cells expressed CD44. Expression of ABCG2, CD90, and CD13 was significantly higher in SP cells than in MP cells. MP = main population; SP = side population. $^{**}P < 0.01$.

TABLE 2. Tumorigenicity of HCCLM3 Side Population (SP) and Main Population (MP) Cells Injected Into Nude Mice

No. of Cells Injected	No. of Animals Showing a Tumor at Injection Site*	
	SP Cells	**MP Cells**
5×10^3	1	0
5×10^4	3	0
5×10^5	5	2

* For each number of cells shown in the left column, 5 animals were injected with that number of SP cells on the left side of the back and with the same number of MP cells on the right side of the back.

The enzyme activity of COX-2 was tested by measuring levels of 1 of its main products, PGE2, in the culture medium. In the presence of 25 or 50 μM celecoxib, SP cells produced significantly less PGE2 than control cells (Figure 6B). This further supported the importance of COX-2 in hepatic CSC migration and invasion.

COX-2 Inhibitor Celecoxib Inhibits SP Cell Migration and Invasion in Part by Up-Regulating PDCD4 and PTEN

We used qRT-PCR and Western blotting to compare PDCD4 and PTEN expression in SP cells in the presence or absence of celecoxib. Treatment with celecoxib increased the levels of PDCD4 and PTEN mRNA (Figure 6E) and protein (Figure 6F) in a dose-dependent manner. These results suggest
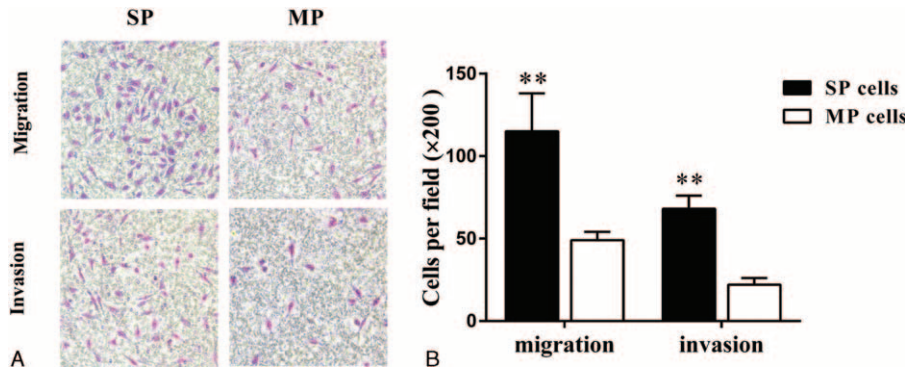
FIGURE 3. In a transwell assay, (A,B) SP cells from the HCCLM3 line showed significantly greater migration and invasion activity than MP cells. Magnification, 200 ×. Data are shown as mean ± SD. **$P < 0.01$.

that celecoxib may inhibit SP cell migration and invasion in part by up-regulating PDCD4 and PTEN.

DISCUSSION

The present study provides in vitro evidence that COX-2 helps drive the migration and invasion ability of HCC SP cells, and that it may do so in part by down-regulating PDCD4 and PTEN. Treating SP cells with the COX-2 inhibitor celecoxib reverses the pro-metastatic effects of COX-2 overexpression through at least 2 potential mechanisms: down-regulating COX-2 and up-regulating PDCD4 and PTEN expression. These insights provide valuable molecular clues into the processes that drive HCC recurrence and metastasis, significantly extending previous work hinting at a role for COX-2 in migration and invasion of various cancers, including HCC.[22]

Recent research increasingly implicates CSCs as the source of metastasis in HCC and other cancers. CSCs initiate and sustain tumor growth, translocating from the primary tumor to distant tissues, where they give rise to new tumors.[5,6] SP cells within tumors show CSC-like characteristics and can be a valuable system for understanding metastasis. In agreement with a previous report,[10] we show here that SP cells of the HCCLM3 cell line exhibit the CSC-like properties of high sphere-forming ability, strong resistance to 5-Fu and CDDP, and abundant surface expression of various CSCs markers, including CD90,[23] CD13,[24] and ABCG2.[25] Our findings are consistent with the idea that CSCs are enriched in the SP subpopulation and that SP cells drive HCC invasion and metastasis, as proposed for several other cancers.[26,27]

FIGURE 4. COX-2 expression in SP and MP cells from the HCCLM3 line. (A) qRT-PCR showed a significantly higher level of COX-2 mRNA in SP cells than in MP cells. (B) Western blotting showed higher levels of COX-2 protein in SP cells than in MP cells. COX-2 = cyclooxygenase-2; MP = main population; qRT-PCR = quantitative real-time PCR; SP = side population. **$P < 0.01$.

We found COX-2 to be expressed at higher levels in the HCCLM3 cell line than in the Huh7 cell line, which may help to explain the much stronger stem cell-like phenotype of the HCCLM3 line.[19] We found that COX-2 is highly expressed in SP cells. This result extends the list of CSCs in which COX-2 is overexpressed, which includes CSCs in glioblastoma,[11,16] osteosarcoma,[12] breast cancer,[13] nasopharyngeal cancer,[14] and medulloblastoma.[15] We demonstrated that COX-2 overexpression increased the proportion of SP cells in HCC cultures, and that this overexpression increased chemoresistance, migration, and invasion by SP cells. These results are consistent with reports that COX-2 up-regulation enhances stem cell-like phenotype of nasopharyngeal CSCs,[14] radioresistance of medulloblastoma and glioblastoma stem-like cells,[15,16] self-renewal of breast CSCs,[17] and proliferation of glioma stem cells.[18] It would be intriguing to knock down COX-2 expression to see whether it weakens the stem cell-like phenotype. Indeed, studies in nasopharyngeal carcinoma cell lines CNE1 and CNE2 suggest that knocking down COX-2 expression using short hairpin RNA reduces the proportion of SP cells and weakens other CSC-like characteristics.[14] These findings suggest that COX-2 is an essential regulator of CSCs.

Metastasis is a multi-stage, complex process involving multiple proteins. PDCD4 is a tumor suppressor that inhibits metastasis of cancer cells, including HCC.[20] It works by inhibiting the expression and/or activity of invasion-related proteins such as MAP4K1[28] and Akt[29] and by up-regulating metastasis-suppressor genes such as TIMP2[30] and E-cadherin.[31] PTEN is a phosphoinositide phosphatase originally identified as a multifunctional tumor suppressor that is frequently lost in various cancers, including HCC.[32] PTEN can affect the Akt and ERK signaling pathways, which are associated with cell proliferation, differentiation, and cell migration and invasion in HCC.[21] Consistent with this, we found that COX-2 significantly enhanced migration and invasion of SP cells in part by down-regulating PDCD4 and PTEN (Figure 5B and C). These findings in HCC are consistent with a previous study in HCC SP cells suggesting that siRNA-mediated silencing of PDCD4 and PTEN increases cell migration and invasion.[33] Further research is needed to elucidate exactly how COX-2 influences PDCD4 and PTEN expression.

Our findings suggest the potential of celecoxib for selectively killing CSCs in order to reduce the risk of recurrence and metastasis, such as in HCC patients following curative treatment. Celecoxib has already been approved as a chemopreventive drug that can significantly reduce the risk of cancer in
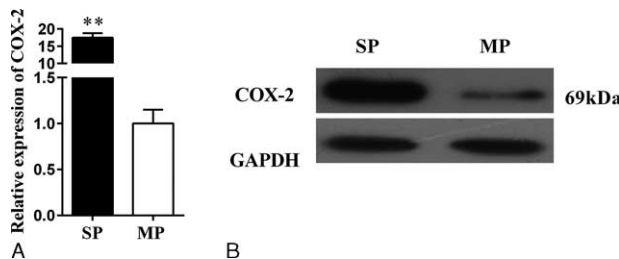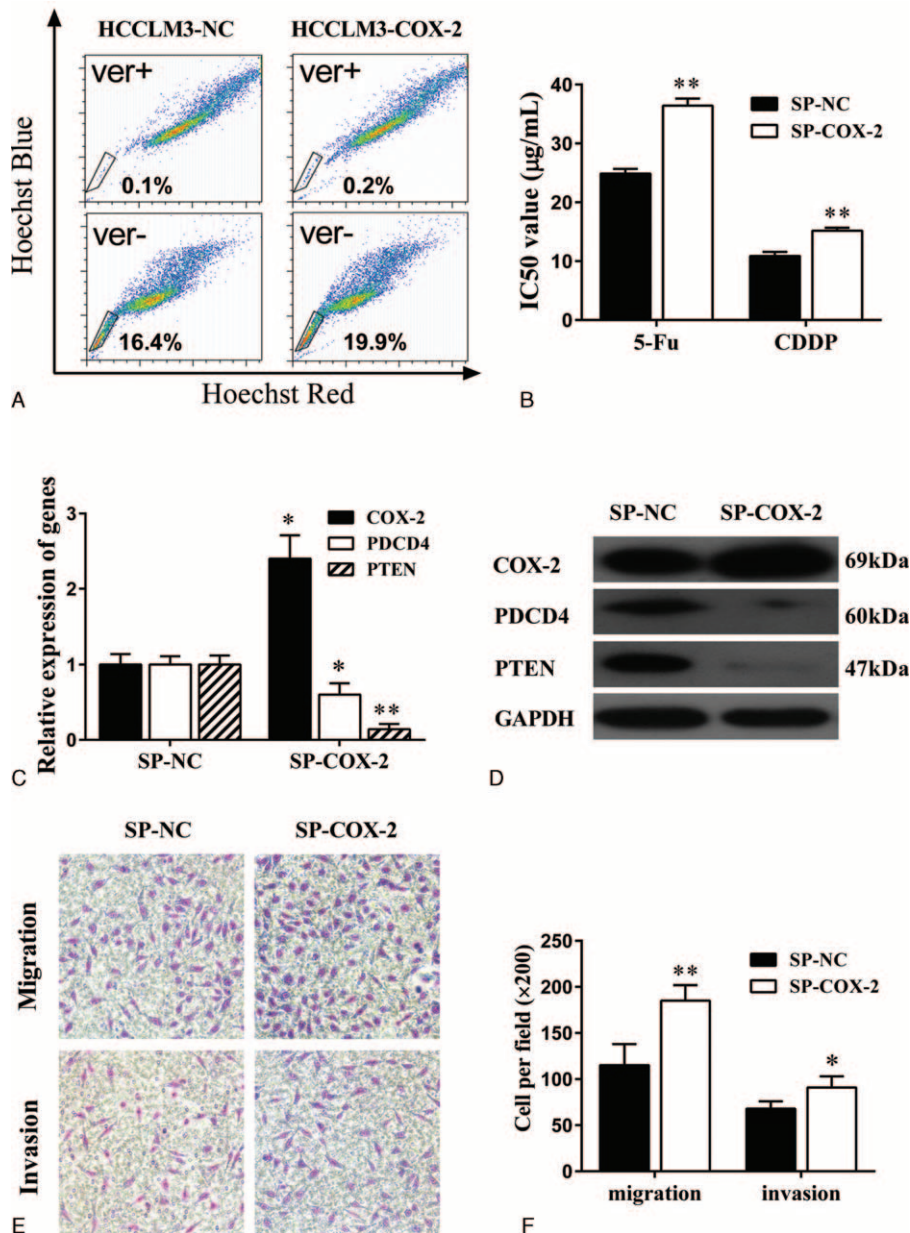
FIGURE 5. COX-2 overexpression enhanced migration and invasion of SP cells in part by down-regulating PDCD4 and PTEN. A stable HCCLM3 cell line constitutively overexpressing COX-2 (HCCLM3-COX-2) was constructed by transducing HCCLM3 cells with a lentiviral COX-2 expression plasmid also encoding green fluorescent protein (LV-COX-2-GFP). In parallel, a negative control cell line (HCCLM3-NC) was prepared by transducing HCCLM3 cells with empty vector (LV-GFP). (A) Flow cytometry showed that SP proportions were significantly higher in the HCCLM3-COX-2 line than in the HCCLM3-NC line. (B) SP cells from HCCLM3-COX-2 were consistently more resistant to 5-Fu and CDDP than SP cells from HCCLM3-NC. (C, D) qRT-PCR and Western blotting showed that COX-2 overexpression was associated with down-regulation of PDCD4 and PTEN mRNA and protein. (E, F) COX-2 overexpression in SP cells increased cell migration and invasion. Magnification, ×200. COX-2 = cyclooxygenase-2; MP = main population; qRT-PCR = quantitative real-time PCR; SP = side population. $^*P < 0.05$, $^{**}P < 0.01$.

the colon,[34] lungs,[35] breasts,[36] and ovaries.[37] Previous work suggests that the antineoplastic effects of celecoxib are due to inhibition of cell proliferation and angiogenesis, stimulation of apoptosis, and induction of an immune response.[38] Celecoxib can also inhibit sphere formation by colorectal CSCs[39]; reduce the self-renewal ability and increase apoptosis in glioma stem cells[18]; sensitize various CSCs to radiotherapy, including glioblastoma,[16] medulloblastoma,[15,40] melanoma, and oral

carcinoma[41]; and suppress cancer stemness and progression of HCC.[42] We found that celecoxib significantly suppressed SP cell migration and invasion. The present study is the first to provide direct evidence that celecoxib can exert antineoplastic effects by acting on SP cells of tumors. Several studies have indicated that celecoxib may exert its anticancer effect in part by decreasing expression of COX-2 and production of PGE2.[43,44] Here we extend those studies by providing evidence
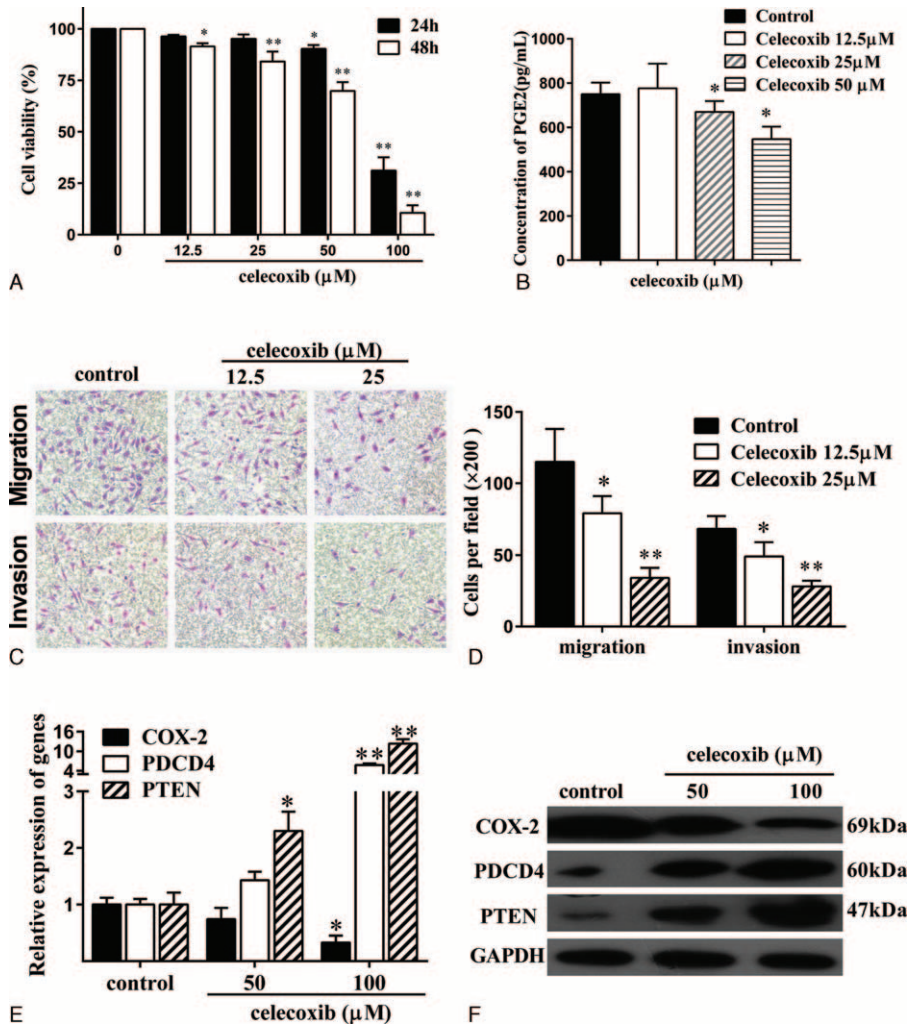
FIGURE 6. The selective COX-2 inhibitor celecoxib inhibited SP cell migration and invasion and may exert these effects by down-regulating COX-2 and up-regulating PTEN and PDCD4. (A) Celecoxib significantly inhibited SP cell proliferation in a dose- and time-dependent manner. (B) Celecoxib significantly inhibited prostaglandin E2 (PGE2) production in SP cells. (C, D) Celecoxib significantly suppressed SP cell migration and invasion in a dose-dependent manner. Magnification, ×200. (E, F) Analysis by qRT-PCR and Western blotting revealed that celecoxib treatment significantly decreased expression of COX-2 mRNA and protein, while increasing expression of PDCD4 and PTEN mRNA and protein in a dose-dependent manner. COX-2 = cyclooxygenase-2; MP = main population; qRT-PCR = quantitative real-time PCR; SP = side population. $^{*}P < 0.05$, $^{**}P < 0.01$.

that celecoxib inhibits hepatic SP cell migration and invasion by down-regulating COX-2 expression, inhibiting COX-2 enzyme activity, and up-regulating PDCD4 and PTEN. These findings not only highlight the therapeutic potential of celecoxib for acting specifically on CSCs, which are resistant to most commonly used chemotherapeutics, but they also further support the importance of COX-2 in SP cell migration and invasion.

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