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CXCL11 promotes self-renewal and tumorigenicity of α2δ1$^+$ liver tumor-initiating cells through CXCR3/ERK1/2 signaling

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ABSTRACT

Tumor-initiating cells (TICs), which are responsible for sustaining tumor growth and recurrence, rely on several regulatory factors. However, the mechanism of inflammation-related molecules in the acquisition and maintenance of TIC properties in hepatocellular carcinoma (HCC) remains elusive. We previously demonstrated that the voltage-gated calcium channel α2δ1 subunit is a functional surface marker of HCC TICs. Here, we found that the expression of an inflammation-related molecule C-X-C motif chemokine 11 (CXCL11) was significantly upregulated in α2δ1$^+$ HCC TICs and that CXCL11 induced the expression of stem cell-related genes, such as *BMI1*, *NANOG*, *MDR1*, *ABCG2*, and *CACNA2D1*. Furthermore, CXCL11 could promote the acquisition and maintenance of self-renewal, tumorigenic, and chemoresistance properties of α2δ1$^+$ HCC TICs by activating the extracellular signal-regulated kinase (ERK1/2) through its affinity receptor CXCR3. Collectively, our results suggest that CXCL11 may positively regulate the stemness of α2δ1$^+$ HCC TICs via ERK1/2 activation through an autocrine signaling pathway.

1. Introduction

Hepatocellular carcinoma (HCC), the fifth most commonly diagnosed malignancy, is one of the deadliest cancers associated with chemoresistance and high frequency of tumor relapse and metastasis [1,2]. HCC is partly associated with the existence of tumor-initiating cells (TICs) or cancer stem cells (CSCs), which possess stem cell-like properties, including the ability to undergo self-renewal and differentiation [3,4]. These cells may be characterized by the expression of their cell surface markers, such as α2δ1 (isoform 5), CD13, CD133, CD90, or epithelial cell adhesion molecule (EpCAM) [5–9]. The properties of TICs may be regulated by several factors, such as signaling pathways, epigenetic alterations, and tumor microenvironment niche.

Recent studies have indicated that many inflammation-related molecules are risk factors associated with the development of HCC, and these molecules may be engaged in the acquisition and subsequent maintenance of TIC properties [10,11]. For examples, IL-6, TGF-β1, and cathepsin S can promote stem cell-like properties of HCC either through paracrine or autocrine signaling [12–14]. However, the identification of new inflammation-related molecules involved in the regulation of HCC TICs is needed for a better management of HCC.

C-X-C motif chemokine ligand 11 (CXCL11) or interferon (IFN)-inducible T cell chemoattractant (I-TAC) belongs to a superfamily of chemotactic cytokines and participates in inflammatory reactions by selectively recruiting the activated T cells to inflammation sites [15]. Furthermore, it was shown that CXCL11 was associated with tumor progression and prognosis [16,17]. However, its involvement in the regulation of TICs, especially HCC TICs, is yet unclear. On the basis of our earlier findings [5,18], we analyzed the differences in gene expression between the non-tumorigenic Hep-11 cells and TIC-enriched Hep-12 cells and found that CXCL11 was overexpressed in Hep-12 cells, indicative of its participation in the maintenance of the stem cell-like properties of HCC α2δ1$^+$ TICs. In the present study, we confirmed that CXCL11 expression was significantly upregulated in HCC α2δ1$^+$ TICs and that it played a crucial role in the acquirement and subsequent maintenance of self-renewal and tumorigenic abilities of HCC α2δ1$^+$ TICs through the CXCL11/C-X-C motif chemokine receptor 3 (CXCR3)/extracellular signal-regulated kinase 1/2 (ERK1/2) signaling axis via autocrine signaling.

2. Materials and methods

2.1. Cell lines and cell culture

The HCC cell lines HepG2 and PLC/PRF/5 were purchased from the American Type Culture Collection (ATCC, Manassas, VA), while HuH-

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7 cell line was obtained from the Japan Society for the Promotion of Science (Tokyo, Japan). Hep-11 and Hep-12 cell lines were described in our earlier report [18]. All cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Grand Island, NY, USA) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. RNA extraction and quantitative reverse transcription - polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using RNeasy® Mini Kit according to the manufacturer's instruction (QIAGEN, Valencia, CA, USA). For mRNA detection, cDNAs were synthesized from 2 μg of total RNA using oligo-d $(T)_{15}$ primers and Moloney murine leukemia virus reverse transcriptase (MMLV; Invitrogen, Carlsbad, CA, USA). The cDNA products were used as templates for qRT-PCR using SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) on Applied Biosystems 7500 real-time PCR system. Sequences of all primers are listed in Supplemental Table 1. Results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level for mRNA measurement. Fold difference of target mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method where $\Delta C_t = C_t$ (target) $- C_t$ (reference).

2.3. Protein extraction and western blot analysis

Proteins were extracted from cells using radioimmunoprecipitation assay buffer (Suolaibo Biotechnology Co. Ltd, Shanghai, China) supplemented with complete protease and phosphatase inhibitor cocktail (Roche, Mannheim, Germany). Proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gels and transferred by electroblotting onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, CA). The PVDF membranes were probed with primary antibodies listed in Supplemental Table 2, and incubated with a secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Signals were detected with Immobilon™ Western Chemiluminescent HRP substrate (Millipore).

2.4. Vector construction, cell transfection, and stable cell line establishment

The coding sequence (CDS) of human *CXCL11* gene with restrictive endonuclease sites *Bam*HI and *Xho*I at the 5′-end and 3′-end, respectively, was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and subsequently cloned into the lentivirus shuttle vector plenti6 (Invitrogen). For the knockdown of *CXCL11* and *CXCR3* expression, short-hairpin RNAs (shRNAs) against *CXCL11* and *CXCR3* were designed and cloned into the lentiviral vector pSIH1-puro. The sequences of human *CXCL11*-specific 21-mer shRNAs were 5′-GCTGTGATATTGT GTGCTACA-3′ (shRNA1) and 5′-GCCTAAATCCCAAATCGAAGC-3′ (shRNA2). The sequences of human CXCR3-specific 21-mer shRNAs were 5′-GAGAACTTCAGCTCTTCCTAT-3′ (shRNA1) and 5′-—CCCTCTT CAACATCAACTTCT-3′ (shRNA2). Scrambled shRNA (sequence: CGAG AAGCGCGATCACATG) was used as a negative control. Lentiviral constructs were transfected with ViraPower Packaging Mix (Invitrogen) into 293FT cells to generate lentivirus. Cells infected with virus were selected using 5 μg/mL of blasticidin (Invitrogen) or 2 μg/mL of puromycin (Thermo Fisher Scientific). The pool of antibiotic-resistant cells was used for subsequent assays.

2.5. Soft agar assay

About 1,000 cells were suspended in 0.5 mL of 0.3% (w/v) Noble agar (Difco, Detroit, MI) culture medium and seeded in a 24-well plate with solidified basal layer agar (0.5 mL of 0.5% agar per well; four wells per group). After 2–3 weeks, colonies larger than 50 μm were counted and images were recorded with a stereomicroscope (Olympus, Tokyo, Japan).

2.6. Spheroid formation assay

About 100 dissociated cells were seeded in each well of Ultra-Low attachment 96-well plates (Corning Incorporated Life Sciences, Acton, MA) and cultured in Dulbecco's modified Eagle's medium/F12 (Invitrogen) supplemented with 1% methylcellulose (Sigma-Aldrich, St Louis, MO), 20 ng/mL of epidermal growth factor, 20 ng/mL of basic fibroblast growth factor, 10 ng/mL of hepatocyte growth factor (HGF; PeproTech, Rocky Hill, NJ), and 1 × B27 (Invitrogen) in a humidified atmosphere of 5% CO_2 at 37 °C. After 2–3 weeks, the spheres larger than 100 μm were counted under a stereomicroscope (Olympus, Tokyo, Japan).

2.7. Colony formation assays

About 500 transfected cells were seeded in six-well plates and cultured in RPMI-1640 medium supplemented with 10% FBS at 37 °C in a humidified incubator. After 2 weeks, colonies were fixed with 100% methanol for 30 s and subsequently stained with 0.1% crystal violet for 5 min, washed with 1 × phosphate-buffered saline (PBS), and photographed. ImageJ software was used for quantification of colonies.

2.8. Cell viability assay

The viability and chemosensitivity of cells were determined using Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). Briefly, transfected cells were seeded in 96-well plates at a density of 5×10^3 cells/well with 100 μL of complete medium and incubated with different concentrations of doxorubicin (0, 0.2, 0.5, 1, 2, and 5 μM).

At indicated time points, 10 μL of CCK-8 solution was added to each well and incubated at 37 °C for 4 h. Optical density (OD) values were measured at 450 nm using a microplate reader (iMark, Bio-Rad, Hercules, CA, USA). The mean of three to six replicates for each drug concentration was used to calculate inhibition rate. The half maximal inhibitory concentration (IC50) was calculated by GraphPad Prism 6.0 software.

2.9. Flow cytometry

Cells were dispersed and resuspended into sterile 1 × PBS buffer and labeled with either monclonal mouse anti−α2δ1 (isoform 5) antibody (Mab1B50-1) [5] after pre-labeling with Lightning-Link™ Fluorescein Labeling Kit (Innova Biosciences Ltd, Cambridge, UK) or isotype-matched mouse IgG3 at 4 °C for 40 min. The labeled cells were washed twice with sterile 1 × PBS buffer and sorted with FACSAria II flow cytometer (BD Biosciences, San Jose, CA).

2.10. Tumorigenicity assay in NOD/SCID mice

For assessment of tumor formation abilities, cells at indicated numbers were suspended in 100 μL of RPMI-1640 medium and Matrigel (BD Biosciences, Bedford, MA, USA) and subcutaneously transplanted into the armpit of 4- to 6-week-old female non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (NOD.CB17-Prkdc^{scid}/NcrCrl, Vital River, Beijing, China). Tumor formation was weekly monitored. After 7–8 weeks, mice were sacrificed and the tumors were dissected. All animal protocols were performed after the approval of the Peking University Cancer Hospital Animal Care and Use Committee.

2.11. Statistical analysis

All data were analyzed using GraphPad Prism 6.0 software. Differences between each group were assessed by *t*-test unless otherwise

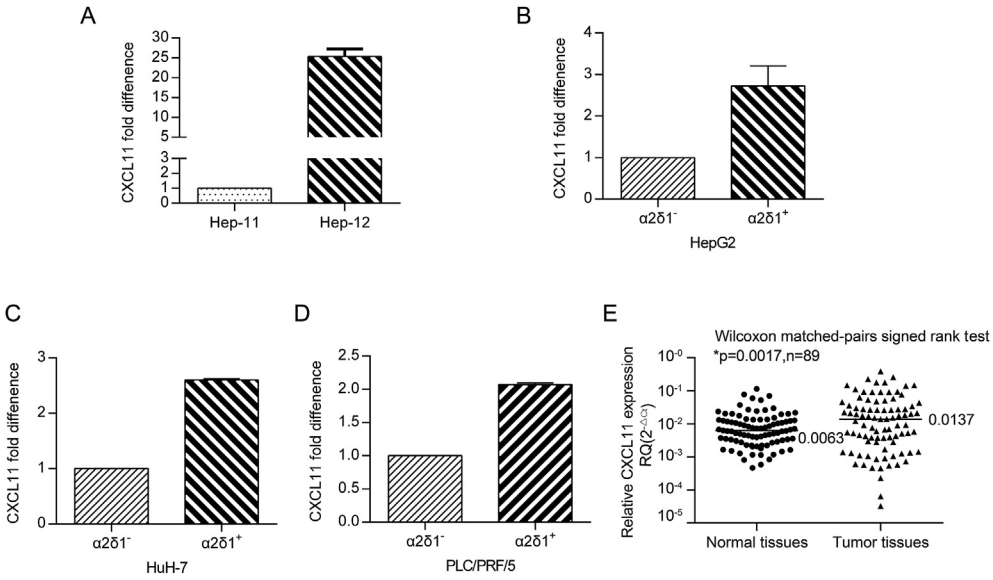

Fig. 1. The expression of *CXCL11* is upregulated in α2δ1$^+$ HCC TICs and HCC tissues. (A) The expression of *CXCL11* in TIC-enriched Hep-12 cells and non-tumorigenic Hep-11 cells was determined by qRT-PCR. Data are presented as fold difference over Hep-11 cells, which was defined as 1 (calibrator). Error bars indicate S.D. (B)–(D) The expression level of *CXCL11* in α2δ1$^+$ TICs sorted from HepG2, HuH-7, and PLC/PRF/5 HCC cell lines was compared with respective α2δ1$^-$ subpopulations. Data are presented as fold difference of α2δ1$^+$ TICs over respective negative counterparts, which was defined as 1 (calibrator). Error bars indicate S.D. (E) The expression of *CXCL11* in HCC tissues and paired normal tissues adjacent to tumors was analyzed by qRT-PCR. Horizon lines indicate the median values of each group.

specified. A value of $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. The expression of CXCL11 is upregulated in α2δ1$^+$ HCC TICs and HCC tissues

Our previous unpublished RNA-seq data indicated that *CXCL11* was upregulated significantly in the TIC-enriched Hep-12 cells compared with the non-tumorigenic Hep-11 cells. To confirm this result, we analyzed the expression of *CXCL11* in the two cells lines using qRT-PCR. Consistent with the RNA-seq data, the expression of *CXCL11* mRNA was upregulated as many as about 25 folds in Hep-12 cells compared with that in Hep-11 cells (Fig. 1A). To validate the higher expression of *CXCL11* mRNA in HCC TICs, we detected *CXCL11* expression in α2δ1$^+$ HCC TICs sorted from HepG2, HuH-7, and PLC/PRF/5 HCC cell lines. As shown in Fig. 1B–D, the purified α2δ1$^+$ HCC TICs expressed significantly higher *CXCL11* level than their negative counterparts. The analysis of *CXCL11* expression in 89 paired primary HCC tissues also revealed the remarkably upregulated expression of *CXCL11* in the tumor tissues than in their adjacent normal tissues (Fig. 1E). These data confirm that *CXCL11* expression was upregulated in α2δ1$^+$ HCC TICs as well as in primary HCC tissues.

3.2. CXCL11 upregulates the expression of stem cell-related genes

To detect the effect of CXCL11 expression on the levels of stem cell-related genes, we knocked down the expression of CXCL11 in α2δ1$^+$ HCC TIC-enriched Hep-12 cells and analyzed the expression of stem cell-related molecules, including BMI1, NANOG, MDR1, ABCG2, and CACNA2D1 by Western blot. The knockdown of CXCL11 expression in Hep-12 cells remarkably downregulated the expression of these stem cell-related molecules (Fig. 2A and B). To verify these results contradictorily, we overexpressed CXCL11 in HuH-7 and PLC/PRF/5 cells and tested the expression of stem cell-related genes. The results (Fig. 2C–F) showed that the overexpression of CXCL11 distinctly increased the levels of these stem-related genes. Collectively, these data suggest that CXCL11 positively regulated the expression of stem cell-related genes.

3.3. CXCL11 expression is sufficient and necessary for the acquisition and maintenance of self-renewal property of α2δ1$^+$ HCC TICs

The above results prompted us to investigate whether CXCL11 plays any role in the acquisition and/or maintenance of self-renewal property of α2δ1$^+$ HCC TICs. We performed a sphere formation assay to evaluate the effects of CXCL11 on α2δ1$^+$ HCC TICs and found that the downregulation of CXCL11 expression significantly decreased the spheroid formation ability of TIC-enriched Hep-12 cells (Fig. 3A and B). Similar inhibitory effects on sphere formation property were observed after the infection of α2δ1$^+$ HCC TICs sorted from HuH-7 and PLC/PRF/5 cells with *CXCL11* shRNA lentiviruses (Fig. 3C–F). These results suggest that CXCL11 was necessary for the maintenance of the self-renewal capability of α2δ1$^+$ HCC TICs.

To address whether CXCL11 is sufficient to induce TIC-like phenotypes, we induced ectopic expression of CXCL11 in HuH-7 and PLC/PRF/5 cells and performed the spheroid formation assay to investigate whether these cells could acquire high *in vitro* self-renewal ability. As shown in Fig. 3G–J, the ectopic expression of CXCL11 in HuH-7 and PLC/PRF/5 cells remarkably enhanced their spheroid formation efficiency. Furthermore, these spheroids could be clonally expanded in subsequent serial propagation with increased efficiency after their dissociation into single cells.

Taken together, these data demonstrate that CXCL11 was sufficient to reprogram HCC cells into TIC-like cells with high self-renewal ability and was necessary for the maintenance of self-renewal property of α2δ1$^+$ HCC TICs.

3.4. CXCL11 promotes tumorigenicity of HCC TICs

To validate whether CXCL11 is sufficient and necessary for the acquisition and maintenance of tumorigenicity in HCC TICs, the α2δ1$^+$ HCC TIC-enriched Hep-12 cells with CXCL11 expression knockdown were assayed for their growth abilities in soft agar and tumorigenicity in NOD/SCID mice. Soft agar assay revealed that the downregulation of CXCL11 expression significantly inhibited the colony formation ability of Hep-12 cells as compared with the control cells (Fig. 4A). In addition, CXCL11-kncokdown Hep-12 cells showed a conspicuously reduced ability of tumor initiation (Fig. 4B), consistent with the results of the *in vitro* soft agar assay. These data demonstrate that CXCL11 was necessary for the maintenance of the tumor formation property of α2δ1$^+$ HCC TICs.

Furthermore, the ectopic expression of CXCL11 in HuH-7 and PLC/PRF/5 cells significantly enhanced their ability to initiate hepatosphere formation *in vitro* after cultivation in soft agar medium (Fig. 4C and D). These HCC cells overexpressing CXCL11 exhibited an enhanced ability to initiate tumors upon subcutaneous injection of as few as 100 cells in

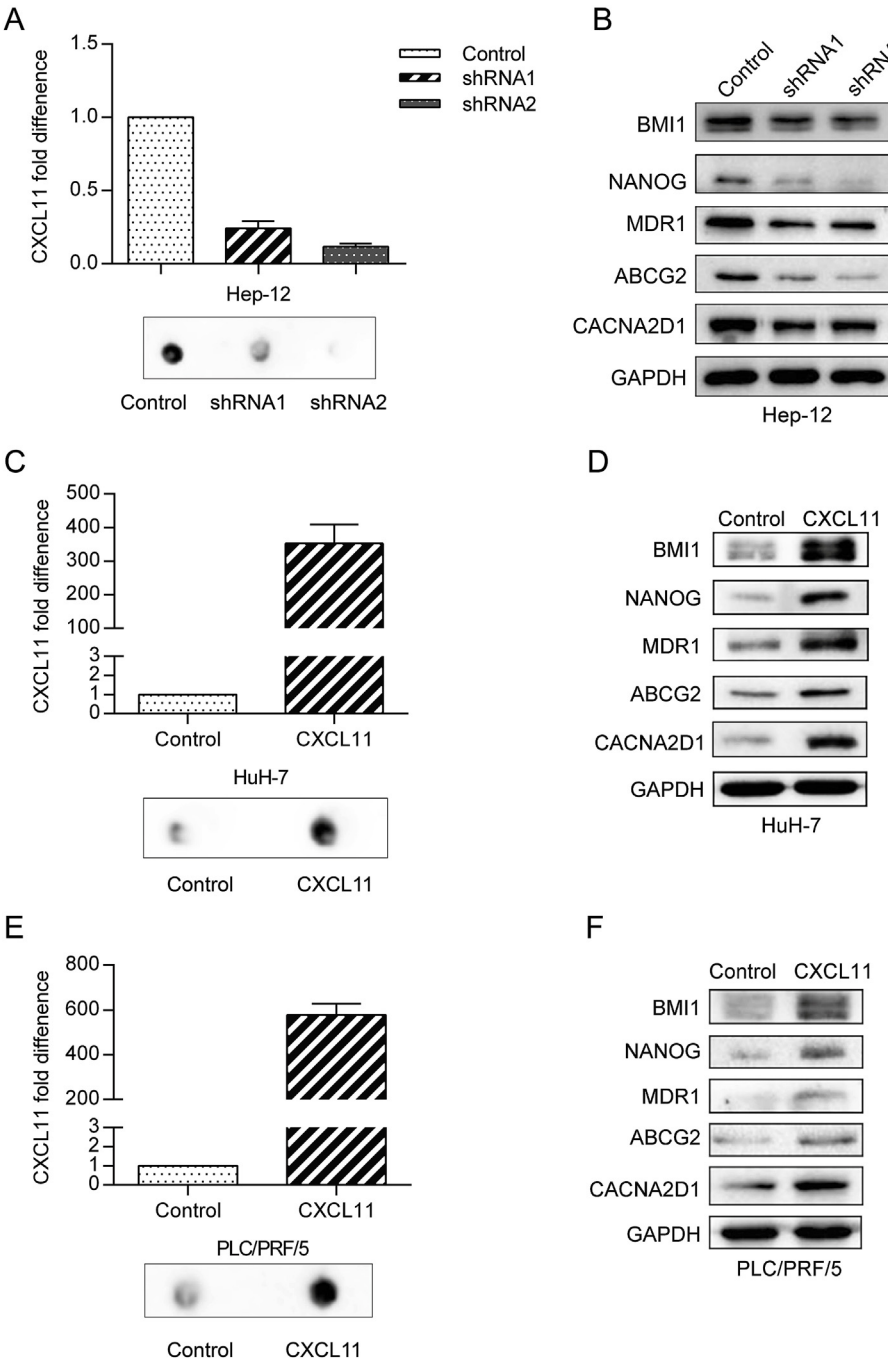

Fig. 2. CXCL11 upregulates the expression of stem cell-related genes. (A) The effects of shRNA-mediated knockdown of CXCL11 on Hep-12 cells were analyzed by qRT-PCR and dot blot at both mRNA and protein levels, respectively. (B) Western blot analysis of the expression of indicated stem cell-related genes in Hep-12 cells infected with CXCL11 shRNAs or control lentivirus. (C) The expression of CXCL11 in HuH-7 cells and their culture medium following treatment with CXCL11 or control lentivirus were determined by qRT-PCR and dot blot at both mRNA and protein levels, respectively. (D) The expression of stem cell-related genes was compared between HuH-7 cells overexpressing CXCL11 and control cells transfected with vector alone using western blot assays. (E) The expression of CXCL11 in PLC/PRF/5 cells and culture medium following infection with CXCL11 or control lentivirus were determined by qRT-PCR and dot blot at both mRNA and protein levels, respectively. (F) Western blot results showing the expression of stem cell-related genes in PLC/PRF/5 cells overexpressing CXCL11.

NOD/SCID mice as compared with control group (Fig. 4E and F), indicating that CXCL11 was sufficient for the acquisition of tumor formation ability in TIC-like cells. Therefore, CXCL11 promoted tumorigenicity of HCC TICs.

3.5. CXCL11 enhances the proliferation and drug resistance ability of HCC TICs

TICs play an important role in the continuous growth and chemoresistance of malignant tumors. To assess the effects of CXCL11 on the proliferation and drug resistance properties of HCC TICs, we performed colony formation and CCK-8 assays. As shown in Fig. 5A–C, the downregulation of CXCL11 expression not only remarkably attenuated the proliferation ability but also decreased the chemoresistance to common chemotherapeutic agents, such as doxorubicin (ADM) in TIC-enriched Hep-12 cells.

The overexpression of CXCL11 in HuH-7 and PLC/PRF/5 cells led to a significant increase in their proliferation ability and strengthened their resistance to doxorubicin, resulting in higher colony formation rate and IC50 values as compared with the control cells ($P < 0.05$, Fig. 5D–I). These results show that CXCL11 could reprogram HuH-7 and PLC/PRF/5 cells into TIC-like cells to enhance their growth and chemoresistance properties. Therefore, CXCL11 regulated the proliferation and drug resistance ability of HCC TICs.

3.6. CXCL11 promotes stem cell-like properties of HCC cells through its receptor CXCR3 and ERK1/2 signaling

As inflammation factors may exert their functions through an autocrine signaling pathway, we investigated the signaling pathways

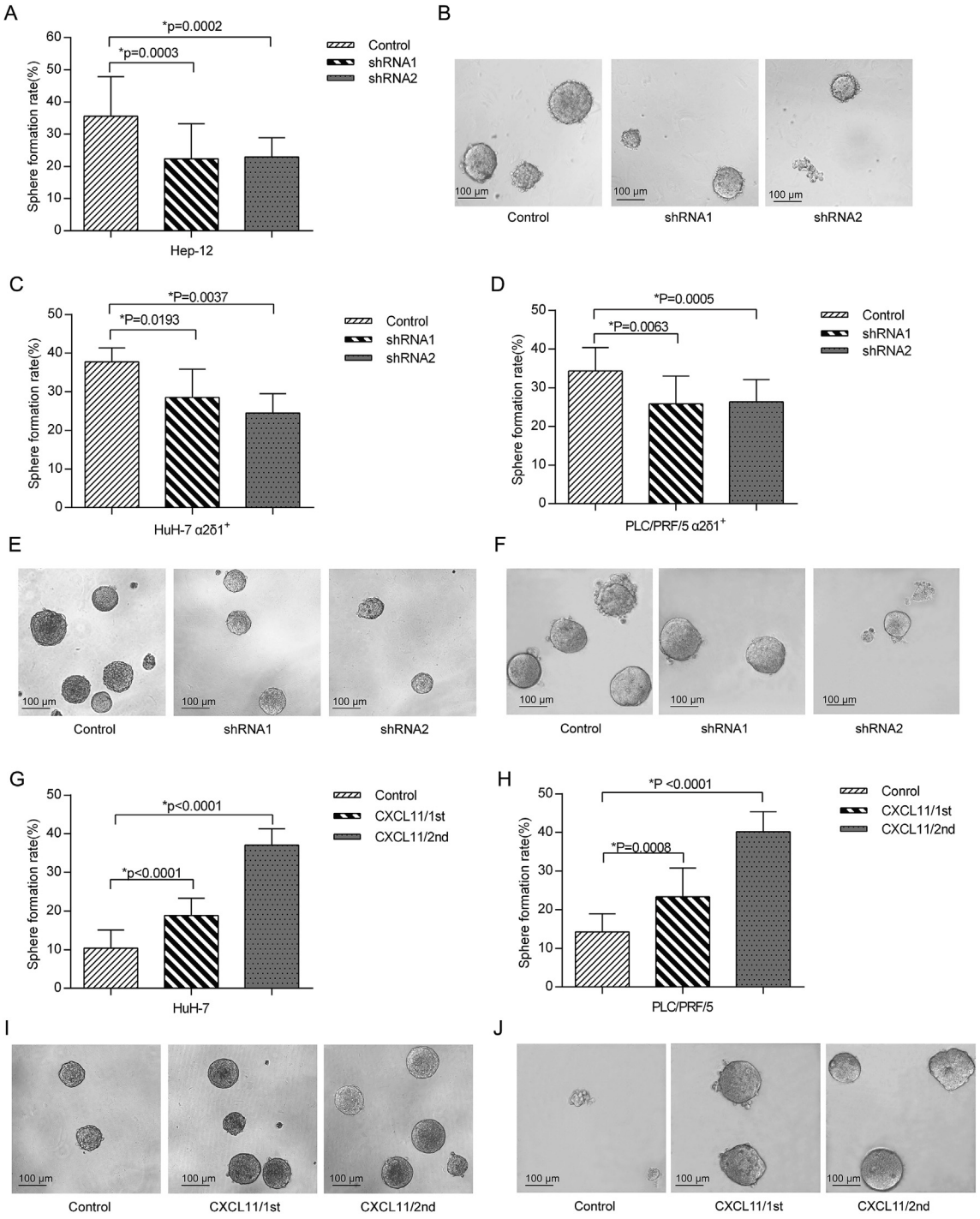

Fig. 3. CXCL11 expression is sufficient and necessary for the acquisition and maintenance of self-renewal properties of α2δ1$^+$ HCC TICs. (A)–(B) Histograms (A) and representative photographs (B) showing the changes in the spheroid formation ability of Hep-12 cells and the spheroids formed by Hep-12 cells after CXCL11 knockdown. About 100 cells per well were seeded (n = 6). Spheroids (≥100 μm) were counted under a stereomicroscope. (C)–(D) Histograms showing the spheroid formation ability of α2δ1$^+$ cells sorted from HuH-7 (C) and PLC/PRF/5 cells (D) and infected with lentiviruses harboring *CXCL11* shRNAs or control shRNAs. About 100 cells per well were plated (n = 6). Spheroids (≥100 μm) were counted under a stereomicroscope. (E)–(F) Representative photographs demonstrating the spheroids formed by α2δ1$^+$ cells sorted from HuH-7 (E) and PLC/PRF/5 cells (F) infected with the lentiviruses harboring *CXCL11* shRNAs or control shRNAs. (G)–(H) Histograms showing the spheroid formation efficiency of HuH-7 (G) and PLC/PRF/5 cells (H) overexpressing CXCL11. CXCL11/1st and CXCL11/2nd refer to primary spheroids and secondary spheroids, respectively, formed by CXCL11-overexpressing HuH-7 or PLC/PRF/5 cells. About 100 cells per well were plated (n = 6). Spheroids (≥100 μm) were counted under a stereomicroscope. (I)–(J) Representative photographs demonstrating the spheroids formed by CXCL11-overexpressing HuH-7 (I) and PLC/PRF/5 cells (J).

underlying the roles of CXCL11 in the acquisition and maintenance of HCC TICs properties. Our previous study indicates that ERK1/2 phosphorylation plays a vital role in the maintenance of properties of HCC TICs [5]. Furthermore, CXCL11 has been shown to induce the phosphorylation of ERK1/2 in intestinal myofibroblasts and peripheral blood lymphocyte (PBL) [19]. Therefore, CXCL11 may regulate the stem cell-like properties of HCC cells through the activation of ERK1/2. To test this hypothesis, we checked if the phosphorylation of ERK1/2

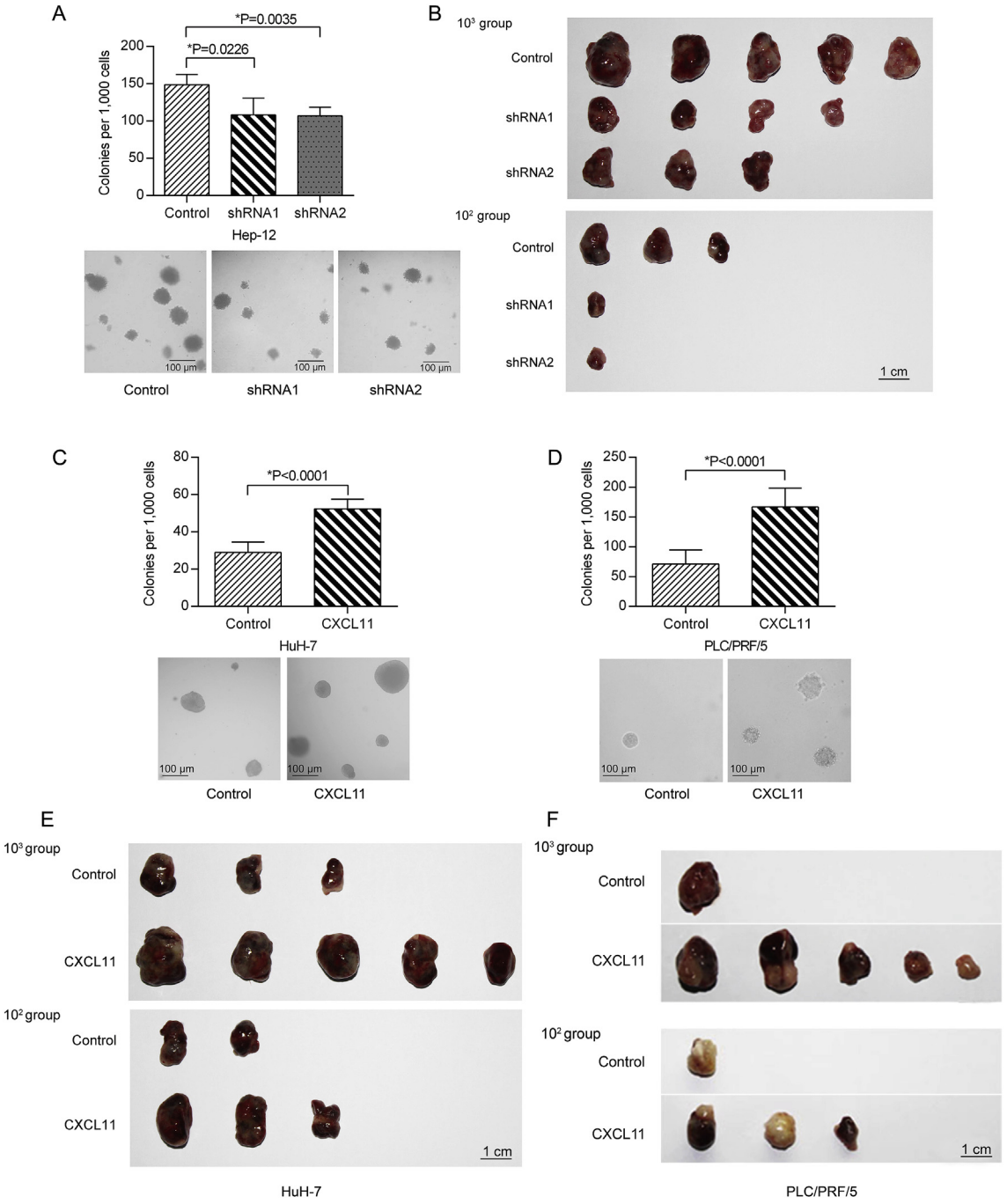

Fig. 4. CXCL11 promotes tumorigenicity of HCC TICs. (A) The colony formation efficiency and representative photographs of Hep-12 cells with stable downregulation of CXCL11 expression in soft agar. A total of 1,000 cells per well were seeded into 0.3% agar. Error bars indicate S.D. (B) The tumor formation ability of Hep-12 cells stably infected with CXCL11 shRNAs or control shRNAs lentiviruses. A total of 10^3 or 10^2 cells/site were subcutaneously transplanted into NOD/SCID mice (n = 5). (C)–(D) Histograms and representative photographs showing the colony formation efficiency of HuH-7 (C) and PLC/PRF/5 cells (D) stably overexpressing CXCL11. A total of 1,000 cells/well were plated into 0.3% agar. Error bars indicate S.D. (E)–(F) The tumor formation ability of HuH-7 (E) and PLC/PRF/5 (F) cells infected with CXCL11 lentiviruses. A total of 10^3 or 10^2 cells/site were subcutaneously transplanted into NOD/SCID mice (n = 5).

was involved in the roles of CXCL11 in HCC TICs. As shown in Fig. 6, the downregulation of CXCL11 expression in Hep-12 cells reduced the level of ERK1/2 phosphorylation, while CXCL11 overexpression in HuH-7 and PLC/PRF/5 cells increased the level of ERK1/2 phosphorylation (Fig. 6A). The inhibition of ERK1/2 phosphorylation in HuH-7 cells overexpressing CXCL11 with a p-ERK1/2 inhibitor SCH772984 not only significantly suppressed their self-renewal ability but also decreased the expression of stem cell-related genes (Fig. 6B–E). A similar phenomenon was observed in PLC/PRF/5 cells (Fig. 6F–I). These data confirm that CXCL11 may promote the stem cell-like properties of HCC cells through ERK1/2 activation.

CXCL11 mainly plays its role depending upon the manner in which it interacts with its receptor. CXCR3 is an selective high affinity receptor of CXCL11 [20]. To detect whether the high phosphorylation level of ERK1/2 was a consequence of the interaction between CXCL11 and CXCR3, we first inhibited the interplay between CXCL11 and CXCR3 through the use of a small molecule inhibitor AMG487 and analyzed the level of ERK1/2 phosphorylation using Western blotting. As shown in Fig. 6J, the phosphorylation of ERK1/2 induced by CXCL11 dramatically reduced following the treatment with CXCR3 inhibitor

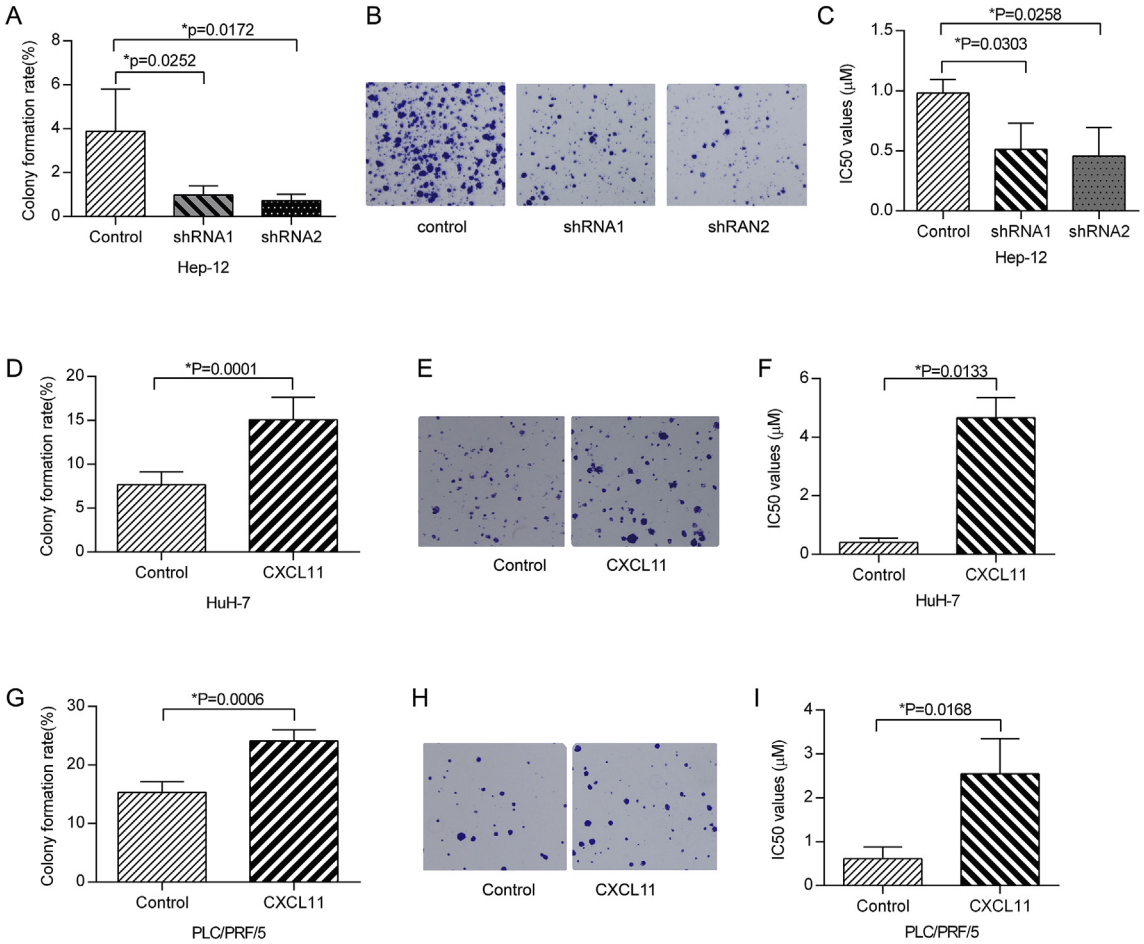

Fig. 5. CXCL11 enhances the proliferation and drug resistance ability of HCC TICs. (A)–(B) Plate colony formation assay was performed to assess the proliferation ability of Hep-12 cells with stable knockdown of CXCL11 expression. A total of 500 cells/well were seeded into six-well plates (n = 6). Error bars indicate S.D of three independent experiments. (C) Histograms showing chemoresistance of Hep-12 cells to doxorubicin after CXCL11 knockdown. The data are shown as mean ± SD of three independent experiments and statistical significance of the results was calculated by the Student's *t*-test. (D)–(E) Histograms and representative photographs showing the proliferation ability of HuH-7 cells overexpressing CXCL11. A total of 500 cells/well were seeded into six-well plates (n = 6). Error bars indicate S.D of three independent experiments. (F) Histograms indicating resistance of HuH-7 cells to doxorubicin after CXCL11 upregulation. The data are shown as mean ± SD of three independent experiments and statistical significance of the results was calculated by the Student's *t*-test. (G)–(H) The analysis of the proliferation ability of PLC/PRF/5 cells following CXCL11 overexpression using plate colony formation assay. About 500 cells/well were seeded into six-well plates (n = 6). Error bars indicate S.D of three independent experiments. (I) Histograms showing the chemoresistance of PLC/PRF/5 cells to doxorubicin after stable overexpression of CXCL11. The data are shown as mean ± SD of three independent experiments and statistical significance of the results was calculated by the Student's *t*-test.

AMG487. Similar results were observed after CXCR3 knockdown in Hep-12 cells as well as in HuH-7 and PLC/PRF/5 cells overexpressing CXCL11 (Fig. 6K). These results suggest that the high ERK1/2 phosphorylation level could be achieved through the interaction between CXCL11 and CXCR3.

In summary, the above data reveal that CXCL11 promotes stem cell-like properties of HCC cells through its receptor CXCR3 and the subsequent activation of the downstream ERK1/2 signaling.

4. Discussion

It is well established that many inflammation-related molecules are actively involved in HCC [21–24]. Accumulating evidence also suggests that the immune cell components or inflammation-related molecules participate in the regulation of HCC TICs [13,25,26], thereby improving our understanding of the roles and molecular mechanisms of the immune microenvironment in the regulation of HCC TICs. In the present study conducted based on our previous reports [5,18,27], we confirmed that the expression of the chemokine CXCL11 was significantly upregulated in $\alpha2\delta1^+$ HCC TICs and that CXCL11 promoted the acquisition and maintenance of stemness properties of $\alpha2\delta1^+$ HCC TICs through the activation of ERK1/2 following interaction with its affinity receptor CXCR3.

CXCL11 is a major chemoattractant for effector T cells [20]. Increasing evidence has supported the role of CXCL11 in the regulation of the oncogenic process in various types of human cancers. In patients with colorectal cancer (CRC), the expression of CXCL11 in the serum and tumor samples was significantly high, indicating that CXCL11 is a key cytokine interlinking inflammation and tumor development [28]. Chronic inflammation of liver has been a well-known microenvironment fostering HCC development. CXCL11 plays an important role not only in the pathogenesis of chronic hepatitis C (CHC) and its correlation with hepatic inflammation but also in HCC development [15,22,24,29,30]. However, little is known about its biological roles in the regulation of HCC TICs. Herein, we demonstrate that CXCL11 level was remarkably higher in HCC cancer tissues than in the matched normal tissues, consistent with the previously reported data [30]. Furthermore, in comparison with $\alpha2\delta1^-$ counterparts, $\alpha2\delta1^+$ HCC TICs express higher level of CXCL11, which contributes to the maintenance of the expression of the stem cell-related genes such as *BMI1*, *NANOG*, *MDR1*, *ABCG2*, and *CACNA2D1* and sustenance of the self-renewal and tumorigenic properties of $\alpha2\delta1^+$ HCC TICs. This

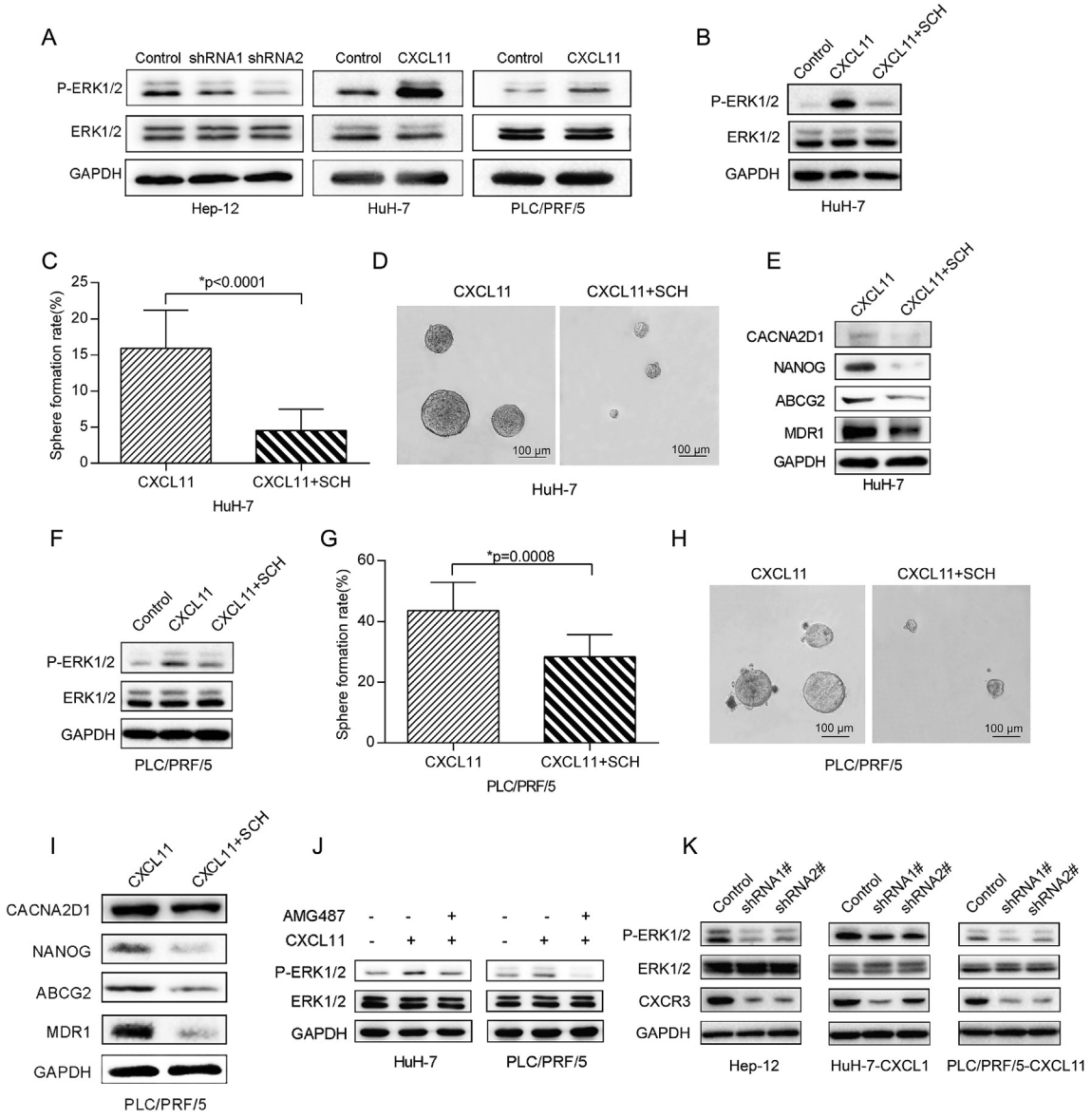

Fig. 6. CXCL11 promotes the stem cell-like properties of HCC cells through its receptor CXCR3 and ERK1/2 signaling. (A) Western blot analysis for the expression of ERK1/2 and p-ERK1/2 after CXCL11 downregulation in Hep-12 cells and CXCL11 overexpression in HuH-7 and PLC/PRF/5 cells. (B) Western blot analysis to evaluate the inhibitory effects of a p-ERK1/2 inhibitor SCH772984 on the expression of ERK1/2 and p-ERK1/2 (Selleck company, S7101S7101, 5 μM, 48 h) in HuH-7 cells. (C)–(D) Histograms (C) and representative photographs (D) showing the effects of SCH772984 on the spheroid formation ability of HuH-7 cells overexpressing CXCL11. (E) Western blot analysis for the evaluation of the expression of stem cell-related genes in HuH-7 cells overexpressing CXCL11 after p-ERK1/2 inhibition. (F) The inhibitory effects of the p-ERK1/2 inhibitor SCH772984 (Selleck company, S7101S7101, 5 μM, 48 h) on the expression of ERK1/2 and p-ERK1/2 in PLC/PRF/5 cells were assessed by western blotting. (G)–(H) Histograms (G) and representative photographs (H) indicating the effects of SCH772984 on the spheroid formation ability of CXCL11-overexpressing PLC/PRF/5 cells. (I) Western blot assay showing the expression of stem cell-related genes in PLC/PRF/5 cells overexpressing CXCL11 after p-ERK1/2 inhibition. (J) Western blot analysis of the expression of ERK1/2 and p-ERK1/2 in HuH-7 and PLC/PRF/5 cells pretreated with CXCR3 inhibitor AMG487 (APExBIO Company, B3266, 10 μM, 30 min), followed by treatment with CXCL11 (10 nM, 10 min). (K) After CXCR3 knockdown in Hep-12 cells as well as HuH-7 and PLC/PRF/5 cells overexpressing CXCL11, Western blot was performed to evaluate the expression of ERK1/2 and p-ERK1/2.

observation improves our understanding of the effects of CXCL11 on the regulation of HCC TICs, and strengthens the information on the role of inflammation-related molecules in HCC development.

The autocrine pathway regulation of inflammation-related molecules in cancer has been previously described [7,26]. In the present study, an autocrine mechanism of CXCL11 in the regulation of HCC TICs was revealed, wherein the overexpressed CXCL11 by $\alpha2\delta1^+$ HCC TICs was shown to interact with its affinity receptor CXCR3 expressed on HCC TICs to induce the activation of ERK1/2 and subsequently regulate the stem cell-like properties. These findings are consistent with those previously reported, wherein the activated mitogen-activated protein kinase (MAPK) positively participated in the regulation of HCC TICs [5,31]. In particular, these results still enrich the regulation mechanism of inflammation-related molecules in HCC TICs. As other signaling pathways, such as phosphoinositide 3-kinase (PI3K) signaling pathway may also be involved in CXCL11/CXCR3 signaling [32], further studies are warranted to clarify the involvement of these signaling pathways in the regulation of CXCL11 for the maintenance of cancer stem cell-like properties of $\alpha2\delta1^+$ HCC TICs.

In summary, our study identifies that $\alpha2\delta1^+$ HCC TICs express high levels of CXCL11, which contributes to the acquisition and maintenance of self-renewal, tumorigenic, and chemoresistance properties of $\alpha2\delta1^+$ HCC TICs via interaction with its receptor CXCR3 and activation of the downstream ERK1/2. The results established herein may contribute to

the understanding of the molecular mechanisms underlying the malignant transformation resulting from the non-resolving inflammation and would encourage the development of prognostic and therapeutic strategies for liver cancer. Future studies should address more comprehensive inflammation events in the development of $\alpha2\delta1^+$ cell-induced HCC.

Conflicts of interest

All authors declare no conflict of interest.

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Appendix A. Supplementary data

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