

# CXCR7 contributes to the aggressive phenotype of cholangiocarcinoma cells

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## ABSTRACT

Development of cholangiocarcinoma (CCA) is dependent on a cross-talk with stromal cells, which release different chemokines including CXCL12, that interacts with two different receptors, CXCR4 and CXCR7. The aim of the present study was to investigate the role of CXCR7 in CCA cells. CXCR7 is overexpressed by different CCA cell lines and in human CCA specimens. Knock-down of CXCR7 in HuCCT-1 cells reduced migration, invasion, and CXCL12-induced adhesion to collagen I. Survival of CCA was also reduced in CXCR7-silenced cells. The ability of CXCL12 to induce cell migration and survival was also blocked by CXCR7 antagonist, CCX733, a CXCR7 antagonist. Similar effects of CXCR7 activation were observed in CCLP-1 cells and in primary iCCA cells. Enrichment of tumor stem-like cells by a 3D culture system resulted in increased CXCR7 expression compared to cells grown in monolayers, and genetic knockdown of CXCR7 robustly reduced sphere formation both in HuCCT-1 and in CCLP-1 cells. In HuCCT-1 cells CXCR7 was found to interact with β-arrestin 2, which was necessary to mediate CXCL12-induced migration, but not survival. In conclusion, CXCR7 is widely expressed in CCA, and contributes to the aggressive phenotype of CCA cells, inducing cell migration, invasion, adhesion, survival, growth and stem cell-like features. Cell migration induced by CXCR7 requires interaction with β-arrestin 2.

## 1. Introduction

Cholangiocarcinoma (CCA) is the second most common primary liver malignancy [1,2] but only limited information is available on its

pathobiology, in spite of its poor prognosis and low response to current therapies [3,4]. CCA is characterized by the presence of neoplastic ductal structures surrounded by a considerable amount of fibrous stroma, comprised of myofibroblasts, small vessels and lymphoid cells.

**List of abbreviations:** AT, arsenic trioxide; H69, SV40-immortalized human cholangiocytes; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NT, non targeting; shRNA, short hairpin RNA; siRNA, small interfering RNA; NHC, human non-malignant cholangiocyte; HiBECS, Human intrahepatic biliary epithelial cells

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The presence of stromal tissue favors tumor progression and invasion of the normal hepatic parenchyma [5]. Secretion of several soluble mediators and expression of their cognate receptors has been shown to play a key role in tumor-stroma interactions [5,6]. Myofibroblasts derived from activated hepatic stellate cells (HSC) are the major contributors to collagen synthesis in chronic liver diseases [7], and have also been shown to promote CCA progression *in vitro* and *in vivo* [8], mostly through secretion of mediators which ultimately regulate the biology of CCA cells [5].

CXCL12 is a member of the CXC chemokine subfamily secreted by stromal fibroblasts, endothelial cells and, within the liver, HSC [9]. Studies in different types of tumor indicate that CXCL12 has a key role in promoting invasion and migration of cancer cells [10,11]. The G protein-coupled receptor CXCR4 has been considered for many years the only receptor for CXCL12 [12]. Recent evidence indicates that CXCR7, another seven-transmembrane domain receptor, binds to CXCL12 with even higher affinity [13]. CXCR7 also binds to CXCL11, another chemokine family member [14]. Interestingly, CXCR7 may behave as a decoy (non-signaling) or signaling receptor depending on the cell type [12]. CXCR7 is expressed by many different tumor cells, and has been implicated in cell motility, adhesion, and angiogenesis [15,16]. Because CXCR7 expression has been associated with tumor aggressiveness, CXCR7 has been proposed as a prognostic marker in some types of cancer [17,18].

The molecular mechanisms of CCA progression are poorly understood, and this limits the development of effective therapies for this highly aggressive tumor. Here we show that CXCR7 is widely expressed in CCA, and its interaction with CXCL12 modulates the aggressive phenotype of CCA cells through intracellular signaling involving  $\beta$ -arrestin 2.

## 2. Material and methods

### 2.1. Materials

RPMI media was from Invitrogen (Carlsbad, CA, USA). Enhanced chemiluminescence (ECL) reagents and nitrocellulose membrane Hybond-C extra were from Amersham Pharmacia Biotech (Cologno Monzese, Milano, Italy). Immobilon Western reagents and ECM cell adhesion array kit were from Millipore Corporation (Billerica, MA, USA). Recombinant CXCL12 was purchased from R&D (Minneapolis, MN, USA). CCX733, CCX771 and CCX704 were kindly provided by Dr. Mark Penfold (Chemocentryx, Mountain View, CA, USA). Anti-CXCR7 antibody used for immunoprecipitation was from Biolegend (San Diego, CA, USA). Antibodies against CXCR4,  $\beta$ -arrestin 2, PARP and anti-CXCR7 antibodies used for Western blotting were from Abcam (Cambridge, United Kingdom). Immunohistochemistry was performed with an anti-CXCR7 Ab purchased from Proteintech (Rosemont, IL, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), arsenic trioxide (AT), monoclonal antibodies against  $\beta$ -actin and vinculin were purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

### 2.2. Cell culture

The CCA cell lines used in this study were kindly provided by Dr. A.J. Demetris, University of Pittsburgh (HuCCT-1, CCLP-1, SG231,) and Dr. G. Alpini, Temple, TX (MzCha1 and TFK1). The primary culture of normal human cholangiocyte, NHC, was provided By Dr. J.M. Banalles [19]. HiBECs were purchased by ScienCell Research laboratories (Carlsbad, Germany).

Cells were cultured according to conditions described elsewhere [20]. Experiments were performed when cells reached about 90% confluence and after serum deprivation for 24 h.

### 2.3. Migration and invasion assays

Migration of HuCCT cells was assayed using modified Boyden chambers, essentially as described elsewhere [21,22]. Briefly, 20,000 cells were seeded in wells equipped with 8- $\mu$ m pore filters (Millipore Corp, MA, USA) and coated with rat tail collagen (20  $\mu$ g/ml) (Collaborative Biomedical Products, Bedford, USA) on the bottom side. Chemoattractants were loaded in the lower chamber, while inhibitors, if present, were added to the upper chamber together with the cells. After 16 h incubation, cells migrated to the underside of the filters were fixed, stained with Giemsa, mounted and counted at 40 $\times$  magnification. All experiments were performed in duplicate. Each duplicate assay was repeated at least 3 times on separate occasions. Data are the average of cell counts obtained in 12 randomly chosen high-power fields (HPF). The procedure of the invasion assay was similar to the chemotaxis assay, but filters were coated on the top side with Matrigel (150  $\mu$ g/ml) (BD Biosciences, MA, USA) and incubation was prolonged for 24 h.

### 2.4. Cell survival

The test was performed using MTT assay, as previously described [23]. 10,000 cells/well were seeded in 96-well plates, incubated in complete medium for 24 h and serum-deprived for additional 24 h. Cells were then stimulated under different conditions. To measure cell viability, MTT solution (10  $\mu$ l/well of a 5 mg/ml solution in PBS) was added to the cells the last hour of incubation time and the reaction was stopped by adding 20  $\mu$ l of lysis solution (20% (w/v) SDS, 50% (v/v) *N,N*-dimethylformamide, 2% (v/v) acetic acid, 25 mM HCl, pH 4.7). Absorbance was read at 570 nm, in a Thermo Scientific Multiskan FC.

### 2.5. Immunohistochemistry

Twenty-three paraffin-embedded human liver tissue specimens were analyzed for the expression of CXCR7 by immunohistochemistry. All specimens were obtained after surgical resection and collected in the tissue bank of the Center for Autoimmune Liver Diseases, IRCCS Istituto Clinico Humanitas (Rozzano, Italy) after obtaining informed consent and approval of the local ethics committee. The investigated specimens included two samples of normal liver tissue and 21 specimens of CCA. Normal liver tissue was obtained from patients undergoing partial hepatectomy for a single colorectal cancer metastasis. In these cases, CXCR7 expression was evaluated in tissue distant at least 5 cm from the metastasis. All specimens of CCA were from previously untreated patients. Histology of the primary tumors was reviewed by pathologists experienced in liver tumors and classified according to the TNM staging system (Table 1).

For immunohistochemistry, paraffin sections were deparaffinized in xylene and rehydrated in graded ethanol to deionized water. Sections were then washed in 0.0005% PBS/Tween and subjected to endogenous peroxidase blocking with 3% H<sub>2</sub>O<sub>2</sub>, for 20 min at room temperature. Background Sniper buffer was used to block unspecific binding (Biocare Medical, Concord CA). Immunostaining was performed with anti-CXCR7 antibodies (dilution 1:100). Sections were then washed in PBS-Tween and incubated with MACH1 Universal HRP-Polymer Detection kit (Biocare Medical, Concord CA) for 30 min at room temperature. The reaction was finally developed in Biocare's Betazoid DAB and counterstained with hematoxylin-eosin (DAKO, Glostrup, Denmark).

The proportion of CXCR7-positive cell was classified into four grades (0,  $\leq$ 1%; 1, 1–30%; 2, 31–50%; 3, 51–100%). The staining intensity was graded from 0 to 3 (0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining). The score in each section analyzed was obtained adding the two grades.

**Table 1**

Clinical and histopathological features of human tissue specimens used for evaluation of CXCR7 expression. Histological tumor scoring was performed by expert pathologists. The proportion of positive cells was classified into four grades (0, < 1%; 1, 1–30%; 2, 31–50%; 3, 51–100%). Staining intensity was arbitrarily graded from 0 to 3 (0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining). After addition of the two grades, a total staining score for each section was obtained.

| Case | Gender | Cancer tissue scoring | Grade | TNM      | Angioinvasion | Lymphatic metastases |
|------|--------|-----------------------|-------|----------|---------------|----------------------|
| 1    | F      | 4+/5+                 | G2    | pT2bNxM1 | N             | Y                    |
| 2    | M      | 2+                    | G2    | N/A      | N             | N                    |
| 3    | F      | 3+                    | G2    | pT2aN0Mx | Y             | N                    |
| 4    | M      | 4+                    | G3    | pT1N1    | N             | Y                    |
| 5    | M      | 4+                    | G3    | N/A      | Y             | N                    |
| 6    | M      | 0                     | G3    | pT3N0Mx  | N             | N                    |
| 7    | M      | 5+                    | G2    | pT1N0    | N             | N                    |
| 8    | M      | 4+                    | G2    | pT2N0    | Y             | N                    |
| 9    | M      | 4+/5+                 | G1    | pT1      | N             | N                    |
| 10   | M      | 4+/5+                 | G2    | pT2N0M1  | N             | N                    |
| 11   | F      | 2+                    | G2    | pT2N0    | Y             | N                    |
| 12   | F      | 0                     | G3    | N/A      | Y             | N                    |
| 13   | F      | 4+                    | G2    | pT3Nx    | N             | N                    |
| 14   | F      | 4+                    | G1    | pT1      | N             | N                    |
| 15   | F      | 4+                    | G2    | pT1N0    | N             | N                    |
| 16   | F      | 4+                    | G3    | pT2bN0   | Y             | N                    |
| 17   | M      | 4+                    | G2    | N/A      | N             | N                    |
| 18   | F      | 2+                    | G2    | pT2aN0Mx | Y             | N                    |
| 19   | F      | 4+                    | G3    | pT1      | N             | N                    |
| 20   | F      | 2+                    | G1    | pT1N0    | N             | N                    |
| 21   | M      | 4+                    | G2    | pT1N1Mx  | N/A           | Y                    |

## 2.6. iCCA primary cell cultures

The use of human materials and the research protocol were approved by the Ethics Committees of the Policlinico Umberto I, University Hospital, Rome, Italy. Primary cell cultures were prepared, as previously described [24,25], from specimens of human iCCA obtained from patients submitted to surgical resection. Primary cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> with H69 medium [24,25] and cultured for 3 passages before use.

## 2.7. RNA isolation and real time quantitative PCR

Total RNA was isolated using the RNeasy kit (QIAGEN Sciences, MD) according to the manufacturer's recommendations, then 400 ng of RNA were examined by RTQ-PCR. FAM-labeled probes and primers for specific genes or for the housekeeping gene GAPDH were purchased from Applied Biosystems. Relative gene expression was calculated as  $2^{-\Delta Ct}$  ( $\Delta Ct = Ct$  of the target gene minus  $Ct$  of GAPDH) [11].

## 2.8. RNA interference transfection assay

Transient gene silencing was performed by using human  $\beta$ -arrestin 2 validated siRNA and a non-specific-control siRNA (Custom SMARTpool siRNA Design Service of Dharmacon, Inc., Lafayette, CO). Transfection was performed using Lipofectamine RNAiMAX and Opti-MEM I reduced serum medium according to the manufacturer's instructions (Invitrogen). Western blot analysis was performed to confirm silencing. RNA interference was also performed by an Amara cell line Nucleofector kit L (Lonza, Basel, Switzerland) [26] using two shRNA plasmid constructs (10  $\mu$ g/10<sup>6</sup> cells) for human CXCR7 to obtain long term silencing. A control non-targeting (NT) shRNA plasmid construct expressing an shRNA sequence with no significant homology to any mammalian transcript (Sigma Chemical Co. St. Louis, MO) was used as control (10  $\mu$ g/10<sup>6</sup> cells). Treatment for 3 days with 0.5  $\mu$ g/ml of puromycin was performed to select the transfected cells.

## 2.9. Western blot analysis

Cells were lysed at 4 °C with lysis buffer (1% Triton X-100, 50 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l sodium orthovanadate, 2 mmol/l PMSF, and 1 mmol/l each of leupeptin and pepstatin). After 30 min of lysis, cellular extracts were centrifuged 10 min at 12,000g, and the supernatant was used for Western blot experiments as detailed elsewhere [23].

## 2.10. Immunoprecipitation assay

300  $\mu$ g of total protein were incubated with specific antibodies or control immunoglobulins at 4 °C for 2 h, as previously described [27]. Immune complexes were recovered by adding protein A-Sepharose beads (Amersham Pharmacia Biotech, Sweden) by overnight incubation with gentle shaking at 4 °C. After washing three times in buffer (50 mM Tris-HCl, 0.5 mM NaCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> and 0.1% Tween 20), the beads were analyzed by SDS-Page and Western blotting as described above [27].

## 2.11. Cell adhesion assay

Adhesion assays were performed as described elsewhere [28]. Briefly, flat-bottom 96-well plates were coated with type I collagen (10  $\mu$ g/mL) overnight at 4 °C. Wells were then washed and incubated at 37 °C for 2 h with heat-denatured bovine serum albumin to block nonspecific binding sites. Subsequently, 10,000 cells/well (pretreated with 100 ng/ml CXCL12 when required) were seeded and incubated for 1 h at 37 °C. Nonadherent cells were carefully removed by washing 3 times with DMEM. Adherent cells were fixed and stained with 0.5% crystal violet. Cell density was determined measuring the absorbance at 540 nm.

## 2.12. Cell proliferation assay

Cell proliferation was performed using a colorimetric immunoassay based on the measurement of BrdU incorporation. 15,000 cells/well were seeded in 96-well plates, incubated in complete medium for 24 h and serum-starved for additional 24 h. The assay was performed following the manufacturer's protocol (Roche, Basel, Switzerland).

## 2.13. Sphere assay

Cells were grown in anchoring-independent conditions with selective serum-free DMEM/F12 medium supplemented with  $1 \times B27$  supplement minus vitamin A (Life Technologies), 20 ng/ml human recombinant epidermal growth factor, and 20 ng/ml bFGF (R&D System) for 15 days, as previously described [29]. To calculate sphere-forming efficiency, CCA cells were single-cell sorted into 96-well plates coated with an Ultra-Low Attachment Surface (Corning) using a FACS Aria (BD Biosciences). After 10 days, the amount of spheres formed was quantified, and expressed as the average number of formed sphere per microscopic field (20 $\times$ ) over five fields.

## 2.14. Cell growth assay

10<sup>4</sup> CCLP-1 cells were plated in 24-well dishes and incubated in medium deprived of serum for 24 h. Cells were exposed to different stimuli and counted in triplicate at 36 h.

## 2.15. Human samples

iCCA samples from the San Sebastian (Spain) cohort were studied. All the samples were obtained from the Basque Biobank of the Donostia University Hospital. CXCR7 expression was measured by real-time qPCR. Research protocols were approved by the Clinical Research

Ethics Committees of supporting institutions, and all patients signed written consents for the use of their samples for biomedical research. Total RNA was isolated from human liver samples (i.e. CCA tumors and surrounding liver tissue) using Tri-Reagent® (Sigma-Aldrich Co) following the manufacturer's instructions. Then, total RNA was reverse transcribed using the SuperScript Vilo cDNA Synthesis Kit (Invitrogen), according to manufacturer's instructions. qPCR was performed using the iQ™ SYBR® Green Supermix (Bio-Rad) using the following primer sequences: CXCR7: 5'-CTAGAGGCTCCTTCTGCAGTG-3' (forward) and 5'-GCAGATCCATCGTTCTGAGGC-3' (reverse); glyceraldehyde-3-phosphate dehydrogenase 5'-CCAAGGTCATCCATGACAAC-3' (forward) and 5'-TGTCTATACCAAGGAATGAGC-3' (reverse). Gene expression was determined using the  $\Delta$ CT method and GAPDH was used as internal control for normalization of data. mRNA levels are represented as the percent increase over data obtained in normal surrounding liver tissue, set at 100.

### 2.16. Statistical analysis

Data in bar graphs represent mean  $\pm$  SEM of at least three independent experiments. Statistical analysis was performed using Student's *t*-test. *P* values  $\leq 0.05$  were considered significant.

## 3. Results

### 3.1. CCA tissues and cell lines express CXCR7

We first analyzed CXCR7 immunoreactivity in 21 individual samples of CCA and in 2 samples of normal liver. The characteristics of the patients are reported in Table 1. In normal liver, CXCR7 expression was clearly detectable in bile ducts (Fig. 1A). In all CCA specimens, a diffuse cytoplasmic staining for CXCR7 was clearly evident in tumor cells (Fig. 1A) with the exception of two cases (Table 1). Notably, in the surrounding non-neoplastic tissue, bile ducts and endothelium retained remarkably high CXCR7 expression. We also compared CXCR7 gene expression in cultured normal human cholangiocytes (NHC), SV-40 immortalized H69 human cholangiocytes (non-malignant), and in human CCA cells, isolated from 4 patients with iCCA (Fig. 1B). A marked and significant increase in CXCR7 expression was evident in iCCA cells compared to non-tumor cells (Fig. 1B). Expression of CXCR4 tended to be higher in iCCA cells, although the results did not reach statistical significance due to the high variability of expression. Similarly,  $\beta$ -arrestin 2 expression (Fig. 1B) was higher in cells from patients with iCCA. In contrast, iCCA cells did not express detectable signal for CXCL12, as previously reported in HuCCT-1 cells [11].

We next evaluated whether different CCA cell lines express CXCR7. All intrahepatic (HuCCT-1, SG231, CCLP1) and extrahepatic (MzCha, TFK1) CCA cell lines tested were found to express CXCR7 (Fig. 1C) at protein level. Of note, CXCR7 expression tended to be higher in all lines derived from intrahepatic CCA, in comparison to cells derived from extrahepatic tumors or HiBECs, an immortalized normal biliary epithelial cell line.

To establish a possible correlation between CXCR7 expression and tumor characteristics, gene expression was measured in samples obtained from patients with iCCA. In 15 patients expression levels in both tumor and surrounding non-tumoral tissue were available. Average CXCR7 expression was higher in iCCA, and the difference was borderline significant ( $100 \pm 28$  vs.  $356 \pm 151\%$ ,  $P = 0.067$ ). Moreover, CXCR7 expression tended to be higher in larger ( $> 50$  mm) vs. smaller tumors, and in moderate/poorly differentiated vs. well-differentiated tumors (data not shown). However, these latter differences were not significant due to the low number of patients and the high variability in CXCR7 gene expression. Similarly, we could not analyze the possible correlation with outcome.

### 3.2. CXCR7 inhibition contributes to CXCL12-induced adhesion, migration and invasiveness of HuCCT-1 cells

We next evaluated the biologic characteristics relevant for the progression of CCA in HuCCT-1 cells, silencing CXCR7 with two specific shRNA (Fig. 2A). CXCL12-mediated adhesion to culture wells coated with type I collagen was reduced in cells silenced for CXCR7 (Fig. 2B). Moreover, CXCL12-induced chemo-invasion was also significantly reduced (Fig. 2C). Because similar effects were observed with both shRNAs, in the next set of experiments we decided to use only one shRNA specific for CXCR7. Experimental knockdown of CXCR7 expression resulted in reduced gene expression of MMP2 and MMP9, two metalloproteinases important for CCA invasiveness [30] and of VEGF (Fig. 2D).

Silencing of CXCR7 significantly reduced CXCL12-elicited migration (Fig. 3A). To provide additional support to this effect, we performed experiments using CCX733, a specific CXCR7 antagonist used in different tumors [31–33]. CCX733 prevented CXCL12-induced migration (Fig. 3B), while CCX704, a compound structurally similar to CCX733 but inactive towards CXCR7, did not exert any significant actions (Fig. 3C).

### 3.3. CXCR7 inhibition reduces survival of HuCCT-1 cells

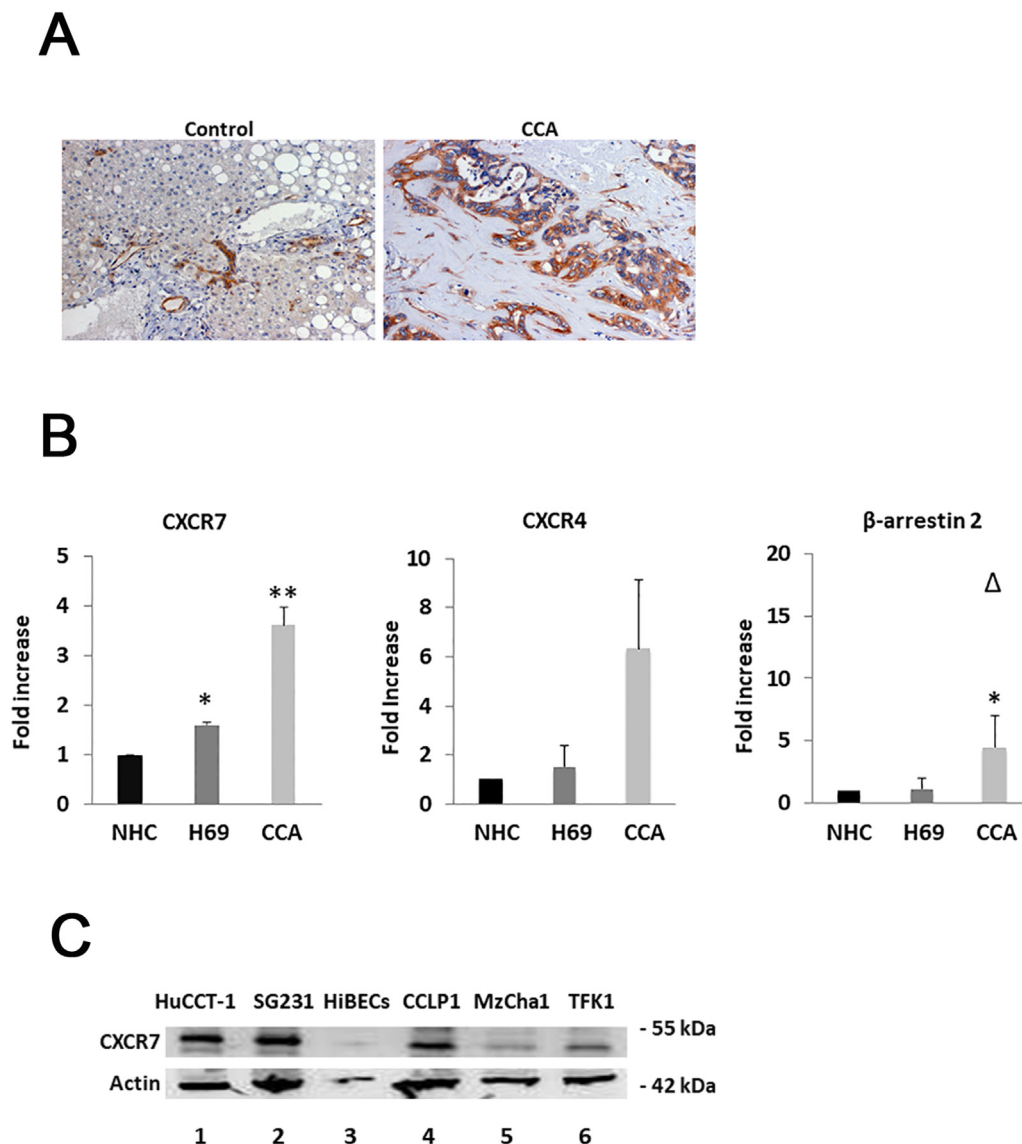
We then analyzed the effect of CXCR7 knock down on survival of HuCCT-1 cells. Increased survival induced by exposure to FBS (Fig. 4A) was less evident when cells were silenced for CXCR7. Moreover, CXCR7 silencing prevented BrdU incorporation indicating a proliferative role of CXCR7 signaling (Fig. 4E). In agreement with these results, in CXCR7-silenced cells, cleaved PARP, an indicator of the apoptotic process, was more abundant than in control cells (Fig. 4B), while total PARP decreased. These data indicate that CXCR7 contributes to mediate anti-apoptotic effects and to induce cell proliferation (Fig. 4A, B and E). To further demonstrate the role of CXCR7 in mediating cell survival, we induced apoptosis exposing CCA cells to arsenic trioxide (AT). Decreased viability induced by this drug was reverted by co-incubation with CXCL12. In cells silenced for CXCR7 (Fig. 4C) or exposed to the CXCR7 inhibitor, CCX733 (Fig. 4D) the rescue induced by CXCL12 was no longer evident, indicating that CXCR7 activation is necessary to maintain cell survival.

### 3.4. CXCR7 inhibition contributes to CXCL12-induced migration, invasiveness, growth and survival of CCLP-1 cells

To support the data obtained in HuCCT-1 cells, we performed additional experiments in CCLP-1, another iCCA line, using both genetic and pharmacological strategies to inhibit CXCR7 activity. Also in these cells, CXCL12-induced migration, invasion and growth (Fig. 5), and these biologic actions were reduced after genetic knockdown of CXCR7 (Fig. 5B, C, D). Of note, the ability of CXCL12 to rescue the pro-apoptotic effects of AT was blocked both by CXCR7 silencing or pretreatment with CCX 771, a specific CXCR7 antagonist (Fig. 5E).

### 3.5. CXCR7 inhibition contributes to CXCL12-induced migration of primary iCCA cells

To further expand the significance of these findings in the context of CCA, we conducted experiments in iCCA58 and iCCA60 cells, two primary cell lines derived as described in the Methods section. Both these cells expressed CXCR7 at levels comparable to those observed in HuCCT-1 (Fig. 6A). In both types of cells, CXCL12 induced an increase in migration that was significantly reduced by preincubation with CCX711 (Fig. 6B–C).



**Fig. 1.** CXCR7 expression in human liver tissue and CCA cells.

[A]: expression of CXCR7 in normal human liver tissue (left panel) and CCA (right panel). In normal liver CXCR7 expression is present in bile ducts and blood vessels, while in CCA nearly all tumor cells express this receptor. [B]: Total RNA from normal human cholangiocytes (NHC), the human immortalized cholangiocyte line H69, and CCA cells derived from 4 patients with intrahepatic CCA was analyzed for CXCR7, CXCR4 and  $\beta$ -arrestin 2 gene expression by RT-PCR. RNA expression is represented as fold-increase compared to control conditions. \* $P \leq 0.05$  vs. NHC, \*\* $P \leq 0.05$  vs. NHC and H69. In the  $\beta$ -arrestin 2 panel, the CCA barogram represents the mean of 3 patients, with 'Δ' showing the value of an outlier [C]: cell lysates of different CCA lines were subjected to Western blot analysis for CXCR7. All lines were found to express CXCR7. Equal gel loading was assessed by actin blotting. Molecular weight markers are indicated on the right.

### 3.6. Role of CXCR7 in CXCL12-induced migration in hepatocarcinoma cells

Previous data indicate that CXCR7 is expressed by hepatocarcinoma (HCC) and by HCC cells [34–36]. To establish whether regulation of the biology of liver tumor cells was unique to CCA, we conducted additional experiments in HuH7 and HepG2, two well-established HCC cell lines. Both lines expressed CXCR7 at the mRNA level (Supplementary Fig. 1A). Moreover, activation of CXCR7 by CXCL12 induced cell migration, which was significantly reduced by preincubation with CCX733 or CCX771, two antagonists of CXCR7 (Supplementary Fig. 1B–C).

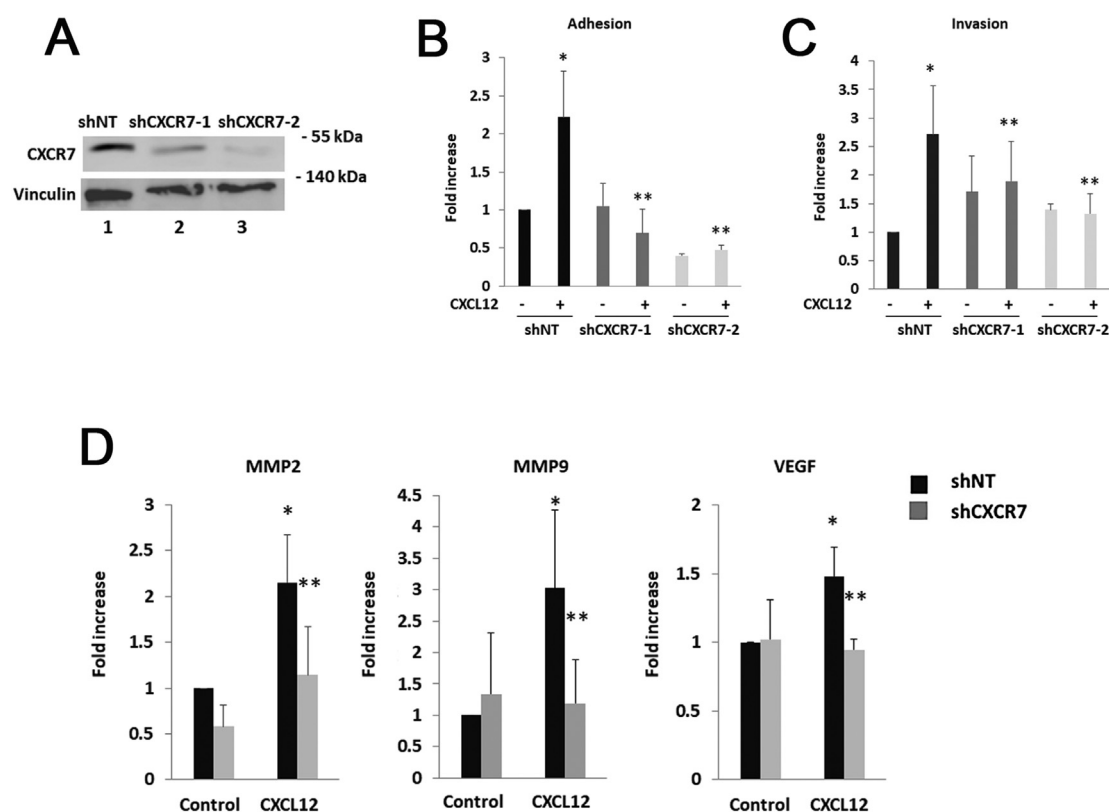
### 3.7. 3D cultures of CCA overexpress CXCR7

We next analyzed the potential impact of CXCR7 activation in a 3D sphere system used as a functional tool to select for tumor stem-like cells. Spheres originated from HuCT-1 cells had higher CXCR7 expression compared to parental cells grown in monolayer (Fig. 7A). More importantly, genetic inhibition of CXCR7 robustly reduced sphere formation by HuCT-1 and CCLP-1 cells (Fig. 7B). These data indicate that CXCR7 expression is associated with the maintenance of a stemness-like status in CCA cells, further supporting CXCR7 involvement in the aggressive phenotype of these cancer cells.

### 3.8. $\beta$ -Arrestin 2 associates with CXCR7 and mediates CXCL12-induced migration

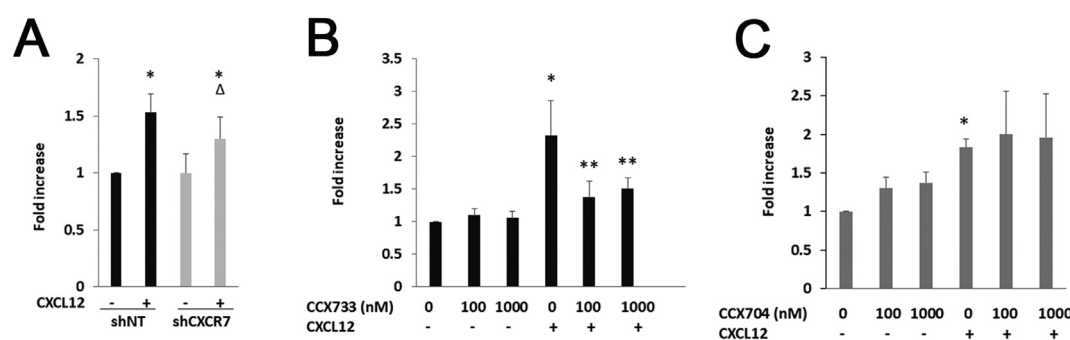
$\beta$ -arrestin 2 has been shown to mediate the internalization and signaling of CXCR4 and CXCR7 in different cellular systems [31]. We examined the potential interaction between  $\beta$ -arrestin 2 and CXCR7 in HuCT-1 cells. Immunoprecipitation of  $\beta$ -arrestin 2 followed by Western blotting for CXCR7 showed that these molecules are associated in baseline conditions, and this association is further increased upon CXCL12 treatment (Fig. 8A). In addition, CXCR4, the other receptor for CXCL12, was shown to co-immunoprecipitate with CXCR7, although no differences were found comparing unstimulated cells with cells exposed to CXCL12 (Fig. 8B).

To investigate the functional role of  $\beta$ -arrestin 2 in CXCL12 signaling, HuCT-1 cells were silenced using specific siRNA (Fig. 8C). Knockdown of  $\beta$ -arrestin 2 significantly reduced the ability of CXCL12 to induce CCA cell migration (Fig. 8D), while no changes were observed in the effects of CXCL12 on cell survival (Fig. 8E). These data indicate that  $\beta$ -arrestin 2 participates in CXCR7 signaling in HuCT-1 cells, but is not required for all biologic actions triggered by this receptor.



**Fig. 2.** CXCR7 is involved in CCA cell adhesion, invasion and expression of soluble mediators.

[A]: HuCCT-1 cells were silenced for CXCR7 as reported in Materials and Methods, using two different shRNAs (shCXCR7-1 and shCXCR7-2) or with non-targeting (NT) shRNA (lane 1). Equal loading of the gel was assessed by the expression of vinculin (representative of  $n = 3$ ). [B, C]: HuCCT-1 cells silenced for CXCR7 as described in [A] were incubated in the presence or absence of 100 ng/ml CXCL12, as indicated. Cell adhesion on type I collagen [B] and invasion of matrigel-coated filters [C] was assessed as indicated in Materials and Methods. The number of cells migrated is represented as fold-increase compared to control conditions.  $*P \leq 0.05$  vs. control,  $**P \leq 0.05$  vs. shNT ( $n = 3$ ). [D]: Total RNA from cells silenced for CXCR7 (shCXCR7-2) incubated in the presence or absence of CXCL12 for 8 h (left and middle panels) or 24 h (right panel) was analyzed for expression of *MMP2*, *MMP9*, and *VEGF* by RT-PCR. RNA expression is represented as fold-increase compared to control conditions.  $*P \leq 0.05$  vs. unstimulated control,  $**P \leq 0.05$  vs. shNT ( $n = 3$ ).



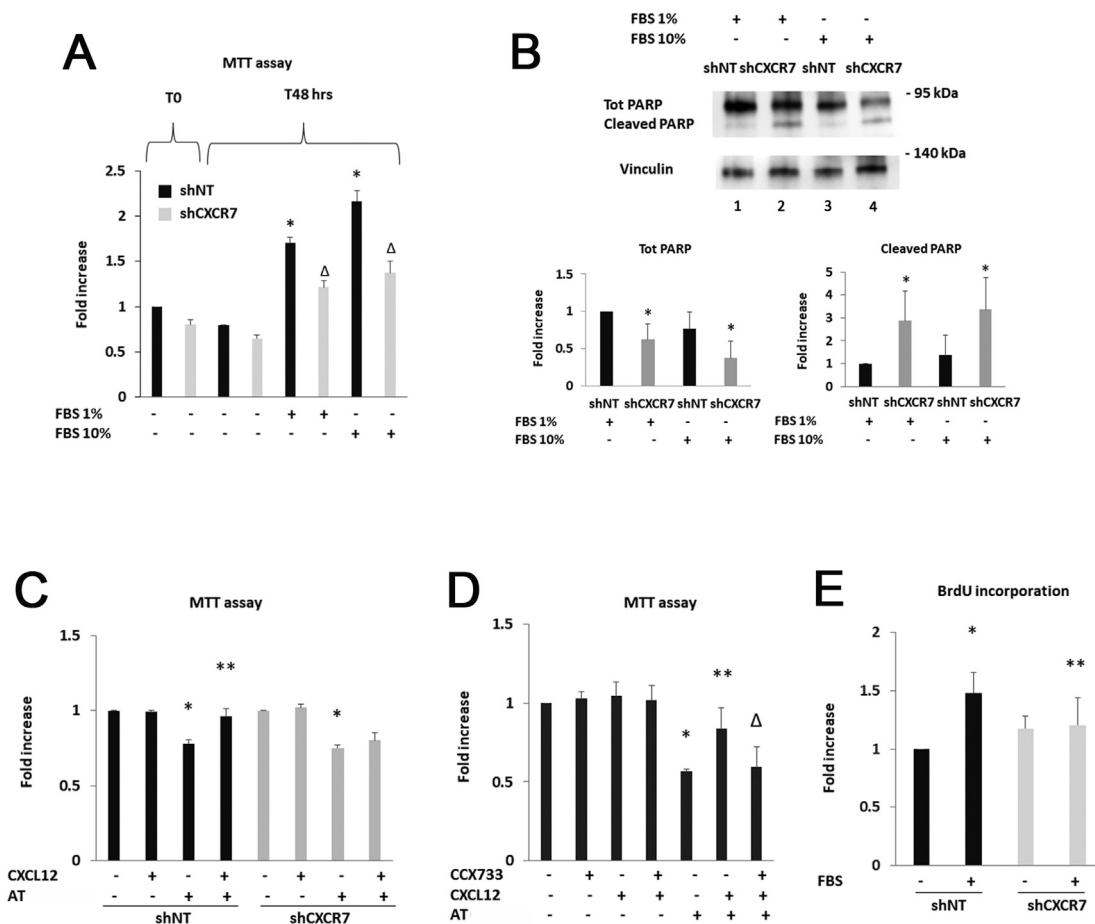
**Fig. 3.** Genetic knockdown or pharmacological antagonism of CXCR7 blocks CXCL12-induced migration.

[A] HuCCT-1 cells were silenced for CXCR7 or with non-targeting (NT) shRNA, and incubated in the presence or absence of 100 ng/ml CXCL12, as indicated. Cell migration was measured as indicated in Materials and Methods. The number of cells migrated is represented as fold-increase compared to control conditions.  $*P \leq 0.05$  vs. unstimulated control;  $\Delta P \leq 0.05$  vs. shNT ( $n = 3$ ). [B]: HuCCT-1 cells were incubated in the presence or absence of 100 ng/ml CXCL12, with or without a 30 min pretreatment with different concentrations of the CXCR7 antagonist, CXC733, and cell migration was measured.  $*P \leq 0.05$  vs unstimulated control;  $**P \leq 0.05$  vs. CXCL12 ( $n = 3$ ). [C]: The experiment was performed as described in panel [B], using CXC704, an inactive compound structurally similar to CXC733.  $*P \leq 0.05$  vs. unstimulated control ( $n = 3$ ).

#### 4. Discussion

No effective therapies are available for surgically-untreatable CCA, and more detailed information on the biology of this tumor has the potential to identify novel therapeutic approaches, which represent a clear unmet need in oncology. CCA is characterized by an abundant stromal component, which modulates the biology of cancer cells through a complex intercellular cross-talk, also involving soluble

mediators. Chemokines have been shown to regulate the aggressive phenotype of many types of cancer, including CCA [37], inducing a number of biologic actions via interaction with cognate receptors. In this study, we provide evidence for the first time that CXCR7, which binds the chemokine CXCL12, contributes to numerous and relevant phenotypic characteristics of CCA cells, potentially favoring cancer progression. Of note, CXCR7 was found to be present in many different CCA cell lines, where its abundance was higher than in a line of non-



**Fig. 4.** Genetic knockdown or pharmacological antagonism of CXCR7 reduces CCA survival and proliferation.

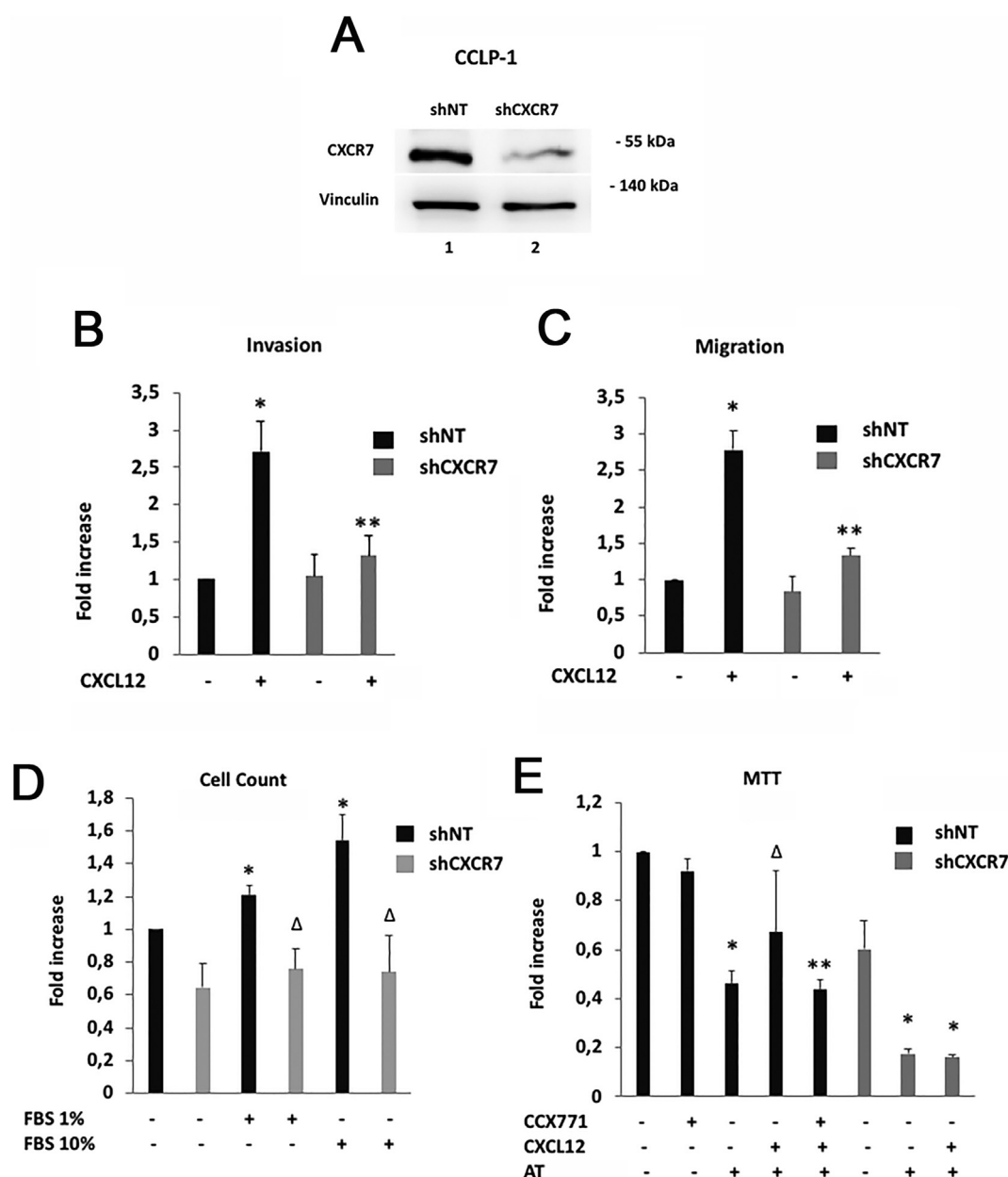
[A]: HuCCT-1 cells were silenced for CXCR7 or with non-targeting (NT) shRNA, and incubated in the presence or absence of different concentrations of FBS, as indicated. Cell survival was evaluated at time 0 and after 48 h, and is represented as fold-increase compared to control conditions.  $*P \leq 0.05$  vs. unstimulated control at 48 h;  $\Delta P \leq 0.05$  vs. shNT ( $n = 3$ ). [B]: HuCCT-1 cells were silenced for CXCR7 or with non-targeting (NT) shRNA, and incubated in the presence or absence of different concentrations of FBS, as indicated, for 24 h. Western blot analysis with an antibody recognizing both total PARP and cleaved PARP was performed as indicated in Materials and Methods. Equal loading of the gel was assessed by GAPDH expression. Densitometry measured in four different experiments is shown in the lower panel.  $*P \leq 0.05$  versus shNT. [C]: HuCCT-1 cells were silenced for CXCR7 or with non-targeting (NT) shRNA, in the presence or absence of 0.05 mM arsenic trioxide (AT) and/or 100 ng/ml CXCL12, as indicated. Cell survival was measured by MTT assay.  $*P \leq 0.05$  vs. unstimulated control;  $**P \leq 0.05$  vs. AT alone ( $n = 3$ ). [D]: HuCCT-1 cells were incubated in the presence or absence of 0.05 mM arsenic trioxide (AT), with or without a 30 min pretreatment with 100 nM CCX733, and cell survival was measured by MTT assay.  $*P \leq 0.05$  vs. unstimulated control;  $**P \leq 0.05$  vs. AT alone ( $n = 3$ )  $\Delta P \leq 0.05$  vs. AT + CXCL12. [E]: HuCCT-1 cells were silenced for CXCR7 or with non-targeting (NT) shRNA, and incubated in the presence or absence of 10% FBS, as indicated, for 24 h. Cell proliferation was assessed by BrdU incorporation. Cells BrdU incorporation is represented as fold-increase compared to control conditions.  $*P \leq 0.05$  vs. unstimulated control;  $**P \leq 0.05$  vs. shNT ( $n = 3$ ).

transformed biliary epithelial cells. CXCR7 gene expression was also more abundant in cells isolated from patients with iCCA than in non-tumor cholangiocytes. Expression of the *CXCR7* gene tended to be higher in tumor tissue than in the surrounding non-tumoral specimen, although statistical significance was only borderline due to wide dispersion of the data and the limited number of patients. Finally, CXCR7 immunostaining was present in 19/21 surgical samples of CCA, also in this case with different levels of expression. The widespread expression of CXCR7 in cultured cells and in specimens collected from patients with CCA suggests a high biological relevance of this receptor. Moreover, while no data regarding CXCR7 expression in CCA have been previously reported, this receptor has been shown to mediate acquisition of an aggressive phenotype in many tumor cells and in activated tumor-associated endothelial cells [15,18,32,33,38].

In HuCCT-1 cells, a well-established cellular model of CCA, down-regulation of CXCR7 by gene silencing reduced the ability of CXCL12 to increase migration, invasion of a basement-membrane like matrix, and adhesion to collagen-coated surfaces. In addition, CXCR7 was found to be involved in survival and proliferation of HuCCT-1 in response to

CXCL12, both in the presence or absence of mitogenic factors. The ability of CXCR7 to modulate the biology of CCA was confirmed in another iCCA line, CCLP-1, and in two primary cells derived from patients with iCCA, demonstrating the general relevance of these findings. Of note, activation of CXCR7 was also found to regulate the migration of HCC cell lines. This latter observation is in agreement with previously reported data indicating widespread expression of CXCR7 in HCC cell lines, where activation of the receptor mediates proangiogenic signals, proliferation and invasion [34–36]. Additionally, CXCR7 was found to be over-expressed in metastatic HCC and to correlate with a poor outcome [35]. Taken together, these lines of evidence suggest that the biological relevance of this receptor may be a general feature of hepatic cancer.

We found that gene expression of *MMP9* and *MMP2* was upregulated by the CXCL12/CXCR7 axis, similarly to what was observed in other types of cancer [35,39]. These effects are relevant in view of the fact that extracellular matrix (ECM) remodeling by matrix metalloproteinases (MMPs) [30] plays a relevant role in the biology of CCA, a cancer with high metastatic potential. Furthermore, gene expression of



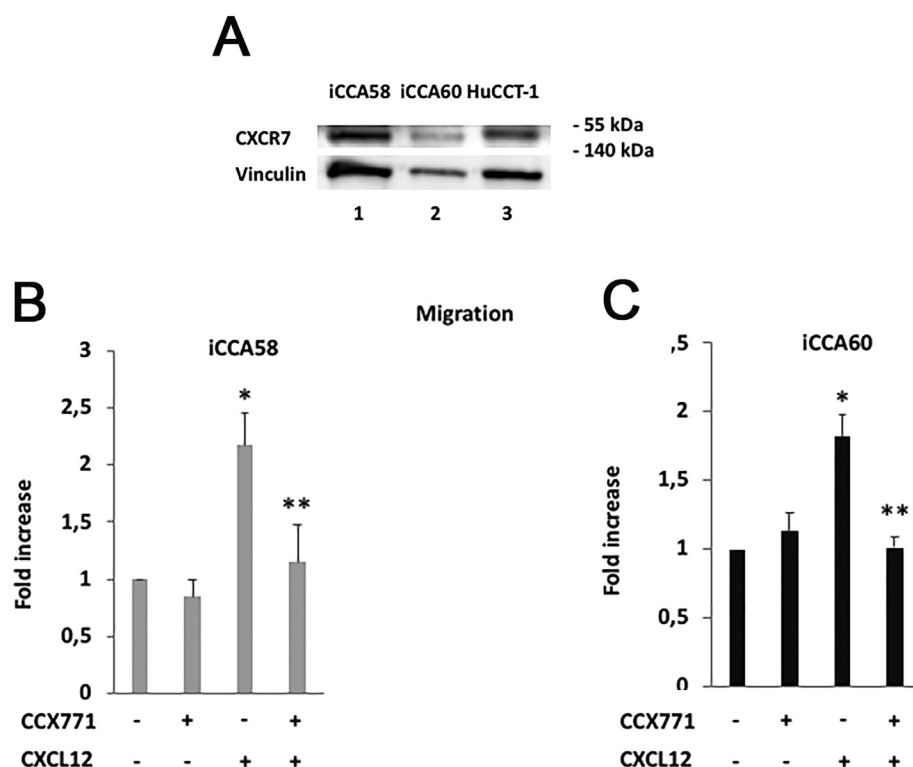
**Fig. 5.** Genetic knockdown of CXCR7 reduces CCA migration, invasion, growth and survival of CCLP-1 cells.

[A]: CCLP-1 cells were silenced for CXCR7 or with non-targeting (NT) shRNA and CXCR7 expression was measured by Western blotting as described in Materials and Methods. as indicated. Equal loading of the gel was assessed by the expression of vinculin (representative of  $n = 3$ ). [B, C]: CCLP-1 cells were silenced for CXCR7 or with non-targeting (NT) shRNA, and incubated in the presence or absence of 100 ng/ml CXCL12, as indicated. Cell invasion and migration were measured as indicated in Materials and Methods. The number of cells migrated is represented as fold-increase compared to control conditions.  $*P \leq 0.05$  vs. unstimulated control;  $**P \leq 0.05$  vs. shNT ( $n = 3$ ). [D]: CCLP-1 cells were silenced for CXCR7 or with non-targeting (NT) shRNA and incubated in the presence or absence of 1% or 10% FBS, as indicated. Cell count was measured after 36 h of incubation.  $*P \leq 0.05$  vs. unstimulated control;  $\Delta P \leq 0.05$  vs. shNT ( $n = 3$ ). [E]: CCLP-1 cells were silenced for CXCR7 or with non-targeting (NT) shRNA, and incubated in the presence or absence of 0.05  $\mu$ M arsenic trioxide (AT) and/or 100 ng/ml CXCL12, as indicated. shNT cells were also incubated in the presence or absence of AT and with or without a 30 min pretreatment with 100 nM CX771, a specific CXCR7 antagonist. Cell survival was measured by MTT assay.  $*P \leq 0.05$  vs. unstimulated control;  $\Delta P \leq 0.05$  vs. AT alone;  $**P \leq 0.05$  vs. AT + CXCL12 ( $n = 3$ ).

VEGF, that can modulate CCA cell migration [40] and induce angiogenesis, was increased by CXCL12 administration in a CXCR7-dependent fashion. Importantly, the critical role of CXCR7 in mediating these actions was confirmed with the use of a pharmacologic inhibitor of this receptor. This observation adds translational value to our findings, also considering that seven transmembrane, G-protein linked receptors may be good targets for drug development [41].

Until recently, the actions of CXCL12 were believed to be mediated by activation of CXCR4, which is also expressed on CCA cell lines where it regulates migration and survival [11]. More recently, CXCR7 has

been unequivocally identified as an additional receptor mediating CXCL12's downstream signals [15]. Moreover, CXCR7 shows a 10-fold higher binding affinity for CXCL12 than CXCR4 [13]. Thus, it is possible that many of the actions elicited by CXCL12 in cancer cells and attributed uniquely to CXCR4 actually require the contribution of CXCR7. These observations may have an impact on the eventual development of therapeutic strategies involving this chemokine system. The combined involvement of CXCR7 and CXCR4 suggests that drugs targeting both receptors or interfering with ligand's binding could be more effective compared to blockade of a single receptor. Additional preclinical and



**Fig. 6.** Pharmacological antagonism of CXCR7 blocks CXCL12-induced migration of primary iCCA cells.

[A]: Primary cell lines derived from two different patients affected by iCCA (iCCA58 and iCCA60) were analyzed for CXCR7 expression in comparison to HuCCT-1 (positive control). Equal loading of the gel was assessed by the expression of vinculin. [B, C]: iCCA58 and iCCA60 were incubated in the presence or absence of 100 ng/ml CXCL12, with or without a 30 min pretreatment with the CXCR7 antagonist CCX771 (100 nM). Cell migration was measured as described in Materials and Methods. \* $P \leq 0.05$  vs unstimulated control; \*\* $P \leq 0.05$  vs. CXCL12 ( $n = 3$ ).

clinical studies are required.

We have also provided information on the signaling mechanisms activated by CXCR7 in CCA cells, and specifically on the role of  $\beta$ -arrestin 2. In other cells, CXCR7-mediated effects may be dependent or independent of  $\beta$ -arrestin 2 activation [42]. In HuCCT-1, CXCR7 and  $\beta$ -arrestin 2 were found to interact in a time- and ligand-dependent fashion. More important,  $\beta$ -arrestin 2 was necessary for CXCL12-induced chemotaxis, as previously reported in other cell types [43,44], but dispensable for CXCR7-mediated cell proliferation. As  $\beta$ -arrestin 2 may be itself a target for therapy, these lines of information may be helpful in understanding the fine tuning of signaling downstream the CXCL12/CXCR7 axis in this cancer cell type.

A very intriguing result of this study is related to the possible modulation of the cancer stem cell compartment by CXCR7. Cancer stem cells (CSCs) are critical for tumor initiation, progression and resistance to treatments, driving to recurrence after surgery and metastatic behavior [29,45]. We used an in vitro model of anchorage-independent growth to enrich the CCA cell culture of stem-like cells, growing in spheroids. Expression of CXCR7 was higher in CCA stem-like cells, suggesting a role of CXCR7 in the acquisition of stemness features.

More important, after CXCR7 knock-down, CCA cells were less able to form spheres, reinforcing the importance of this receptor in CCA aggressiveness. Further studies will be needed to identify the molecular mechanisms elicited by CXCR7 in CCA stem-like cells.

In conclusion, we have elucidated for the first time that CXCR7, highly expressed in CCA, plays a key role in the most important biological effects that lead to CCA aggressiveness. These results may contribute to identify potential new targets for cancer therapy, in consideration of the lack of effective therapies for CCA treatment.

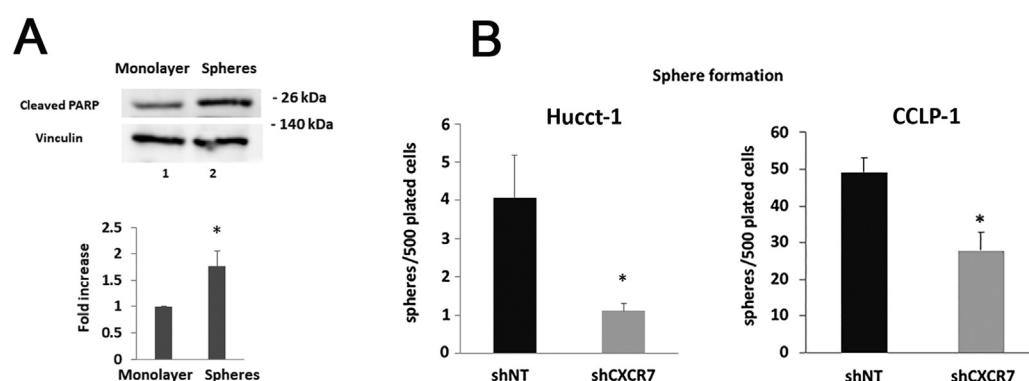
Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbdis.2019.04.020>.

## Conflicts of interest statement

The authors have declared that no competing interest exists.

## Financial support

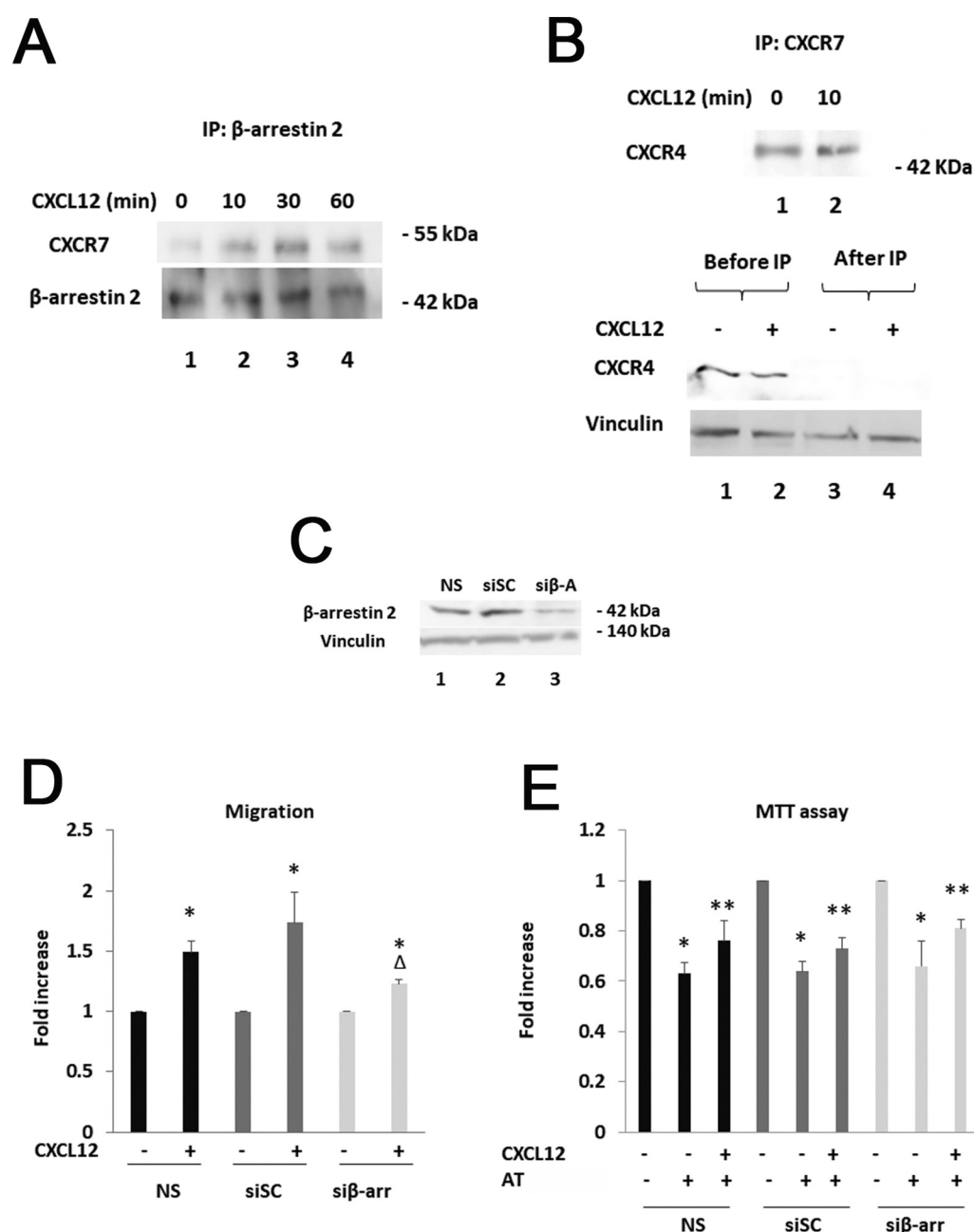
This work was financially supported by Associazione Italiana per la Ricerca sul Cancro (AIRC) grant IG 2015 id. 17786 and by the



**Fig. 7.** CXCR7 expression contributes to the acquisition of stem cell-like features in HuCCT-1 cells.

[A]: Cell lysates from HuCCT-1 cells cultured in monolayer or after sphere formation were analyzed for CXCR7 expression using Western blot analysis. Equal loading was assessed by the expression of vinculin. Densitometry values of three different experiments is indicated below. \* $P \leq 0.05$  vs. monolayer. [B]: HuCCT-1 or CCLP-1 cells were silenced for CXCR7 or with non-targeting (NT) shRNA and the number of spheres formed was measured as described in

Materials and Methods. Each experiment was performed in triplicate. Mean  $\pm$  SD of 4 independent experiments, \* $P \leq 0.05$  versus shNT.



**Fig. 8.** Interaction between CXCR7 and β-arrestin 2 mediates CCA cell migration but not survival.

[A]: HuCCT-1 cells were incubated with 100 ng/ml CXCL12 for different periods of time, as indicated. Cell lysates were immunoprecipitated with an antibody against β-arrestin 2 and used for Western blot analysis under non-reducing conditions to detect CXCR7 or β-arrestin 2. [B]: (Upper panel) HuCCT-1 cells were incubated with 100 ng/ml of CXCL12 for 10 min, and cell lysates were immunoprecipitated with an antibody against CXCR7 and used for Western blot analysis under non-reducing conditions to detect CXCR4 (Lower panel). Western blot for CXCR4 was conducted with cell lysates collected before and after the immunoprecipitation as control of the IP experiment (representative of  $n = 3$ ). [C]: HuCCT-1 cells were silenced for β-arrestin 2 as indicated in Materials and Methods, or with scrambled (SC) siRNA. Equal loading of the gel was assessed by the expression of vinculin (representative of  $n = 3$ ). [D]: HuCCT-1 cells silenced for β-arrestin 2 as described in [C] were incubated in the presence or absence of 100 ng/ml CXCL12, as indicated, and cell migration was measured. The number of cells migrated is represented as fold-increase compared to control conditions. \* $P \leq 0.05$  vs. unstimulated control; Δ  $P \leq 0.05$  vs. siSC cells treated with CXCL12 ( $n = 3$ ). [E]: The experiment was performed as in [D], and cell survival was measured by MTT assay in cells exposed to 0.05 μM arsenic trioxide (AT) in the presence or absence of 100 ng/ml CXCL12. Cell survival is represented as fold-increase compared to control conditions. \* $P \leq 0.05$  vs. unstimulated control; \*\* $P \leq 0.05$  vs. AT alone ( $n = 3$ ).

University of Florence.

#### Transparency document

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