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CXC Chemokine Receptor 4 is Essential for Maintenance of Renal Cell Carcinoma-Initiating Cells and Predicts Metastasis

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ABSTRACT

In many solid tumors, cancer stem cells (CSC) represent a population with tumor-initiating, self-renewal, and differentiation potential, which can be identified by surface protein markers. No generally applicable markers are yet known for renal cell carcinoma (RCC). Two RCC cell lines (RCC-26, RCC-53) were found to differ widely in their capacity to form spheres in vitro and to establish tumors in mice, potentially reflecting differences in CSC content. A subpopulation expressing the CXC chemokine receptor 4 (CXCR4) was present only in the more tumorigenic cell line RCC-53. When grown as spheres, most of the RCC-53 cells were CXCR4-positive, expressed stem cell-associated transcription factor genes at elevated levels, and were more resistant toward the tyrosine kinase inhibitors sunitinib, sorafenib, and pazopanib. Sorted CXCR4-positive cells exhibited greater capacity for sphere formation and tumor growth-inducing potential in vivo than CXCR4-negative cells. Significantly, higher CXCR4 mRNA levels in primary RCC tumors from patients with localized but not disseminated disease predicted shorter survival. Downregulation of CXCR4 expression by small interfering RNA (siRNA) or pharmacological inhibition by AMD3100 compromised tumor sphere formation, viability of CXCR4-positive cells, and increased their responsiveness toward tyrosine kinase inhibitors. In conclusion, CXCR4 identifies a subpopulation of tumor-initiating cells in RCC cell lines and plays a role in their maintenance. The relative insensitivity of such cells to tyrosine kinase inhibitors might contribute to the development of therapy resistance in RCC patients. Future therapies therefore could combine blockade of the CXCR4 signaling pathway with standard therapies for more effective treatments of metastatic RCC. STEM CELLS *2013;31:1467–1476*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Renal cell carcinoma (RCC) accounts for 3% of all adult malignancies worldwide, with increasing incidence. Up to 30% of RCC patients have metastases at the time of diagnosis and metastases develop metachronously in 20%–40% of patients undergoing partial or radical nephrectomy [1]. Metastatic RCC is resistant to both conventional chemotherapy and radiotherapy. The clinical response rate is very low and the 5-year survival of patients with metastatic RCC is less than 10%.

Over the last decade the development of targeted molecular therapies, as both first and second-line treatments, has substantially improved the prognosis for patients with metastatic RCC. These include receptor tyrosine kinase inhibitors, monoclonal antibodies, and mammalian target of rapamycin (mTOR) inhibitors [2]. These agents are mostly directed against signaling pathways that foster angiogenesis [3].

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Although, some tumors show regression, most patients develop therapy resistance over time [4].

There is increasing evidence that the capacity of tumor growth resides in a small subpopulation of cells, termed cancer-initiating cells or cancer stem cells (CSCs; [5, 6]). Such cell populations were recently identified in breast [7], brain [8], prostate [9], ovarian [10], gastric [11], colon [12], pancreatic [13], head and neck [14], and liver cancers [15], and in melanoma [16]. Like normal stem cells, these cells are characterized by their ability for self-renewal and their capacity to form serially transplantable tumors, which recapitulate the heterogeneous tumor phenotype in immunodeficient mice. CSCs have been identified and isolated based on expression of various proteins such as CD44, CD133, aldehyde dehydrogenase 1 (ALDH1), and ATP-binding cassette transporters (e.g., ABCG2) [17]. Their clinical importance is underscored by their higher resistance to chemotherapy and radiation therapy [18], which may explain in part the failure of therapies in patients with solid tumors that lead to disease relapse after initial remission. Efficient therapies therefore must include elimination of CSCs as well as of their proliferating daughter cells. In a number of preclinical studies, various therapeutic approaches to kill CSCs have been tested, such as blockade of CSC maintenance pathways and induction of cell differentiation [18–21].

Stem-like tumor cells have also been identified in RCC. Addla et al. [22] characterized a Hoechst 33342 dye side population in malignant renal epithelial cells derived from primary RCC which were enriched for cells with high proliferative capacity and stem cell-like properties. In addition, cultivation of cells from a RCC cell line as spheres enriched a cell population which exhibited many features of CSCs, including higher expression of stemness genes and resistance to chemotherapeutic agents, as well as stronger tumorigenicity in comparison to adherently growing parental cells [23]. Furthermore, CD105 which is present on normal renal mesenchymal stem cells was used to enrich cells with stem cell characteristics from primary RCC. These showed expression of stemness genes, tumor sphere formation, and enhanced tumorigenicity in severe combined immunodeficiency (SCID) mice [24]. Interestingly, CD105-expressing CSCs from renal carcinomas were amenable to differentiation into nontumorigenic cells with epithelial characteristics using interleukin 15, which might have therapeutic relevance [19].

To identify CSC markers in RCC that can possibly be used as therapeutic targets, we screened a number of known surface molecules that are overexpressed in tumor-initiating cells for differential expression in two RCC cell lines, RCC-26 and RCC-53. These two lines were established from primary tumors of patients with clear cell RCC [25, 26]. RCC-26 was derived from a patient with stage I disease in whom only a single brain metastasis appeared after nephrectomy following a disease-free interval of 9 years. RCC-53 was established from a patient with stage IV clear cell carcinoma and is characterized by its high proliferative capacity. Therefore, we reasoned that these cell lines may differ in their CSC content, providing a model to study cancer stem cells in RCC.

Materials and Methods

Ethics Statement

Animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany; approval ID 55.2-1-54-2531-44-10). The study of tissues from patients was approved by the local ethics committee (Project 214/04).

Antibodies and Reagents

The following fluorophore-conjugated mouse anti-human monoclonal antibodies were used for flow cytometry: allophycocyanin-labeled CD105 (43A4E1, Miltenyi Biotech GmbH, Bergisch-Gladbach, Germany, http://www.miltenyibiotech.com), CD117 (YB5.B8, BD Biosciences, Heidelberg, Germany, http://www.bdbiosciences.com), fluorescein isothiocyanate-conjugated CD29 (TS2/16, eBiosciences, Frankfurt, Germany, http://www.ebiosciences.com), CD44 (SFF-2, Bender MedSystems, Vienna, Austria), CD146 (541-10B2, Miltenyi), phycoerythrin (PE)-labeled CD24 (SN3 A5-2H10, eBiosciences), CD73 (AD2, Miltenyi), CD90 (5E10, BD Biosciences), CD133/2 (AC141, Miltenyi), CXCR1 (5A12, BD Biosciences), CXCR4 (12G5, BD Biosciences), and PE-Cy7-labeled CD34 (4H11, eBiosciences). For immunohistology, rabbit anti-CXCR4 (ab2074, Abcam, Cambridge, U.K., http://www.abcam.com) or mouse anti-human CXCR4 (44716, R&D Systems, Wiesbaden-Nordenstedt, Germany, http://www.rndsystems.com), rabbit anti-POU5F1 (ab19857, Abcam), and rabbit anti-pan-cytokeratin (#18-0059, Invitrogen, Life Technologies GmbH, Darmstadt, Germany, http://www.invitrogen.com) antibodies were used. The CXCR4 antagonist AMD3100 was from Merck-Millipore (Darmstadt, Germany, http://www.millipore.com); the protein tyrosine kinase inhibitors sorafenib, *p*-toluene sulfonate salt, sunitinib, maleate salt, and pazopanib were purchased from LC Laboratories (Woburn, MA, http://www.lclabs.com).

Cell Lines

RCC-26 was derived from a patient with stage I disease (pT1N0M0G2) [25] and RCC-53 from a patient with stage IV disease (pT2N1MxG2-3) [26]. SK-RC-17 cells were a kind gift from J. Vissers, Nijmegen. Cells were maintained in standard medium, consisting of RPMI 1640 supplemented with 10% fetal bovine serum (FBS, "Gold," heat-inactivated, PAA Laboratories GmbH, Cölbe, Germany, http://www.paa.com), 1% minimal essential medium (MEM) nonessential amino acids, 1 mM sodium pyruvate, and 2 mM L-glutamine (Invitrogen).

Sphere Formation Assay

Tumor spheres were generated in serum-free sphere medium consisting of Dulbecco's modified Eagle's medium (DMEM)/F12, 1% insulin-transferrin-selenium-X (Invitrogen), 2% B27 (Invitrogen), 20 ng/mL epidermal growth factor (EGF; Sigma-Aldrich Chemie, München, Germany, http://www.sigmaaldrich.com), 20 ng/mL basic fibroblast growth factor (bFGF; Sigma-Aldrich Chemie). Cells were seeded in ultra-low attachment flasks or plates (Corning Costar, Amsterdam, The Netherlands, http://www.corning.com) and cultured for up to 7 days. The number of spheres was evaluated after 4 days to avoid miscalculation due to sphere aggregation. For immunofluorescence staining of RCC-26 spheres, cells were washed once with phosphate buffered saline (PBS) and stained with PE-labeled anti-CXCR4 antibody for 30 minutes at room temperature. Subsequently, cells were washed once again and transferred to chamber slides (Thermo Scientific, Schwerte, Germany, http://www.thermoscientific.com) for fluorescence microscopy. Photos were taken with a Leica DM IRBE microscope using XnView software (Leica Microsystems GmbH, Wetzlar, Germany, http://www.leica.com).

Flow Cytometry and Fluorescence-Activated Cell Sorting

For flow cytometry, live cells were dissociated with Accutase, washed once with PBS (supplemented with 1% FBS, 25 mM HEPES, 4 mM EDTA) and stained with conjugated antibodies

according to the manufacturers' recommendations. Cells were analyzed using the FACSCalibur (Becton Dickinson, San Jose, CA, http://www.bd.com). Unstained cells served as negative control. For calculation of the percentage of labeled cells, gates were set to contain less than 0.01% cells of the negative control. A minimum of 1×10^4 viable cell events were recorded per sample. BD CellQuest software (version 4.0.2) was used for data acquisition, and data were processed using FlowJo software (version 8.8.6; Tree Star, Ashland, OR, http://www.treestar.com). For discrimination of live/dead cells, 7-aminoactinomycin D or propidium iodide was applied. For determination of aldehyde dehydrogenase, the ALDEFLUOR kit from ALDAGEN (StemCell Technologies, Grenoble, France, http://www.stemcell.com) was used with diethylaminobenzaldehyde treatment as negative control. For fluorescence-activated cell sorting (FACS), 2×10^7 cells were stained with 200 μL PE-labeled anti-CXCR4 antibody in 1 mL PBS for 30 minutes at 4°C, washed with PBS, and kept on ice (60–120 minutes) until separation. The CXCR4 positive and negative cell populations were sorted on a MoFlo Legacy (Beckman Coulter, Miami, FL, http://www.beckmancoulter.com).

Drug Sensitivity Assay

For drug sensitivity testing, spheres were dissociated using Accutase and a cell strainer with 40 μm nylon mesh (BD Biosciences). Adherent and sphere-derived cells were seeded in 96-well plates and incubated overnight. The medium was then exchanged with drug- or solvent-containing medium. Drugs were dissolved in dimethyl sulfoxide and further diluted with standard medium. Cell viability was assessed after 24 hours drug treatment using the CellTiter-Blue Cell Viability Assay (Promega, Mannheim, Germany, http://www.promega.com). Measurement of fluorescence at 560(20) excitation/590(10) emission was done using the FLUOstar OPTIMA microplate reader (BMG LABTECH, Ortenberg, Germany, http://www.bmglabtech.com) and the OPTIMA software version 2.0.

Tumor Induction in Immunocompromised Mice

Cells were resuspended in DMEM/F12 and mixed with ice cold Matrigel Basement Matrix High Concentration (BD Biosciences) 1:1 and kept on ice. One hundred microliters were injected subcutaneously into the right and left dorsal sides of 6–9-week-old female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice NOD.CB17-*Prkdc*^{scid}/J (Charles River, Sulzfeld, Germany, http://www.criver. com) using a 1 mL syringe with a 23-gauge needle. Injection was done under short term isoflurane (Forene, Abbott, Heidelberg, Germany, http://www.abbott.com) anesthesia. Mice were housed at the animal facility of the Walter Brendel Center, Ludwig-Maximilians University. Tumor size was measured using a digital caliper, and tumor volume was calculated as length $\times$ width^2/2.

Immunohistology

For immunocytological staining, spheres and cells were washed and embedded in Tissue-Tek O.C.T. Compound (Weckert-Labortechnik, Würzburg, Germany, http://www.weckert-labortechnik.de). Cryosections (7 μm) from shock frozen RCC primary tumors, spheres, cell pellets, and xenograft tumors were mounted on Super Frost Ultra Plus slides (Menzel, Braunschweig, Germany, http://www.menzel.de), air-dried, and stored at −20°C. Cryosections were fixed for 10 minutes in acetone, and endogenous peroxidase was blocked by incubation with 3% H_2O_2, 10% methanol in PBS, or BLOXALL Blocking Solution (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) for 5 minutes at room temperature. Subsequently, cells or tissue sections were incubated with polyclonal rabbit anti-CXCR4 antibody (1:300, Abcam), mouse anti-human CXCR4 (R&D Systems), polyclonal rabbit anti-POU5F1 antibody (1:100, Abcam), or rabbit anti-pan-cytokeratin antibody (1:400, Invitrogen) for 2 hours at room temperature, followed by 1 hour incubation with ImmPRESS reagent peroxidase-coupled rabbit or mouse anti-IgG antibody (Vector Laboratories) and stained by incubation with 3-amino-9-ethylcarbazole and hematoxylin.

Quantitative Real Time Reverse Transcriptase Polymerase Chain Reaction

Total RNA was isolated from 10 to 20 10 μm tissue cryosections and tumor cells, grown either adherently or under sphere forming conditions for 7 days, using QIAshredder columns and the RNeasy Mini Kit (Qiagen, Hilden, Germany, http://www1.qiagen.com). RNA integrity was controlled by capillary electrophoresis using RNA 6000 Pico Assay and Bioanalyzer 2100 (Agilent Technology, CO Springs, http://www.home.agilent.com). One microgram of total RNA was used for cDNA syntheses by reverse transcription (RT) using the Reverse Transcription System (Promega). Complementary DNA (cDNA) was amplified by quantitative polymerase chain reaction (PCR) with the LightCycler 2.0 System and the LightCycler FastStart DNA Master^{PLUS} SYBR Green I Kit (Roche, Basel, Switzerland, http://www.roche.com). The relative amount of selected cDNAs was determined using the primers listed in Supporting Information Table S1. Human GAPDH and β-actin cDNA were quantified using LightCycler Primer Sets according to the manufacturer's protocol (SearchLC GmbH, Heidelberg, Germany, http://www.search-lc.com). Cycling conditions were as follows: 10 minutes, 95°C initial denaturation, 40 cycles, 10 seconds, 95°C denaturation, 10 seconds, 60°C annealing, and then followed by 16 seconds, 72°C extension. The relative amounts of cDNA were normalized for the GAPDH or β-actin cDNA content of the samples as follows: relative normalized amount of cDNA = $2^{\text{Cp housekeeping gene}}/2^{\text{Cp gene of interest}}$ (arbitrary units, AU) whereby Cp represents the crossing point where a fluorescence value of one is reached.

Transfection with siRNA

CXCR4 expression was reduced by transfecting cells with a mixture of two double-stranded siMAX siRNA with UU-3' overhangs, CXCR4-1 (sense, 5'-UAAAAUCUUCCUGCCC ACC-3') and CXCR4-2 (sense, 5'-GGAAGCUGUUGGCUG AAAA-3') from Eurofins MWG Operon (Ebersberg, Germany, http://www.eurofinsdna.com). Control lacZ siRNA (sense, 5'-UUAUGCCGAUCGCGUCACA-3') was a kind gift by A. Herbst, Department of Medicine II, University of Munich, Munich, Germany. The siRNAs were transfected into RCC-53 cells at a final concentration of 50 nM using Lipofectamine RNAiMAX Reagent (Invitrogen). After 24 hours, cells were harvested for determination of the siRNA knockdown efficiency by quantitation of CXCR4 expression by flow cytometry and in sphere formation assays.

Statistical Analysis

Significance (p) of differences was calculated using the unpaired two-tailed Mann–Whitney U test or the Student's t test in the GraphPad Prism5 software package. Outcome was evaluated using the Kaplan-Meier method and the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. CXCR4 mRNA levels larger than the median values were classified as high. p values below .05 were considered to be significant.

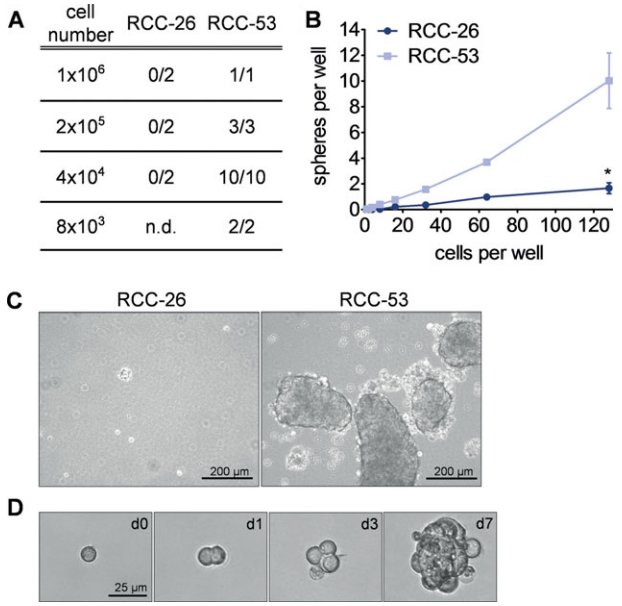

Figure 1. RCC-53 cells exhibit higher tumor and tumor sphere formation capacity than RCC-26 cells. **(A)**: RCC-26 cells are not able to form tumors in NOD/SCID mice, whereas RCC-53 cells are highly tumorigenic. The indicated number of cells were injected s.c. into NOD/SCID mice. The number of tumors palpable after 16 weeks and the number of mice or injection sites are listed. n.d., not done. **(B, C)**: RCC-53 cells form numerous large spheres under nonadherent conditions, while RCC-26 shows only poor sphere formation. The indicated numbers of cells were seeded in 96-well (12 wells per cell concentration) (B) or 1×10^4 cells in six-well low attachment plates (C) and the number of spheres was determined after 4 and 7 days, respectively. The representative results of one out of three experiments with similar results are shown. Mean values and SEM are indicated. *, $p < .05$. **(D)**: Spheres originate from single cells and do not result from cell aggregation. RCC-53 cells were cultivated in 96-well low attachment plates and the same sphere was photographed at the indicated days.

Results

RCC-53 Cells Exhibit a Higher CSC Content Than RCC-26 Cells

The cell lines RCC-26 and RCC-53 were established from two patients, who exhibited slow and rapid disease progression, respectively. We reasoned that the difference in tumor aggressiveness might be reflected in the CSC content of the derived cell lines. To test this assumption, we analyzed the tumor-initiating potential of these two cell lines. RCC-53 cells readily formed tumors at all cell numbers tested, including only 8,000 cells, when subcutaneously transplanted into NOD/SCID mice. In contrast, RCC-26 cells did not grow even when 1×10^6 cells were injected. This suggested they had a lower CSC content (Fig. 1A). To further substantiate this finding, we determined the tumor sphere formation capability of RCC-26 and RCC-53 by cultivating the cell lines under nonadherent conditions in the presence of bFGF and EGF in serum-free medium. The extent of sphere formation is considered to correlate positively with the CSC content of tumor cell populations [27]. At all cell densities tested, RCC-53 cells were capable of forming significantly more and larger spheres than RCC-26 cells (Fig. 1B, 1C). Sphere formation was not due to cell aggregation since sphere formation was found to originate mostly from single cells (Fig. 1D). These observations suggested that RCC-53 cells contained more stem cell-like cells capable of tumor initiation, sphere formation, and conversion into differentiated progeny.

CXCR4 Is a Candidate CSC Marker That Predicts Survival in RCC Patients Without Metastasis

We then evaluated putative stem cell markers by flow cytometry for differential expression on RCC-26 and the more aggressive RCC-53 cells. CD24, CD29, CD44, CD73, and CD146 were highly expressed on both cell lines and, therefore, were not further considered as putative CSC markers for RCC (data not shown and Supporting Information Fig. 1). CXCR1, CD34, CD90, CD105, and CD133 were either expressed at very low levels or did not show preferential expression in RCC-53 cells, as seen with CD133 as an example (Figs. 2A; Supporting Information Fig. 1). ALDH1 activity, as measured by the ALDEFLUOR assay, was highly variable but predominantly found in RCC-53 cells. It was not pursued further as a CSC marker (data not shown). In contrast, CD117 and the chemokine receptor CXCR4 were expressed in 0.01% and 0.8% of RCC-26 and 2% and 5% of RCC-53 cells, respectively (Fig. 2A; Supporting Information Fig. 1). They were thereby the only markers tested, that were preferentially expressed in RCC-53 cells at levels that would be consistent with CSCs, which comprise only a minor tumor cell population.

CSCs are considered to drive progression and metastasis of tumors. Therefore, we expected a high CSC content as estimated by the amount of marker gene mRNA to correlate with a worse prognosis of tumor patients. To corroborate our contention that CXCR4 might represent a CSC marker for RCC, we determined *CXCR4* mRNA levels in 54 clear cell RCC primary tumors and 34 metastases. High CXCR4 levels were more commonly found in primary tumors of patients with lymph node or distant metastases at the time of diagnosis ($p = .034$ and $p = .029$, respectively; Supporting Information Table S2). Using Kaplan-Meier analyses and the median *CXCR4* mRNA content as a cut-off, we found longer cancer-specific survival of RCC patients with tumors exhibiting low *CXCR4* expression. Interestingly, this was only true for patients without metastases at the time of surgical removal of the primary tumor (Fig. 2B, left panel; $p = .005$). Such correlation was found neither for patients with synchronous metastasis ($p = .304$; Fig. 2B, middle panel) nor for RCC patients whose *CXCR4* mRNA content of metastatic tissue was used for Kaplan-Meier analyses ($p = .954$; Fig. 2B, right panel). In addition, *CXCR4* mRNA levels in the primary tumor represent an independent prognostic factor for M0/M1 patients. Patients with above median *CXCR4* mRNA levels have a worse prognosis (hazard ratio 4.1; 95% confidence interval 1.2–14.8; $p = .03$; Supporting Information Table S3).

Immunohistology revealed that low and high levels of *CXCR4* mRNA in primary tumors correlated with the amount of CXCR4 protein in tumor cells as well as with the number of CXCR4-positive cells which represented only a minority of tumor cells even in tumors with high *CXCR4* mRNA levels (Fig. 2C).

CXCR4$^+$ Cells Are Enriched in RCC Tumor Spheres and Are Capable of Tumor Initiation

Sphere formation culture conditions are considered to allow preferential growth of CSCs; thus CSC marker-expressing cells should be enriched in spheres. To test whether CXCR4 can serve as a CSC marker in RCC spheres, we analyzed CXCR4 expression on RCC-26, RCC-53, and the RCC cell line SK-RC-17 by flow cytometry after dissociation of spheres. Although the sphere formation potential of the three

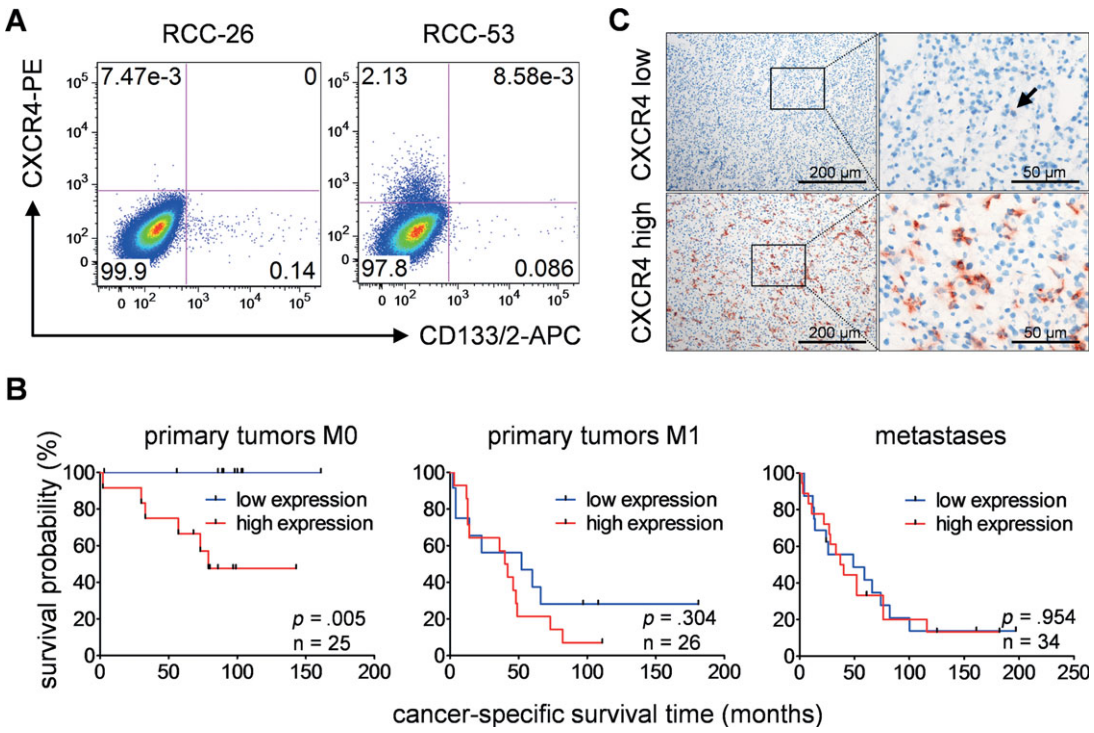

Figure 2. ICXCR4 expression correlates with aggressive tumor cell growth of RCC-53 cells and poor prognosis in RCC patients without metastases. **(A)**: The nontumorigenic RCC-26 cell line lacks a CXCR4$^+$ subpopulation in contrast to RCC-53 cells. Exponentially growing RCC-26 and RCC-53 cells were harvested and stained with fluorescently labeled anti-CXCR4 and anti-CD133/2 antibodies. The numbers in the quadrants indicate the fraction of cells in %. Note, that the content of CXCR4$^+$ cells but not that of CD133/2$^+$ cells correlates with tumor-initiation capacity of the cell lines. **(B)**: The *CXCR4* mRNA content of primary tumors predicts survival of patients without (M0) but not with metastasis (M1). The relative *CXCR4* mRNA content of RCC primary tumors and metastases was determined by quantitative polymerase chain reaction. Cancer-specific survival probabilities were calculated according to Kaplan-Meier using the respective median of *CXCR4* mRNA content as a cut-off. Survival of M0 patients with high *ICXCR4* expression (red line) was significantly shorter than for patients with low *ICXCR4* mRNA levels (blue line). Neither *ICXCR4* mRNA levels in primary tumors nor in metastases of M1 patients correlated significant with survival. The *p* values (*p*) and the number of patients (*n*) are indicated. **(C)**: Low and high *ICXCR4* mRNA expression correlates with CXCR4$^+$ tumor cells in primary RCC as shown by immunohistology using polyclonal rabbit anti-human CXCR4 antibody. Two representative samples out of six are shown. The boxed areas are enlarged in the right panel. The arrow points to a single CXCR4$^+$ cell. Abbreviations: APC, allophycocyanin; CXCR4, CXC chemokine receptor 4; PE, phycoerythrin.

cell lines varied widely with respect to number and size of spheres formed, the fraction of CXCR4$^+$ cells in dissociated spheres increased strongly in all three cell lines in comparison to adherently growing cells (6.6–364-fold; Fig. 3A, 3B). This increase in CXCR4$^+$ cells was transient and the content of CXCR4$^+$ cells returned nearly to the levels observed in standard cultures upon cultivation under adherent conditions for 3 days in the presence of serum (Fig. 3A, bottom panel). This is in line with CSCs being able to re-establish a hierarchy of stem cells and more differentiated cells upon cultivation under standard conditions. The strong enrichment of CXCR4$^+$ cells was also evident when CXCR4-expressing cells were detected by immunofluorescence in RCC-26 spheres despite the extremely low level of such cells in adherently grown RCC-26 cells (Fig. 3C). In contrast, cells expressing the putative RCC CSC marker CD105 [24] were not enriched after sphere formation in these cell lines but rather similar (RCC-53) or smaller CD105$^+$ cell fractions (RCC-26, SK-SC-17) were observed (Fig. 3A, 3B). Similarly, reduction of CD105-expressing cells after sphere formation was observed by Zhong et al. [23] in another RCC cell line. Interestingly, this loss of CD105 expression was reversed in RCC-26 and SK-RC-17 cells when sphere cells were cultivated under adherent growth conditions for 3 days (Fig. 3A, bottom panel). To assess the enrichment of cells with stem cell-like properties in spheres relative to adherently grown cells, we analyzed the transcriptional activity of *CXCR4* and genes typically expressed in stem cells in both populations. As expected, *CXCR4* mRNA levels were nearly 4- and 20-fold higher in SK-RC-17 and RCC-53 spheres, respectively, than in the corresponding adherent cells. In addition, the "stemness" genes *NANOG*, *POU5F1* (also known as *OCT3/4*), and *SOX2* showed elevated expression in spheres of both cell lines, whereas *CTNNB1* (encoding β-catenin) and the vimentin gene (*VIM*) not known to be selectively expressed in stem cell-like cells showed no enhanced expression in spheres (Fig. 3D). Interestingly, *MYC* known to be involved in uncontrolled proliferation and anchorage-independent growth in RCC [28] either was not or only slightly higher expressed in spheres (Fig. 3D). For RCC-53 cells, upregulation of CXCR4 and POU5F1 expression in spheres could be confirmed at the protein level visible as strong cytoplasmic/membranous and nuclear immunohistological staining, respectively. On the other hand, expression of the epithelial marker cytokeratin appeared to be downregulated in spheres relative to adherently grown cells (Fig. 3E).

To test the sphere formation and tumor-initiating potential of CXCR4$^+$ RCC cells more directly, we isolated CXCR4$^+$ RCC-53 cells by flow cytometry after labeling with a CXCR4-specific fluorescent antibody. CXCR4$^+$ cells formed fivefold more spheres and grew significantly faster than CXCR4$^-$ cells in immune compromised NOD/SCID mice

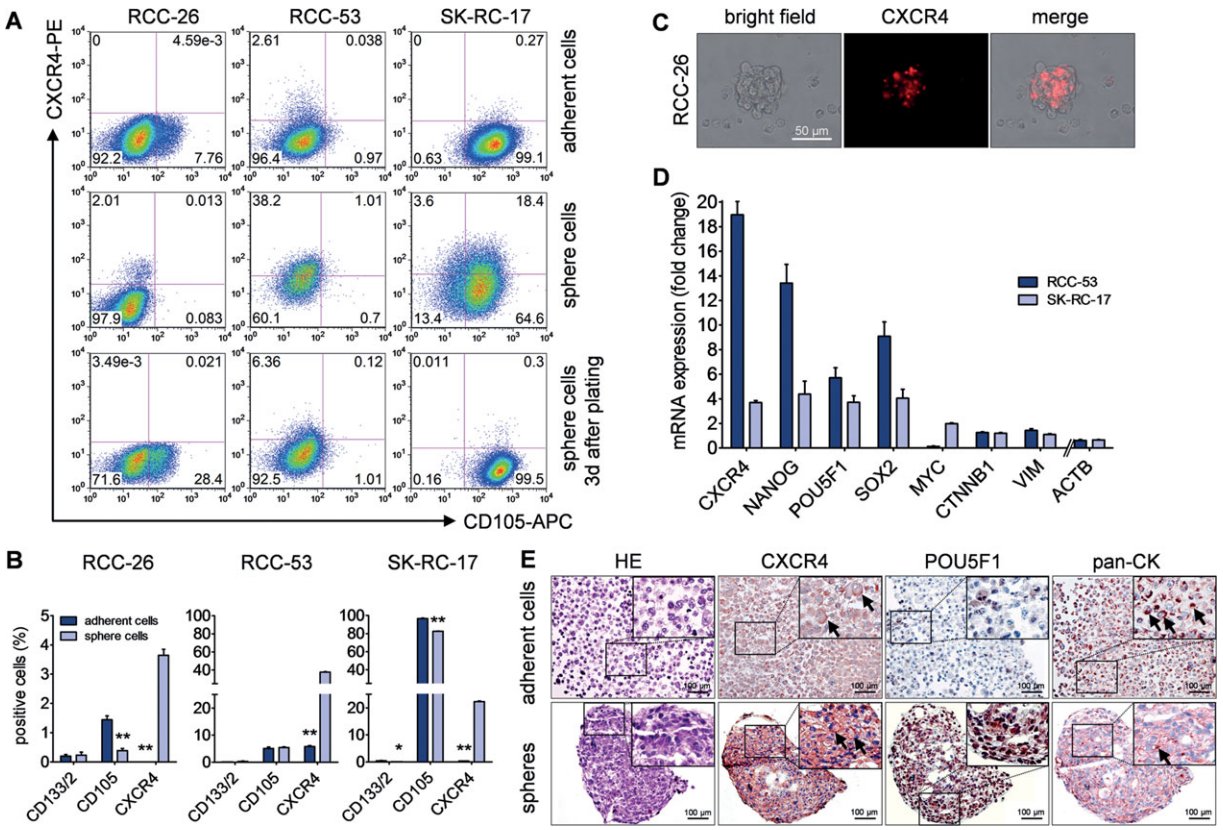

Figure 3. CXCR4$^+$ cells are enriched in tumor spheres. **(A, B)**: The fraction of CXCR4$^+$ RCC cells is transiently increased by sphere formation compared with corresponding adherent cells. RCC-26, RCC-53, and SK-RC-17 cells were grown under sphere formation conditions for 7 days, dissociated, and stained with anti-CXCR4 and anti-CD105 or anti-CD133/2 antibodies either directly after sphere dissociation or after cultivation under adherent conditions for 3 days. The numbers in the quadrants indicate the fraction of cells in %. In comparison, adherently growing cells were analyzed. The experiments were repeated twice and the fractions of positive cells are shown as means and SEM. *, $p < .05$; **, $p < .01$. **(C)**: CXCR4$^+$ RCC-26 cells can only be detected in spheres by immunofluorescence staining with a PE-labeled anti-CXCR4 antibody. **(D, E)**: Stemness genes are preferentially expressed in spheres. RCC-53 cells were grown under sphere formation or under adherent conditions for 7 days. (D) mRNA were quantitated by real time PCR and normalized for the β-actin mRNA (ACTB) content of the samples. The ratios mRNA levels$_{\text{spheres}}$/mRNA levels $_{\text{adherent cells}}$ are depicted (fold change). ACTB mRNA ratios are plotted without normalization. Results are shown as means and SEM ($n = 3$). (E): For detection of CXCR4, POU5F1 and cytokeratin cryosections of embedded cells and spheres were stained with specific mouse monoclonal (CXCR4) and rabbit polyclonal antibodies (POU5F1, pan-cytokeratin). Arrows indicate strongly labeled cells. Note enhanced cell surface/cytoplasmic and nuclear staining for CXCR4 and POU5F1, respectively, as well as reduced cytokeratin expression in spheres in comparison to adherently grown cells. Abbreviations: APC, allophycocyanin; CXCR4, CXC chemokine receptor 4; CK, cytokeratin; HE, hemalaun-eosin; PE, phycoerythrin.

(Fig. 4A, 4B). As in primary RCC, CXCR4$^+$ cells represented only a minority of the tumor cells in xenografts as shown by immunohistology (Fig. 4C).

CXCR4 Function Is Needed for Maintenance of CSCs

It has been described that signaling through CXCR4 triggered by interaction with its ligand CXCL12 induces chemotaxis as well as a number of other cellular processes, including proliferation and survival [29]. To test whether CXCR4 is functional, we preincubated RCC-53 cells for 7 days with the antagonistic inhibitor AMD3100 and tested their sphere formation capacity by cultivation for another 7 days under nonadherent conditions. AMD3100-pretreated cells formed threefold fewer spheres and contained 25% fewer CXCR4-expressing cells compared with solvent-treated cells despite the fact that RCC-53 cells treated with AMD3100 for 3 days showed a slightly elevated viability in comparison with untreated cells (Fig. 5A, 5B, 5C, left panel). In addition, the viability of RCC-53 cells from dissociated spheres was reduced by 40% in comparison to sphere cells cultured in the absence of the antagonist (Fig. 5C, right panel). Furthermore, reduction of CXCR4 expression by siRNA treatment of RCC-53 cells led to a decrease in sphere formation (Fig. 5D, 5E).

From these data, we concluded that long-term abrogation of CXCR4 signaling interferes with the sphere formation capacity of CXCR4$^+$ RCC-53 cells but not with cell replication and/or viability of bulk RCC-53 cultures.

CXCR4$^+$ Cells Are More Resistant to Multikinase Inhibitors and CXCR4 Inhibition Leads to Higher Drug Sensitivity

Since CSCs have been demonstrated to be more resistant to chemotherapy and radiation therapy and thus might contribute to drug resistance and tumor recurrence, we analyzed the sensitivity of RCC-26 and RCC-53 cells derived from dissociated tumor spheres toward sorafenib, sunitinib, and pazopanib in comparison to parental cells in adherent cultures. The drug concentrations were selected to inhibit the viability of parental RCC-26 and RCC-53 cells by 60%–90% (Supporting Information Fig. S2). These experiments revealed that sphere-derived RCC-26 and RCC-53 cells which are enriched in CXCR4$^+$ cells (Fig. 3) exhibit a 1.5–2.3-fold and 1.5–7.5-fold

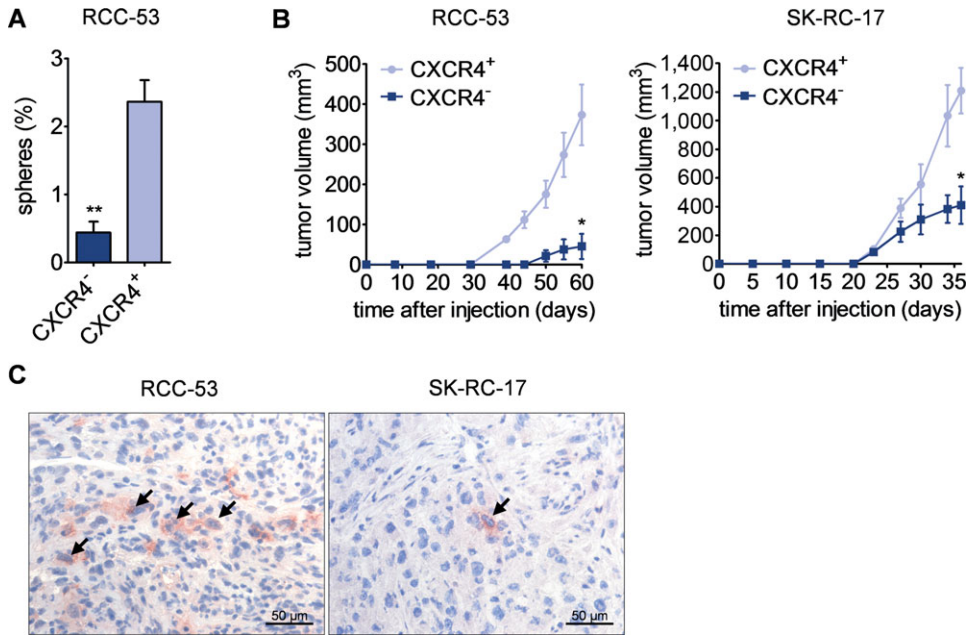

Figure 4. CXCR4$^+$ cells display cancer stem cell characteristics. **(A)**: Sphere forming capacity resides in the CXCR4$^+$ cell population. RCC-53 cells were separated into CXCR4^{high} (CXCR4$^+$) and CXCR4^{low} (CXCR4$^-$) cells by fluorescence-activated cell sorting and the fraction of cells (in %) capable of sphere formation within 7 days was determined. Results are shown as means and SEM; $n = 3$; **, $p < .01$. **(B)**: CXCR4$^+$ cells are more tumorigenic than CXCR4$^-$ cells. 8,000 and 2,500 RCC-53 and SK-RC-17 cells, respectively, were injected s.c. into NOD/SCID mice ($n = 4$–6 injection sites). Results are shown as means and SEM; $n = 2$–3 mice with two injection sites each; *, $p < .05$. **(C)**: CXCR4$^+$ cells in RCC-53 and SK-RC-17 xenograft tumors represent a minority of tumor cells as in primary RCC as shown by staining with polyclonal rabbit anti-human CXCR4 antibody. Arrows indicate CXCR4$^+$ tumor cells. Abbreviation: CXCR4, CXC chemokine receptor 4.

higher viability than parental cells after treatment with the higher drug doses for 1 day (Fig. 6A).

Interestingly, although AMD3100 alone did not inhibit parental cells, pretreatment with AMD3100 followed by coincubation of the CXCR4 antagonist and the kinase inhibitors pazopanib, sunitinib, and sorafenib reduced the cell viability of RCC-53 further, especially at kinase inhibitor doses higher than IC_{50} concentrations (Fig. 6B; Supporting Information Fig. S2).

Discussion

Emergence of resistant tumor cells following classical chemotherapy and radiation therapy or treatment with small molecule drugs leading to disease relapse has been attributed to selection of genetic tumor cell variants. More recently, evidence is accumulating that a small population of tumor cells with stem cell-like properties is also instrumental in conferring drug resistance [30]. CSCs are considered to drive progression and metastasis of tumors. Consistent with this notion, a high CSC content as estimated by the amount of marker gene mRNA or the number of CSC marker-positive cells identified by immunohistology was commonly found to correlate with a worse prognosis of tumor patients [31–34]. Consequently, such stem-like cells need to be considered as targets for efficient cancer therapies [18]. In order to be able to monitor the efficiency of such therapies, CSCs must be reliably identified using suitable markers. Although surface expression of CD105 has been used to enrich for tumor-initiating cells in RCC [19, 24], this marker was not suitable for use in all RCC cell lines to identify CSC ([23] and this work).

We used a novel approach to identify putative CSC markers in RCC by comparing expression of candidate cell surface proteins in two cell lines which were derived from tumors with different aggressive behaviors. This was reflected by the short and long survival of the patients, as well as by the different tumorigenic potentials of these cell lines in NOD/SCID mice, suggesting differences in content of tumor-initiating cells. Out of nine putative CSC markers tested, CXCR4 was found to be the most reliable marker. Several lines of evidence indicate that CXCR4 marks CSC in RCC. We have found that CXCR4$^+$ cells represented a small distinct population, which was enriched in tumor spheres, established using three RCC cell lines. In contrast, expression of CD133, a common CSC marker in a wide variety of other solid tumors, was restricted to a small fraction of cells whose number did not increase with tumor sphere formation. This is in line with the observation that the numbers of CD133$^+$ cells in RCC specimens did not directly or even inversely correlate with patient survival [35, 36]. RCC-53 cells from spheres expressed characteristic stemness genes, like the transcription factor-encoding *POU5F1*, *SOX2*, and *NANOG* and contained reduced levels of epithelial cytokeratins, which is considered typical for CSCs exhibiting a more immature mesenchymal phenotype [23, 37]. Most importantly, CXCR4$^+$ cells from both RCC-53 and SK-RC-17 RCC cells formed tumor spheres and initiated tumor growth in immunodeficient NOD/SCID mice more readily. Interestingly, CD105 which has been used in two other studies as a CSC marker in RCC [19, 24] was only marginally coexpressed in the CXCR4$^+$ cell population of RCC-26 and RCC-53 sphere cells (0.6% and 2.6%, respectively) but represented a major subpopulation in CXCR4$^+$ SK-RC-17 sphere cells (84%; Fig. 3A). This may indicate that CD105/endoglin which serves as an accessory protein in transforming growth factor-β receptor complexes is not essential for tumor initiation or marks a rare, highly tumorigenic subpopulation of RCC tumor-initiating cells in some cell lines [38, 39]. Expression of CXCR4 in CSC has also been

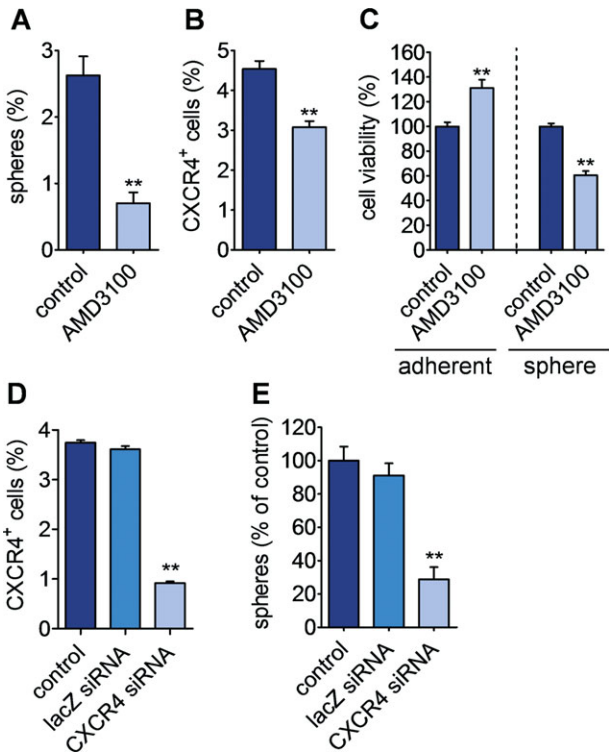

Figure 5. CXCR4 receptor function is necessary for sphere formation. AMD3100 inhibits sphere formation **(A)** and reduces the fraction of CXCR4$^+$ cells **(B)**. RCC-53 cells were treated with 10 μM AMD3100 for 7 days prior to determination of the fraction of cells (in %) capable of sphere formation in the absence of antagonist. **(C)**: Sphere-derived (right panel) but not adherent cells are sensitive to CXCR4 inhibition (left panel). Ten μM AMD3100 was added for 72 hours to adherently growing parental or sphere-derived cells, prior to cell viability testing. Decrease of CXCR4 by siRNA leads to reduced sphere formation **(D, E)**. Results are shown as means and SEM; $n = 3$; **, $p < .01$. Abbreviations: CXCR4, CXC chemokine receptor 4; siRNA, small interfering RNA.

reported for a number of cell lines from other solid tumors including glioma, prostate, hepatocellular, and non-small cell lung carcinomas as well as from breast, colon, and pancreas cancers [34, 37, 40-46]. Therefore, CXCR4 seems to represent one of the most prevalent CSC markers in solid tumors.

It becomes more apparent that in addition to playing an important role in metastasis to target organs [37, 47], CXCR4 and its ligand CXCL12 (also known as stromal-derived factor 1) are indispensable for maintenance and growth of CSCs in vitro and in vivo in various tumors, such as breast, prostate, non-small cell lung, and hepatocellular carcinoma and RCC cells, as shown by silencing of expression by siRNA and pharmacological inhibition of CXCR4 ([40, 41, 43, 46] and this work). Furthermore, our data suggest that CXCR4-expressing cells exhibit a more mesenchymal phenotype which has also been described for invasive tumor cells undergoing epithelial–mesenchymal transition [48]. CXCR4-expressing RCC cells might, therefore, be the driving force also for invasion. Indeed, it has been reported that CXCR4 signaling stimulates the production of matrix metalloproteases, like MMP9, and thus could contribute to invasion and dissemination of CXCR4-expressing CSCs [49]. Interestingly, high expression of MMP9 in primary RCC has been demonstrated to be associated with poor prognosis of RCC patients [50]. The properties of CXCR4$^+$ CSCs also seem to be relevant in RCC patients, since high levels of *CXCR4* mRNA or higher content of CXCR4$^+$ cells were associated with a poor prognosis ([36, 51] and this work). Interestingly, only M0 patients with high *CXCR4* mRNA content in their primary tumors, indicating a higher number of CSCs, were more likely to succumb to metastasis, accompanied by a shorter survival time. In contrast, high *CXCR4* mRNA levels in primary tumors of patients with established metastases at the time of diagnosis or in synchronous or metachronous metastases had only a marginal or no influence on the patient outcome (Fig. 2). This suggests that high numbers of CXCR4$^+$ CSCs in RCC facilitate invasion and dissemination of tumor cells and once metastasis has occurred, the CSC content has little influence on disease progression.

Multikinase inhibitors like sorafenib, sunitinib, and pazopanib are commonly used today as second and third line therapeutics in RCC patients with advanced disease [52]. In the highly vascularized RCC tumors, these inhibitors are thought to exert their antitumor effect mainly by antiangiogenesis because of their targeting preference for receptors such as vascular endothelial growth factor receptors, platelet-derived growth factor receptors, and others involved in promoting tumor vascularization [53]. However, these growth receptors probably also play a direct role in promotion of RCC growth [54]. We have observed that CXCR4-expressing RCC cells are more resistant to the receptor tyrosine kinase inhibitor drugs sorafenib, sunitinib, and pazopanib. This is in line with observations by D'Alterio et al. [55] who found that high

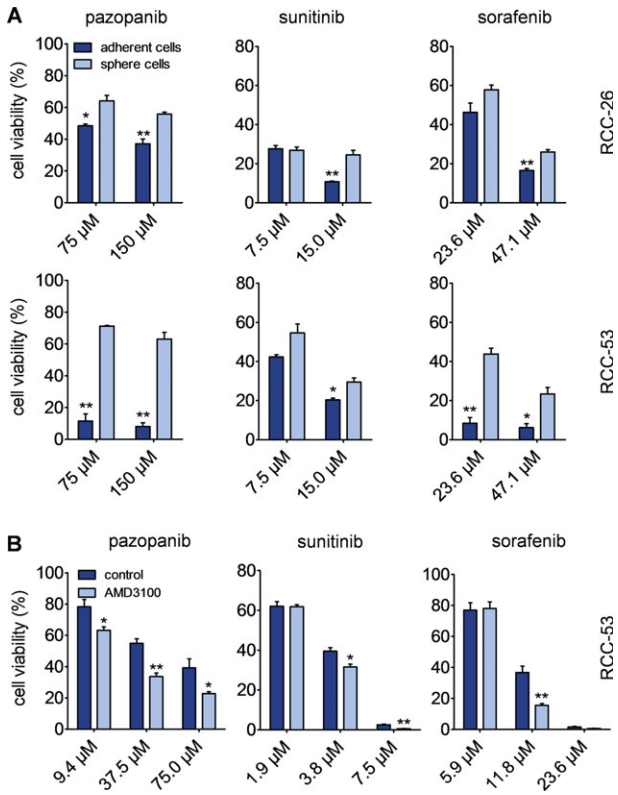

Figure 6. Spheres cells are more drug-resistant than adherent cells. **(A)**: RCC-26 and RCC-53 cells were grown for 7 days under sphere formation conditions. Dissociated spheres and control cells were cultured under adherent conditions and treated with pazopanib, sunitinib, and sorafenib for 24 h. (B): RCC-53 cells were pretreated or not with 10 μM AMD3100 for 7 days and then the indicated drugs together with AMD3100 were added for another 72 hours. Control cells were treated with the drugs alone. Cell viability was assessed by CellTiter-Blue Cell Viability Assay. Cotreatment was more effective than kinase inhibitor treatment alone. The results are shown as means and SEM; $n = 3$; *, $p < .05$; **, $p < .01$.

CXCR4 expression in primary tumors from RCC patients with metastatic disease, as evaluated by immunohistology, predicts a poor response to first-line treatment with sunitinib. Associations between higher resistance to endocrine therapy and CXCR4 signaling have been reported for breast cancer stem cells [41, 56]. Furthermore, in pancreatic tumor cells, gemcitabine resistance was found to be conferred by CXCR4 signaling [57]. In tamoxifen-resistant breast cancer cells, CXCL12-CXCR4 signaling stimulated the expression of the aryl hydrocarbon receptor, a ligand-dependent transcription factor, which led to expression of genes encoding xenobiotic-metabolizing enzymes and multidrug resistance proteins like cytochrome P450 and ABCG2, respectively [41].

Our data suggest that the CXCR4-expressing tumor-initiating cells in RCC cell lines represent CSCs in primary RCC. This is mainly based on the finding that $CXCR4^+$ tumor cells can also be found in primary RCC at variable frequencies, whereby high frequencies correlate with a worse prognosis. However, the stem cell properties and tumor-initiating potential of these cells still have to be demonstrated. In addition, it would be interesting to know whether RCC cell lines selected for drug resistance display higher tumor-initiating capacity, which is the scope of future experiments.

Conclusion

In conclusion, we have demonstrated for the first time that CXCR4 also represents a marker for tumor-initiating cells in RCC. CXCR4 appears to be indispensable for the maintenance of RCC CSCs and to confer drug resistance as well as invasive and metastatic properties. This was highlighted by its strong prognostic power for RCC patients without metastases at the time of diagnosis. Therefore, targeting the CXCR4 signaling pathway may be a promising therapeutic approach to suppress or eliminate CSCs in RCC [43, 58]. However, early therapeutic interference with CXCR4 signaling seems to be critical for therapeutic benefit, since it appears to be insignificant once metastasis has occurred.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

References

1 Janzen NK, Kim HL, Figlin RA et al. Surveillance after radical or partial nephrectomy for localized renal cell carcinoma and management of recurrent disease. Urol Clin North Am 2003;30:843–852.
2 Di Lorenzo G, Autorino R, Sternberg CN. Metastatic renal cell carcinoma: Recent advances in the targeted therapy era. Eur Urol 2009;56:959–971.
3 Negrier S, Raymond E. Antiangiogenic treatments and mechanisms of action in renal cell carcinoma. Invest New Drugs 2012;30:1791–1801.
4 Sosman JA, Puzanov I, Atkins MB. Opportunities and obstacles to combination targeted therapy in renal cell cancer. Clin Cancer Res 2007;13:764s–769s.
5 Reya T, Morrison SJ, Clarke MF et al. Stem cells, cancer, and cancer stem cells. Nature 2001;414:105–111.
6 Vermeulen L, de Sousa e Melo F, Richel DJ et al. The developing cancer stem-cell model: Clinical challenges and opportunities. Lancet Oncol 2012;13:e83–e89.
7 Al-Hajj M, Wicha MS, Benito-Hernandez A et al. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 2003;100:3983–3988.
8 Singh SK, Clarke ID, Terasaki M et al. Identification of a cancer stem cell in human brain tumors. Cancer Res 2003;63:5821–5828.
9 Collins AT, Berry PA, Hyde C et al. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 2005;65:10946–10951.
10 Bapat SA, Mali AM, Koppikar CB et al. Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. Cancer Res 2005;65:3025–3029.
11 Haraguchi N, Utsunomiya T, Inoue H et al. Characterization of a side population of cancer cells from human gastrointestinal system. Stem Cells 2006;24:506–513.
12 Ricci-Vitiani L, Lombardi DG, Pilozzi E et al. Identification and expansion of human colon-cancer-initiating cells. Nature 2007;445:111–115.
13 Li C, Heidt DG, Dalerba P et al. Identification of pancreatic cancer stem cells. Cancer Res 2007;67:1030–1037.
14 Prince ME, Sivanandan R, Kaczorowski A et al. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc Natl Acad Sci USA 2007;104:973–978.
15 Yang ZF, Ngai P, Ho DW et al. Identification of local and circulating cancer stem cells in human liver cancer. Hepatology 2008;47:919–928.
16 Fang D, Nguyen TK, Leishear K et al. A tumorigenic subpopulation with stem cell properties in melanomas. Cancer Res 2005;65:9328–9337.
17 Baccelli I, Trumpp A. The evolving concept of cancer and metastasis stem cells. J Cell Biol 2012;198:281–293.
18 Zhou BB, Zhang H, Damelin M et al. Tumour-initiating cells: Challenges and opportunities for anticancer drug discovery. Nat Rev Drug Discov 2009;8:806–823.
19 Azzi S, Bruno S, Giron-Michel J et al. Differentiation therapy: Targeting human renal cancer stem cells with interleukin 15. J Natl Cancer Inst 2011;103:1884–1898.
20 Ginestier C, Liu S, Diebel ME et al. CXCR1 blockade selectively targets human breast cancer stem cells in vitro and in xenografts. J Clin Invest 2010;120:485–497.
21 Dubrovska A, Kim S, Salamone RJ et al. The role of PTEN/Akt/PI3K signaling in the maintenance and viability of prostate cancer stem-like cell populations. Proc Natl Acad Sci USA 2009;106:268–273.
22 Addla SK, Brown MD, Hart CA et al. Characterization of the Hoechst 33342 side population from normal and malignant human renal epithelial cells. Am J Physiol Renal Physiol 2008;295:F680–F687.
23 Zhong Y, Guan K, Guo S et al. Spheres derived from the human SK-RC-42 renal cell carcinoma cell line are enriched in cancer stem cells. Cancer Lett 2010;299:150–160.
24 Bussolati B, Bruno S, Grange C et al. Identification of a tumor-initiating stem cell population in human renal carcinomas. FASEB J 2008;22:3696–3705.
25 Schendel DJ, Gansbacher B, Oberneder R et al. Tumor-specific lysis of human renal cell carcinomas by tumor-infiltrating lymphocytes. I. HLA-A2-restricted recognition of autologous and allogeneic tumor lines. J Immunol 1993;151:4209–4220.
26 Djafarzadeh R, Noessner E, Engelmann H et al. GPI-anchored TIMP-1 treatment renders renal cell carcinoma sensitive to FAS-meditated killing. Oncogene 2006;25:1496–1508.
27 Pastrana E, Silva-Vargas V, Doetsch F. Eyes wide open: A critical review of sphere-formation as an assay for stem cells. Cell Stem Cell 2011;8:486–498.

28 Tang SW, Chang WH, Su YC et al. MYC pathway is activated in clear cell renal cell carcinoma and essential for proliferation of clear cell renal cell carcinoma cells. Cancer Lett 2009;273:35–43.
29 Duda DG, Kozin SV, Kirkpatrick ND et al. CXCL12 (SDF1alpha)-CXCR4/CXCR7 pathway inhibition: An emerging sensitizer for anticancer therapies? Clin Cancer Res 2011;17:2074–2080.
30 Creighton CJ, Li X, Landis M et al. Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. Proc Natl Acad Sci USA 2009;106:13820–13825.
31 Li T, Su Y, Mei Y et al. ALDH1A1 is a marker for malignant prostate stem cells and predictor of prostate cancer patients' outcome. Lab Invest 2010;90:234–244.
32 Nakata S, Campos B, Bageritz J et al. LGR5 is a Marker of poor prognosis in glioblastoma and is required for survival of brain cancer stem-like cells. Brain Pathol 2013;23:60–72.
33 Su Y, Qiu Q, Zhang X et al. Aldehyde dehydrogenase 1 A1-positive cell population is enriched in tumor-initiating cells and associated with progression of bladder cancer. Cancer Epidemiol Biomarkers Prev 2010;19:327–337.
34 Zhang SS, Han ZP, Jing YY et al. CD133+CXCR4+ colon cancer cells exhibit metastatic potential and predict poor prognosis of patients. BMC Med 2012;10:85.
35 Costa WH, Rocha RM, Cunha IW et al. CD133 immunohistochemical expression predicts progression and cancer-related death in renal cell carcinoma. World J Urol 2012;30:553–558.
36 D'Alterio C, Cindolo L, Portella L et al. Differential role of CD133 and CXCR4 in renal cell carcinoma. Cell Cycle 2010;9:4492–4500.
37 Hermann PC, Huber SL, Herrler T et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell 2007;1:313–323.
38 Barbara NP, Wrana JL, Letarte M. Endoglin is an accessory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor-beta superfamily. J Biol Chem 1999;274:584–594.
39 Sanz-Rodriguez F, Guerrero-Esteo M, Botella LM et al. Endoglin regulates cytoskeletal organization through binding to ZRP-1, a member of the Lim family of proteins. J Biol Chem 2004;279:32858–32868.
40 Dubrovska A, Elliott J, Salamone RJ et al. CXCR4 expression in prostate cancer progenitor cells. Plos One 2012;7:e31226.
41 Dubrovska A, Hartung A, Bouchez LC et al. CXCR4 activation maintains a stem cell population in tamoxifen-resistant breast cancer cells through AhR signalling. Br J Cancer 2012;107:43–52.
42 Ehtesham M, Mapara KY, Stevenson CB et al. CXCR4 mediates the proliferation of glioblastoma progenitor cells. Cancer Lett 2009;274:305–312.
43 Jung MJ, Rho JK, Kim YM et al. Upregulation of CXCR4 is functionally crucial for maintenance of stemness in drug-resistant non-small cell lung cancer cells. Oncogene 2013;32:209–221.
44 Miki J, Furusato B, Li H et al. Identification of putative stem cell markers, CD133 and CXCR4, in hTERT-immortalized primary nonmalignant and malignant tumor-derived human prostate epithelial cell lines and in prostate cancer specimens. Cancer Res 2007;67:3153–3161.
45 Ping YF, Yao XH, Jiang JY et al. The chemokine CXCL12 and its receptor CXCR4 promote glioma stem cell-mediated VEGF production and tumour angiogenesis via PI3K/AKT signalling. J Pathol 2011;224:344–354.
46 Yang W, Wang C, Lin Y et al. OV6(+) tumor-initiating cells contribute to tumor progression and invasion in human hepatocellular carcinoma. J Hepatol 2012;57:613–620.
47 Kim M, Koh YJ, Kim KE et al. CXCR4 signaling regulates metastasis of chemoresistant melanoma cells by a lymphatic metastatic niche. Cancer Res 2010;70:10411–10421.
48 Brabletz T, Jung A, Spaderna S et al. Opinion: Migrating cancer stem cells—An integrated concept of malignant tumour progression. Nat Rev Cancer 2005;5:744–749.
49 Furusato B, Mohamed A, Uhlen M et al. CXCR4 and cancer. Pathol Int 2010;60:497–505.
50 Cho NH, Shim HS, Rha SY et al. Increased expression of matrix metalloproteinase 9 correlates with poor prognostic variables in renal cell carcinoma. Eur Urol 2003;44:560–566.
51 Staller P, Sulitkova J, Lisztwan J et al. Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. Nature 2003;425:307–311.
52 Negrier S, Raymond E. Antiangiogenic treatments and mechanisms of action in renal cell carcinoma. Invest New Drugs 2012;30:1791–1801.
53 Huang D, Ding Y, Li Y et al. Sunitinib acts primarily on tumor endothelium rather than tumor cells to inhibit the growth of renal cell carcinoma. Cancer Res 2010;70:1053–1062.
54 Xu L, Tong R, Cochran DM et al. Blocking platelet-derived growth factor-D/platelet-derived growth factor receptor beta signaling inhibits human renal cell carcinoma progression in an orthotopic mouse model. Cancer Res 2005;65:5711–5719.
55 D'Alterio C, Portella L, Ottaiano A et al. High CXCR4 expression correlates with sunitinib poor response in metastatic renal cancer. Curr Cancer Drug Targets 2012;12:693–702.
56 Rhodes LV, Short SP, Neel NF et al. Cytokine receptor CXCR4 mediates estrogen-independent tumorigenesis, metastasis, and resistance to endocrine therapy in human breast cancer. Cancer Res 2011;71:603–613.
57 Singh S, Srivastava SK, Bhardwaj A et al. CXCL12-CXCR4 signalling axis confers gemcitabine resistance to pancreatic cancer cells: A novel target for therapy. Br J Cancer 2010;103:1671–1679.
58 Domanska UM, Kruizinga RC, Nagengast WB et al. A review on CXCR4/CXCL12 axis in oncology: No place to hide. Eur J Cancer 2013;49:219–230.

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