CXCL12 Chemokine Expression Suppresses Human Pancreatic Cancer Growth and Metastasis

Ishan Roy¹, Noah P. Zimmerman¹, A. Craig Mackinnon², Susan Tsai³, Douglas B. Evans³, Michael B. Dwinell¹*

¹Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, Wisconsin, United States of America, ²Department of Pathology, Medical College of Wisconsin, Milwaukee, Wisconsin, United States of America, ³Department of Surgery, Medical College of Wisconsin, Milwaukee, Wisconsin, United States of America

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is an unsolved health problem with nearly 75% of patients diagnosed with advanced disease and an overall 5-year survival rate near 5%. Despite the strong link between mortality and malignancy, the mechanisms behind pancreatic cancer dissemination and metastasis are poorly understood. Correlative pathological and cell culture analyses suggest the chemokine receptor CXCR4 plays a biological role in pancreatic cancer progression. In vivo roles for the CXCR4 ligand CXCL12 in pancreatic cancer malignancy were investigated. CXCR4 and CXCR7 were consistently expressed in normal and cancerous pancreatic ductal epithelium, established cell lines, and patient-derived primary cancer cells. Relative to healthy exocrine ducts, CXCL12 expression was pathologically repressed in pancreatic tissue specimens and patient-derived cell lines. To test the functional consequences of CXCL12 silencing, pancreatic cancer cell lines stably expressing the chemokine were engineered. Consistent with a role for CXCL12 as a tumor suppressor, cells producing the chemokine were increasingly adherent and migration deficient in vitro and poorly metastatic in vivo, compared to control cells. Further, CXCL12 reintroduction significantly reduced tumor growth in vivo, with significantly smaller tumors in vivo, leading to a pronounced survival advantage in a preclinical model. Together, these data demonstrate a functional tumor suppressive role for the normal expression of CXCL12 in pancreatic ducts, regulating both tumor growth and cellular dissemination to metastatic sites.

Citation: Roy I, Zimmerman NP, Mackinnon AC, Tsai S, Evans DB, et al. (2014) CXCL12 Chemokine Expression Suppresses Human Pancreatic Cancer Growth and Metastasis. PLoS ONE 9(3): e90400. doi:10.1371/journal.pone.0090400

Editor: Jeffrey K. Harrison, University of Florida, United States of America

Received November 11, 2013; Accepted January 29, 2014; Published March 4, 2014

Copyright: © 2014 Roy et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported in part by grants from the A Healthier Wisconsin and the MCW Cancer Center. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: We have the following interests. Dr. Dwinell is a recipient of a patent (8,404,640) for the use of CXCL12 in the treatment of malignant cancers and is a co-founder of Protein Foundry, LLC. There are no further patents, products in development or marketed products to declare. This does not alter our adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors. The title of the patent is: "Method of Diagnosing and Treating Colon Cancer".

* E-mail: mwdwinell@mcw.edu

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an unrelenting form of human cancer, with no effective technique for early diagnosis or treatment, and an incidence nearly equal to mortality [1,2]. Most patients, at the time of diagnosis, already have locally advanced or metastatic disease, making surgical resection of the primary tumor of limited therapeutic value [3]. Current pharmacological therapies for PDAC are inadequate as the vast majority of pancreatic cancer patients die of micro- or macro-metastatic disease [4]. An improved understanding of the biologic mechanisms underlying the aggressiveness of PDAC is needed. Although recent studies have determined the molecular factors involved in the progression of PDAC [5–7], little is known about the biologic mechanisms regulating cell motility and, in turn, metastasis. Chemotactic cytokines, or chemokines, play key roles in cellular migration, and are capable of coordinating multiple aspects of the cell migration machinery. Through activation of their receptors, chemokines regulate several facets of normal physiology, including leukocyte trafficking, epithelial cell migration necessary for wound closure, tissue vascularization, and organ development during embryogenesis [8–10]. Previously, our lab revealed the importance of the dual expression of the chemokine CXCL12 and its cognate receptor CXCR4 in enterocyte migration, wound healing, and angiogenesis [11–16].

Several studies have implicated a role for chemokine receptors, in particular CXCR4 and CCR7, in cancer progression and metastasis [9,17–22]. CXCR4 expression has been linked with the malignancy of over 20 different cancers [17,23]. We have previously demonstrated that CXCL12 is expressed in normal epithelial cells in intestine and mammary glands, but is epigenetically silenced in human breast and colorectal cancer [24,25]. This pattern of CXCL12 gene repression results in tumor cells whose chemokine receptor profiles mirror that of highly mobile leukocytes, which express the receptors CXCR4 and CCR7 but not the ligand. We showed that re-expression of CXCL12 decreased the ability of breast and colorectal cancer cells to metastasize [24–26]. Several studies have extended these seminal findings to show beneficial effects of autocrine CXCL12 in breast, lung, gastric, and head and neck cancers [27–30]. Loss of CXCL12 expression in osteosarcoma and breast cancer patient tumor specimens was correlated with poorer prognosis [31–34]. In
pancreatic cancer, conflicting and incomplete reports suggest either that CXCL12 expression is elevated or that CXCL12 is not expressed in the vast majority of patients [35,36]. Thus while a growing body of evidence suggests a tumor-suppressive role for CXCL12 in human cancer malignancy, its mechanistic roles in PDAC remain poorly understood.

The prevailing model of cancer metastasis is that malignant tumors spread to distant tissues through the over-expression of CXCR4 [22,37,38], thereby sensitizing malignant cells to spread in response to distant gradients of CXCL12 produced by metastatic destination organs. This model has also been used to explain PDAC metastasis, as several reports document the expression of CXCR4 by pancreatic carcinoma cell lines and human tissue [39–42]. However none of these studies have rigorously examined the parallel expression of CXCL12 or CXCR7 in the context of CXCR4 or defined expression of any of the three components of this chemokine axis in normal pancreatic epithelium. The objectives of our study were to determine the expression profile and functional role of the CXCL12-CXCR4-CXCR7 axis in both healthy normal and malignant pancreas. Herein, our data demonstrate that while receptor expression is increased, CXCL12 expression is significantly diminished in resected PDAC tissue and a battery of pancreatic cancer cell lines compared to normal pancreata. CXCL12 expression was transiently restored using inhibitors of epigenetic repression. Stable reintroduction of CXCL12 in PDAC cells prevented directed cell migration and hepatic metastasis and slowed tumor growth in vitro and in vivo, resulting in increased survival in preclinical models.

**Materials and Methods**

**Ethics statement**

Exempt, de-identified normal pancreata, PDAC tissue specimens, and unique patient-derived PDAC cells were obtained from the Surgical Oncology Biorepository using a Medical College of Wisconsin (MCW) Institutional Review Board #4 approved protocol. Tissues and cells within the biobank were obtained from patients following signed written informed consent and are coded and de-identified, with personal identifying information not shared with research investigators. Preclinical mouse xenograft studies were completed using protocols and procedures documented in an established Animal Use Application(AUA076) approved by the MCW Institutional Animal Care and Use Committee and in compliance with guidelines established by Office of Laboratory Animal Welfare and in accordance with Guide for the Care and Use of Laboratory Animals. All mice were housed under pathogen-free conditions, received food and water ad libitum, and maintained in a 10hr:14-hr light/dark cycle in a temperature-controlled room (25±2°C). Animals were euthanized at the completion of the study or following signs of distress or poor body condition as assessed by trained laboratory personnel and confirmed by MCW Biomedical Resource Center staff veterinarians.

---

**Figure 1.** Aberrant chemokine ligand and receptor expression in human pancreatic ductal adenocarcinoma. Serial sections of normal and diseased pancreatic tissue were stained for CXCL12, CXCR4, CXCR7, and CK19. CXCL12 staining apparent in normal exocrine ducts was diminished in PDAC tissue. CXCR4 staining increased in PanIN and PDAC relative to normal ductal epithelium. CXCR7 expression was variable in normal epithelium, PanIN lesions, and PDAC. (A) Normal tissue from a single patient with healthy pancreas represents observations from 25 different normal tissues from 21 individual patients. (B) PDAC tissue from one patient represents observations from 82 different tissues from 29 different patients. 1000× magnification represents inset box at 200×. (C) Staining was quantified by blinded scoring of serial sections in relation to CK19 staining. (***) denotes P<0.001. doi:10.1371/journal.pone.0090400.g001
ians to ameliorate pain and distress associated with tumor formation.

**Human pancreatic cancer cell lines**

Panc1 (CRL-1469), MiaPaCa2 (CRL-1220), Capan2 (HTB-80), and HPAFII (CRL-1997) cell lines were purchased from the ATCC. Panc1 and MiaPaCa2 cell lines were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS). The Capan2 and HPAFII cell lines were maintained in 10% (v/v) FBS supplemented McCoy’s 5A or MEM medium, respectively. In some experiments, cells grown in normal medium were treated daily with 5-aza-2'-deoxycytidine (5-aza) or Trichostatin-A (EMD Biosciences). MiaPaCa2 cell lines were transfected with Firefly-luciferase under zeocin selection. Resultant luciferase-expressing MiaPaCa2 cells were subsequently transfected with plasmids encoding either eGFP or human CXCL12, and clones grown under neomycin selection conditions [26]. Cell lines were de-identified of all identification parameters from individual consenting patients with pancreatic cancer and were confirmed to be of PDAC origin following DNA karyotype and protein expression analysis.

**Human pancreatic tissue specimens**

Normal ducts and PanIN lesions were isolated exclusively from adjacent normal tissue discards of organ transplantation or pancreatic operations not involving PDAC or other exocrine malignancies. Disease status of those normal and tumor tissues was confirmed by a board-certified pathologist. A total of 25 tissues from 21 different patients were used for normal analyses. A total of 82 tissues from 29 different patients were used for tumor analyses. Of the 29 patients providing PDAC tissue, 11 patients had received neo-adjuvant therapy.

**RT-PCR**

RNA was isolated from cells and tissue specimens using the RNAeasy kit (Qiagen) and treated with DNase (Ambion),

![Figure 2](https://example.com/figure2.png)

**Figure 2. CXCL12 expression in normal pancreas is decreased in dysplastic epithelium.** Representative 200× images of the same (A) normal or (B) pancreatic ductal adenocarcinoma (PDAC) tissues shown at 1000× magnification in Figure 1. Representative serial section images of a pancreatic intestinal neoplasm (PanIN) lesion at (C) 200× or (D) 1000× magnification. Serial tissue sections were immunostained with antibodies to CK19, CXCL12, CXCR4, and CXCR7, or the isotype controls. n = 25 different normal tissues from 21 individual patients, 82 different tissues from 29 different PDAC patients, or 19 pathologically confirmed PanIN lesions.

doi:10.1371/journal.pone.0090400.g002
Intra-epithelial Neoplasms (PanINs) [48–51]. Amplified to detect genomic DNA contaminants [12]. Studies and bioluminescence imaging were previously established [47], with 1×10^6 cells injected into the pancreata of SCID mice and disease progression monitored. Mice were removed from the study when the tumor size reached 1000 mm^3 volume and 1×10^6 p/sec/cm^2/stereadian. Three mice engrafted with CXCL12-expressing cells were removed for veterinary non-study reasons due to cage-infiliting.

**Immunohistochemical studies**

Unstained slides were generated from pancreatic tumor specimens and CXCL12 immunostained using an antibody from R&D Systems (tab/50). Cytokeratin-19 (CK19) (ab7754), CXCR4 (ab2074), CXCL7 (ab30899 & ab72100), and Ki-67 (ab15580) were detected using antibodies from Abcam. Visualization was done using horseradish-peroxidase-conjugated secondary antibodies and the 3,3'-diaminobenzidine-Peroxidase Substrate Kit (Vector Labs). To avoid background interference slides were not counterstained. Protein expression levels were scored using a standard 4-point scale [0 = absent, 1 = weak, 2 = mixed, and 3 = strong staining intensity] [44]. Analyses were independently completed by an investigator blinded to the disease status and immunostaining protocol. Secreted CXCL12 protein from supernatant of pancreatic cancer cells cultured in serum-free media was detected by our previously established sandwich ELISA method using antibodies from R&D Systems (monoclonal mouse and human CXCL12 (MA850) and goat anti-human CXCL12 (BAF310) [24]. Cell surface CXCR4 or CXCR7 was detected using the aforementioned antibodies (Abcam) along with FITC-conjugated secondary antibodies using our previously established method [43]. Briefly, cells were cultured in normal growth medium, lifted using enzyme-free dissociation buffer, washed using ice-cold sterile PBS, and then incubated in a blocking solution containing BSA and secondary antibody serum. After blocking, cells were first incubated with primary antibody and then with FITC-conjugated secondary antibody. Finally, cells were fixed and analyzed by flow cytometry (LSR II, BD Biosciences).

**In vitro tumorigenesis assays**

Cells were plated to the upper well with chemo-attractants added to the bottom well and transwell migration or chemoinvasion enumerated in a representative set of images taken after fluorescent staining as described previously [43,45]. The upper well membrane was coated with collagen in migration assays and with Matrigel (BD Biosciences) in invasion assays. Panc1 and HPAFII cell migration was measured after 24 hour stimulation in transwells while MiaPaCa2 cell migration was measured after 6 hours.

Initial proliferation and apoptosis of PDAC cell lines was defined using the Viacount flow cytometric assay (Millipore), or the caspase-3/7 glo assay (Promega). Briefly, cells were plated in 10% (v/v) serum-containing medium, and once adherent (overnight) were switched to serum-free medium. Cell cycle analysis was done using propidium iodide staining and flow cytometric analysis, as done previously [43]. In some experiments cells were grown in 1% (v/v) serum-containing media after 24 hours of serum starvation.-Gemcitabine (GEM), a well-established chemotherapeutic drug in pancreas cancer patients, was used as a positive control for decreased growth and increased apoptosis.

**In vivo studies and bioluminescence imaging**

An established heterotopic intrasplenic injection model [46] was used to assess metastatic homing and extravasation in the liver. Six-week-old immunocompromised SCID mice were anesthetized and 1×10^6 MiaPaCa2 luciferascells were injected into the spleen through a lateral wall excision and tumor growth and metastasis monitored using bioluminescence imaging every 7 days using an previously described approach [26]. After 28 days, mice were sacrificed and tumor formation in the spleen and liver measured ex vivo using bioluminescence imaging. An orthotopic model was employed as previously established [47], with 1×10^6 cells injected into the pancreata of SCID mice and disease progression monitored. Mice were removed from the study when the tumor size reached 1000 mm^3 volume and 1×10^6 p/sec/cm^2/stereadian. Three mice engrafted with CXCL12-expressing cells were removed for veterinary non-study reasons due to cage-infiliting.

**Statistical analyses**

Multiple comparisons between groups were analyzed using a one-way ANOVA and a Dunnett post-hoc analysis used to identify pair-wise differences (GraphPad Prism 4). Paired analyses were calculated using either a Mann-Whitney or log-rank test where appropriate. Statistical significance was defined as P≤0.05.

**Results**

**Reciprocal CXCL12, CXCR4, and CXCR7 expression in pancreatic ductal adenocarcinoma**

The functions of CXCR4, CXCR7 or CXCL12 in pancreatic cancer progression remain based on limited analyses without simultaneous analysis of all three chemokine signaling components in both normal and diseased tissue. Immunostaining of serial tissue sections with specific antibodies, revealed robust CXCL12 staining on normal ductal epithelial cells, with those same epithelia also staining positive for CXCR4 and CXCR7 (Fig. 1A & Fig. 2A). CXCL12 chemokine expression was specifically restricted to the ductal compartment and absent from acinar and endocrine cells, both of which stained for the chemokine receptors CXCR4 and CXCR7 (data not shown). Incubation of parallel sections with isotype control antibodies confirmed specificity (Fig. 2A). We next examined expression in pre-malignant pancreatic lesions, known as Pancreatic Intra-epithelial Neoplasms (PanINs) [48–51]. As shown in Figure 2C-D, CK19+ PanINs expressed both CXCR4 and CXCR7. By comparison, CXCL12 staining was more variable, with mixed to markedly decreased expression in PanINs compared to normal pancreatic ducts (Fig. 2C-D). In a limited analysis, no significant differences in CXCL12 expression could be detected between PanIN1, PanIN2, and PanIN3 lesions.

Analysis of PDAC tumor tissue revealed further, and more pronounced, extinction of CXCL12 in CK-19+ tumor cells (Fig. 1B, Fig. 2B). In heterogeneous tumor tissue containing both malignant and normal glands, CXCL12 staining was diminished in malignant cells, but preserved in adjacent normal ductal cells (data not shown). Analysis of serial tissue sections revealed that malignant tissue with low levels of CXCL12 had a reciprocal increase in CXCR4, as well as CXCR7, staining (Fig. 1B, Fig. 2B). Quantification of the CXCL12, CXCR4, and CXCR7 immunostaining intensity confirmed the significant decrease in CXCL12 expression during progression from normal to precursor PanIN to malignant pancreatic tissue (Fig. 1C). In contrast, CXCR4 expression was elevated in PanINs and PDAC compared to normal ducts (Fig. 1C). Scoring of CXCR7 expression revealed a biphasic pattern with lower expression occurring at the PanIN stage and higher expression recurring in PDAC (Fig. 1C). When the patients were subdivided into groups dependent on whether or not they had received neo-adjuvant therapy, patients receiving standard-of-care regimens of gemcitabine or FOLFIRINOX therapy [52–54] had significantly (P≤0.05) higher scores for CXCL12 expression (0.82±0.18) compared to patients not receiving neoadjuvant therapy (0.43±0.09).
CXCL12 expression and epigenetic regulation in pancreatic cancer cell lines

To further characterize chemokine – receptor expression and identify an appropriate model for studying the function of CXCL12 in PDAC, primary patient-derived and established pancreatic cancer cell lines were analyzed through RT-PCR using our previously optimized primer sets for CXCL12, CXCR4, and CXCR7 [24,43]. RT-PCR indicated consistent lack of CXCL12 transcript in patient-derived primary as well as Panc1, MiaPaCa2, Capan2, and HPAFII cell lines (Fig. 3A–B). In agreement with the immunohistochemical analyses, CXCR4 mRNA was present in 7 of the 8 PDAC cell lines. Flow cytometry confirmed the cell-surface localization of CXCR4 and CXCR7 on representative cell lines (Fig. 3C). To ascertain if the lack of CXCL12 expression reflected epigenetic silencing, cells were treated with titrated doses of the DNA methyltransferase inhibitor 5-aza. The inhibitor rescued CXCL12 mRNA expression in representative cell lines (Fig. 3D). Treatment with the histone deacetylase inhibitor Trichostatin-A also restored expression of CXCL12 in Capan2 cells (Fig. 3E). Based upon our prior investigations examining the specific mechanisms of CXCL12 promoter hypermethylation in colorectal and breast cancers [24,25], these data suggest that CXCL12 expression is regulated through epigenetic mechanisms in pancreatic cancer as well.

Cumulatively, these data indicate CXCL12 gene repression in human tissue and a battery of new and established PDAC cell lines. The MiaPaCa2 cell line was identified as an ideal model, as its CXCL12 expression pattern mimics that observed in malignant human PDAC tissue.

Figure 3. CXCL12 expression in human pancreatic ductal adenocarcinoma cell lines. RT-PCR analysis revealed that (A) patient-derived pancreatic cancer cell lines (#1, #2, #3, #4) or (B) established cell lines lacked expression of CXCL12 and maintained expression of CXCR4. CXCR7 mRNA was present in 3 of 8 PDAC lines. Flow cytometric detection of surface CXCR4 or CXCR7 protein expression. Cell lines treated seven days with a concentration curve of (D) 5-aza-2-deoxycytidine (5-aza) restored expression of CXCL12. Lines treated 4 days with a concentration curve of (E) Trichostatin-A (TSA) restored CXCL12 mRNA expression in Capan2 cells.

doi:10.1371/journal.pone.0090400.g003

CXCL12 expression and epigenetic regulation in pancreatic cancer cell lines

To further characterize chemokine – receptor expression and identify an appropriate model for studying the function of CXCL12 in PDAC, primary patient-derived and established pancreatic cancer cell lines were analyzed through RT-PCR using our previously optimized primer sets for CXCL12, CXCR4, and CXCR7 [24,43]. RT-PCR indicated consistent lack of CXCL12 transcript in patient-derived primary as well as Panc1, MiaPaCa2, Capan2, and HPAFII cell lines (Fig. 3A–B). In agreement with the immunohistochemical analyses, CXCR4 mRNA was maintained in 7 of the 8 PDAC cell lines, while CXCR7 expression was more variable (Fig. 3A–B). Flow cytometry confirmed the cell-surface localization of CXCR4 and CXCR7 on representative cell lines (Fig. 3C). To ascertain if the lack of CXCL12 expression reflected epigenetic silencing, cells were treated with titrated doses of the DNA methyltransferase inhibitor 5-aza. The inhibitor rescued CXCL12 mRNA expression in representative cell lines (Fig. 3D). Treatment with the histone deacetylase inhibitor Trichostatin-A also restored expression of CXCL12 in Capan2 cells (Fig. 3E). Based upon our prior investigations examining the specific mechanisms of CXCL12 promoter hypermethylation in colorectal and breast cancers [24,25], these data suggest that CXCL12 expression is regulated through epigenetic mechanisms in pancreatic cancer as well.

Cumulatively, these data indicate CXCL12 gene repression in human tissue and a battery of new and established PDAC cell lines. The MiaPaCa2 cell line was identified as an ideal model, as its CXCL12-CXCR4-CXCR7 expression pattern mimics that observed in malignant human PDAC tissue.
CXCL12 re-expression in human PDAC cells disrupted chemotaxis, increased adhesive potential, and decreased hepatic metastasis

The conventional paradigm for chemokine mediated metastasis of cancer cells is that expression of CXCR4 is pro-metastatic. This model stems from the ability of exogenous CXCL12 to stimulate migration of cancer cells in vitro [13,45,55]. As expected, measurement of the native migration potential of several CXCL12-deficient pancreatic cell lines revealed that CXCR4-expressing PDAC cells migrate towards acute CXCL12 stimulation (Fig. 4A). Importantly, Panc1 and MiaPaCa2 cells migrated towards CXCL12 in biphasic-concentration dependent manner consistent with current understanding of chemotactic migration [55,56]. The HPAFII cell line which lacked surface expression of either CXCR4 or CXCR7 was unable to migrate in response to CXCL12 treatment (Fig. 4A). Panc1 cells also invaded an extracellular matrix in response to acute exogenous CXCL12 stimulation (Fig. 4B).

Figure 4. PDAC cells migrate and invade following acute exogenous CXCL12 stimulation. (A) Panc1 and MiaPaCa2 cells migrated towards exogenous gradients of CXCL12 in a range from 1 nM to 1000 nM under serum-free conditions (*), (**), and (***) denote P<0.05, P<0.01, and P<0.001, respectively, in comparison to unstimulated cells (NS). Receptor-null HPAFII cells did not migrate in response to CXCL12. (B) CXCL12 directs Panc1 cell chemoinvasion into a three-dimensional Matrigel plug. The positive control (+) was 10% serum-containing medium. (*), (**), and (***) denote P<0.05, P<0.01, and P<0.001, respectively, in comparison to unstimulated cells (NS).

doi:10.1371/journal.pone.0090400.g004

CXCL12 Is a Multifunctional Tumor Suppressor

Figure 5. Double transfectant Firefly luciferase and GFP- or CXCL12-expressing MiaPaCa2 cells. Luciferase levels in control GFP or three different (31, #2, #3) CXCL12-expressing MiaPaCa2 clones as measured by spectrophotometer (A) or IVIS-100 biophotonic imager (B). Levels of CXCL12 were measured by ELISA (C) in established PDAC cell lines as well as GFP- and CXCL12-expressing MiaPaCa2-luciferase clones. (D) CXCL12-secreted by transfected MiaPaCa2-luciferase clones #1 and #2 stimulated U937 chemotaxis. Cells treated with neutralizing antibody to CXCL12 (aL12) confirmed the specificity of U937 chemotaxis. Values in A, C, and D are mean±SEM, n=2–3.

doi:10.1371/journal.pone.0090400.g005
We believe that the pro-metastatic response of PDAC cells is due to the silencing of expression of CXCL12. To test this we introduced expression of the chemokine, using doublestable plasmid integration, into MiaPaCa2 cells. Cells were first stably transfected with firefly-luciferase and then transfected with additional genes using a second selection reagent. Several CXCL12-expressing clones were generated along with a control clone transfected with eGFP, each exhibiting equivalent levels of luciferase activity (Fig. 5A–B). Importantly, lack of chemokine production was confirmed by ELISA in Panc1, MiaPaCa2, Capan2, and HPAFII cell lines (Fig. 5C). Functional chemokine production in CXCL12-expressing clones compared to GFP-expressing clones was validated by ELISA and transwell migration of U937 cells (Fig.5C–D). MiaPaCa2 cells secreting CXCL12 demonstrated a significant reduction in migratory response to TGF-β or CXCL12, both known inducers of in vitro PDAC cell migration (Fig.6A–D). Given the importance of adhesive potential in the metastatic abilities of cancer cells, cell adhesion was next measured. CXCL12 positive cells were significantly more adherent to tissue culture plastic compared to CXCL12-null cells. Untreated cells = (−), neutralizing antibody for CXCL12 activity = (αL12), and a positive control = 1 ng/mL of EGF (+). (##) denotes P<0.01 compared to unstimulated GFP cells, n = 7.

doi:10.1371/journal.pone.0090400.g006

We next sought to determine whether the simultaneous increased adhesive and decreased migratory phenotypes in PDAC cells would suppress metastatic spread in vivo using a heterotopic xenograft mouse model. CXCL12-expressing and CXCL12-null cells were injected into the spleen of SCID mice. Over 28 days, in vivo tracking of cells indicated that, compared with control cells, there was a significant alteration in the ability of CXCL12-expressing MiaPaCa2 cells to disseminate away from the initial site of inoculation (Fig.7A–B). Ex vivo analysis indicated that luminescence at the site of splenic inoculation was unchanged, while mice injected with PDAC that produce CXCL12 had significantly lower hepatic tumor burden than mice injected with GFP-control cells (Fig.7C–E). These data suggest that CXCL12 specifically alters the ability of pancreatic cancer cells to home to the liver, an organ with high expression of CXCL12 and a common metastatic destination of PDAC patients.

Concomitant CXCL12, CXCR4 and CXCR7 expression decreased tumor cell proliferation and metastasis and prolonged survival in a preclinical PDAC model

Previously, we had observed that re-expression of CXCL12 in colorectal cancer cells lead to increased anoikis, detachment based apoptosis [24,43]. We tested whether re-introduction of CXCL12 into PDAC cells would change their apoptotic or proliferative potentials. In serum-free conditions, an increase in apoptosis was observed.

![Figure 6. Chronic CXCL12 production decreased migration potential and increased cell:substrate adhesion.](image)

- (A) Transwell migration assays revealed significantly reduced chemotaxis of CXCL12-expressing clones (#1 and #2) compared to ligand null (GFP) cells. Attractants were serum-free media (−), 10% serum (+), or 10 nM CXCL12 (L12) in serum-free media. (SS and $$$) denote P<0.01 and P<0.001, respectively, compared to 10 nM CXCL12-stimulated GFP cells, n = 5.
- (B) CXCL12 re-expression diminished TGF-β (5 ng/mL)-induced chemotaxis relative to the CXCL12-null cells. (###) denotes P<0.01 compared to TGF-β-stimulated GFP cells, n = 4.
- (C) & (D) Representative images of experiments in (A) and (B) respectively.
- (E) CXCL12-expressing cells were significantly more adherent to tissue culture plastic compared to CXCL12-null cells. Untreated cells = (−), neutralizing antibody for CXCL12 activity = (αL12), and a positive control = 1 ng/mL of EGF (+). (##) denotes P<0.01 compared to 10% serum-stimulated control cells. (*), (**), and (***) denote P<0.05, P<0.01 and P<0.001, respectively, compared to unstimulated GFP cells, n = 7.

doi:10.1371/journal.pone.0090400.g006
detected in adherent CXCL12-expressing pancreatic cancer cells when compared with GFP-expressing controls, though this response was attenuated with the addition of serum (Fig. 8A–B). Using an established model for studying detachment-induced apoptosis, cells were cultured on Poly-HEMA [43]. As with adherent cells, apoptosis was increased in non-adherent CXCL12-positive PDAC cells compared to controls, but again the addition of serum resulted in no change in apoptosis (Fig. 8C–D). Cell number remained unchanged between non-adherent CXCL12-positive and negative cell populations (Fig. 8C–D).

Given the minimal change in anoikis-sensitivity in CXCL12-expressing PDAC cells in serum containing conditions, we next tested their growth potential. In marked contrast to our data in human colorectal or breast cancers [24–26,43], population growth of CXCL12 expressing PDAC was significantly decreased compared to chemokine deficient cells (Fig. 9). Slower growth of CXCL12-expressing cells was observed in both serum-free and serum-containing conditions (Fig. 9A–B). Decreased population growth was restricted to adherent cells as there was no measurable change in the number of cells detected in the supernatant of either population (data not shown). Two separate CXCL12-expressing clones demonstrated doubling times significantly greater than the doubling time observed in GFP-expressing cells (Fig. 9B).

We hypothesized that the decrease in growth potential from forced CXCL12 expression was due to cell cycle arrest. Using propidium iodide staining, we measured a ~50% decrease in the percent of CXCL12-expressing pancreatic cancer cells in the G2 phase of cell cycle, compared to GFP-expressing controls (Fig. 9C–D). These data, along with the cell counting study, suggest that CXCL12-expressing cells have decreased proliferation related to cell cycle arrest prior to entering the G2 phase.

Finally, the in vivo functional role of CXCL12 in PDAC progression was modeled using an orthotopic xenograft technique. As expected, MiaPaCa2 luciferase cells consistently metastasized through hematogenous routes, with mice succumbing to metastatic tumor burden in a 90 to 140 day window (Figure 10). A survival study was conducted wherein control GFP or experimental CXCL12-secreting cells were injected directly under the pancreatic capsule and tumor progression tracked using bioluminescence imaging. Overall, mice injected with CXCL12-expressing cells were found to have a significantly increased survival advantage compared to control mice (Fig. 10A). While control mice had significant tumor burden by day 141, the majority of mice implanted with CXCL12-expressing PDAC remained alive, with little to no apparent tumor. The presence of CXCL12 increased survival, with a hazard ratio of 4.9. Weekly tracking measurements of total radiance in the animals revealed a significant decrease in percent change of tumor burden throughout the course of the study (Fig. 10B). Over time, mice with tumor cells producing CXCL12 had lower tumor burden when assessed at early, middle, and later time points as visualized by bioluminescent imaging at days 7, 49, and 98, respectively (Fig. 10C–D).

Ex vivo analysis confirmed that CXCL12 expression was a significant impediment to PDAC progression. Tumor wet-weight was significantly lower in mice with CXCL12 compared to GFP control cells (Fig. 10E). Consistent with the delayed cell-cycle arrest

---

Figure 7. Decreased liver metastasis of circulating CXCL12-expressing PDAC cells. (A) Representative bioluminescence images of mice xenografted with GFP- or CXCL12-expressing cells at implantation (Day 0) or study endpoint (Day 28). (B) Whole-body in vivo radiance over time of GFP- and CXCL12-mice. (C) Ex vivo radiance of excised spleen (C), reflecting tumor cells at the site of injection, or the metastatic destination (D), reflecting decreased hepatic metastasis of CXCL12-expressing cells relative to GFP-cells. (**P denotes 0.01, n = 4–5. (E) Representative H&E images showing pronounced tumor mass in the liver of control (GFP), relative to experimental (CXCL12) xenografted mice. doi:10.1371/journal.pone.0090400.g007

CXCL12 Is a Multifunctional Tumor Suppressor

PLOS ONE | www.plosone.org 8 March 2014 | Volume 9 | Issue 3 | e90400
observed in vitro, immunohistochemistry revealed that CXCL12, CXCR4, and CXCR7-expressing tumors had decreased focal expression of Ki-67 compared with control tumors, indicating lower proliferative potential in vivo (Fig.10F–G). Increased survival in mice with CXCL12-expressing PDAC was correlated not only with decreased primary tumor proliferation but also with the pronounced absence of tumor metastasis (Fig.11). Specifically, hematogenous metastasis to the liver and lung was significantly abrogated (Fig.11A–C), in addition to decreased lymphatogenous metastasis to the mesenteric lymph nodes (Fig.11D). Cumulatively, these in vivo data reflect the tumor suppressive potential of CXCL12 expression in PDAC cells.

Discussion

Our findings demonstrate a seminal role for the homeostatic epithelial expression of CXCL12 in suppressing PDAC tumorigenesis. The conventional paradigm for chemokine involvement in malignancy stems from the correlation between elevated chemokine receptor levels, in particular CXCR4, and increased migration in cell culture [17]. The initial model suggested that cancer cells hijack CXCR4, allowing the malignant cell to follow endocrine gradients of ligand produced by distant tissues [38]. Subsequently, our work in both colorectal and breast cancer has shifted the perspective on the role of the CXCL12-CXCR4 axisin...
metastasis. We first established that autocrine expression of CXCL12 in normal intestinal and breast epithelial cells is epigenetically down-regulated by hypermethylation of the promoter region of its gene [24,25]. Retention of CXCR4 expression in CXCL12 silenced colorectal or breast cancer cells resulted in increased metastatic potential. With over 75% of patients diagnosed with either local or distant metastasis [57], PDAC is a highly aggressive form of cancer in need of more effective therapies. Although CXCR4 expression has been previously correlated with human PDAC, we show here that human PDAC malignancy is halted following re-expression of its cognate ligand CXCL12. Specifically, CXCL12 interrupted both primary tumor growth, through cell-cycle arrest, and cancer cell metastasis, leading to increased overall survival in diseased animals. These inhibitory mechanisms are distinct from those we defined in colorectal or mammary carcinoma and suggest CXCL12 is a tumor suppressive cellular brake limiting PDAC malignancy.

Work from our laboratory was among the first to show a beneficial preclinical effect of autocrine CXCL12 on carcinoma malignancy [24–26,43,56]. Autocrine CXCL12 has similar preclinical effects in lung cancer progression [28] and has been correlated with positive clinical outcomes in patients with osteosarcoma or breast cancers [31,34]. In human colon carcinoma cells we determined that re-expression of CXCL12 restored sensitivity to detachment-induced apoptosis, a key molecular brake preventing dissemination of cancer cells [26,43]. In contrast, in breast cancer we found that while autocrine CXCL12 decreased hematogenous spread of tumor cells, it also increased proliferation of the primary mammary fat-pad-engrafted tumor [25]. Shown here, human pancreatic cancer cells were strikingly different from colon and breast cancer in that reintroduction of autocrine CXCL12 expression decreased the growth and migration potential in vitro, while simultaneously decreasing growth and metastasis of PDAC cells in vivo. In contrast, there was minimal change in anoikis-sensitivity of CXCL12-expressing PDAC cells. Thus, CXCL12 appears to be broadly effective in limiting cancer progression, invasion, and metastasis and this benefit results from cell-type specific mechanisms. The tumor suppressive properties of CXCL12 may be translatable to the clinic as we have shown that administration of the recombinant protein provides an equally strong survival benefit in a preclinical colon cancer model [56].

Our study is the first to comprehensively study the expression of CXCL12, CXCR4, and CXCR7, a newly characterized member of the signaling axis, in normal and malignant pancreatic tissue. Congruent with its near ubiquitous organ expression [58], we found strong expression of CXCL12 in normal pancreatic tissue, localized largely to ductal epithelial cells. By contrast, CXCR4 and CXCR7 were evident in exocrine ducts, acini, and endocrine islets. Further, there was mixed expression of CXCL12, CXCR4, and CXCR7 in PanIN precursor lesions. These results suggest potential roles for CXCL12 and its receptors CXCR4 and CXCR7 in normal physiology. The exclusive expression of CXCL12 in normal pancreatic ductal epithelial cells, relative to acinar cells or

Figure 9. CXCL12 expression results in decreased proliferation of PDAC cells. Population growth of adherent GFP and CXCL12-expressing MiaPaCa2 cells was assessed using the Viacount reagent and flow cytometric cell counting (A–B). CXCL12-expressing cells starved for 24 hours and cultured in both 0% serum (A) or 1% serum (B) containing medium were found to have decreased population growth. (B) Two CXCL12-expressing clones (#1, #2) were compared to GFP alone or GFP + gemcitabine (GEM) controls in 1% serum containing medium. Doubling time of adherent clones (T2) was calculated using a linear regression of the data to determine slope and the intercept at y = 106, with increased T2 observed in both CXCL12-expressing clones. (C–D) Propidium Iodide cell cycle analysis revealed a decrease in percentage of cells in the G2 phase in CXCL12-expressing cells compared to GFP controls. (*), (**), and (***) denote P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001 respectively in comparison to control cells (GFP). Values are mean ± SEM, n = 4–5.

doi:10.1371/journal.pone.0090400.g009
endocrine islets, suggests the ligand may have homeostatic contributions to the exocrine pancreas. Further explorations into their physiologic roles in the pancreas, however, are hindered by embryonic lethality of CXCL12, CXCR4 and CXCR7 knockout mice [59–62]. Likewise, there is no established normal human pancreatic epithelial ductal cell line available for mechanistic studies in vitro.

CXCL12 expression was markedly absent, and CXCR4 and CXCR7 expression were more pronounced in human PDAC tumor cells. These data, using our well-established technique for analyzing chemokine expression [24,25], settles conflicting results between two previous studies, one that reported CXCL12 was over-expressed in human PDAC [35] and the other that showed that CXCL12 was absent in human PDAC tumor cells [36]. Both reports, however, were limited to malignant tissue and did not include the normal or benign tissue controls analyzed herein. Our immunostaining approach allowed for the discrimination between CXCL12, CXCR4, and CXCR7 expression levels within adenocarcinoma, tumor-associated fibroblasts, endothelium, and normal ducts and revealed a pronounced decrease in chemokine within the malignant ductal cell. Moreover, the histopathological decrease in CXCL12 observed in the tissues was recapitulated at the mRNA and protein level in several primary patient-derived as well as established PDAC cell lines. In their prior report, Liang and colleagues [35] used an immunostaining amplification kit to report elevated levels of CXCL12 in human pancreas cancer. Given the use of identical antibodies in both Liang and our studies, the resulting differences likely reflect the differing immunohistochemical staining procedures, in which the amplification kit likely overwhelmed detection limits for the constitutively produced and secreted CXCL12 protein. We additionally noted that CXCL12 expression appeared higher in tumor tissue from patients that had received neo-adjuvant therapy, a therapeutic regimen that improves survival in PDAC patients undergoing surgical resection [52–54]. It is tempting to speculate that the increase in CXCL12 expression reflects a direct effect of the gemcitabine and/or FOLFIRINOX treatment regimens. However, the effect of those drugs on CXCL12 transcript or...
protein expression was not directly assessed. Alternatively, the neoadjuvant treatment preferentially kills tumor cells lacking CXCL12, resulting in the observed increase in chemokine ligand expression. Our data support that of Zhong et al., and indicate that CXCL12 expression is pathologically diminished in human pancreatic cancer compared to healthy human pancreatic exocrine ductal epithelium.

Our work is the first to demonstrate that pancreatic cancer cells, like numerous other cell types, migrate towards exogenous sources of chemokine in a biphasic-concentration dependent manner [55,56,63]. As has been shown in previous in vitro experiments, exogenously administered concentrations of CXCL12 close to 10 nM stimulate chemotactic migration of cells through the monomeric form of the chemokine, while higher doses close 100 or 1000 nM lead to cytostasis through the predominant dimeric form of the chemokine. Endogenous or autocrine CXCL12 expression disrupted the ability of pancreatic cancer cells to migrate and home to distant sites of metastasis. Intriguingly, CXCL12 also altered non-migratory functions of pancreatic cancer cells, with stable re-expression of the ligand decreasing population growth via cell-cycle arrest in vitro and correspondingly smaller tumor xenografts with decreased proliferation markers in vivo. Importantly, re-introduction of CXCL12 resulted in a marked improvement in survival of tumor-bearing mice. The hazard ratio from the survival study was 4.9, reflecting the powerful effect CXCL12 expression imposed on altering the rate of tumor-induced mortality. These results in pancreatic cells suggest a tissue specific, tumor-suppressive role for CXCL12 that is adaptive to the unique programming of particular organs. Our data suggest that CXCL12 plays an integral role in the tumor progression which characterizes PDAC. In total, our studies reveal novel aspects of CXCL12 function in cancer biology and identify this gene as a multifunctional tumor suppressor that affects both PDAC growth and metastasis.

Acknowledgments

We thank Jenny Grewal and Qing Lai for expert technical assistance and appreciate Dr. Michael Wendt’s (Purdue University) critical evaluation of the manuscript.

Author Contributions

Conceived and designed the experiments: IR MBD. Performed the experiments: IR NPZ ACM. Analyzed the data: IR ACM DBE MBD. Contributed reagents/materials/analysis tools: ACM ST MBD. Wrote the paper: IR ACM DBE MBD.

References

1. Koorstra JB, Hustinx SR, Offerhaus GJ, Maitra A. (2008) Pancreatic carcinogenesis. Panreatology 8: 110–125.
2. Maitra A, Hruban RH. (2008) Pancreatic cancer. Annu Rev Pathol 3: 157–183.
3. Hidalgo M. (2010) Pancreatic cancer. N Engl J Med 362: 1605–1617.
4. Haeno H, Gonen M, Davis M, Herman J, Iacobuzio-Donahue C, et al. (2012) Computational modeling of pancreatic cancer reveals kinetics of metastasis suggesting optimum treatment strategies. Cell 148: 362–373.
5. Jonas S, Zhang X, Pauvros DW, Lin JC, Leary RJ, et al. (2008) Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science 321: 1801–1806.
6. Hingorani SR, Wang L, Mahani AS, Combs C, Dermenadzh TB, et al. (2005) Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. Cancer Cell 7: 469–483.
7. Pasca di Magliano M, Sekine S, Ermilov A, Ferris J, Dlugosz AA, et al. (2006) Hedgehog/ras interactions regulate early stages of pancreatic cancer. Genes & Development 20: 3161–3173.
8. Ahul K, Abbas, Andrew H. Lichtman. (2010) Basic immunology: Functions and disorders of the immune system. : Saunders/Eleveic, 2010. 312 pages p.
9. Raman D, Baugher PJ, Thu YM, Richmond A. (2007) Role of chemokines in tumor growth. Cancer Lett 236: 157–165.
10. Teacher BA, Fricker SP. (2010) CXCL12 (SDF-1)/CXCR4 pathway in cancer. Clin Cancer Res 16: 2927–2931.
11. Heidemann J, Ogawa H, Rafiee P, Lugering N, Maaser C, et al. (2004) Mucosal angiogenesis regulation by CXCR4 and its ligand CXCL12 expressed by human intestinal microvascular endothelial cells. Am J Physiol Gastrointest Liver Physiol 286: G1059–68.
12. Dwinnell MB, Ogawa H, Barrett KE, Kagnoff MF. (2004) SDF-1/CXCL12 regulates cAMP production and ion transport in intestinal epithelial cells via CXCR4. Am J Physiol Gastrointest Liver Physiol 286: G044–50.

Figure 11. Distant metastasis in an orthotopic xenograft model of pancreatic cancer. Ex vivo bioluminescence analysis revealed significantly decreased metastasis to the liver (A), lung (C), and mesenteric lymph nodes (D) of CXCL12-expressing cells compared to GFP-controls. Representative biophotonic images of hepatic metastases are shown in panel B. (***) and (****) denote statistically significant \( P \leq 0.01 \) and \( P \leq 0.001 \), respectively, differences between CXCL12-expressing and control tumor engrafted mice. \( n = 8–10 \) mice in each group.

doi:10.1371/journal.pone.0090400.g011
37. Zlotnik A. (2006) Involvement of chemokine receptors in organ-specific metastasis. Nature 440: 56–60.

36. Zhong W, Chen W, Zhang D, Sun J, Li Y, et al. (2012) CXCL12/CXCR4 axis regulates EMT and angiogenesis in breast cancer cells. J Pathol 226: 2222–2227.

35. Liang JJ, Zhu S, Bruggeman R, Zaino RJ, Evans DB, et al. (2010) High levels of CXCL12 are associated with shorter survival in patients with head and neck squamous cell carcinoma. J Surg Oncol 102: 108–114.

34. Mirisola V, Zuccarino A, Bachmeier BE, Sormani MP, Falter J, et al. (2009) Simultaneous CXCR4/SDF-1 signaling is important for tumor growth and progression in human pancreatic cancer cells. Int J Cancer 124: 1385–1394.

33. Kobayashi T, Tsuda H, Moriya T, Yamasaki T, Kikuchi R, et al. (2010) CXCR4 expression correlates with serum CXCL12 and secretion regulates colorectal carcinoma cell anoikis through bim-mediated intrinsic apoptosis. PLoS One 5: e12895.

32. Ramos E, Camargo A, Braun K, Slowik R, Cavalli I, et al. (2010) Simultaneous CXCL12/CXCR4 chemokine expression and secretion regulates colorectal carcinoma cell anoikis through bim-mediated intrinsic apoptosis. PLoS One 5: e12895.

31. Adams EJ, Green JA, Clark AH, Yousem JH. (1999) Comparison of different scoring systems for immunohistochemical analysis of CXCL12 expression in patients with pancreatic cancer. Journal of Gastrointestinal Surgery 16: 784–792.

30. Veldkamp CT, Ziarek JJ, Gravel S, Veldkamp CT, Takekoshi T, et al. (2011) Monomeric and dimeric CXCL12 inhibit metastasis through distinct CXCR4 interactions and signaling pathways. Proc Natl Acad Sci U S A 108: 17653–17658.

29. Clatot F, Picquenot J, Choussy O, Goue’rant S, Moldovan C, et al. (2011) The role of CXCR4 in the progression of epithelial ovarian cancer. Cancer Res 71: 9194–9203.

28. Hassan S, Ferrario C, Saragovi U, Quenneville L, Gaboury L, et al. (2009) The chemokine receptor CXCR4 is required for comedonecrosis in colon carcinoma micrometastases. Cancer Research 69: 3833–3839.

27. Wendt MK, Johanesen PA, Ang DC, Neujahr M, Sheng L, et al. (2008) Epigenetic silencing of CXCL12 increases the metastatic potential of mammary carcinoma cells. Oncogene 27: 1461–1471.

26. Wendt MK, Druy RJ, Vongsa RA, Dwinell MB. (2008) Constitutive CXCL12 expression induces anoikis in colorectal cancer cells. Gastroenterology 135: 508–517.

25. Hassan S, Ferrario C, Saragovi U, Quenneville L, Gaboury L, et al. (2009) The chemokine receptor CXCR4 is required for comedonecrosis in colon carcinoma micrometastases. Cancer Research 69: 3833–3839.

24. Zeelenberg IS, Ruuls-Van Stalle L, Roos E. (2003) The chemokine receptor CXCR4 is required for comedonecrosis in colon carcinoma micrometastases. Cancer Research 63: 3833–3839.

23. Clatot F, Picquenot J, Choussy O, Goue’rant S, Moldovan C, et al. (2011) The role of CXCR4 in the progression of epithelial ovarian cancer. Cancer Res 71: 9194–9203.

22. Zlotnik A. (2008) New insights on the role of CXCR4 in cancer metastasis. Contrib Microbiol 13: 191–199.

21. Cabioglu N, Gong Y, Islam R, Broglio KR, Sneige N, et al. (2007) Expression of CXCL12 is associated with improved survival in patients with pancreatic cancer. J Pathol 215: 211–213.

20. Maitra A, Fukushima N, Takaori K, Hruban RH. (2005) Precursors to invasive pancreatic cancer. Methods Mol Med 103: 1–13.

19. Dwinell MB, Johanesen PA, Wendt MK, Dwinell MB. (2005) CXCL12 is a multifunctional tumor suppressor. Cancer J 11: 260–264.

18. Shirozu M, Nakano T, Inazawa J, Tashiro K, Tada H, et al. (1995) Structure and chromosomal localization of the human stromal cell-derived factor 1 (SDF1) gene. Genomics 17660.

17. Hruban RH, Iacopinzo-Domaña C, Wellington RE, Goggins M, Kern SE. (2002) E- cadherin is a negative prognostic factor for cancer cell invasion and lymph node metastasis in colorectal carcinoma. J Surg Res 107: 1761–1767.

16. Hwang S, Zimmerman NP, Agle KA, Turner JR, Kumar SN, et al. (2012) E- cadherin is a collective shear stress measurement and is regulated by the chemokine CXCL12 protein during restitution. Journal of Biological Chemistry 287: 22222–22240.

15. Dwinell MB, Johanesen PA, Turner JR, Kumar SN, et al. (2012) E- cadherin is a collective shear stress measurement and is regulated by the chemokine CXCL12 protein during restitution. Journal of Biological Chemistry 287: 22222–22240.

14. Zimmerman NP, Vongsa RA, Faherty SL, Salzman NH, Dwinell MB. (2011) Targeted intestinal epithelial deletion of the chemokine receptor CXCR4 reveals important roles for extracellular-regulated kinase-1/2 in restitution. Lab Invest 91: 1049–1055.

13. Smith JM, Johanesen PA, Wendt MK, Binion DG, Dwinell MB. (2005) CXCL12 activation of CXCR4 regulates mucosal host defense through stimulation of epithelial cell migration and promotion of intestinal barrier integrity. Am J Physiol Gastrointest Liver Physiol 288: G316–26.

12. Zimmerman NP, Vongsa RA, Faherty SL, Salzman NH, Dwinell MB. (2011) Targeted intestinal epithelial deletion of the chemokine receptor CXCR4 reveals important roles for extracellular-regulated kinase-1/2 in restitution. Lab Invest 91: 1049–1055.

11. Wendt MK, Druy RJ, Vongsa RA, Dwinell MB. (2008) Constitutive CXCL12 expression induces anoikis in colorectal cancer cells. Gastroenterology 135: 508–517.

10. Hassan S, Ferrario C, Saragovi U, Quenneville L, Gaboury L, et al. (2009) The influence of tumor-host interactions in the stromal cell-derived factor-1/CXCR4 ligand/receptor axis in determining metastatic risk in breast cancer. Am J Pathol 175: 66–73.

9. Tessema M, Klange DM, Yingling CM, Do K, Van Neste L, et al. (2010) Re-expression of CXCL14, a common target for epigenetic silencing in lung cancer, induces tumor necrosis. Oncogene 29: 5159–5170.

8. Clatot F, Picquenot J, Choussy O, Goue’rant S, Moldovan C, et al. (2011) Intratumoural level of SDF-1 correlates with survival in head and neck squamous cell carcinoma. Oral Oncol 47: 1062–1068.

7. Zhi Y, Chen J, Zhang S, Chang X, Ma J, et al. (2012) Down-regulation of CXCL12 by DNA hypermethylation and its involvement in gastric cancer metastatic progression. Dig Dis Sci 57: 650–659.

6. Baumhoer D, Smida J, Zillner S, Rosenm M, Atkinson MJ, et al. (2012) Strong expression of CXCL12 is associated with a favorable outcome in osteosarcoma. Mod Pathol 25: 522–528.

5. Ramos E, Camargo A, Braun K, Sowik R, Cavalli I, et al. (2010) Simultaneous CXCL12 and ESR1/ERα island hypermethylation correlates with poor prognosis in sporadic breast cancer. BMC Cancer 10: 23.

4. Kobayashi T, Tsuda H, Moriya T, Yamasaki T, Kikuchi R, et al. (2010) Expression pattern of stromal cell-derived factor-1 chemokine in invasive breast cancer is correlated with estrogen receptor status and patient prognosis. Breast Cancer Res Treat 123: 733–745.

3. Mirisola V, Zacarino A, Bachmeier BE, Sormani MP, Falter J, et al. (2009) CXCL12/SDF1 expression by breast cancers is an independent prognostic marker of disease-free and overall survival. Eur J Cancer 45: 2379–2387.

2. Liang JJ, Zhu S, Bruggeman R, Zaino RJ, Evans DB, et al. (2010) High levels of expression of human stromal Cell-Derived factor-1 are associated with worse prognosis in patients with stage II pancreatic ductal adenocarcinoma. Cancer Epidemiology Biomarkers & Prevention 19: 2290–2296.

1. Zhong W, Chen W, Zhang D, Sun J, Li Y, et al. (2012) CXCL12/CXCR4 axis plays pivotal roles in the organ-specific metastasis of pancreatic adenocarcinoma: A clinical study. Exp Ther Med 4: 363–369.