Pancreatic Cancer Cell Migration and Metastasis Is Regulated by Chemokine-Biased Agonism and Bioenergetic Signaling

Ishan Roy1, Donna M. McAllister1,2, Egal Gorse1, Kate Dixon1, Clinton T. Piper1, Noah P. Zimmerman1,3, Anthony E. Getschman4, Susan Tsai3,5, Dannielle D. Engle6, Douglas B. Evans3,5, Brian F. Volkman4, Balaraman Kalyanaraman2,3, and Michael B. Dwinell1,3

Abstract

Patients with pancreatic ductal adenocarcinoma (PDAC) invariably succumb to metastatic disease, but the underlying mechanisms that regulate PDAC cell movement and metastasis remain little understood. In this study, we investigated the effects of the chemokine gene CXCL12, which is silenced in PDAC tumors, yet is sufficient to suppress growth and metastasis when re-expressed. Chemokines like CXCL12 regulate cell movement in a biphasic pattern, with peak migration typically in the low nanomolar concentration range. Herein, we tested the hypothesis that the biphasic cell migration pattern induced by CXCL12 reflected a biased agonist bioenergetic signaling that might be exploited to interfere with PDAC metastasis. In human and murine PDAC cell models, we observed that nonmigratory doses of CXCL12 were sufficient to decrease oxidative phosphorylation and glycolytic capacity and to increase levels of phosphorylated forms of the master metabolic kinase AMPK. Those same doses of CXCL12 locked myosin light chain into a phosphorylated state, thereby decreasing F-actin polymerization and preventing cell migration in a manner dependent upon AMPK and the calcium-dependent kinase CAMKII. Notably, at elevated concentrations of CXCL12 that were insufficient to trigger chemotaxis of PDAC cells, AMPK blockade resulted in increased cell movement. In two preclinical mouse models of PDAC, administration of CXCL12 decreased tumor dissemination, supporting our hypothesis that chemokine-biased agonist signaling may offer a useful therapeutic strategy. Our results offer a mechanistic rationale for further investigation of CXCL12 as a potential therapy to prevent or treat PDAC metastasis. Cancer Res, 75(17): 3529–42. ©2015 AACR.

Introduction

With an overall survival rate of only 6%, pancreatic cancer remains a poorly addressed health problem relative to other commonly diagnosed malignancies (1). The vast majority of patients with pancreatic ductal adenocarcinoma (PDAC) ultimately succumb to metastatic disease (2). Standard chemotherapies for PDAC provide a minimal improvement in survival for patients, particularly those with advanced stages of disease. The inability to chemotherapeutically target advanced PDAC reflects, in part, a current paucity of knowledge regarding the mechanisms driving metastasis. While data from transgenic mice suggested PDAC cell dissemination can occur early in disease progression (3), sequencing and modeling analysis after rapid autopsy of patients revealed that dissemination and metastasis of PDAC occurs subsequent to primary tumor development (4). Although no mechanism is known, human tissue analysis implicates the signaling protein Smad4 in multifocal metastasis of PDAC (5). However, the specific extracellular cues that regulate the invasion, migration, and metastatic homing of PDAC cells into distant tissues have yet to be identified.

As soluble/secreted proteins, chemokines, or chemotactic cytokines, direct cell movement in a concentration-dependent manner. In normal physiology, chemokines direct cell trafficking of a wide variety of cell types. Dysregulated expression of chemokines, or their cognate G-protein–coupled receptors (GPCRs), is associated with pathologic cell migration in many diseases (6). Solid tumor progression has been linked with elevated expression of the chemokine receptor CXCR4 (7). Recently, we have uncovered epigenetic repression of the chemokine ligand CXCL12 in colorectal, mammary, and pancreas cancer, in parallel with increased expression of its cognate receptor CXCR4. Silencing of the ligand enables homing of CXCR4-expressing cancer cells to metastatic sites rich in CXCL12 (8–10). Subsequent histopathologic studies have also linked diminished CXCL12 expression with worse prognosis in gastric cancer, osteosarcoma, and breast cancer (11–13). Our more recent study in pancreatic cancer demonstrated that re-expression of CXCL12 inhibits PDAC cell growth as
well as cell migration (10). Chemokines such as CXCL12 stimulate migration in a biphasic manner, with 10 nmol/L optimally stimulating epithelial cell movement and ≥100 nmol/L unable to induce migration (14–18). In contrast, chemokine signaling through the G protein stimulates intracellular calcium flux over a broader concentration range (e.g., 1–1,000 nmol/L; refs. 16, 18). Importantly, the 1 to 1,000 nmol/L concentration range is comparable to concentrations of CXCL12 physiologically available in circulation and human tissue (15, 19–21). The disparity between these two downstream signaling effects represents a longstanding conundrum in GPCR function. Lefkowitz and colleagues first observed that distinct agonists of the same β-adrenergic receptor can elicit different signals and functions that are either G-protein–dependent or β-arrestin signaling–mediated and termed this functional selectivity as “biased agonism” (22). Thus, as defined by Reiter and colleagues, GPCR agonists can fall into three categories, G-protein–biased, β-arrestin–biased, or balanced, in which both G-proteins and arrestin are recruited and signaled. A preliminary report by Lefkowitz and colleagues also showed that the chemokines CCL19 and CCL21 could elicit differential β-arrestin–biased agonist signaling when binding to the chemokine receptor CCR7 (23).

Prior reports had shown that in physiologic solutions, chemokines were capable of reversibly forming multimeric structures, in a concentration-dependent manner (14, 24, 25). For CXCL12, at lower concentrations, the protein remains monomeric, but as the concentration increases, the presence of dimeric structures predominate (25). These data lead to the hypothesis that the dimeric and trimolecular structures may possess functional selectivity for the biphasic migration response observed for CXCL12. Subsequently, in a series of prior reports using engineered variants of CXCL12, we discovered that the unique oligomeric forms of the ligand can function as biased agonists after binding to CXCR4 (26, 27), with monomeric CXCL12 eliciting ‘balanced’ signaling and migration, whereas dimeric CXCL12 stimulated ‘G-protein–biased’ signaling without eliciting migration. However, further investigation into a downstream signaling mechanism to link the lack of migration with the G-protein–biased agonism stimulated by dimeric CXCL12 is necessary.

Recent studies of PDAC illustrate that there is a shift in metabolic flux toward anabolic pathways that facilitate nutrient synthesis (28) and that PDAC cells rely on glutamine metabolism to drive oncogene-dependent growth (29–31). However, little is known regarding the metabolic or bioenergetic profiles of migrating PDAC cells. Likewise, although hormone-dependent calcium signaling and cell polarity regulation have been associated with bioenergetic signaling through the homeostatic regulator AMP kinase (AMPK; refs. 32, 33), the potential relationship between cell metabolism and chemotaxis is unexplored. Herein, we establish that the decline in chemotactic migration at elevated CXCL12 concentrations reflects CXCR4-dependent AMPK and myosin light chain (MLC) phosphorylation. The lack of migration at high concentrations of CXCL12 was replicated using the engineered CXCL12 dimeric ligand. These are the first data demonstrating that receptor activation by a dimeric chemokine ligand can enhance a specific signaling pathway as a result of biased agonism. The direct stimulation of a nonmotile state by the dimeric ligand we herein have termed “ataxis.” Broadly, our work unveils an important energy signaling mechanism underlying chemotaxis and reveals a viable strategy for pharmaceutical targeting of cancer metastasis.

### Materials and Methods

#### Reagents

AICAR, LPA, AMD3100, metformin, and compound C were purchased from Sigma. BAPTA-AM, ionomycin, STO-609, and pertussis toxin were purchased from EMD Biosciences Calbiochem. The mitochondrial inhibitor Mito-CP was produced and used as previously published (34). Antibodies against total or phosphorylated AMPK, MLC, LKB1, and MYPT1 were purchased from Cell Signaling Technologies. The wild-type, locked monomer, and locked dimer variants of CXCL12 were expressed and purified as previously described (27, 35). The CXCL12 locked dimer (CXCL12-LD) mutations are L36C and A65C and create a dimeric protein. The CXCL12 locked monomer (CXCL12-LM) mutations are L55C and I58C. The engineered locked dimer or locked monomer proteins are structurally indistinguishable from native dimeric and monomeric protein, respectively, and retain full CXCR4 receptor-binding capability. Proteins were expressed in Escherichia coli and cells were lysed by French press. Fusion protein was purified through nickel chromatography and refolded by infinite dilution and ULP1 protease was used to cleave the 6XHIS-Tag. Cation exchange and high-performance liquid chromatography was used for final purification.

#### Cells

The human pancreatic carcinoma cells Panc1 (CRL-1469) and MiaPaCa2 (CRL-1420) were purchased from the ATCC. Patient-derived PDAC MCV512, corresponding to MCV4 from our prior report, were obtained from the Medical College of Wisconsin Surgical Oncology Biobank using institutional review board–approved protocols and cultured as previously published (10). The cell lines K8282 and K8484 were derived from the original Kras<sup>SLG-G12D/+; p53R172H/+; Pdx<sup>Cre</sup> (KPC) mice on the mixed 129/SvJae/C57BL/6 background and were the kind gift of Dr. Kenneth Olive (Columbia University, New York, NY). CI1199, CI1242, CI1245, and DT10022 cell lines were derived from KPC mice in which each of the founder mutant mice had been backcrossed to the C57BL/6 genetic background. KPC cells were maintained in high-glucose DMEM with 10% (v/v) FBS (Life Technologies Inc.). The Pan-02 cell lines were provided by the National Cancer Institute Cell Repository (Bethesda, MD) and maintained in RPMI-1640 with 10% (v/v) FBS.

#### Orthotopic xenograft model

SCID mice (c<sup>-Pr<sub>d</sub>ls<sup>x<sup>md</sup>l</sub>sd</sup>, Charles Rivers Laboratories) were anesthetized and orthotopically implanted with either 10<sup>6</sup> Panc1 or MiaPaCa2 cells stably expressing firefly luciferase and tumor formation tracked in vivo by bioluminescent imaging (Lumina IVIS 100, Perkin Elmer) using our previously published technique (10). At 7 days postimplantation, mice were sorted into vehicle or treatment groups with equivalent average luminescence and treated twice weekly thereafter with 200 μl intraperitoneal injections of PBS or 5 μmol/L recombinant CXCL12 protein. Mice in the Panc1 model were allowed to survive until humanely euthanized when morbid, in accordance with an Institutional Animal Care and Use Committee-approved protocol, whereas MiaPaCa2 xenografted mice were sacrificed on day 70. Ex vivo analysis was performed with individual luminescence measurements of the liver, lung, and adjacent lymph nodes. The peritoneal cavity was visually inspected and imaged post-mortem to detect potential peritoneal movement of tumor cells.
Energetic flux assay
Changes in bioenergetic flux in pancreatic cancer cells were measured using Seahorse Bioscience XF96 Extracellular Flux Analyzer (Seahorse Bioscience). MiaPaCa2 cells were first plated overnight in Seahorse plates and then equilibrated in unbuffered, serum-free medium containing only 5.5 mmol/L glucose and 4 mmol/L L-glutamine for 3 hours. Prior to the injection of chemokine into each well, 8 baseline measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were taken and averaged as a time zero energy measurement. Chemokines were then automatically dispensed into wells, with 8 measurements taken over 1 hour to determine basal energetic flux, after which glycolytic and oxidative stress tests were employed using the inhibitors, oligomycin, dinitrophenol, and antimycin A. Stress test inhibitors were injected sequentially, with three measurements taken after each individual treatment. Oligomycin was used to measure ATP-linked OCR and reserve ECAR, dinitrophenol was used to measure reserve OCR, and antimycin A was used to correct for nonspecific flux.

Immunoblotting
Cells were plated to 80% confluency in 60-mm dishes and then starved 24 hours for transfected cells or 5 hours for stimulated cells. Stimulations were performed in serum-free medium and inhibitors placed on cells 1 hour before stimulation. After stimulation, cells were washed twice in cold PBS and lysed using a modified RIPA buffer. Lysates were normalized for protein concentration, size separated using reducing SDS-PAGE, electrophoresed on polyvinylidene difluoride membranes (Millipore), and then probed using primary and horseradish peroxidase-conjugated secondary antibodies. Proteins were visualized by chemiluminescence with autoexposure and quantified by densitometric analysis using the FluorChem HD2 from Cell Biosciences. The optical densities of the proteins in immunoblots from independent experiments were obtained by densitometry.

Flow cytometry
Cells are grown to 80% confluence, then lifted using Enzyme-Free Dissociation Buffer (Invitrogen), and washed twice using cold PBS. Cells were incubated in a 1% (w/v) BSA blocking solution, incubated for 1 hour in primary antibody on ice, washed twice, and then incubated for 1 hour in fluorophore-conjugated secondary antibody. Cells were then fixed in 2% (w/v) paraformaldehyde and fluorescence intensity measured on a BD-LSR II flow cytometer and analyzed by FlowJo software (BD Biosciences).

Immunofluorescence
Immunofluorescence analysis was performed as published previously (36). Briefly, cells were plated overnight at 50% confluence on glass coverslips, serum-starved 2 hours, and then treated. After treatment, cells were fixed using paraformaldehyde, blocked and permeabilized, and then incubated overnight at 4°C with a phosphor-specific antibody for MLIC. Cells were then washed and incubated with a goat anti-rabbit Alexa Fluor-488-conjugated antibody and phalloidin-AlexaFluor-594 for 2 hours at room temperature, counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 15 minutes, and coverslipped. Microscopy was performed and fluorescence intensity measured using the Nikon Eclipse Ti microscope and software at ×600 magnification under oil immersion. Percent of cells with stress fiber formation was quantified with images blinded for treatment and then scored by a separate investigator for presence of stress fiber or cortical actin.

RT-PCR
RNA was isolated using the RNA Easy Kit from Qiagen and treated with DNase I to remove gDNA contaminants. Conversion to cDNA was performed by priming with random hexamers and the SuperScript II cDNA Synthesis Kit (Life Technologies). PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining and ultraviolet imaging. Primers for the promoter region of the mannose-binding lectin gene were used to test for gDNA contaminant, while primers for the GAPDH transcript were used as a loading control. Amplification of chemokine receptors and chemokine was done using the following primers (5’–3’): mouse CXCL12 forward—ACCTCCTTGCTGTCTCTGGTGCTCC, mouse CXCL12 reverse—GGTGGGCTCTGCGATGTCGGCTCTC, mouse CXCR4 forward—TGATCCACGCCCACCAAGTCA, mouse CXCR4 reverse—TGAACACCACTCACCACACGCC, mouse CXCR7 forward—GGAGCTGTACGCCCTACCG, and mouse CXCR7 reverse—CTTAGGCTTGATATTCCACC.

CXCR4 depletion
shRNA sequences complementary to CXCR4 transcript, sequences 4052 (#1) and 4055 (#2), were purchased from the Open Biosystems dataset preinserted into the LL3.7-Puro vector. scRNA sequences 4052 (#1) and 4055 (#2), were purchased from the Open Biosystems dataset preinserted into the LL3.7-Puro vector. shRNA sequences complementary to CXCR4 transcript, sequences 4052 (#1) and 4055 (#2), were purchased from the Open Biosystems dataset preinserted into the LL3.7-Puro vector. lentiviral particles were constructed by transfecting each plasmid with viral accessory plasmids, pVSVG, pPRE, and pRRE into HEK-293T cells using Mirus reagent (Mirus). After overnight transfection, medium was exchanged for harvest media and cells cultured for 48 hours. Harvested viral particles were then collected, filtered, and stored for later use. MiaPaCa2 cells were grown to about 50% confluence and then transduced with harvested mock, scramble, or CXCR4-encoding lentiviral particles using polybrene for 24 hours. Transduced cells were then selected using puromycin for 7 days, after which stable protein depletion was measured by both immunoblotting and flow cytometry.

Calcium flux
Calcium mobilization was measured using the Fluo-4-AM cell permeable dye (Invitrogen) according to manufacturer’s directions. Cells were plated to 90% confluence in 96-well plates and then serum-starved for 2 hours the next day. Cells were washed with calcium/magnesium-free Dulbecco’s PBS, loaded with Fluo-4-AM for 30 minutes at 37°C, and then 10 minutes at room temperature. Stimulants, diluted in calcium/magnesium-free HBSS, were then loaded into auto-injectors of the Victor Wallac. Background readings were taken before stimulant injection (3 readings), and then 30 readings every 5 seconds were taken postinjection with ionomycin as a positive control.

Transwell migration
Cells were grown to confluence in Transwell inserts (Corning Costar). The upper well of the Transwell membrane was coated with collagen I (15 µg/mL) and then incubated in 2% (w/v) BSA/PBS until cells were ready for plating. Cells were lifted using enzyme-free buffer, washed, and plated to the upper well, with chemoattractants added to the bottom well in serum-free media. MiaPaCa2 and MCW512 cell migration (10³ cells plated)
CXCL12 had less dissemination, relative to vehicle treatment (10, 27, 37), we found that mice treated twice weekly with in vivo the protein, CXCL12 would function as a biologic response mod-
ifier of PDAC malignancy. Combining our previously established pancreatic orthotopic xenograft models with a therapeutic regimen and window defined in our previous studies using the protein in vivo for colorectal cancer and melanoma (10, 27, 37), we found that mice treated twice weekly with CXCL12 had less dissemination, relative to vehicle treatment (Fig. 1A and B). Ex vivo luminescence of metastatic destination organs, including liver, lung, and proximal lymph nodes, showed that CXCL12 treatment significantly lowered overall tumor dissemination, compared with controls (Fig. 1C and D). Notably, decreased metastasis was separately observed in both the MiaPaCa2 and Panc1 xenograft models, without any concomitant increase in peritoneal cavity dissemination. These data on the ant metastatic effects of CXCL12 mirror our prior report in colorectal cancer and melanoma (27).

**CXCL12 alters pancreatic cancer bioenergetic rates and AMPK signaling**

One explanation for the antimetastatic effect of CXCL12 in vivo is that exogenous chemokine disrupts the concentration gradients necessary for chemotactic migration of cancer cells. On the basis of our observation that CXCL12 regulates in vitro pancreatic cell migration in a biphasic manner, wherein low nanomolar doses (≤10 nmol/L) stimulate migration whereas higher doses (≥100 nmol/L) are “ataxic” and unable to evoke migration, we hypothesized that the change from CXCL12 from a pro-to an antimetastatic ligand reflected functional selectivity or biased agonist signaling through the CXCR4 receptor (10). The mechanisms for functional selectivity of CXCL12 through CXCR4 are unknown. Using a Seahorse-XF analyzer, we first tested the notion that the biphasic migratory response to CXCL12 reflected changes in energy metabolism. A declining, dose-dependent trend in basal oxygen consumption and glycolysis rates in MiaPaCa2 cells stimulated with CXCL12 was measured (Supplementary Fig. S1; Fig. 2A and B). Significant decreases in ATP-linked oxidative phosphorylation, basal glycolysis, and reserve glycolytic capacity were observed in MiaPaCa2 cells stimulated with the ataxic 1,000 nmol/L dose of CXCL12, compared with vehicle control (Fig. 2A and B). When
oxygen consumption and glycolytic rates were visualized together, an overall declining trend in cellular energy was observed as the concentration of CXCL12 increased (Fig. 2C).

We next predicted that CXCL12-dependent changes in cell energetics may be linked to altered activity of AMPK, a known regulator of metabolic stress. Immunoblot analysis demonstrated that CXCL12 dose dependently stimulated phosphorylation at the activating threonine-172 site of AMPK (Fig. 2D). Nonmotile 100 and 1,000 nmol/L doses of CXCL12 stimulated significant and sustained AMPK phosphorylation through 1 hour (Fig. 2D). CXCL12 regulation of migration and AMPK activity was next tested in a previously characterized patient-derived PDAC cell line (10). As predicted, patient-derived MCW512 cells migrated in response to exogenous CXCL12 stimulation in a biphasic fashion,
with peak chemotaxis at 10 nmol/L CXCL12 (Supplementary Fig. S1C). As with MiaPaCa2 cells, 1,000 nmol/L CXCL12 stimulated significant increase in AMPK phosphorylation in patient-derived cells (Supplementary Fig. S1D). Together, these data demonstrate that ataxic doses of CXCL12 stimulate prolonged AMPK activity in human PDAC cells.

To address whether CXCL12-induced AMPK activity was species-dependent, we next acquired a battery of mouse-derived PDAC cell lines and first analyzed chemokine and receptor expression. RT-PCR analysis showed that 4 of 7 mouse lines lacked appreciable CXCL12 transcript, and 4 of 7 mouse lines expressed CXCR4 transcript, and each line expressed CXCR7 mRNA (Supplementary Fig. S2A). Immunoblot and flow cytometric analyses confirmed protein expression of CXCR4 (Supplementary Fig. S2B–S2D). Of the 7 cell lines, FC1199 and Pan-02 most closely mimicked the expression pattern of CXCL12, CXCR4, and CXCR7 that we noted in human PDAC (10). Given its novel isolation from the primary tumor of C57BL/6 KRas<sup>12D/L</sup>/p53<sup>R172H</sup>/Pdx<sup>Cre</sup>/ (KPC) mouse model of PDAC, we identified the FC1199 cell line as a suitable model for further study. FC1199 cells had increased AMPK activity in a dose-dependent fashion after CXCL12 stimulation (Fig. 2E). Consistent with the human cell lines, CXCL12 stimulated AMPK phosphorylation was significantly increased 1 hour after stimulation with 100 or 1,000 nmol/L, but not with 1 or 10 nmol/L doses, in FC1199 cells (Fig. 2E). These data indicate that CXCL12-induced AMPK activity is a species-independent event.

In sum, our observations indicate that CXCL12 regulates bioenergy consumption rates as well as energy-sensing signaling in a dose-dependent manner. The optimal cell migratory dose of CXCL12 leads to transient AMPK activity and minimal change in glycolysis or oxidative phosphorylation. In contrast, doses exceeding 100 nmol/L CXCL12, which do not evoke cell migration, lead to sustained (>1 hour) AMPK activity and decreased glycolysis and oxidative phosphorylation.

**CXCL12 stimulates AMPK through CXCR4 and G<sub>a<i>i</i></sub> calcium signaling**

As our previous studies demonstrated that CXCL12-induced biphasic migration is mediated through CXCR4 binding (27), rather than CXCR7, we asked whether the AMPK phosphorylation stimulated by CXCL12 is dependent on CXCR4 signaling. As a first approach, immunoblot and flow cytometric analyses confirmed depletion of CXCR4 in 2 MiaPaCa2 clones (Supplementary Fig. S3A and S3B). Transwell migration assays then showed that the control and scramble sequence–expressing cells migrated toward 10 nmol/L CXCL12, whereas CXCR4-knockdown clones were unable to migrate in response to chemokine stimulation (Supplementary Fig. S3C and S3D). We subsequently assessed AMPK activity in those CXCR4-knockdown cells. As shown in Supplementary Fig. S3E and S3F, at the short time point of 15 minutes, CXCL12 stimulated little, if any, AMPK phosphorylation in CXCR4-depleted clones, whereas the scramble-transduced cells ably responded to nonmotility-inducing doses of chemokine. We next tested whether AMPK activation was due to G<sub>a<i>i</i></sub> protein–coupled signaling. Consistent with elevated CXCL12 signaling in a biased agonist fashion, immunoblot analysis showed that AMPK phosphorylation was decreased after pretreatment with either pertussis toxin (Supplementary Fig. S3G) or the small-molecule CXCR4 antagonist AMD3100 (Supplementary Fig. S3H). These data demonstrate that AMPK phosphorylation in response to the ataxic >100 nmol/L doses of CXCL12 reflects activation of the canonical CXCR4–G<sub>a<i>i</i></sub> signaling mechanism.

Prior reports exploring the regulation of AMPK activity have shown that T172 can be phosphorylated by either the cell polarity regulator LKB1 or calcium-dependent calmodulin kinase kinase 2 (CamKK2). While LKB1 activity was not increased by stimulation with CXCL12 (Supplementary Fig. S4E), the CamKK2-specific inhibitor STO-609 reversed AMPK phosphorylation induced by 1,000 nmol/L CXCL12 (Fig. 3E and F). In total, our experiments examining CXCL12 downstream signaling in human and murine PDAC cells demonstrate that AMPK activity driven by CXCL12 is mediated through receptor-dependent intracellular calcium flux and CamKK2 activity.

**AMPK regulates the CXCL12 biphasic migration response**

RAPTOR, the established regulator of mTOR (mTORC1) signaling, is a key downstream target for AMPK-mediated phosphorylation. While AMPK is conventionally thought to regulate mTOR activity in the context of cell growth, we did not detect any altered RAPTOR signaling (data not shown), consistent with other studies demonstrating non–growth-related AMPK signaling (33). The repeated cycling of MLC phosphorylation and subsequent dephosphorylation is essential for the perpetual lever-like action that myosin exhibits on F-actin fibers, allowing cells to contract, relax, and then migrate (38, 39). Activation of AMPK has been proposed to affect MLC activity by preventing the dephosphorylation that occurs on the regulatory unit of MLC (40, 41). Mirroring our previous studies in intestinal epithelial cells (36), immunoblot analysis of MiaPaCa2 cells showed that the chemotactic dose lead to peak phosphorylation of MLC at 15 minutes, which then returned to baseline levels by 60 minutes; a similar temporal pattern in MLC phosphorylation was observed with the migration-positive control (Fig. 4A). In contrast, both ataxic CXCL12 stimulation and the AICAR-positive control evoked a sustained increase in MLC phosphorylation over 60 minutes (Fig. 4B).

We hypothesized that differential MLC phosphorylation in response to CXCL12 was due to AMPK downstream signaling. The AMPK inhibitor compound C reversed MLC phosphorylation induced by 1,000 nmol/L CXCL12 (Fig. 4C). Previous studies indicated that AMPK regulates MLC by inhibitory phosphorylation of myosin phosphatase 1 (MYPT1; refs. 42, 43), an enzyme responsible for dephosphorylating MLC. We thus predicted that ataxic doses of CXCL12 would activate AMPK and in turn inhibit...
MYPT1 function. In a time course, CXCL12 lead to inhibitory phosphorylation of T853 of MYPT1 between 15 and 60 minutes (Supplementary Fig. S5). We then examined the AMPK dependence of CXCL12 regulation of MYPT1 and showed that compound C significantly reversed the ataxic CXCL12 or AICAR-induced inhibitory phosphorylation of MYPT1 (Fig. 4D), suggesting that elevated levels of CXCL12 disrupt MLC activity through AMPK.

We next questioned whether the CXCL12-stimulated change in MLC phosphorylation significantly altered the assembly of cell migration machinery through AMPK. Immunofluorescence microscopy revealed that after stimulation with ataxic doses of

Figure 3.
CXCL12-stimulated AMPK activity is CamKK2-dependent. Calcium mobilization was probed using the Fluo-4 membrane permeable fluorescent dye and measured on a Victor Wallac plate reader continuously for 180 seconds following stimulation with 1, 10, 100, or 1,000 nmol/L CXCL12 and the ionomycin (IM)-positive control. A and B, MiaPaCa2 cells were preincubated in ion-free buffer to assess intracellular calcium mobilization and a representative time curve (A) and measurement of maximal flux achieved (B) are shown. **P < 0.01; ***P < 0.001 in comparison to vehicle control. n = 4. C and D, the experiment was repeated with cells preincubated in buffer containing calcium ions to assess total calcium intracellular and extracellular mobilization, and a representative time curve (C) and measurement of maximal flux achieved (D) are shown. **P < 0.01; ***P < 0.001 in comparison to vehicle control. Values = mean ± SE. n = 5. E and F, MiaPaCa2 or FC1199 cells were pretreated with the CamKK2 inhibitor STO-609 and then stimulated with 10 or 1,000 nmol/L CXCL12 for 15 minutes. Protein lysates were then taken and immunoblot analysis performed to assess AMPK activity. Densitometric analysis revealed that 1,000 nmol/L CXCL12 or, as detected on a separate gel, ionomycin, induced AMPK phosphorylation was significantly inhibited using STO-609 in both MiaPaCa2 and FC1199 cells. *P < 0.05 comparison ± STO-609. Values = mean ± SE. n = 3.
Figure 4.
Elevated doses of CXCL12 induce AMPK-dependent locking of MLC–actin migration machinery through sustained phosphorylation of MLC and MYPT1. A and B, MiaPaCa2 cells were stimulated with chemotactic (10 nmol/L) and ataxic (1,000 nmol/L) doses of CXCL12, along with 1 μg/mL lysophosphatidic acid (LPA) or 100 nmol/L AICAR as positive controls, over a time course of 5, 15, 30, and 60 minutes. Lysates were then collected and probed for phosphorylated and total levels of MLC protein. CXCL12 (10 nmol/L) and the migration control LPA stimulated pMLC in a biphasic time-dependent manner, peaking within 15 minutes. (Continued on the following page.)
CXCL12, cells were rounded with few cytoplasmic projections, consistent with the nonmigratory phenotype (Fig. 5E). By comparison, the chemotactic dose of CXCL12 led to more cell spreading, with abundant filopodia and lamellipodia formation (Fig. 5E). Consistent with our immunoblot analysis, after 1-hour stimulation, immunofluorescence microscopy revealed that CXCL12 (1,000 nmol/L) mimicked AICAR, stimulating high pMLC that plateaued through 60 minutes. C and D, pretreatment with the AMPK inhibitor compound C (Com. C) abrogated phosphorylation of both MLC and MYPT1 (T853) stimulated by 1,000 nmol/L CXCL12 or AICAR at 30 minutes, confirmed by densitometric analysis. *P ≤ 0.05 in comparison of stimulation ± pretreatment with the AMPK inhibitor compound C. Biphasic chemotactic Transwell migration stimulated by CXCL12 was abolished in cells pretreated with compound C for 30 minutes. The 10% FBS used as a positive control stimulated equal cell migration irrespective of compound C pretreatment. Representative images of high-powered fields of Transwell membranes from each condition. ***, P ≤ 0.001 in comparison to vehicle control; ###, P ≤ 0.001 compared with 1,000 nmol/L CXCL12 + Compound C. Values = mean ± SE. n = 5.

Pancreatic cancer cell migration is dependent on metabolic activity and CXCL12 biphasic regulation of migration is AMPK-dependent. A and B, in 0.5% serum-containing media, both basal and 10 nmol/L CXCL12-induced Panc1 migration were decreased in a dose-dependent fashion by a combination of a mitochondrial inhibitor Mito-CP and the glycolytic inhibitor 2-DG, mixed with cells in the upper chamber of the Transwell 30 minutes prior to migration (A). A combination of metformin and 2-DG significantly decreased 10 nmol/L CXCL12-induced migration of MiaPaCa2 cells (B). n = 5–6; *, P ≤ 0.05; **, P ≤ 0.01 compared with unstimulated (–) control; #, P ≤ 0.05 compared with 10 nmol/L CXCL12-stimulated cells. C and D, MiaPaCa2 cells were stimulated with 1, 10, 100, or 1,000 nmol/L doses of CXCL12 with or without pretreatment with the AMPK inhibitor compound C. Biphasic chemotactic Transwell migration stimulated by CXCL12 was abolished in cells pretreated with compound C for 30 minutes. The 10% FBS used as a positive control stimulated equal cell migration irrespective of compound C pretreatment. Representative images of high-powered fields of Transwell membranes from each condition. ***, P ≤ 0.001 in comparison to vehicle control; ###, P ≤ 0.001 in comparison between 100 and 1,000 nmol/L CXCL12 ± compound C. Values = mean ± SE. n = 4 biologic replicates.

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ataxic, and not the chemotactic, dose significantly increased levels of phosphorylated MLC in treated cells (Fig. 4F). In parallel, PDAC cells stimulated with 10 nmol/L CXCL12 had a higher percentage of actin stress fibers, whereas actin in 1,000 nmol/L CXCL12-treated cells was organized primarily in cortical bundles (Fig. 4G). Finally, compound C pretreatment of cells stimulated with 1,000 nmol/L CXCL12 reversed MLC phosphorylation and in turn increased formation of actin stress fibers and cell spreading (Fig. 4E–G).

We then sought to establish a link between metabolism and migration. We first showed in a Transwell migration assay, blocking glycolysis with 2-deoxyglucose (2-DG) and inhibiting mitochondria function with our previously established inhibitor MitoCP (34), that abrogation of metabolic activity decreased both basal migration and chemokine–directed migration of MiaPaCa2 cells in a concentration-dependent manner (Fig. 5A). We then duplicated this result using dual inhibition of metabolic flux with 2-DG and metformin, an established inhibitor of pancreatic cancer cell metabolism (Fig. 5B; ref. 44). Last, we tested the notion that chemotaxis regulated by CXCL12 is dependent on AMPK activity in a MiaPaCa2 Transwell migration assay stimulated with a concentration curve of CXCL12 in the presence or absence of the AMPK inhibitor (Fig. 5C and D). In the absence of AMPK inhibition, CXCL12 stimulated a biphasic pattern of migration consistent with our previously published data (10). However, pretreatment with compound C completely reversed the biphasic pattern and lead to saturable CXCL12-induced migration. Importantly, the introduction of the AMPK inhibitor significantly increased migration at the previously ataxic doses of 100 and 1,000 nmol/L CXCL12 while continuing to permit chemotaxis at the optimal dose of 10 nmol/L CXCL12 (Fig. 5C and D).

Our prior work established not only that CXCL12 exists primarily as a monomer at low concentrations and as a dimer at elevated concentrations but also that an engineered locked dimer CXCL12 protein effectively interfered with colorectal cancer and melanoma metastasis, with increased stability in vivo compared with wild-type protein (27, 37). We tested whether the biophysical dimeric chemokine mechanism might explain CXCL12-induced ataxis through activation of the AMPK bioenergetic signaling mechanism. Using our previously established engineered mutants of CXCL12 locked in either the monomeric or dimeric state (27, 35), we first showed that 10 nmol/L CXCL12-LM stimulated migration of MiaPaCa2 cells equivalent to 10 nmol/L wild-type protein (Fig. 6A). As predicted, 10 nmol/L CXCL12-LD did not elicit significant Transwell migration of MiaPaCa2 cells but did stimulate equivalent calcium mobilization compared with monomer or wild-type protein in both MiaPaCa2 and FC1199 cells (Fig. 6B). Reflecting our observations...
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using 1,000 nmol/L of the wild-type protein, CXCL12-LD stimulated prolonged AMPK activity compared with CXCL12-LM. Furthermore, 1- and 2-hour stimulation with locked dimer CXCL12 resulted in substantially more phosphorylation of AMPK compared with monomeric CXCL12 ligand receptor binding is still emerging. In this study of PDAC, we show that 10 nmol/L CXCL12 stimulates maximal migration (chemotactic) whereas concentrations >100 nmol/L are unable to stimulate migration (ataxic). As we and others have shown for a number of chemokine axes (14, 16–18), over a concentration curve, CXCL12 stimulates migration in a biphasic fashion while eliciting intracellular calcium mobilization in a saturable manner. The decline in chemokine-induced migration at elevated doses has commonly been attributed to receptor desensitization but often without a mechanistic explanation (48). Our data reveal a novel mechanism that explains the lack of migration at elevated concentrations of chemokines through a biased agonist signaling cascade. Here, we show that CXCL12 dose dependently alters glycolysis, oxidative phosphorylation, and promotes activity of the homeostatic metabolism regulator AMPK. We confirmed that this dose-dependent effect on bioenergetic signaling was CXCR4–Goi calcium signaling dependent. While others have demonstrated that small-molecule GPCR agonists are capable of inducing AMPK activity (33) in a calcium-dependent manner, our study is the first to link chemokines, as soluble proteins, with downstream AMPK signaling (Fig. 7). Furthermore, chemotactic CXCL12 concentrations induced cycling of phosphorylation of MLC, facilitating the perpetual motion of stress fiber actin (Fig. 7A) and in turn allowing directional movement (Fig. 7C). In contrast, cells stimulated with ataxic doses of CXCL12 stimulated the AMPK-dependent increase of MYPT1 inhibitory phosphorylation, which blocks MLC phosphorylation/dephosphorylation cycling (Fig. 7B), effectively locking the migration machinery and preventing cell movement (Fig. 7D). While the role of the alternative CXCL12 receptor CXCR7 was not explored, we predict that since CXCR7 signaling is G-protein–independent (27), it will not affect AMPK activity. In addition, as activity of AKT, a known downstream effector of chemokines, has recently been linked to inhibition of AMPK (33), further study into the interplay between AKT and AMPK in chemokine receptor signaling is necessary. Cumulatively, these data support a model wherein elevated concentrations of chemokine ligand, such as might be found once cells have arrived at their chemotactic destination, activate a bioenergetic molecular brake to stop moving. Lefkowitz and colleagues first posited the concept of biased agonism wherein different ligands were capable of distinct signaling through a single GPCR (22). Importantly, their more recent study has shown that β-arrestin, a critical regulator of GPCR biased agonism, also modulates MLC phosphorylation downstream of angiotensin signaling (49). Our recent work identified chemokine oligomerization as a biophysical mechanism for changes in migration potential during chemotaxis and suggested that ligand concentration-dependent biased agonism could explain the associated changes in signaling (25–27). At low nanomolar concentrations, CXCL12 preferentially exists as a monomeric protein, whereas at high nanomolar and micromolar concentrations and in the presence of binding partners, the chemokine forms dimeric structures (25, 26, 50). Using engineered mutants locked into each form, we determined that monomeric CXCL12 was equipotent to the native protein and thus functions as a balanced agonist. In contrast, dimeric CXCL12 was incapable of stimulating migration but retained the equipotent G-protein–biased calcium signaling response as wild-type or

While canonical chemokine signaling occurs through G-protein–dependent calcium mobilization, the precise biochemical mechanisms downstream of chemokine ligand receptor binding are still emerging. In this study of PDAC, we show that 10 nmol/L CXCL12 stimulates maximal migration (chemotactic) whereas concentrations >100 nmol/L are unable to stimulate migration (ataxic). As we and others have shown for a number of chemokine axes (14, 16–18), over a concentration curve, CXCL12 stimulates migration in a biphasic fashion while eliciting intracellular calcium mobilization in a saturable manner. The decline in chemokine-induced migration at elevated doses has commonly been attributed to receptor desensitization but often without a mechanistic explanation (48). Our data reveal a novel mechanism that explains the lack of migration at elevated concentrations of chemokines through a biased agonist signaling cascade. Here, we show that CXCL12 dose dependently alters glycolysis, oxidative phosphorylation, and promotes activity of the homeostatic metabolism regulator AMPK. We confirmed that this dose-dependent effect on bioenergetic signaling was CXCR4–Goi calcium signaling dependent. While others have demonstrated that small-molecule GPCR agonists are capable of inducing AMPK activity (33) in a calcium-dependent manner, our study is the first to link chemokines, as soluble proteins, with downstream AMPK signaling (Fig. 7). Furthermore, chemotactic CXCL12 concentrations induced cycling of phosphorylation of MLC, facilitating the perpetual motion of stress fiber actin (Fig. 7A) and in turn allowing directional movement (Fig. 7C). In contrast, cells stimulated with ataxic doses of CXCL12 stimulated the AMPK-dependent increase of MYPT1 inhibitory phosphorylation, which blocks MLC phosphorylation/dephosphorylation cycling (Fig. 7B), effectively locking the migration machinery and preventing cell movement (Fig. 7D). While the role of the alternative CXCL12 receptor CXCR7 was not explored, we predict that since CXCR7 signaling is G-protein–independent (27), it will not affect AMPK activity. In addition, as activity of AKT, a known downstream effector of chemokines, has recently been linked to inhibition of AMPK (33), further study into the interplay between AKT and AMPK in chemokine receptor signaling is necessary. Cumulatively, these data support a model wherein elevated concentrations of chemokine ligand, such as might be found once cells have arrived at their chemotactic destination, activate a bioenergetic molecular brake to stop moving. Lefkowitz and colleagues first posited the concept of biased agonism wherein different ligands were capable of distinct signaling through a single GPCR (22). Importantly, their more recent study has shown that β-arrestin, a critical regulator of GPCR biased agonism, also modulates MLC phosphorylation downstream of angiotensin signaling (49). Our recent work identified chemokine oligomerization as a biophysical mechanism for changes in migration potential during chemotaxis and suggested that ligand concentration-dependent biased agonism could explain the associated changes in signaling (25–27). At low nanomolar concentrations, CXCL12 preferentially exists as a monomeric protein, whereas at high nanomolar and micromolar concentrations and in the presence of binding partners, the chemokine forms dimeric structures (25, 26, 50). Using engineered mutants locked into each form, we determined that monomeric CXCL12 was equipotent to the native protein and thus functions as a balanced agonist. In contrast, dimeric CXCL12 was incapable of stimulating migration but retained the equipotent G-protein–biased calcium signaling response as wild-type or

Discussion

The CXCL12–CXCR4 chemokine axis has been implicated in numerous cancers, including highly metastatic PDAC, under the conventional model that elevated receptor expression correlates with malignancy (7). However, this model has not translated to successful clinical development of CXCR4-targeted anticancer therapies, suggesting a clear need for a novel pharmacologic approach. In particular, the mechanism underlying the ability of chemokines to control directional cellular migration at different doses is not known. The conventional model for chemokine involvement in cancer metastasis has been oversimplified by CXCR4 leading to increased sensitization and passive migration toward distant tissue sources of CXCL12 such as the liver (45). This current dogma ignores the potential active role that the ligand CXCL12 plays in modulating cancer. Our work in highly malignant PDAC extends the current understanding of chemokines in cancer in 2 important ways. First, while other recent PDAC studies have focused on the role of chemokines in the primary tumor (46, 47), our data herein and in a previous study (10) identify an ataxic, protective role for CXCL12–CXCR4 in metastasis of pancreatic cancer. Second, although numerous studies have defined shifts in metabolism that occur during pancreatic cancer cell growth (29–31), our report is the first to observe the changes in bioenergetic metabolism that occur during pancreatic cancer cell migration. Our data indicate that CXCL12 can function as a balanced or biased agonist for its cognate receptor CXCR4, depending on the chemokine concentration. Elevated doses of CXCL12 effectively activate an AMPK-mediated bioenergetic brake that shifts pancreatic cancer cells into a nonmigratory response. We additionally demonstrate in a preclinical model that CXCL12 inhibits pancreatic cancer metastasis in vivo.
monomeric forms of the ligand (27). As CXCL12 dimer-treated cells were able to migrate in response to CXCR4-independent stimulation, we first termed the dimer effect as "cellular idling" (27). We additionally showed that oligomers of CXCL12 differentially recruited β-arrestin and stimulated phosphorylation of ERK1/2 (27). In this study, we used engineered oligomeric mutants of CXCL12 to demonstrate that the ataxis of pancreatic cancer cells elicited by elevated concentrations of CXCL12 is mirrored with locked dimeric CXCL12. In addition, similar to atactic, that is, >100 nmol/L, doses of wild-type CXCL12, dimeric CXCL12 elicited more prolonged AMPK activity compared with a monomeric variant of the ligand. Thus, these new data establish a mechanistic link between the observed biphasic regulation of migration by CXCL12 and biased agonism elicited by distinct concentrations or oligomeric states of the chemokine. These data are the first to identify a mechanism by which enhanced signaling drives the arrest of cellular migration, which we have termed "ataxis," at elevated physiologic concentrations of the chemokine ligand.

Reexamining the role for CXCL12–CXCR4 in the context of biased signaling sheds important light on chemokine function in malignancy. Under the conventional model, some have suggested that CXCR4 is a prometastatic factor for pancreatic cancer (51), whereas others have shown that CXCL12 can stimulate increased activity of the cell survival factor AKT, implying that the chemokine is a progrowth factor (52). However, nearly all of those studies rely upon a single, typically migratory, dose of CXCL12 to examine signaling outputs and ignore potentially significant functional effects of higher concentrations of CXCL12 previously observed in vivo (20). Along with our previous study (10), these data suggest a model for CXCL12–CXCR4 in pancreatic cancer wherein low chemotactic concentrations elicit cancer cell movement to distant tissues but locally increased ataxic concentrations of the ligand CXCL12 abrogate cancer cell movement through bioenergetic signaling and cytoskeletal dysregulation. Translating this novel signaling mechanism, we found that systemic treatment with wild-type CXCL12 elicits a similar antimetastatic effect against malignant pancreatic cancer cells. Some have suggested that inhibition of CXCR4 is a strategy for blocking PDAC malignancy (46, 47). However, administration of small-molecule antagonists of CXCR4, such as AMD3100, passively blocks cell movement through competitive inhibition, primarily by blocking

Figure 7.
Model for CXCL12 biased agonist regulation of migration through AMPK MLC signaling. A, in PDAC cells stimulated with chemotactic doses of CXCL12 (<10 nmol/L), signaling leads to a balanced cycling of MLC phosphorylation. MLC phosphorylation by MLCK and subsequent dephosphorylation by MYPT1 allows MLC to first bind filamentous actin (F-actin), move the fiber with a lever-like action, and then re-attach to allow the process to begin again for further movement. B, in PDAC cells stimulated with atactic biased doses of CXCL12 (>100 nmol/L), extended AMPK signaling inhibits the activity of MYPT1, leading to imbalanced cycling of MLC phosphorylation. Buildup of phosphorylated MLC prevents the lever-like action of MLC and subsequent re-attachment to F-actin; as a result, F-actin fibers remain immobile. C, at the cellular level, by 60 minutes of stimulation, chemotactic doses of CXCL12 induce direction movement through balanced MLC phosphorylation cycling, subsequent stress fiber actin formation, and filopodia formation. D, atactic doses of CXCL12 cause unbalanced and high levels of phospho-MLC binding, perimembranous cortical localization of actin, and prevent polarized cell contraction and relaxation necessary for directional movement.
one of the two ligand-binding sites on the CXCR4 receptor. Given the CXCR4 antagonist binds only one of two necessary binding sites with micromolar binding affinity, it is perhaps not surprising that AMD3100 failed to provide clinical benefit in long-term treatment of patients with HIV (53). Our report suggests that the CXCL12 protein is an effective alternative to CXCR4 antagonists to inhibit PDAC metastasis. By specifically eliciting the antimigratory downstream signaling components, biased agonist variants of CXCL12 have potential to actively block pancreatic cancer cell movement. Further study is needed to fully uncover the pharmacokinetics/pharmacodynamics of this biologic response modifying drug, although extended biologic therapy for >75 days in our two models of pancreatic cancer herein showed few signs of toxicity. Future studies must also examine how CXCL12 biologic therapy will modulate the immunosurveillance in the pancreatic primary tumor environment. Finally, as pancreatic tumors rich in GAGs (54, 55), which are known to alter chemokine binding, further investigating into the in vivo interaction between recombinant chemokine and extracellular components is necessary.

In sum, this work uncovers a biochemical mechanism for CXCL12 biphasic regulation of migration that links our previous reports in oligomeric chemokine biased agonism (27) and CXCL12 chemotactic signaling (36). We show that ataxic doses of CXCL12 lead to incomplete turnover of migratory signaling and subsequent lacking of migration machinery that is bioenergetic signaling dependent. The cumulative findings of our recent reports underscore the antimetastatic potential of exploiting chemokine biased agonist signaling, address the dire need for identifying strategies to target metastasis, and delineate the importance of future comprehensive preclinical studies of chemokine ligand–specific therapies for pancreatic cancer.

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**References**


Pancreatic Cancer Cell Migration and Metastasis Is Regulated by Chemokine-Biased Agonism and Bioenergetic Signaling

Ishan Roy, Donna M. McAllister, Egal Gorse, et al.