An Undesired Effect of Chemotherapy

GEMCITABINE PROMOTES PANCREATIC CANCER CELL INVASIVENESS THROUGH REACTIVE OXYGEN SPECIES-DEPENDENT, NUCLEAR FACTOR κB- AND HYPOXIA-INDUCIBLE FACTOR 1α-MEDIATED UP-REGULATION OF CXCR4*

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Sumit Arora†, Arun Bhardwaj‡, Seema Singh‡, Sanjeev K. Srivastava‡, Steven McClellan‡, Chaitanya S. Nirodi‡, Gary A. Piazza‡, William E. Grizzle‡, Laurie B. Owen‡, and Ajay P. Singh‡¶

From the †Department of Oncologic Sciences, Mitchell Cancer Institute, University of South Alabama, Mobile, Alabama 36604, the ‡Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama 35294, and the ‡Department of Biochemistry and Molecular Biology, College of Medicine, University of South Alabama, Mobile, Alabama 36688

Background: CXCR4 signaling protects pancreatic cancer cells from gemcitabine toxicity. However, the effect of gemcitabine on this resistance mechanism is unclear.

Results: Gemcitabine up-regulates CXCR4 expression in pancreatic cancer cells and promotes their invasiveness.

Conclusion: CXCR4 signaling serves as a counterdefense mechanism against gemcitabine.

Significance: These findings are significant for the formulation of effective therapeutic strategies against pancreatic cancer.

Recently, we have shown that CXCL12/CXCR4 signaling plays an important role in gemcitabine resistance of pancreatic cancer (PC) cells. Here, we explored the effect of gemcitabine on this resistance mechanism. Our data demonstrate that gemcitabine induces CXCR4 expression in two PC cell lines (MiaPaCa and Colo357) in a dose- and time-dependent manner. Gemcitabine-induced CXCR4 expression is dependent on reactive oxygen species (ROS) generation because it is abrogated by pre-treatment of PC cells with the free radical scavenger N-acetyl-L-cysteine. CXCR4 up-regulation by gemcitabine correlates with time-dependent accumulation of NF-κB and HIF-1α in the nucleus. Enhanced binding of NF-κB and HIF-1α to the CXCR4 promoter is observed in gemcitabine-treated PC cells, whereas their silencing by RNA interference causes suppression of gemcitabine-induced CXCR4 expression. ROS induction upon gemcitabine treatment precedes the nuclear accumulation of NF-κB and HIF-1α, and suppression of ROS diminishes these effects. The effect of ROS on NF-κB and HIF-1α is mediated through activation of ERK1/2 and Akt, and their pharmacological inhibition also suppresses gemcitabine-induced CXCR4 up-regulation. Interestingly, our data demonstrate that nuclear accumulation of NF-κB results from phosphorylation-induced degradation of IκBα, whereas HIF-1α up-regulation is NF-κB-dependent. Lastly, our data demonstrate that gemcitabine-treated PC cells are more motile and exhibit significantly greater invasiveness against a CXCL12 gradient. Together, these findings reinforce the role of CXCL12/CXCR4 signaling in gemcitabine resistance and point toward an unintended and undesired effect of chemotherapy.

Pancreatic cancer remains one of the most lethal malignancies in the United States with a rising incidence and unabated mortality (1, 2). The overall median survival after diagnosis is 2–8 months, and only 3–6% of all pancreatic cancer patients survive 5 years after diagnosis (1, 2). It is estimated that nearly 45,220 Americans will be diagnosed this year with pancreatic malignancy, and over 38,460 will die from this disease, making it as the fourth leading cause of cancer-related death in the United States (2). Surgical resection is the most effective and potentially curative treatment option. However, most pancreatic cancers are diagnosed at a late stage when the disease has already spread to distant sites (3). Under this scenario, systemic chemotherapy is considered as an option, but it provides only modest improvement in patient survival (4, 5). Therefore, further research is warranted to develop novel and effective therapeutic strategies against this devastating disease.

Gemcitabine has been the standard of care drug for pancreatic cancer patients as a single-agent therapy (5). However, most patients do not respond well to gemcitabine-treatment, and those who do respond ultimately develop chemoresistance and exhibit disease progression (6). It is suggested that cellular resistance to gemcitabine is either inherent or acquired during the treatment. However, underlying molecular mechanisms remain poorly defined. Recently, we demonstrated that activation of the CXCL12/CXCR4 signaling axis is involved in conferring chemoresistance to pancreatic cancer cells through potentiation of intrinsic survival mechanisms (7). It has been shown in earlier studies that CXCR4, a chemokine receptor, is aberrantly overexpressed in pancreatic cancer cells, whereas its sole ligand, CXCL12, is produced abundantly by stromal cells (7–9). CXCL12/CXCR4 signaling plays an important role in pancreatic tumor growth, invasion, and metastasis (10, 11). Furthermore, our recent data demonstrated a role of this signaling axis in facilitating bidirectional tumor-stromal interaction through up-regulation of the hedgehog ligand sonic hedgehog (SHH) (12). Because paracrine hedgehog signaling is shown to be important in pathobiology and chemoresistance of pan-

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† To whom correspondence should be addressed: Department of Oncologic Sciences, Mitchell Cancer Institute, University of South Alabama, 1660 Springhill Ave., Mobile, AL 36604-1405. Tel.: 251-445-9843; Fax: 251-460-6994; E-mail: asingh@usouthal.edu.
creatic cancer (4, 13), it appears that CXCL12/CXCR4 signaling impacts pancreatic tumorigenesis and therapeutic outcome through direct as well as indirect mechanisms.

In this study, we examined the effect of gemcitabine on CXCR4 expression in pancreatic cancer cells to explore the possibility of a counterdefense mechanism. Our data demonstrate, for the first time, that gemcitabine induces dose- and time-dependent up-regulation of CXCR4 in pancreatic cancer cells. We also show that gemcitabine-induced CXCR4 expression follows ROS-elicited² activation of the Akt and ERK pathways, which leads to nuclear accumulation of NF-κB and HIF-1α through distinct mechanisms. NF-κB and HIF-1α directly bind to the CXCR4 promoter, and their silencing by RNA interference abrogates gemcitabine-induced CXCR4 up-regulation. Furthermore, our data reveal that gemcitabine-treated pancreatic cancer cells display a remarkable increase in motility and invasiveness against a CXCL12 gradient. Thus, our findings suggest that pancreatic cancer cells utilize CXCR4 up-regulation as a novel counterdefense mechanism to resist gemcitabine-induced apoptosis and may also facilitate tumor cell spread to CXCL12-overexpressing chemoprotective niches.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—Pancreatic cancer cell lines MiaPaCa and Colo357 were obtained and cultured as described previously (12). Validation of cell lines was done by short tandem repeat genotyping and the presence of defined markers (MUC1, MUC4, vimentin, and DPC4). Moreover, cells were monitored continuously for their typical morphology and intermittently tested for mycoplasma using a MycoSensorPCR assay kit (Stratagene, CA) according to the protocol of the manufacturer.

Antibodies and siRNAs—Anti-CXCR4 and anti-HIF-1α antibodies (rabbit polyclonal and mouse monoclonal, respectively) were purchased from Abcam (Cambridge, MA). Antibodies against ERK1/2 (rabbit monoclonal), pERK1/2 (mouse monoclonal), IκB-α (mouse monoclonal), p-IκB-α (Ser-32/36) (rabbit polyclonal), and NF-κB/p65 (rabbit monoclonal) were from Cell Signaling Technology (Beverly, MA). Antibodies against Akt and p-Akt (both rabbit monoclonal) were from Epitomics (Burlingame, CA). β-Actin (mouse monoclonal) antibody was purchased from Sigma-Aldrich (St. Louis, MO). Laminin (mouse monoclonal), α-tubulin (mouse monoclonal), and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All non-target (ON-TARGET plus non-targeting pool) and target-specific (ON-TARGET Plus) siRNAs and transfection reagent were from Dharmacon (Lafayette, CO).

Treatments and Transfections—Cells were cultured in 6- or 12-well plates in complete medium and allowed to grow up to 60–80% confluence level. Thereafter, cells were treated with different doses of gemcitabine (TOCRIS Bioscience, Bristol, UK) for various time intervals (as indicated in the figure legends). To elucidate the role of ROS and specific signaling pathways, cells were pretreated for 1 h with 10 mM N-acetyl-L-cys-2 The abbreviations used are: ROS, reactive oxygen species; NAC, N-acetyl-L-cysteine; PC, pancreatic cancer.
teine (NAC), an antioxidant (Sigma-Aldrich), and 20 μM LY294002 (PI3K inhibitor) and 25 μM PD98059 (ERK inhibitor) (Cell Signaling Technology) alone or in combination. Cells were treated with cyclohexamide (50 μM, Sigma-Aldrich) to examine the effects of gemcitabine on the stability of HIF-1α. For the specific knockdown of NF-κB/p65 and HIF-1α, cells were cultured in 6-well plates and transiently transfected with 50 nm of non-target or target-specific siRNAs using DharmaFECT according to the protocol of the manufacturer.

RNA Isolation and RT-PCR—Total RNA from the cells was isolated using an RNasea purification kit (Qiagen, MD). Subsequently, cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA) following the instructions of the manufacturer. Quantitative real-time PCR was performed in 96-well plates using SYBR Green master mix (Applied Biosystems) on an iCycler system (Bio-Rad). Specific PCR primers were used for amplification of CXCR4, HIF-1α, and GAPDH. The sequences for the PCR primer pairs used were as follows: CXCR4, 5’-GAAGCTGTGTGGCTGAAGAGG-3’ (forward) and 5’-TGAGGTGTTGACA-GCTTTGAG-3’ (reverse); HIF-1α, 5’-TGCAATGGAAGGTATTCGA-3’ (forward) and 5’-TTATACCAACTGAG-GTTGTGATT-3’ (reverse); and GAPDH, 5’-GCTGTTGTCGAAATCTCAA-3’ (forward) and 5’-GGTCAGGGCTTGCAGAAGATA-3’ (reverse). The thermal conditions for real-time PCR assays were as follows. Cycle 1, 95 °C for 10 min and cycle 2 (40X), 95 °C for 10 s and 58 °C for 45 s. Threshold cycle (Ct) values for CXCR4 or HIF1-α were normalized against Ct values for GAPDH, and a relative fold change in expression with respect to a reference sample was calculated by the 2⁻ΔΔCt method.

Measurement of ROS—In situ ROS generation was examined by using a cell-permeable dye, i.e. 2’,7’-dichloro-fluorescin diacetate, which is first converted into a non-fluorescent reduced form 2’,7’-dichloro-fluorescin after cleavage by cellular esterases. Subsequently, 2’,7’-dichloro-fluorescin is oxidized by cellular ROS into a fluorescent form 2’,7’-dichloro-fluorescin. In brief, cells were incubated with 2’,7’-dichloro-fluorescin diacetate (Sigma Aldrich) in regular medium for 30 min at 37 °C. After incubation, dye containing medium was removed, and cells were washed three times with PBS and then resuspended in PBS. Fluorescence was recorded at an excitation/emission wavelength of 485 nm/530 nm by flow cytometry on a FACSCanto Li (BD Biosciences).

Nuclear and Cytoplasmic Fractionation—The preparation of cytoplasmic and nuclear extracts was performed using the nuclear extract kit (Active Motif, Carlsbad, CA) as described previously (14).

Immunoblot Analysis—Immunoblotting was performed as described earlier (14). In brief, total or fractionated protein lysates (30–100 μg) were resolved by electrophoresis on 10% SDS-PAGE and transferred onto a PVDF membrane. The blots were subjected to a standard immunodetection procedure using specific antibodies against various proteins and their respective secondary antibodies and incubated further with chemiluminescent Super Signal West Femto Maximum sensitivity substrate (Thermo Scientific, Logan, UT). The signal was detected using an LAS-3000 image analyzer (Fuji Photo Film
Co., Tokyo, Japan). β-Actin, α-tubulin, and laminin were used as loading controls for total, cytoplasmic, and nuclear protein, respectively.

**ChIP Assay**—Binding of NF-κB/p65 and HIF-1α to the CXCR4 promoter was analyzed by ChIP assay using a ChIP-IT enzymatic kit (Active Motif). Briefly, cells were fixed with paraformaldehyde (37%) for the cross-linking of DNA and protein. Thereafter, enzymatic DNA shearing was performed, and sheared DNA was subjected to immunoprecipitation with anti-NF-κB/p65, anti-HIF-1α, and normal rabbit IgG (as control) antibodies. Following immunoprecipitation, cross-linking was reversed, the proteins were digested by proteinase K, and the DNA was isolated. Isolated ChIPed DNA was then subjected to PCR using specific primers. The primer sets used were as follows: CXCR4 primers for the NF-κB/p65 chip assay, 5′-TCGAAAGCTTATTGCCGCCTACT-3′ (forward) and 5′-CGAGGATCCCCAACAACCTGAAGTTCTG-3′ (reverse); HIF-1α, 5′-TCGTGCCAAAGCTTGTCCCTG-3′ (forward) and 5′-GCGGTAACCAATTCGCGAATAGTGC-3′ (reverse). Primers used for the HIF-1α promoter were as follows: 5′-GAACAAGAGCCCAGACAG-3′ (forward) and 5′-AGGTGCCTGAGGAGCTGAGG-3′ (reverse) flanking the NF-κB binding site. Input DNA (cross-linked chromatin without immunoprecipitation) and negative control Ab-precipitated DNA were used as positive and negative controls, respectively.

**Motility and Invasion Assays**—MiaPaCa and Colo357 cells grown in 6-well plates were treated with gemcitabine (10 μM) for 24 h. Post-treated cells were trypsinized, counted, and plated for motility and invasion assays. For the motility assay, MiaPaCa and Colo357 (1 × 10^5 and 5 × 10^5 cells/well) cells were plated in the top chamber of a non-coated polyethylene teraphthalate membrane (6-well insert, pore size 8 μm, BD Biosciences). For the invasion assay, MiaPaCa (2.5 × 10^4) and Colo357 (1.25 × 10^5) cells were plated in the top chamber of the transwell chamber with a Matrigel-coated polycarbonate membrane (24-well insert, 0.8 μm, BD Biosciences). To the lower chamber, media containing 5% FBS alone or supplemented with CXCL12 (100 ng/ml) was added as a chemoattractant. After 16 h of incubation, cells that remained on the upper surface of the insert membrane were removed with a cotton swab. Cells that had migrated or invaded through the membrane/Matrigel to the bottom of the insert were fixed and stained with a Diff-Quick cell staining kit (Dade Behring, Inc., Newark, DE), mounted on a slide, and photographed. To examine whether...
the enhanced invasion was mediated proteotypically, we used broad-spectrum matrix metalloproteinase and serine protease inhibitors. Gemcitabine-treated (24 h) cells were incubated with either matrix metalloproteinase inhibitor (25 μM, GM6001, EMD Millipore) or serine protease inhibitor (100 μM, AEBSF, EMD Millipore) 30 min prior and during the seeding into the invasion chamber. The number of invaded cells were examined and counted as described.

### Statistical Analysis

All experiments were performed at least three times. The data are expressed as mean ± S.D. Whenever appropriate, the data were also subjected to unpaired two-tailed Student’s t test. *p < 0.05; **p < 0.01. The data show that gemcitabine treatment generates ROS in a time-dependent manner in pancreatic cancer cells.

### RESULTS

#### Gemcitabine Induces CXCR4 Expression in Pancreatic Cancer Cells through Generation of ROS

The effect of gemcitabine on CXCR4 expression was examined in two human pancreatic cancer (PC) cell lines, MiaPaCa and Colo357. After treatment with gemcitabine (1.25–20 μM) for 24 h, we observed a dose-dependent increase in the expression of CXCR4 at mRNA as well as protein levels in both cell lines (Fig. 1A). A 3- to 4-fold up-regulation of the CXCR4 transcript was observed with the lowest dose of treatment that reached over 40-fold at the highest treatment dose (Fig. 1A, upper panel). Similarly, we observed up to 19.5- and 26.5-fold increases in CXCR4 protein levels in MiaPaCa and Colo357 cells, respectively, with increasing doses of gemcitabine (Fig. 1A, lower panel). In a time course assay (0–48 h) with 10 μM of gemcitabine treatment, we detected CXCR4 up-regulation as early as 1 h at the mRNA and protein levels in both cell lines (Fig. 1B). CXCR4 expression increased with exposure time and reached over 30- and 20-fold at the transcript (Fig. 1B, upper panel) and protein levels (lower panel), respectively, after 48 h.

We next examined the role of ROS in gemcitabine-induced expression of CXCR4. Induction of ROS upon gemcitabine treatment has been reported earlier and is considered to be one of the mechanisms for its antitumor activity (15). Therefore, we treated the PC cells with 10 mM NAC (an antioxidant...
and free radical scavenger) 1 h prior to gemcitabine treatment. Protein extracts were made after 0-, 6-, and 24-h treatments, and CXCR4 expression was determined. Our data show that the effect of gemcitabine on CXCR4 expression is abolished in NAC-pretreated PC cells (Fig. 2A). In a separate experiment, we confirmed the efficacy of NAC by examining the ROS levels in gemcitabine-treated cells pretreated with NAC. Our data demonstrated nearly complete abrogation of the gemcitabine-induced ROS increase in NAC-pretreated pancreatic cancer cells (data not shown). To derive further support for a role of ROS in CXCR4 expression, we estimated its levels in gemcitabine-treated PC cells at various (early and late) time durations. The data demonstrate that ROS levels are increased within 10 min of gemcitabine treatment and continue to rise up to 24 h of treatment (Fig. 2B). Altogether, our data suggest that gemcitabine induces the expression of CXCR4 in PC cells through generation of ROS.

**NF-κB and HIF-1α Are Major Players in Gemcitabine-induced Expression of CXCR4**—ROS has been reported to modulate the activity of several transcription factors, including NF-κB and HIF-1α (16). These transcription factors have also been reported in CXCR4 regulation in different cancer types (17, 18). To investigate whether NF-κB and HIF-1α mediate ROS-dependent up-regulation of CXCR4, we monitored the change in their expression and/or subcellular localization following gemcitabine treatment. The data demonstrate that gemcitabine treatment causes a remarkable and time-dependent increase in nuclear levels of NF-κB/p65 (Fig. 3A, upper panel) with a concomitant decrease in cytoplasmic levels (center panel) in both cancer cell lines. Furthermore, we also observed an increase in HIF-1α levels in nuclear (Fig. 3A, upper panel) as well as cytoplasmic (center panel) fractions of PC cell lysates after gemcitabine treatment. Accordingly, although we did not observe a change in total NF-κB levels, we observed an up-regulation of HIF-1α in total cell lysates (Fig. 3A, lower panel). To ascertain whether induction of NF-κB/p65 translocation or HIF-1α expression upon gemcitabine treatment is ROS-dependent, we pretreated the PC cells with NAC for 1 h prior to gemcitabine treatment and examined the effect on NF-κB and HIF-1α. The data clearly demonstrate that pretreat-
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Activation of NF-κB and HIF-1α Involves ROS-mediated Activation of Akt and ERK—The PI3K/Akt and MAPK signaling pathways have been shown to be activated by ROS (16, 19, 21). In other studies, NF-κB and HIF-1α are shown to be the downstream targets of PI3K/Akt and/or ERK (22–24). Thus, to investigate the role of these signaling nodes in gemcitabine-induced activation of NF-κB and HIF-1α, we treated the pancreatic cancer cells with specific pharmacological inhibitors of Akt (LY294002) and ERK (PD98059) prior to stimulation with gemcitabine. Immunoblot analyses demonstrated effective and specific inhibition of gemcitabine-induced phosphorylation of Akt and ERK by their respective pharmacological inhibitors in both cell lines (Fig. 5A, upper panel). Importantly, gemcitabine-induced nuclear expression of NF-κB and HIF-1α was also decreased significantly in cells pretreated with Akt or ERK inhibitors (Fig. 5A, center panel). Moreover, we observed that inhibition of either Akt or ERK led to abrogation of gemcitabine-induced CXCR4 expression, whereas their combined inhibition caused a more potent suppression (Fig. 5A, lower panel). To confirm the involvement of ROS in the activation of ERK and Akt, we examined their activation status in PC cells incubated with NAC 1 h prior to gemcitabine treatment. The data show a suppression of Akt and ERK activation in PC cells pretreated with NAC (Fig. 5B). Together, these findings indi-
cate that ROS activates Akt and ERK, which, in turn, cooperatively mediate gemcitabine-induced activation of NF-κB and HIF-1α and subsequent CXCR4 up-regulation.

**Gemcitabine-induced HIF-1α Up-regulation Occurs at the Transcriptional Level and Is NF-κB-dependent**—We next dissected the mechanisms underlying NF-κB nuclear translocation and HIF-1α up-regulation. For this, we first examined the effect of gemcitabine treatment on IκB-α, a biological inhibitor of NF-κB that keeps it sequestered in the cytoplasm. Our data show that gemcitabine treatment resulted in increased phosphorylation of IκB-α in a time-dependent manner that led to a concomitant decrease in its expression (data not shown). These data suggest that enhanced nuclear accumulation of NF-κB results from gemcitabine-induced destabilization of IκB-α. HIF-1α expression is regulated at transcriptional, translational, and posttranslational levels. As ROS is known to stabilize HIF-1α (25, 26), we examined HIF-1α stabilization in gemcitabine-treated PC cells. For this, we treated the PC cells with gemcitabine (10 μM) for 6 h, followed by 30 min of incubation with the protein synthesis inhibitor cycloheximide (50 μM) with or without additional treatment with NAC (10 mM) for the next 90 min. The rate of HIF-1α decay was monitored by immuno- blot assay. The data demonstrated no change in the pattern of HIF-1α decay in the presence or absence of NAC, which ruled out the possibility that ROS-induced HIF-1α expression occurred through its stabilization (Fig. 6A). To consider another possibility, we examined the expression of HIF-1α at the transcript level by RT-PCR assay in gemcitabine-treated PC cells. Our data show that the expression of HIF-1α is increased at the mRNA level in both cell lines after gemcitabine treatment in a time-dependent manner (Fig. 6B). To understand the underlying molecular mechanism, we surveyed the HIF-1α promoter by *in silico* analyses (transcription element search system) and identified a putative binding sequence for NF-κB/p65 (GGGACTTGCC) at -197/-188 bp in the HIF-1α promoter. Therefore, we examined the role of NF-κB/p65 in HIF-1α transcriptional up-regulation by silencing its expression prior to gemcitabine treatment. The data demonstrate that gemcitabine-induced HIF-1α expression is completely abrogated after silencing of NF-κB/p65 in both cell lines (Fig. 6C). Furthermore, we observed an enhanced binding of the NF-κB to HIF-1α promoter upon treatment with gemcitabine in a

**FIGURE 5.** ROS-induced nuclear localization of NF-κB/p65 and HIF-1α is mediated through the Akt and ERK pathways. A, pancreatic cancer cells were pretreated with Akt inhibitor (LY294002, 20 μM) or ERK inhibitor (PD98059, 25 μM) for 1 h, followed by treatment with gemcitabine (GEM, 10 μM) for either 15 min, 12 h, or 24 h. Total or nuclear protein was isolated, and expression of Akt, p-Akt, ERK, p-ERK (after a 15-min exposure in total lysate), NF-κB/p65 and HIF-1α (after a 12-h exposure in nuclear lysate), and CXCR4 (after a 24-h exposure in total lysate) was examined by immunoblot analysis. β-Actin and laminin were used as loading controls for total and nuclear lysates, respectively. B, cells were pretreated with NAC as described earlier, followed by treatment with gemcitabine (10 μM) for 15 min. Total protein was isolated, and the effects on the expression of Akt, p-Akt, ERK, and p-ERK were examined by immunoblot analysis. β-Actin was used as a loading control. Altogether, the data demonstrate the selective efficacy of inhibitors and indicate that the ROS-induced nuclear localization of both NF-κB/p65 and HIF-1α occurs through the Akt and ERK pathways after gemcitabine treatment.
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FIGURE 6. NF-κB/p65 enhances expression of HIF-1α in pancreatic cancer cells. A, MiaPaCa and Colo357 cells were pretreated with gemcitabine (GEM, 10 μM) for 6 h, followed by cycloheximide (CHX, 50 μM) treatment for 30 min. Thereafter, NAC (10 mM) was added to the wells for different time intervals (15–90 min). As a control, one of the wells treated with gemcitabine and cycloheximide lacked NAC for each time point. Total protein was isolated, and expression of NF-κB and HIF-1α was examined by immunoblot assay. β-Actin was used as an internal control. The data show that there is no effect of the gemcitabine treatment on the stability of HIF-1α. B, cells were treated with gemcitabine (10 μM) for the indicated time intervals, and total RNA was extracted. c-DNA was prepared, and the expression of HIF-1α was analyzed by quantitative RT-PCR. GAPDH was used as an internal control. The data show that gemcitabine induces expression of HIF-1α in a time-dependent manner. Bars represent the mean of triplicates ± S.D. *, p < 0.05; **, p < 0.01. C, cells were transiently transfected with non-target (NT) or NF-κB/p65-targeted siRNAs. Following 48 h of transfection, cells were treated with gemcitabine (10 μM) for the next 12 h. Nuclear protein was isolated and subjected to immunoblot analysis to assess the expression of NF-κB/p65 and HIF-1α. Laminin was used as a loading control. D, pancreatic cancer cells were treated with gemcitabine (10 μM), and proteins and DNA were cross-linked with formaldehyde. Cross-linked chromatin was sheared and immunoprecipitated with an anti-NF-κB/p65 or nonspecific IgG. Immunoprecipitated chromatin was subjected to PCR to amplify the HIF-1α promoter region harboring a putative NF-κB site. The data show an increased binding of NF-κB/p65 to the HIF-1α promoter upon gemcitabine treatment, suggesting its direct involvement in gemcitabine-induced HIF-1α expression.

chromatin immunoprecipitation assay (Fig. 6D). To be more rigorous, we also carried out a PCR using a primer set that amplifies a region in the HIF-1α promoter not harboring a putative NF-κB binding site using ChIPed DNA from gemcitabine-treated cells but observed no amplification (data not shown). Altogether, these findings reveal distinct molecular mechanisms for NF-κB and HIF-1α induction in gemcitabine-treated PC cells.

Gemcitabine-treated Pancreatic Cancer Cells Exhibit Enhanced Motility and Invasion—Having observed CXCR4 up-regulation following gemcitabine treatment, we examined the pathobiological significance of this observation in addition to serving as a counterdefense mechanism. Several lines of evidence suggest that CXCR4 overexpression promotes cancer cell motility and invasion, leading to enhanced metastasis (10, 11). Therefore, we monitored the motility and invasion of gemcitabine-treated PC cells in the presence or absence of CXCL12 (a ligand for CXCR4). As expected, the number of cells migrated in the presence of the CXCL12 chemotactic gradient was increased in both MiaPaCa and Colo357 cells (~1.7-fold) compared with the untreated group (Fig. 7). Similarly, we also observed an increase in invasiveness of MiaPaCa and Colo357 (~2.1-fold) cells against a CXCL12 gradient. However, when the migration and invasiveness of gemcitabine-treated pancreatic cancer cells was examined against a CXCL12 gradient, we observed a dramatic increase in numbers of cells migrated and invaded in both the MiaPaCa (~4.5- and 6.1-fold, respectively) and Colo357 (~4.7- and 7.2-fold, respectively) cell lines (Fig. 7). We next used broad-spectrum matrix metalloproteinase and serine protease inhibitors to confirm that a relative increase in invasiveness is indeed mediated proteolytically. Our data demonstrated that both matrix metalloproteinase and serine protease inhibitors failed to inhibit cell migration (data not shown). Altogether, these data show that gemcitabine treatment enhances CXCL12-induced migration and invasion in PC cells.

DISCUSSION

The CXCL12/CXCR4 signaling axis has emerged as an important determinant of aggressive and drug-resistant phenotypes in several malignancies, including pancreatic cancer (7, 10, 11). Our recent studies revealed that CXCL12/CXCR4 signaling might play important roles in the gemcitabine resistance of pancreatic cancer cells by directly altering the cell survival
pathways or by facilitating tumor-stromal interactions (7, 12). This study provides additional support for the functional significance of this signaling axis as a resistance mechanism by demonstrating an up-regulation of CXCR4 in response to gemcitabine treatment of PC cells. Our data demonstrate that gemcitabine-induced CXCR4 up-regulation is ROS-dependent and is mediated through activation of the Akt and ERK pathways. Transcriptional up-regulation of CXCR4 is eventually facilitated by Akt- and ERK-dependent nuclear accumulation of the NF-κB and HIF-1α transcription factors, which then bind to the CXCR4 promoter to cooperatively induce CXCR4 expression. More importantly, gemcitabine-treated PC cells exhibit enhanced motility and invasion against a CXCL12 gradient. Together, these findings highlight a novel pathobiological significance of CXCR4 and indicate an unintended and undesired effect of chemotherapy.

CXCR4 expression is elevated in a majority of pancreatic cancers and preinvasive neoplastic lesions, suggesting its role in the pathogenesis and progression of pancreatic neoplasia (27, 28). CXCL12, the sole ligand of CXCR4, is also produced abundantly by tumor-associated stromal cells and at common sites of metastasis, thus indicating an important role of CXCL12/CXCR4 signaling in pancreatic cancer progression and metastasis (9, 29). In addition, it has been shown that CXCR4 is expressed by pancreatic cancer stem cells and is essential for their invasive and metastatic properties (30). In this study, we observed that gemcitabine treatment led to a dose- and time-dependent increase in CXCR4 expression. This observation provides additional support for our earlier findings where we showed a role of the CXCL12/CXCR4 signaling axis in gemcitabine resistance (7). Our data suggest that induction of CXCR4 upon gemcitabine treatment could be a counterdefense mechanism of tumor cells to resist apoptosis by promotion of CXCL12-induced cell survival pathways.

Gemcitabine (2’-2’ difluorodeoxycytidine, dFdC) is a nucleoside analog of deoxycytidine. It is a prodrug that, upon cellular
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entry, is phosphorylated by deoxycytidine kinase to form gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTTP). Active phospho forms of gemcitabine then interfere with DNA synthesis through incorporation into DNA, causing cell cycle arrest and subsequent apoptosis (31, 32). It has also been shown that gemcitabine, like certain other anticancer agents, induces ROS, which, according to a recent report, serves as an additional anticancer mechanism of gemcitabine (15). However, our current findings present another possibility. Our data show that gemcitabine-induced CXCR4 expression is ROS-dependent, which is reversed by pretreatment of cancer with the free radical scavenger NAC. If this is true, then gemcitabine-induced ROS actually seems to promote a drug resistance mechanism mediated through CXCR4 up-regulation. Therefore, our data suggest that the role of ROS in the therapeutic efficacy of gemcitabine and clinical outcome needs to be addressed carefully.

It has been suggested that ROS below a threshold level can actually promote cell survival. Indeed, ROS have been linked to cell proliferation and reduced apoptosis through the activation of the ERK1/2 and PI3K/Akt pathways (22). In several other studies, constitutive or induced activation of the ERK and Akt pathways has been associated with chemoresistance of pancreatic cancer cells (33, 34). Accordingly, an inhibition of ERK1/2 or Akt activation has been shown to enhance the sensitivity of pancreatic cancer cells to gemcitabine toxicity (7, 35). In this study, we observed that activation of ERK1/2 or Akt in gemcitabine-treated cells led to enhanced nuclear accumulation of NF-κB and HIF-1α, which then induced CXCR4 expression. Activation of NF-κB transcriptional activity by Akt has been reported previously and has been shown to be essential for oncogenic transformation by PI3K and Akt (12, 36). In a few other studies, ERK1/2 has also been reported to regulate NF-κB (37–39). Therefore, our studies are consistent with these observations and link gemcitabine-induced ROS to CXCR4 expression through activation of the ERK and Akt pathways.

Our data show direct roles of NF-κB/p65 and HIF-1α in gemcitabine-induced up-regulation of CXCR4 expression in pancreatic cancer cells. Interestingly, pancreatic cancer cells utilized different mechanisms for activation of NF-κB/p65 and HIF-1α. Although nuclear accumulation of NF-κB/p65 resulted from phosphorylation-induced degradation of IκBα according to the canonical mechanism, HIF-1α up-regulation was NF-κB/p65-dependent. This is interesting because in previous studies, stabilization of HIF-1α under oxidative stress has been reported as a dominant hypoxic response (26, 40). Nevertheless, in some reports, a role of NF-κB in transcriptional up-regulation of HIF-1α has also been reported (20, 41, 42). Therefore, regardless of the mechanisms of activation, both NF-κB and HIF-1α mediate up-regulation of CXCR4 in gemcitabine-treated cells. This finding is supported by published data in other cancers, where a role of NF-κB and HIF-1α in CXCR4 expression has been reported (17, 18).

Induction of CXCR4 by gemcitabine holds a dual significance. First, it suggests that this may lead to potentiation of survival pathways downstream of CXCR4 and, thus, empower cancer cells to counter gemcitabine-induced apoptosis, providing additional support for our previous finding (7). Second, it suggests that gemcitabine may potentiate the metastasis of pancreatic cancer cells to other CXCL12-overexpressing chemoprotective niches. Consistent with this notion, we observed a marked increase in the invasion and migration of gemcitabine-treated pancreatic cancer against a CXCL12 gradient. Functional significance of the latter, however, needs to be examined in vivo in animal models and gemcitabine-treated patients. Nonetheless, our study has provided evidence for an unintended and undesired effect of chemotherapy in pancreatic cancer cells. Our findings are significant in planning and formulating effective therapeutic strategies against pancreatic cancer for improved clinical outcome.

REFERENCES

1. Long, J., Zhang, Y., Yu, X., Yang, J., LeBrun, D. G., Chen, C., Yao, Q., and Li, M. (2011) Overcoming drug resistance in pancreatic cancer. Expert. Opin. Ther. Targets 15, 817–826.
2. Siegel, R., Naishadham, D., and Jemal, A. (2013) Cancer statistics, 2013. CA—Cancer J. Clin. 63, 11–30.
3. Bardessy, N., and DePinho, R. A. (2002) Pancreatic cancer biology and genetics. Nat. Rev. Cancer 2, 897–909.
4. Olive, K. P., Iacobetz, M. A., Davidson, C. J., Gopinathan, A., McIntyre, D., Honess, D., Madhu, B., Goldgraben, M. A., Caldwell, M. E., Allard, D., Frese, K. K., Denicola, G., Feig, C., Combs, C., Winter, S. P., Ireland-Zecchini, H., Reichelt, S., Howat, W. J., Chang, A., Dhara, M., Wang, L., Rückert, F., Grützmann, R., Pilsarky, C., Izardjenede, K., Hingorani, S. R., Huang, P., Davies, S. E., Plunkett, W., Egorin, M., Hruban, R. H., Whitebread, N., McGovern, K., Adams, J., Iacobuzio-Donahue, C., Griffiths, J., and Tuveson, D. A. (2009) Inhibition of Hedgehog signaling enhances delivery of chemotherapy to a mouse model of pancreatic cancer. Science 324, 1457–1461.
5. Wang, Z., Li, Y., Ahmad, A., Banerjee, S., Azmi, A. S., Kong, D., and Sarkar, F. H. (2011) Pancreatic cancer: Understanding and overcoming chemoresistance. Nat. Rev. Gastroenterol. Hepatol. 8, 27–33.
6. Warsame, R., and Gromoty, A. (2012) Treatment options for advanced pancreatic cancer. A review. Expert. Rev. Anticancer Ther. 12, 1327–1336.
7. Singh, S., Srivastava, S. K., Bhardwaj, A., Owen, L. B., and Singh, A. P. (2010) CXCL12-CXCR4 signalling axis confers gemcitabine resistance to pancreatic cancer cells. A novel target for therapy. Br. J. Cancer 103, 1671–1679.
8. Koshita, T., Hosotani, R., Miyamoto, Y., Ida, I., Tsuji, S., Nakajima, S., Kagawuchi, M., Kobayashi, H., Doi, R., Hori, T., Fujii, N., and Imamura, M. (2000) Expression of stromal cell-derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer. A possible role for tumor progression. Clin. Cancer Res. 6, 3530–3535.
9. Matsu, Y., Ochi, N., Sawai, H., Yasuda, A., Takahashi, H., Funahashi, H., Takeyama, H., Tong, Z., and Guba, S. (2009) CXCL12/IL-8 and CXCL12/SDF-1α co-operatively promote invasiveness and angiogenesis in pancreatic cancer. Int. J. Cancer. 124, 853–861.
10. Teicher, B. A., and Fricker, S. P. (2010) CXCL12 (SDF-1)/CXCR4 pathway in cancer. Clin. Cancer Res. 16, 2927–2931.
11. Sun, X., Cheng, G., Hao, M., Zheng, J., Zhou, X., Zhang, J., Taichman, R. S., Pienta, K. J., and Wang, J. (2010) CXCL12/CXCR4/CXCR7 chemokine axis and cancer progression. Cancer Metastasis Rev. 29, 709–722.
12. Singh, A. P., Arora, S., Bhardwaj, A., Srivastava, S. K., Kadakia, M. P., Wang, B., Grizzle, W. E., Owen, L. B., and Singh, S. (2012) CXCL12/CXCR4 protein signaling axis induces sonic hedgehog expression in pancreatic cancer cells via extracellular regulated kinase- and Akt kinase-mediated activation of nuclear factor κB. Implications for bidirectional tumor-stromal interactions. J. Biol. Chem. 287, 39115–39124.
13. Bailey, J. M., Swanson, B. J., Hamada, T., Eggers, J. P., Singh, P. K., Caffery, T., Ouellette, M. M., and Hollingsworth, M. A. (2008) Sonic hedgehog promotes desmoplasia in pancreatic cancer. Clin. Cancer Res. 14, 5995–6004.
14. Arora, S., Bhardwaj, A., Srivastava, S. K., Singh, S., McClellan, S., Wang, B., and Singh, A. P. (2011) Honokiol arrests cell cycle, induces apoptosis, and...
potentiates the cytotoxic effect of gemcitabine in human pancreatic cancer cells. *PloS ONE* **6**, e25173
15. Donadelli, M., Costanzo, C., Beghelli, S., Scupoli, M. T., Dandrea, M., Bonora, A., Piccinni, P., Budillon, A., Caraglia, M., Scarpa, A., and Palmieri, M. (2007) Synergistic inhibition of pancreatic adenocarcinoma cell growth by trichostatin A and gemcitabine. *Biochim. Biophys. Acta* **1773**, 1095–1106
16. Trachootham, D., Lu, W., Ogasawara, M. A., Niša, R. D., and Huang, P. (2008) Redox regulation of cell survival. *Antioxid. Redox. Signal.* **10**, 1343–1374
17. Oh, Y. S., Kim, H. Y., Song, I. C., Yun, H. J., Jo, D. Y., Kim, S., and Lee, H. J. (2012) Hypoxia induces CXCR4 expression and biological activity in gastric cancer cells through activation of hypoxia-inducible factor-1α. *OncoL. Rep.* **28**, 2239–2246
18. Sung, B., Jhurani, S., Ahn, K. S., Mastuo, Y., Yi, T., Guha, S., Liu, M., and Aggarwal, B. B. (2008) Zerumbone down-regulates chemokine receptor CXCR4 expression leading to inhibition of CXCL12-induced invasion of breast and pancreatic tumor cells. *Cancer Res.* **68**, 8938–8944
19. Park, K. R., Nam, D., Yun, H. M., Lee, S. G., Jang, H. J., Sethi, G., Cho, S. K., and Ahn, K. S. (2011) β-Caryophyllene oxide inhibits growth and induces apoptosis through the suppression of PI3K/akt/mTOR/S6K1 pathways and ROS-mediated MAPKs activation. *Cancer Lett.* **312**, 178–188
20. van Uden, P., Kenneth, N. S., and Rocha, S. (2008) Regulation of hypoxia-inducible factor-1α by NF-κB. *Biochem. J.* **417**, 472–484
21. Xiao, H., Wang, J., Yuan, L., Xiao, C., Wang, Y., and Liu, X. (2013) Chioric acid induces apoptosis in 3T3-L1 preadipocytes through ROS-mediated PI3K/Akt and MAPK signaling pathways. *J. Agric. Food Chem.* **61**, 1509–1520
22. Freuauf, J. P., and Meyskens, F. L., Jr. (2007) Reactive oxygen species. A breath of life or death? *Clin. Cancer Res.* **13**, 789–794
23. Armstrong, L., Hughes, O., Yung, S., Hyslop, L., Stewart, R., Wappler, I., Peters, H., Walter, T., Stojkovic, P., Evans, J., Stojkovic, M., and Lako, M. (2006) The role of PI3K/akt, MAPK/ERK and NFκB signalling in the maintenance of human embryonic stem cell pluripotency and viability highlighted by transcriptional profiling and functional analysis. *Hum. Mol. Genet.* **15**, 1894–1913
24. Du, J., Xu, R., Hu, Z., Tian, Y., Zhu, Y., Gu, L., and Zhou, L. (2011) PI3K and ERK-induced Rac1 activation mediates hypoxia-induced HIF-αalpha expression in MCF-7 breast cancer cells. *PloS ONE* **6**, e25213
25. Calvaní, M., Comito, G., Giannoni, E., and Chiarugi, P. (2012) Time-dependent stabilization of hypoxia inducible factor-1α by different intracellular sources of reactive oxygen species. *PloS ONE* **7**, e38388
26. Jung, S. N., Yang, W. K., Kim, J., Kim, H. S., Kim, E. J., Yun, H., Park, H., Kim, S. S., Choe, W., Kang, L., and Ha, J. (2008) Reactive oxygen species stabilize hypoxia-inducible factor-1α protein and stimulate transcriptional activity via AMP-activated protein kinase in DU145 human prostate cancer cells. *Carcinogenesis* **29**, 713–721
27. Maréchal, R., Demetter, P., Nagy, N., Berton, A., Decaestecker, C., Polus, M., Closset, J., Devière, J., Salmon, I., and Van Laethem, J. L. (2009) High expression of CXCR4 may predict poor survival in resected pancreatic adenocarcinoma. *Br. J. Cancer* **100**, 1444–1451
28. Thomas, R. M., Kim, J., Revelo-Penafiel, M. P., Angel, R., Dawson, D. W., and Lowy, A. M. (2008) The chemokine receptor CXCR4 is expressed in pancreatic intraepithelial neoplasia. *Gut* **57**, 1555–1560
29. Marchesi, F., Monti, P., Leone, B. E., Zerbi, A., Vecchi, A., Piemonti, L., Mantovani, A., and Allavena, P. (2004) Increased survival, proliferation, and migration in metastatic human pancreatic tumor cells expressing functional CXCR4. *Cancer Res.* **64**, 8420–8427
30. Hermann, P. C., Huber, S. L., Herrler, T., Aicher, A., Ellwart, J. W., Guba, M., Bruns, C. J., and Heeschen, C. (2007) Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell* **1**, 313–323
31. Voutsadakis, I. A. (2011) Molecular predictors of gemcitabine response in pancreatic cancer. *World J. Gastrointest. Oncol.* **3**, 153–164
32. Wong, A., Soo, R. A., Yong, W. P., and Innocenti, F. (2009) Clinical pharmacology and pharmacogenetics of gemcitabine. *Drug Metab. Rev.* **41**, 77–88
33. Yokoi, K., and Fidler, I. J. (2004) Hypoxia increases resistance of human pancreatic cancer cells to apoptosis induced by gemcitabine. *Clin. Cancer Res.* **10**, 2299–2306
34. Zhao, Y., Shen, S., Guo, J., Chen, H., Greenblatt, D. Y., Kleeff, J., Liao, Q., Chen, G., Friess, H., and Leung, P. S. (2006) Mitogen-activated protein kinases and chemoresistance in pancreatic cancer cells. *J. Surg. Res.* **136**, 325–335
35. Fryer, R. A., Barlett, B., Galustian, C., and Dalgleish, A. G. (2011) Mechanisms underlying gemcitabine resistance in pancreatic cancer and sensitisation by the iMiD lenalidomide. *Anticancer Res.* **31**, 3574–3576
36. Madrid, L. V., Wang, C. Y., Guttridge, D. C., Schottelius, A. J., Baldwin, A. S. Jr., and Mayo, M. W. (2000) Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-κB. *Mol. Cell. Biol.* **20**, 1626–1638
37. Jiang, B., Xu, S., Hou, X., Pimentel, D. R., Brecher, P., and Cohen, R. A. (2004) Temporal control of NF-κB activation by ERK differentially regulates interleukin-1β-induced gene expression. *J. Biol. Chem.* **279**, 1323–1329
38. Wei, Y. Y., Chen, Y. J., Hsiao, Y. C., Huang, Y. C., Lai, T. H., and Tang, C. H. (2008) Osteoblasts-derived TGF-β1 enhance motility and integrin up-regulation through Akt, ERK, and NF-κB-dependent pathway in human breast cancer cells. *Mol. Carcinog.* **47**, 526–537
39. Wu, C. J., Wang, Y. H., Lin, C. J., Chen, H. H., and Chen, Y. J. (2011) Tetrandrine down-regulates ERK/NF-κB signaling and inhibits activation of mesangial cells. *Toxicol. In Vitro* **25**, 1834–1840
40. Chua, Y. L., Dufour, E., Dassa, E. P., Rustin, P., Jacobs, H. T., Taylor, C. T., and Hagen, T. (2010) Stabilization of hypoxia-inducible factor-1α protein in hypoxia occurs independently of mitochondrial reactive oxygen species production. *J. Biol. Chem.* **285**, 31277–31284
41. Bonello, S., Zähringer, C., BelAlba, R. S., Djordjevic, T., Hess, J., Michiels, C., Kietzmann, T., and Görlach, A. (2007) Reactive oxygen species activate the HIF-1α promoter via a functional NFκB site. *Arterioscler. Thromb. Vasc. Biol.* **27**, 755–761
42. Sun, H. L., Liu, Y. N., Huang, Y. T., Pan, S. L., Huang, D. Y., Guh, J. H., Lee, F. Y., Xu, S., and Teng, C. M. (2007) YC-1 inhibits HIF-1α expression in prostate cancer cells. Contribution of Akt/NFκB signaling to HIF-1α accumulation during hypoxia. *Oncogene.* **26**, 3941–3951