CXCL1/GROα increases cell migration and invasion of prostate cancer by decreasing fibulin-1 expression through NF-κB/HDAC1 epigenetic regulation

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Inflammatory microenvironments play pivotal roles in the development of cancer. Inflammatory cytokines such as CXCL1/GROα exert cancer-promoting activities by increasing tumor angiogenesis. However, whether CXCL1/GROα also plays a role in the progression of prostate cancer, particularly in highly invasive castration-resistant prostate cancer (CRPC), has not been investigated. We explored whether CXCL1/GROα enhances cell migration and invasion in PC-3 and DU145 CRPC. Induction of PC-3 and DU145 cancer progression by CXCL1/GROα is associated with increased AKT activation and IκB kinase α (IKKα) phosphorylation, resulting in nuclear factor-kappaB (NF-κB) activation. Activated NF-κB interacts with histone deacetylase 1 (HDAC1) to form a gene-silencing complex, which represses the expression of fibulin-1D by decreasing the acetylation of histone H3 and H4 on the NF-κB-binding site of the fibulin-1D promoter. Blockade of AKT2 by small hairpin RNA (shRNA) decreases IKKα phosphorylation, NF-κB nuclear translocation and cell migration, indicating that AKT is required in CXCL1/GROα-mediated NF-κB activation and cell migration. In addition, NF-κB and HDAC1 shRNA decrease the effect of CXCL1/GROα on fibulin-1D downregulation, migration and invasion, suggesting that the NF-κB/HDAC1 complex is also involved in CXCL1/GROα-mediated cancer progression. Our findings provide the first evidence that CXCL1/GROα decreases fibulin-1D expression in prostate cancer cells and also reveals novel insights into the mechanism by which CXCL1/GROα regulates NF-κB activation through the AKT pathway. Our results also clearly establish that co-operation of NF-κB and HDAC1 regulates fibulin-1D expression by epigenetic modification. Our study suggests that inhibition of CXCL1/GROα-mediated AKT/NF-κB signaling may be an attractive therapeutic target for CRPC.

Introduction

The incidence of prostate cancer is steadily increasing and is one of the most commonly diagnosed lethal cancers for men worldwide. Androgen-dependent prostate cancer (ADPC) is treatable by hormone therapy and surgery. Unfortunately, most androgen-dependent prostate cancer progresses to an androgen-independent condition (castration-resistant prostate cancer or CRPC) after androgen ablation therapy (1). Patients with CRPC often have a very poor prognosis, due to its high degree of resistance to current chemotherapeutic approaches (2). Therefore, novel treatment strategies are needed to reduce the mortality rate of CRPC.

Matrix invasion is a crucial requirement for cancer metastasis and is regarded largely as a mechanical process dependent on the expression of adhesion molecules and matrix degrading enzymes (3). The architecture and composition of the extracellular matrix (ECM) of the migrating cells determines the degree of resistance the moving cells encounter, which in turn influences the metastatic strategy and efficiency of the invasive cancer cells (4). Moreover, the development of migration and invasive abilities in cancers is associated with the modification of various matrix structures. Fibulins, encoded by FBLN genes, are ECM proteins, which regulate cell apoptosis, adhesion, motility and invasion (5,6). Fibulin-1 has been found to interact with ECM components such as basement membranes and connective tissue matrix fibers (5,7). Furthermore, fibulin-1 may regulate ECM formation and stabilization, which is implicated in processes such as cancer growth, cell migration and invasion (8). Degradation of the fibulin-1 protein modifies its ability to regulate locoregional growth and invasion of ECM barriers by cancer cells (9), whereas overexpression of fibulin-1 or addition of recombinant fibulin-1 protein has been found to decrease cell adhesion, spread, motility and invasiveness of various human cancer cells (5,9). The expression of fibulin-1 is significantly downregulated in prostate cancer (10). Nuclear factor-kappaB (NF-κB) transcription factor has been reported to play important roles in a variety of cellular biological functions, including proliferation, cell differentiation, apoptosis and motility. The activation of NF-κB is a consequence of the inflammatory response or the formation of an inflammatory microenvironment during androgen-independent progression of CRPC (11). NF-κB activation is increased in most androgen-independent prostate cancer cell lines, whereas normal prostate cells or androgen-positive prostate cancers have little or no activated NF-κB (12,13). Constitutive NF-κB activation may replace androgen receptors to ensure cancer progression in an androgen-deprived environment and, consequently, the inhibition of NF-κB may be an attractive target for the treatment of CRPC (14–16). Furthermore, inhibition of NF-κB by genetic knockdown or chemical reagents decreases cancer cell proliferation, invasion and metastasis, and ameliorates the chemotherapy resistance of cancer to anticancer therapies (17,18). Therefore, we hypothesized that NF-κB may play a role in the inflammatory microenvironment-mediated progression of CRPC.

Growing evidence has demonstrated that an inflammatory tumor microenvironment plays a pivotal role in the development of cancer, including tumorigenesis, growth and metastasis. The chemokines, a multifunctional family of small cytokine-like proteins, selectively control the recruitment and migration of lymphocytes to infection or injury sites by chemo-attraction (19). Recent evidence shows that chemokines also play a critical role in neoplastic transformation, cancer progression and angiogenesis, in addition to their role in development and inflammatory responses (20). CXCL1/GROα, a proangiogenic CXC-type chemokine, is present in many cancer types, including breast, lung, pancreatic, colorectal and prostate cancers (21). CXCL1/GROα transactivates epidermal growth factor receptor by proteolytic cleavage of heparin-binding epidermal growth factor-like growth factor, leading to the activation of mitogen-activated protein kinase signaling, resulting in cancer cell proliferation (22). The aim of this study was to determine whether CXCL1/GROα secreted by CRPC induces tumor progression via an autocrine effect and whether matrix protein fibulin-1 is involved in these biological events. Small hairpin RNA (shRNA)-based genetic knockdown was used to investigate the potential role of CXCL1/GROα and its regulatory signaling cascade in prostate cancer progression.
Materials and methods

Cell culture and treatment
Human prostate cancer cells PC-3 and DU145 were obtained from the American Type Culture Collection (Rockville, MD). Both cancer cell lines were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum.

Cell proliferation assay
Cells were treated with 0, 0.01, 0.1, and 10 nM CXCL1/GROα (R&D System Europe, Abingdon, UK) in Dulbecco’s modified Eagle medium containing 1% fetal bovine serum for 72 h. At the end of the assay period, cell proliferation was measured using a premixed water-soluble tetrazolium-1-cell proliferation reagent (Clontech Laboratories, Mountain View, CA). The amount of color produced is directly proportionate to the number of metabolically active cells.

Cell migration and invasion assay
Cell migration was assessed by a scratch wound-healing assay. Cells were allowed to grow into full confluence in 24-well plates. The following day, a uniform scratch was made down the center of the well using a micropipette tip, followed by washing once with phosphate-buffered saline. CXCL1/GROα was added to the respective wells for 24 h. Photographic imaging was performed using a Nikon inverted microscope.

Cell migration and invasion were also assessed by a QCM™ 24-well cell migration assay and Matrigel-coated invasion system (Millipore, Bedford, MA) according to the manufacturer’s instructions.

Immunoblot/Immunoprecipitation
Cells were treated with 10 nM CXCL1/GROα for the indicated times. The cells were then collected and lysed on ice for 15 min in a solution containing 50 mM Tris, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 2 mM Na3VO4, 2 mM ethylene glycol-bis(aminomethyl)-ether)-tetraacetic acid, 12 mM β-glycerophosphate, 10 mM NaF, 16 μg/ml benzamidine hydrochloride, 10 μg/ml phenanthroline, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 1 mM phenylmethylsulfonyl fluoride. The cell lysates were centrifuged at 14,000 g for 15 min, and the supernatant fraction was collected for immunoblot analysis. Equivalent amounts of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10–12%) and transferred to polyvinylidene difluoride membranes. After blocking for 1 h in 5% non-fat dry milk in Tris-buffered saline, the membranes were incubated with the desired primary antibody for 1–16 h. The membranes were then treated with the appropriate peroxidase-conjugated secondary antibody, and the immunoreactive proteins were detected using an enhanced chemiluminescence kit (Millipore, Bedford, MA) according to the manufacturer’s instructions. The primary antibodies used in this study targeted unphosphorylated and phosphorylated AKT, NF-κB, β-actin, vimentin, N-cadherin (GSK3β, vimentin, N-cadherin), fibronectin, fibulin-1, claudin-3, fibronectin and histone deacetylase 1 (HDAC1) antibody, fibulin-1C transfection, the 5′-GGGACTTTCC-3′ immobilized on a 96-well plate. The p65 subunit of NF-κB was determined in a colorimetric reaction using a specific primary antibody and a secondary horseradish peroxidase-conjugated antibody. Spectrophotometric data were expressed as a ratio of absorbance of each experimental condition, compared with control cells exposed to vehicle alone.

Gene knockdown and overexpression
Knockdown of CXCL1/GROα, AKT2, NF-κB (p65) and HDAC1 in the PC-3 and DU145 cell lines was performed using a lentiviral expression system provided by the National RNAi Core Facility (Taipei, Taiwan). The lentiviruses were produced by co-transfecting HEK293T with pLKO-AS2, pLKO-AS2-CXCL1, pLKO-AS2-REL, and pLKO-AS2-HDAC1 shRNA and two packaging plasmids (pCMVDR8.91 and pMD.G); stable clones were established by using puromycin. The efficacy of all shRNA plasmids was assessed by qRT-PCR. Fibulin-1D-transfected PC-3 and DU145 cells were transfected with pCMV or pFibulin-1D plasmid (Origene, Rockville, MD), and stable clones were established by using G418.

Measurement of secreted factors
Supernatants from the PC-3 and DU145 cells were collected, CXCL1/GROα, CXCL5/ENA78 and interleukin-8 levels were quantified using a DuoSet ELISA Development System (R&D Systems) according to the manufacturer’s instructions.

Chromatin IP and Re-chromatin IP
Chromatin IP (ChIP) was performed using an agarose ChIP kit (Pierce, Rockford, IL). Three micrograms of antibody were used to capture the target protein–DNA complex. Immunoprecipitated DNA was analyzed by PCR (Light Cycler, using SYBR Green dye as described qRT-PCR section). The primers were obtained from Qiagen (GPH1009036–05A) (Valencia, CA). Re-ChIP analysis was carried out to assess the simultaneous binding of NF-κB (p50 and p65), and HDAC1 to fibulin-1 promoters. Protein–DNA complexes generated in the first round of chromatin precipitation were washed, incubated with 10 mMol dithiothreitol for 30 min at 37°C and then subjected to a second round of IP and PCR.

Statistical analysis
Data were expressed as means ± standard errors. Statistical comparisons of the results were made using analysis of variance. Significant differences (P < 0.05) between the means of the test groups were analyzed using Dunnett’s test.

Results

CXCL1/GROα increased the migration, invasion and epithelial-to-mesenchymal transition of hormone-independent prostate cancer PC-3 and DU145 cells
We first determined the effect of CXCL1/GROα on the proliferation of hormone-independent prostate cancer PC-3 and DU145 cells. As shown in Figure 1A, after 72h of treatment of the two cancer cell lines, cell proliferation was slightly increased by CXCL1/GROα treatment at a concentration 0.1–10 nM, but not at lower concentrations less than 0.01 nM. Because CXCL1/GROα is a chemokine, which induces chemotaxis in nearby responsive cells [19], we assessed the effect of CXCL1/GROα on the migration and invasion of PC-3 and DU145 cells. As shown in Figure 1B, cancer cells treated with CXCL1/GROα rapidly closed the scratch wounds compared with the control cells. A transwell system also showed that CXCL1/GROα increased the migration and invasion abilities of PC-3 and DU145 cells in a concentration-dependent manner (Figure 1B and 1C). In addition, CXCL1/GROα also caused PC-3 and DU145 cells to undergo epithelial-to-mesenchymal transition (EMT), including the downregulation of epithelial markers (E-cadherin and claudin-3) and upregulation of fibroblast markers (N-cadherin, vimentin, fibronectin and smooth muscle actin (24)) (Figure 1D).

NF-κB DNA-binding assay
NF-κB activity was determined by a Trans-AM enzyme-linked immunosorbent assay (ELISA) kit, which was used according to the manufacturer’s specifications (Active Motif, Carlsbad, CA). Briefly, the transcription factors of nuclear extracts, as prepared by a nuclear extract kit (Active Motif, Carlsbad, CA), were captured by binding to a consensus oligonucleotide (5′-GGGACTTTCC-3′) immobilized on a 96-well plate. The p65 subunit of NF-κB was determined in a colorimetric reaction using a specific primary antibody and a secondary horseradish peroxidase-conjugated antibody. Spectrophotometric data were expressed as a ratio of absorbance of each experimental condition, compared with control cells exposed to vehicle alone.

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**Fig. 1.** The effect of CXCL1/GROα on cell proliferation, migration and EMT in PC-3 and DU145 prostate cancer cell lines. (A) The effect of CXCL1/GROα on cell proliferation. CXCL1/GROα increased cell migration (B), invasion (C) and EMT (D) in PC-3 and DU145 cells. Cell proliferation of CXCL1/GROα was assessed by water-soluble tetrazolium-1 after 72 h of treatment. Results are expressed as the percentage of cell proliferation relative to the proliferation of the control. Cell migration was assessed by wound-healing assay. Quantitative analysis of cell migration and invasion was assessed by QCM™ 24-well cell migration and invasion assay kits after 24 (for migration) and 48 h (for invasion) of chemo-atraction. EMT-related proteins were assessed after 24 h of treatment. The asterisk indicates a significant difference between the control and test groups, as analyzed by Dunnett’s test (*$P < 0.05$). All experiments were performed independently at least three times.
Blockade of CXCL1/GROα decreased PC-3 and DU145 migration

We assessed the role of CXCL1/GROα on the migration of prostate cancer cells by using CXCL1/GROα-specific receptor (CXCR1 and CXCR2) neutralization antibodies. As shown in Supplementary Figure 1A, available at Carcinogenesis Online, the chemotaxis of CXCL1/GROα in both PC-3 and DU145 cells was partially neutralized by treatment with anti-CXCR1 or -CXCR2 antibodies, how it was completely prevented by treatment with anti-CXCR1 plus anti-CXCR2 antibodies (Supplementary Figure 1A, available at Carcinogenesis Online). This suggests that the migration of PC-3 and DU145 cells induced by CXCL1/GROα is dependent on both CXCR1 and CXCR2 receptors.

Next, we assessed the role of endogenous CXCL1/GROα by using CXCL1/GROα shRNA. We selected clones 3 and 7 for PC-3, and clones 1 and 9 for DU145, which exhibited 60–80% inhibition of CXCL1/GROα expression as measured by qRT-PCR and ELISA (Supplementary Figures 1B and 2A, available at Carcinogenesis Online). CXCL1/GROα shRNA did not affect the expression of other chemokines, including CXCL5/ENA78 and interleukin-8 (Supplementary Figure 1C and 1D, available at Carcinogenesis Online). As shown in Figure 2B, knockdown of CXCL1/GROα decreased the migration ability of all CXCL1/GROα shRNA-transfected clones in both cancer cell lines, in contrast with control shRNA-transfected clones.

CXCL1/GROα activated the AKT signaling pathway

CXCR2 is the major receptor for CXCL1/GROα and AKT involved in CXCR2 signaling (25). Therefore, we assessed the effect of CXCL1/GROα on AKT signaling. The results showed that exposure of PC-3 and DU145 cells to CXCL1/GROα resulted in the phosphorylation of AKT and a downstream target, GSKβ, in a time- and concentration-dependent manner (Figure 3A). However, the expressions of AKT and GSK (unphosphorylated form) were unaltered by CXCL1/GROα treatment in both PC-3 and DU145 cells.

We also assessed whether an increase in CXCL1/GROα on the activation of AKT in PC-3 cells was dependent on CXCR1/2. The results showed that CXCR1 plus CXCR2 antibodies completely blocked the phosphorylation of AKT, whereas either CXCR1 or CXCR2 only partially decreased CXCL1/GROα-mediated AKT activation in PC-3 cells (Figure 3B). This suggests that the activation of AKT by CXCL1/GROα occurs in a CXCR1- and CXCR2-dependent manner.

DNA transfection. CXCL1/GROα also increased cell migration and invasion, further supporting that CXCL1/GROα is involved in the regulation of fibulin-D levels (Figure 4F and 4G). In contrast, overexpression of fibulin-1C did not affect cell migration or invasion, and it did not prevent CXCL1/GROα-mediated cell migration and invasion in either PC-3 or DU145 cells (Supplementary Figure 4A to E, available at Carcinogenesis Online)

The NF-κB/HDAC1 complex regulated fibulin-1 expression

The TFsearch computational tool was used to search for transcription factor binding sites in fibulin-1 promoter. Four NF-κB binding sites were found at -5000 to +200 from the transcription start site in the fibulin-1 promoter, suggesting that NF-κB may regulate the expression of fibulin-1. To determine whether NF-κB is involved in CXCL1/GROα-mediated fibulin-1 downregulation, we assessed the effect of CXCL1/GROα on NF-κB activation. As shown in Figure 5A, CXCL1/GROα
caused translocation of NF-κB from the cytosol into the nuclei of both PC-3 and DU145 cells, as determined by subcellular fractionation and immunoblot. The phosphorylation of IKK and IκB also increased in CXCL1/GROα-treated PC-3 and DU145 cells (Figure 5A). In contrast, the level of IκB was reduced after CXCL1/GROα treatment (Figure 5A). CXCL1/GROα also increased the DNA-binding activity of NF-κB in both PC-3 and DU145 cells (Figure 5B). Moreover, ChIP assay also verified that NF-κB bound to the promoter of fibulin-1 after CXCL1/GROα treatment (Figure 5C).

NF-κB has been reported to be associated with HDAC1, decreasing the expression of anti-inflammatory factors (27). To test whether NF-κB recruits HDAC1, we first assessed the interaction of NF-κB (p65) and HDAC1 by IP. As shown in Figure 5D, CXCL1/GROα increased the association of NF-κB (p65) with HDAC1. The acetylation of histone H3 and H4 on the NF-κB-binding region of fibulin-1 promoter decreased after CXCL1/GROα treatment (Figure 5E). Moreover, re-ChIP data further supported the simultaneous binding of NF-xB (p65 and p50), and HDAC1 to fibulin-1 promoters (Figure 5F). This suggests that NF-xB/p65/p50/HDAC1 co-operation is involved in the regulation of fibulin-1 transcription.

The role of NF-xB and HDAC1 on CXCL1/GROα-mediated fibulin-1 downregulation and cell migration in PC-3 and DU145 cells

We then assessed whether CXCL1/GROα decreases fibulin-1 expression in an NF-xB- and HDAC1-dependent manner. As shown in Figure 6A, Supplementary Figure 5A and 5B, available at Carcinogenesis Online, in comparison with control shRNA, NF-xB (p65) and HDAC1 shRNA reduced NF-xB and HDAC1 expressions by approximately by 80%. Inhibition of NF-xB (p65) expression decreased CXCL1/GROα-induced fibulin-1 downregulation at messenger RNA and protein levels, cell migration and invasion (Supplementary Figure 5C, available at Carcinogenesis Online; Figure 6B to D). Similarly, knockdown of HDAC1 also decreased CXCL1/GROα-induced fibulin-1 downregulation, together with cell migration, and invasion (Figure 6B to D).
Fig. 4. CXCL1/GROα decreased the expression of fibulin-1. CXCL1/GROα reduced the expression of fibulin-1 at messenger RNA (A) and protein levels (B). (C) CXCL1/GROα reduced the levels of fibulin-1D but not fibulin-1C. Upregulation of fibulin-1D decreased the migration (D) and invasion (E) ability of PC-3 and DU145 cells. Overexpression of fibulin-1 inhibited CXCL1/GROα-mediated cell migration (F) and invasion (G). Cells were treated with CXCL1/GROα (10 nM) for 24 h. The expression of fibulin-1 and its splicing variants was assessed by qRT-PCR. The expression of fibulin-1 was assessed by immunoblot assay. PC-3 and DU145 cells were transfected with pCMV or pFibulin-1D plasmid, and stable clones were established by G418. Cell migration and invasion were assessed by QCM™ 24-well cell migration and invasion assay kits after 24 h for migration and 48 h for invasion of chemo-attraction. All experiments were performed independently at least three times. The asterisk indicates a significant difference between the control and test groups, as analyzed by Dunnett’s test (*P < 0.05).
Fig. 5. CXCL1/GROα increased the activation of NF-κB. (A) CXCL1/GROα activated NF-κB. (B) CXCL1/GROα increased the DNA-binding activity of NF-κB. (C) The binding of NF-κB on fibulin-1 promoter. (D) The interaction of NF-κB and HDAC1. (E) The deacetylation of histone H3 and H4 by HDAC1 on fibulin-1 promoter. (F) Re-ChIP assay revealed simultaneous binding of NF-κB and HDAC1 to the promoters of fibulin-1 genes. Cells were treated with CXCL1/GROα (10 nM) for the indicated times or various concentrations of CXCL1/GROα for 1 h, and the protein levels (50 μg total protein) were assessed by immunoblot assay. The interaction of NF-κB and HDAC1 was assessed by IP, and the binding of NF-κB and HDAC1 on fibulin-1 promoter determined by ChIP and re-ChIP assay. Positive control for ChIP: the binding of RNA polymerase II in glyceraldehyde-3-phosphate dehydrogenase promoter. The control for the histone acetylation assay was the acetylation of H3 and H4 on NF-κB binding regions of the BRMS1 gene. All experiments were performed independently at least three times. The asterisk indicates a significant difference between the control and test groups, as analyzed by Dunnett’s test (*P < 0.05).
Moreover, the acetylation of histone H3 and H4 on the NF-κB-binding region of fibulin-1 promoter was also recovered by HDAC1 shRNA (Figure 6E). This suggests that NF-κB and HDAC1 are involved in prostate cancer progression by decreasing fibulin-1 expression.

Because AKT has been shown to be involved in cell migration due to its activation of NF-κB by activating IκKα (28), we investigated the role of AKT on CXCL1/GROα-induced IκKα phosphorylation...
The fibulins are a family of secreted glycoproteins, which have been demonstrated to modulate EMC structure and mediate certain cell signaling transductions (31,32). Fibulins have also been shown to modulate cell morphology, growth, adhesion and motility (6,33).

CXCL1/GROα-mediated signaling in prostate cancer

Fig. 7. Scheme of the proposed mechanism of CXCL1/GROα on prostate cancer progression. Prostate cancer expresses a high level of CXCL1/GROα, which in turn enhances the migration, invasion and EMT of prostate cancer cells. CXCL1/GROα activates AKT and recruitment of NF-κB and HDAC, which in turn decreases transcription of fibulin-1D, a repressor of cell migration.

Discussion

CXCL1/GROα is overexpressed in CRPC and plays an important role in prostate cancer progression (19,29,30). In this study, we showed that CXCL1/GROα, found in prostate cancer cell lines PC-3 and DU145, increased both cell migration and invasion (Figure 1B and 1C). CXCL1/GROα was found to slightly increase cell proliferation (Figure 1A). In addition, CXCL1/GROα increased EMT in PC-3 and DU145 cells by dramatically decreasing epithelial markers (E-cadherin and claudin-3) and increasing mesenchymal markers (N-cadherin, vimentin, fibronectin and smooth muscle actin) (Figure 1D). CXCL1/GROα treatment induced AKT phosphorylation (Figure 3A), subsequently increasing IKKα phosphorylation, resulting in NF-κB activation (Figure 5A). However, blocking upstream AKT or NF-κB by shRNA transfection effectively reversed CXCL1/GROα-mediated cell migration and invasion (Figure 6C and 6D), suggesting a critical role of AKT/NF-κB in the progression of CRPC.

The fibulins are a family of secreted glycoproteins, which have been demonstrated to modulate EMC structure and mediate certain cell signaling transductions (31,32). Fibulins have also been shown to modulate cell morphology, growth, adhesion and motility (6,33). The FBLN1 gene transcript is processed into several isoforms, termed fibulin-1A–D, by alternative splicing. It has been suggested that fibulin-1 may act as a cancer suppressor, and its expression, particularly that of fibulin-1D, has been found to be reduced in several types of cancer (31,32). Overexpression of fibulin-1D in cancer cells has been shown to decrease anchorage-independent growth and invasive potential, and delays cancer formation in vivo (34). In contrast, the oncogenic role of fibulin-1C has been demonstrated to be involved in estrogen-promoted carcinogenesis in ovarian and breast cancers (23,35).

In this study, we found that treating PC-3 and DU145 cells with CXCL1/GROα dramatically decreased the expression of fibulin-1D, but not fibulin-1C (Figure 4C and Supplementary Figure 2, available at Carcinogenesis Online). Ectopically expressed fibulin-1D reduced the migration and invasion potential of PC-3 and DU145 cells (Figure 4D and 4E), which supports the hypothetical tumor suppressive role of fibulin-1D. In addition, overexpression of fibulin-1D decreased the effects of CXCL1/GROα on cell migration and invasion (Figure 4F and 4G), suggesting that the downregulation of fibulin-1D is an important event in CXCL1/GROα-mediated cell migration and invasion.

NF-κB has been reported to modulate the expressions of several genes whose products are associated with several aspects of tumor development, including cell proliferation, differentiation, migration, invasion, apoptosis and angiogenesis (36,37). Cancer cells in which NF-κB is constitutively active are highly metastatic and inhibition of NF-κB activity in these cells greatly decreases their invasiveness (38,39). Recent studies have also reported that the inflammatory condition of the cancer microenvironment enhances NF-κB-mediated tumor growth and metastasis (40). NF-κB increases the progression of cancer, affecting cell migration, invasion and metastasis by decreasing E-cadherin expression and Snail nuclear translocation (41). Chromatin modifying cofactors play a crucial role in the expression of the NF-κB target gene. NF-κB can directly recruit HDAC1 and HDAC2 to form a genetic silencing complex, which represses the expression of some specific genes (27,42,43). This study is the first to demonstrate that CXCL1/GROα increases the nuclear translocation of NF-κB, which forms a NF-κB/HDAC1 complex (Figure 5A, 5D and 5F) and in turn decreases the expression of fibulin-1D by decreasing...
histone acetylation (Figure 5E). Inhibition of NF-xB or HDAC1 alone by shRNA decreased fibulin-1D downregulation (Figure 6B). Indeed, genetic blockade of HDAC1 also prevents the deacetylation of histone H3 and H4 and downregulation of fibulin-1 induced by CXCL1/GROα (Figure 5E), suggesting that co-operation of NF-xB with HDAC1 is required for fibulin-1D regulation. Moreover, NF-xB shRNA also reduced the effects of CXCL1/GROα on cell migration and invasion (Figure 6C and 6D), suggesting that NF-xB/HDAC1 plays a crucial role in CXCL1/GROα-mediated cell progression.

AKT, serine–threonine kinase, is an important regulatory factor in the control of cell proliferation, survival and migration (44,45). AKT regulates the activity of NF-xB by phosphorylating IκB, which in turn causes phosphorylation and degradation of IκB (28,46). We found that treatment of PC-3 and DU145 cells with CXCL1/GROα resulted in increased phosphorylation of the AKT cascade (AKT and GSK3β) (Figure 3A). Inhibition of AKT with AKT shRNA abrogated IκB phosphorylation (Supplementary Figure 6B, available at Carcinogenesis Online). Also, selective inhibition of AKT decreased the effects of CXCL1/GROα on cell migration, invasion and fibulin-1D downregulation (Supplementary Figure 6B to D, available at Carcinogenesis Online), suggesting that the activation of AKT plays a critical role in CXCL1/GROα-mediated NF-xB activation and cell migration.

Taken together, our findings provide the first evidence that CXCL1/GROα decreases fibulin-1 expression in prostate cancer and also reveals novel insights into the mechanism by which CXCL1/GROα regulates NF-xB activation through the AKT pathway. Our results also clearly establish that co-operation of NF-xB and HDAC1 regulates fibulin-1 expression by epigenetic modification in prostate cancer cells (Figure 7). In light of these findings, inhibition of CXCL1/GROα/AKT/NF-xB signaling is an attractive therapeutic target for hormone-refractory prostate cancer.

Supplementary material
Supplementary Figures 1–6 can be found at http://carcin.oxfordjournals.org/

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