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Megakaryocyte-Derived IL-8 Acts as a Paracrine Factor for Prostate Cancer Aggressiveness through CXCR2 Activation and Antagonistic AR Downregulation

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Abstract

Prostate cancer is the fifth leading cause of cancer-related mortality in men, primarily because of treatment resistance, recurrence, and metastasis. In the present study, we investigated the role of paracrine interleukin-8 (IL-8) in the antagonistic expression of IL-8 and androgen receptor (AR), and the contribution of IL-8 to prostate cancer aggressiveness. In hormone-responsive LNCaP cells that do not express IL-8, recombinant IL-8 treatment significantly increased expressions of IL-8, CXC chemokine receptor 2 (CXCR2), matrix metalloproteinase (MMP)-2/9, Snail, and vimentin. IL-8 treatment significantly decreased AR and E-cadherin expression. IL-8-induced gene expression changes were suppressed by navarixin, a CXCR1/2 inhibitor, and gallein, a Gβγ inhibitor. In PC-3 androgen-refractory prostate cancer cells, IL-8 knockdown reduced expressions of CXCR2, MMP-2/9, Snail, and vimentin, and increased AR and E-cadherin expressions at the mRNA and protein levels. Co-culture with MEG-01 human megakaryocytic cells secreting high levels of IL-8 induced gene expression changes in both LNCaP and PC-3 cells, similar to those induced by IL-8 treatment. The altered gene expressions were accompanied by significant activation of transcription factor Snail in LNCaP and PC-3 cells. Treatment with the CXCR blocker navarixin inhibited the invasion of PC-3 cells but not LNCaP cells. However, invasion induced by MEG-01 was inhibited by navarixin in both LNCaP and PC-3 cells. The collective findings demonstrate that IL-8 enhances CXCR2 expression, which antagonistically regulates AR expression. More importantly, through changes in IL-8/CXCR2-regulated gene expression, IL-8 induces antiandrogen therapy resistance and epithelial-mesenchymal transition in prostate cancer.

Key Words: Prostate cancer, Interleukin-8 (IL-8), CXCR2, Androgen receptor (AR), Megakaryocyte, Microparticle

INTRODUCTION

Prostate cancer (PC) ranks fourth in cancer incidence worldwide (Sung *et al*., 2021). Although most PCs initially respond to androgen deprivation therapy (ADT), they soon develop resistance to treatment and progress to androgen-refractory PC (ARPC) (Kirby *et al*., 2011).

Recurrent and metastatic ARPCs (mARPC) are heterogeneous with respect to driver gene mutations and mechanisms of treatment resistance. Depending on the expression of androgen receptor (AR), mARPC is classified into three phenotypes: AR-null, AR-low, and AR-high (Labrecque *et al*., 2019). mARPC growth and progression involve both AR-dependent and AR-independent mechanisms (Saraon *et al*., 2014): AR-dependent mechanisms include AR overexpression, gene mutations, and changes in AR co-regulators. AR-independent survival and growth involve deregulation of apoptosis due to altered receptor tyrosine kinase signaling, loss of phosphatase and tensin homolog (PTEN) function, and B-cell lymphoma 2 (Bcl-2) overexpression.

AR is a transcription factor that regulates the expression of various genes including interleukin-8 (IL-8), a chemoattractant cytokine (Maynard *et al*., 2020). Because PC often occurs in the periphery of the prostate gland where prostate atrophic inflammation occurs (De Marzo *et al*., 2007; Sfanos *et al*., 2018), there is no doubt that the inflammatory cytokine IL-8 plays a critical role in prostate tumorigenesis and acquisition of resistance to antiandrogen therapy and aggressive

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phenotypes (Araki *et al*., 2007; Maynard *et al*., 2020). However, IL-8 is also highly expressed in many other solid tumors (Xie, 2001). In addition to the transcriptional repressor role of AR in IL-8 expression, the opposite direction of regulation has also been proposed, in which IL-8 transfection in hormone-responsive PC cells induces a decrease in AR levels along with increased motility and resistance to androgen therapy (Araki *et al*., 2007), similar to the reported actions of other inflammatory cytokines (Culig *et al*., 2005; Chang *et al*., 2014). As a secretory cytokine, IL-8 from cancer cells stimulates its high-affinity receptors, CXCR1 and CXCR2, which are members of the CXC chemokine receptor family (Holmes *et al*., 1991; Murphy and Tiffany, 1991). CXCR1 and CXCR2 are widely co-expressed in immune cells, but CXCR2 is more likely to be involved in chronic inflammation, angiogenesis, and inflammation-driven tumorigenesis (Vandercappellen *et al*., 2008; Jamieson *et al*., 2012; Hertzer *et al*., 2013). CXCR2 activation and downstream signaling may be involved in the regulation of IL-8 and AR expression. However, the detailed mechanism(s) remain unclear.

IL-8 is also supplied to cancer cells as a paracrine factor. In addition to inflammatory cells in tumor microenvironment (TME), megakaryocytes/platelets secrete IL-8. Platelets floating in the bloodstream enter tumor tissue through the blood supply network required by the growing tumor, adhere to tumor cells, become activated, and accumulate in the TME. These observations along with the greater abundance and availability of platelets compared to inflammatory cells indicate that platelets potentially have a greater impact on the parenchyma and tumor-dependent stroma (Ratajczak *et al*., 2006). Within the tumor tissue, platelets promote micrometastases (Nierodzik and Karpatkin, 2006) and plasma levels of megakaryocyte-derived microparticles have been proposed as a poor outcome in ARPC patients (Helley *et al*., 2009).

In the present study, we investigated whether autocrine and megakaryocyte-derived paracrine IL-8 induce different effects on the invasive ability of hormone-responsive and hormone-refractory PC cells and whether the CXCR2 activation signal is the master regulator of IL-8 and AR expression levels.

MATERIALS AND METHODS

Cell line and cell culture

LNCaP and PC-3 human PC cell lines were purchased from the Korean Cell Line Bank (Seoul, Korea). The MEG-01 human megakaryocytic cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Life Technologies, Waltham, MA, USA) and 1% penicillin–streptomycin (Life Technologies) at 37°C in a 5% CO_2/95% air humidified incubator.

Measurement of relative levels of cytokines

To determine the relative levels of cytokines produced by PC cells, a membrane-based antibody array was performed using the Proteome Profiler Human XL Cytokine Array Kit (ARY022B; R&D Systems, Minneapolis, MN, USA). Briefly, protein extracts from LNCaP and PC-3 cells were collected using lysis buffer provided in the kit. Cell extracts (200 μg) were added to each membrane array and incubated for 16 h. The array blots were then analyzed using a model LAS-4000 mini luminescent image analyzer (Fuji, Tokyo, Japan). The blot density was quantified using HLImage++ software (Western Vision Software, Bountiful, UT, USA).

Protein extraction and immunoblotting

Total protein from LNCaP and PC-3 cells was extracted using radioimmune precipitation assay cell lysis buffer containing a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA) and quantified using bicinchoninic acid (BCA) reagent (Pierce, Thermo Fisher Scientific). Protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Protran nitrocellulose blotting membranes (Amersham Life Science, Buckinghamshire, UK). Membranes were blocked using 5% bovine serum albumin dissolved in Tris-buffered saline (TBS)-Tween 20 (TBST), followed by incubation with primary antibody for 16 h at 4°C. Membranes were then washed with TBST, incubated with secondary antibody for 1 h, washed again with TBST, and analyzed using the aforementioned LAS-4000 mini device. Antibodies against matrix metalloproteinase (MMP)-2 and MMP-9 were obtained from Invitrogen (Carlsbad, CA, USA). IL-8, AR, E-cadherin, Snail, and vimentin antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). CXCR2 antibody was obtained from Abcam (Cambridge, UK). β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cancer cell invasion assay

For invasion assay, LNCaP and PC-3 cells (5×10^4 cells/cm^2) were seeded into 8.0 μm pore Falcon culture inserts coated with 10% Matrigel (growth factor reduced) for 2 h at 37°C. The cancer cell-loaded insert was mounted onto 24-well companion plates and incubated for 24 h at 37°C. The next day, after the cells in the insert were fixed using methanol, hematoxylin-eosin staining was performed. The stained insert membranes were visualized using an inverted microscope (Nikon, Tokyo, Japan) at 100× magnification. The captured images were used to count the number of invading cells. To measure the effect of co-culture, MEG-01 cells were placed in the bottom wells.

Gene silencing with small interfering RNA (siRNA)

LNCaP and PC-3 cells were transfected with 100 nM siRNA of non-target (NT) or specific to *CXCL8* (Merck, Darmstadt, Germany) using the transfection media Opti-MEM (Gibco, Thermo Fisher Scientific) and DharmaFECT transfection reagent (Dharmacon, Thermo Fisher Scientific). After 72 h of transfection, cells were either used for other treatments or collected for the extraction of total proteins and mRNA.

Determination of MEG-01-derived microparticle migration and viable cancer cell number

For visualization of diffused MEG-01-derived microparticles, MEG-01 cells were stained with 5 μM of the cell-tracking red fluorescent dye CMTPX (Thermo Fisher Scientific). Stained MEG-01 cells were seeded into a 1 μm pore-sized Falcon culture insert. Each insert was mounted on 24-well companion plates seeded with LNCaP or PC-3 cells. After 24 h of incubation, migrating microparticles of MEG-01 toward cancer cells in the well were visualized using fluorescence microscopy at 200× magnification (Olympus, Tokyo, Japan).

To determine the number of cancer cells in the bottom well, cells were trypsinized. The single-cell suspension was mixed with trypan blue (0.4%) and viable cells were counted using a hemocytometer.

Transcription factor (TF) activation profiling array

To simultaneously measure the activities of multiple TFs, a Cancer Stem Cell TF activation profiling plate array (FA-1004; Signosis, Santa Clara, CA, USA) was used. Briefly, biotin-labeled probes containing consensus sequences of TF DNA-binding sites were incubated with nuclear extracts that were prepared by nuclear extraction kit (SK-0001; Signosis) for 30 min at 25°C. The TF/probe complex mixtures were separated by spin column purification. The bound probes were detached from the complex using elution buffer and centrifuged at 9,800 ×*g* for 2 min. After the eluents were denatured at 98°C for 5 min, the denatured sample was added to TF hybridization buffer. The resulting mixture (100 μL) was added to each well of the hybridization plate, and the plate was sealed with aluminum adhesive and incubated at 42°C for 16 h. The captured DNA probe was further detected using a streptavidin-horseradish peroxidase conjugate. Endpoint luminescence readings of the samples were observed using Fluostar omega (BMG Labtech, Ortenberg, Germany).

Cell proliferation assay

LNCaP and PC-3 cells were seeded in 96-well plates at a density of 2.5×10^4 cells/mL in serum-starved conditions (1% FBS). After 24 h, the cell medium was changed to 10% serum-containing medium with various concentrations (0.1, 0.3, 1, 3, 10, 30, and 100 μM) of enzalutamide (Selleckchem, Houston, TX, USA), navarixin (Selleckchem), or MMP-2/9 inhibitor (Sigma-Aldrich, St. Louis, MO, USA). After 48 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye solution (5 mg/mL; Merck, Kenilworth, NJ, USA) was added to the wells and incubated for 4 h at 37°C. The media were then discarded, and 200 μL of dimethyl sulfoxide (DMSO) was added to each well (Duksan, Ansan, Korea) to solubilize formazan crystals. After 30 min of incubation, the absorbance was measured at 540 nm using a microplate reader (BMG Labtech).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total mRNAs was extracted from LNCaP and PC-3 cells using TRIzol reagent (Ambion, Thermo Fisher Scientific). cDNA was synthesized using the GoScript™ Reverse Transcriptase kit (Promega, Madison, WI, USA). qPCR was performed using SYBR Green PCR reagent (QIAGEN, Valencia, CA, USA) with primers against *CXCL8*, *CXCR1*, *CXCR2*, *AR*, *MMP2*, *MMP9*, *VIM*, *CDH1*, *SNAI1*, or *GAPDH* (Bioneer, Daejeon, Korea). The primer sequences used are listed in Table 1.

Enzyme-linked immunosorbent assay (ELISA) for cytokine measurement

Human CXCL1, CXCL5, and IL-8 levels secreted from MEG-01 cells alone or in combination with cancer cells were measured using Quantikine ELISA kits (DGR00B, DX000, and D8000C, respectively; R&D Systems). The cell culture supernatant was used for the assay. The colorimetric endpoint absorbance of the samples after the assay was recorded at 450 nm using a microplate reader (Spectrostar Nano; BMG Labtech).

Table 1. List of primer sequences used for qRT-PCR

Gene		Primer sequences
AR	Forward	5'-GACACCGACACTGCCTTAC-3
	Reverse	5'-TAGGGCTGGGAAGGGTCTAC-3
CDH1	Forward	5'-GTCTCTCTCACCACCTCCACAG-3
	Reverse	5'-CAGACAGAGTGGGGAAAATGTA-3
CXCR1	Forward	5'-CGTTTGTTCCCGACGAGAAG-3'
	Reverse	5'-CAGCGGATGCCATTGTGAT-3'
CXCR2	Forward	5'-CAGCGACCCAGTCAGGATTTA-3
	Reverse	5'-ACCAGCATCACGAGGGAGTTT-3
IL-8	Forward	5'-AGAGTGATTGAGAGTGGACC-3'
	Reverse	5'-ACTTCTCCACAACCCTCTG-3'
MMP2	Forward	5'-ACCGCAAGTGGGGCTTCTGC-3
	Reverse	5'-CGTGGCCAAACTCGTGGGCT-3
MMP9	Forward	5'-TTTGACAGCGACAAGAAGTG-3
	Reverse	5'-CAGGGCGAGGACCATAGAGG-3
SNAIL	Forward	5'-GCCTAGCGAGTGGTTCTTCT-3
	Reverse	5'-TAGGGCTGCTGGAAGGTAAA-3
VIM	Forward	5'-GAAGAGAACTTTGCCGTTGAAG-3
	Reverse	5'-ACGAAGGTGACGAGCCATT-3
GAPDH	Forward	5'-ACCACAGTCCATGCCATCAC-3'
	Reverse	5'-TCCACCACCCTGTTGCTGTA-3'

Statistical analysis

Data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Student-Newman-Keuls comparison (GraphPad Prism 8.0 software, GraphPad Software, San Diego, CA, USA) to calculate differences between groups using GraphPad Prism 8.0. A p-value <0.05 were considered statistically significant.

RESULTS

Autocrine IL-8 upregulates the IL-8-CXCR1/2 axis and downregulates AR

To determine the differential expression of IL-8 between hormone-responsive (LNCaP) and ARPC (PC-3) cells, we performed cytokine array analysis. Compared to LNCaP cells, which mainly expressed DKK-1, PAI-1, and Lcn-2, PC-3 cells expressed more than 20 times higher level of various cytokines (Fig. 1A). Notably, chemokine family members ENA-78 (CXCL5), GRO-α (CXCL1), and IL-8 were highly increased in PC-3 cells, with IL-8 being the most highly expressed (Fig. 1A). Differential expression of IL-8 was confirmed by immunoblotting (Fig. 1B). IL-8 levels were proportional to MMP-2/9 and mesenchymal gene products, Snail and vimentin, but inversely proportional to AR and E-cadherin expression levels (Fig. 1B). To reveal that gene expression patterns are responsible for the aggressive behavior of ARPC, we performed an invasion assay under conditions of IL-8 supply or IL-8 blockage. Treatment of LNCaP cells with recombinant human IL-8 in serum-free conditions stimulated cell invasion, which was similar to the invasion of cells in the presence of IL-8 and 1% serum (Fig. 1C). Treatment of PC-3 cells with anti-IL-8 antibody significantly blocked PC-3 cell invasion (Fig. 1D), indicating that autocrine IL-8 induces ARPC cell invasion.

Despite the differential expression of IL-8, CXCR2 expres-

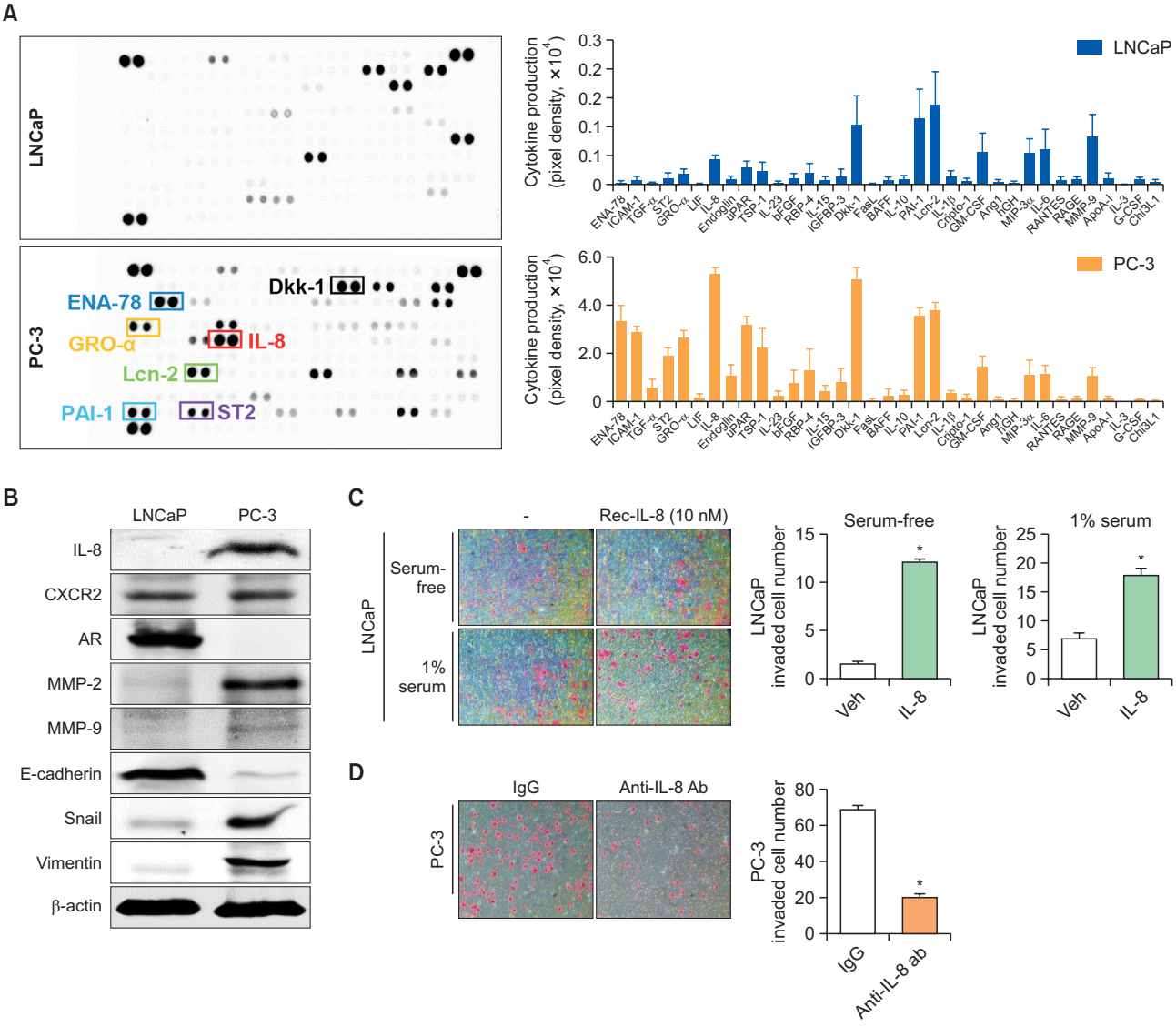

Fig. 1. Differentially expressed IL-8 enhances invasion of both hormone-responsive (LNCaP) and ARPC (PC-3) cells. (A) Cytokine proteome array of LNCaP and PC-3 cells. The blots are representative of three independent experiments. The relative density of the blots was quantified using HLImage++ software. The top 25 significantly upregulated cytokines in each cell line are plotted. (B) Immunoblot showing basal level expression differences of IL-8, CXCR2, and AR between LNCaP and PC-3 cells. (C) Invasion assay of LNCaP cells treated with recombinant IL-8 (10 nM) for 24 h in serum-free or 1% serum-containing media. The bar graphs represent the means ± SEM from three independent experiments. *p<0.05, compared to vehicle-treated group. (D) Invasion assay of PC-3 cells treated with IgG or IL-8-neutralising antibody for 24 h. The bar graphs represent the means ± SEM from three independent experiments. *p<0.05, compared to IgG-treated group.

sion in LNCaP and PC-3 cells was not significantly different (Fig. 1B). We then examined whether the invasive ability of ARPC was solely due to the differential expression of IL-8, rather than receptor levels. Interestingly, LNCaP cells responded to exogenously applied IL-8, with increased IL-8 and CXCR2 expression, which was suppressed by navarixin, a CXCR1/2 inhibitor, and gallein, a Gβγ inhibitor (Fig. 2A). The expression of MMP-2/9 and epithelial-mesenchymal transition (EMT)-related genes also changed in a similar manner. Silencing the highly expressed IL-8 in PC-3 cells significantly reduced the expression of CXCR2, MMP-2/9, Snail, and vimentin, with an increase in E-cadherin at both mRNA levels (Supplementary Fig. 1), and protein levels (Fig. 2B). The decrease in CXCR2 expression after IL-8 knockdown was 84%, which was much greater than the 44% reduction in CXCR1 (Supplementary Fig. 1).

Platelet-derived IL-8 induces proliferation and invasion of PC cells

Next, we examined the paracrine action of IL-8 on ARPC invasion. Cancer cells interact with a variety of cells in the TME. However, platelets may be the main contact cells because they are immediately available and constitutively present in the blood supplying the tumor tissue. Platelets secrete cyto-

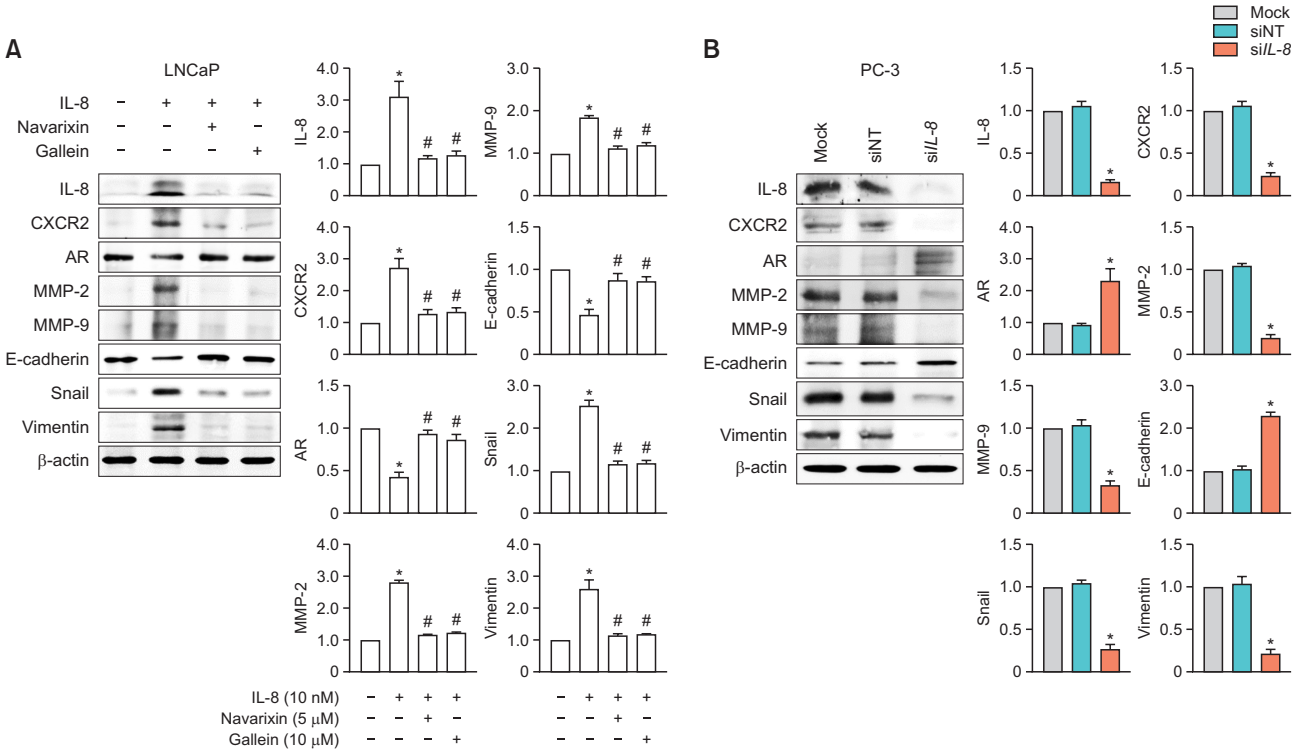

Fig. 2. IL-8-induced CXCR2 activation regulates the expression of IL-8, AR, and EMT genes. (A) Immunoblots of IL-8, CXCR2, AR, and EMT genes in LNCaP cells treated with IL-8 (10 nM) for 24 h in serum-free media in the absence and presence of navarixin (5 μM) or gallein (10 μM). Blot density was quantified as fold change, and bar graphs represent mean ± SEM from three independent experiments. **p*<0.05, compared to vehicle-treated group. #*p*<0.05 compared to IL-8-treated group. (B) Proteins extracts of PC-3 cells transfected with non-target (NT) or IL-8-specific siRNA for 72 h were immunoblotted. The bar graphs represent the means ± SEM from three independent experiments. **p*<0.05, compared to the Mock-treated group.

kines in two ways: in a soluble form from α-granules, and in the form of a microvesicle called platelet microparticles (PMP) (Weyrich *et al*., 2003; Denis *et al*., 2005). In human plasma, PMPs are mainly derived from megakaryocytes. Therefore, the MEG-01 megakaryocytic cell line was used as a model cell line for the source of PMP in this study. To examine the effect of MEG-01-derived cytokines on cancer cells, culture inserts with a 1 μm pore size were used for co-culture with MEG-01. In this co-culture, PMP was observed in cancer cells in the bottom well (Fig. 3A). The level of PMP diffused to the bottom well where LNCaP or PC-3 cells were placed was not different between the two. However, the co-culture significantly induced proliferation of both cancer cell types (Fig. 3B). In the opposite means of co-culture, MEG-01 cells induced invasion of upper chamber cancer cells (Fig. 3C). In addition, the decreased invasion of PC-3 cells induced by IL-8 knockdown was restored to the control level by treatment with MEG-01 (Fig. 3D). In MEG-01 cells, IL-8 secretion was approximately four times higher than the other chemokines, CXCL1 and CXCL5 (Fig. 3E). The secreted IL-8 level in the supernatant of co-culture of LNCaP cells with MEG-01 increased more than 20 times, while the fold change in IL-8 level by co-culture of PC-3 cells with MEG-01 was small, but significant (Fig. 3F).

Co-culture with MEG-01 upregulates IL-8/CXCR2 expression and downregulates AR in PC cells

Co-culture with megakaryocytic MEG-01 cells induced increased expression of IL-8, CXCR2, Snail, and vimentin at the mRNA (Fig. 4A) and protein (Fig. 4B) levels, while MEG-01 co-culture suppressed AR and E-cadherin expression in both LNCaP and PC-3 cells. The changes in gene expression induced by MEG-01 were similar to those induced by exogenous IL-8 stimulation in LNCaP cells. In the TF analysis, MEG-01 significantly activated HIF-1, Nanog, Snail, and Twist-1 in both LNCaP and PC-3 cells, with Snail being the most highly activated (Fig. 4C).

Differential effects of AR and IL-8/CXCR2 on proliferation and invasion of PC

Because platelets induced PC proliferation and invasion, which was accompanied by up- and downregulation of IL-8/CXCR2 and AR, we then investigated which gene was associated with the phenotype. AR-high LNCaP cells were sensitive to enzalutamide, an AR inhibitor, whereas AR-null PC-3 cells were less sensitive. Compared to enzalutamide, the CXCR inhibitor navarixin and MMP-2/9 inhibitor were less effective in inhibiting the proliferation of both LNCaP and PC-3 cells (Fig. 5A). In contrast, enzalutamide failed to inhibit the invasion of both LNCaP and PC-3 cells, regardless of whether the cells were treated with MEG-01 (Fig. 5B). In contrast, in the absence of MEG-01 co-culture, navarixin inhibited the invasion of PC-3 cells, but not LNCaP cells. Moreover, navarixin inhibited the MEG-01-induced invasion of both LNCaP and PC-3 cells (Fig. 5B). These results indicate that IL-8 induced PC

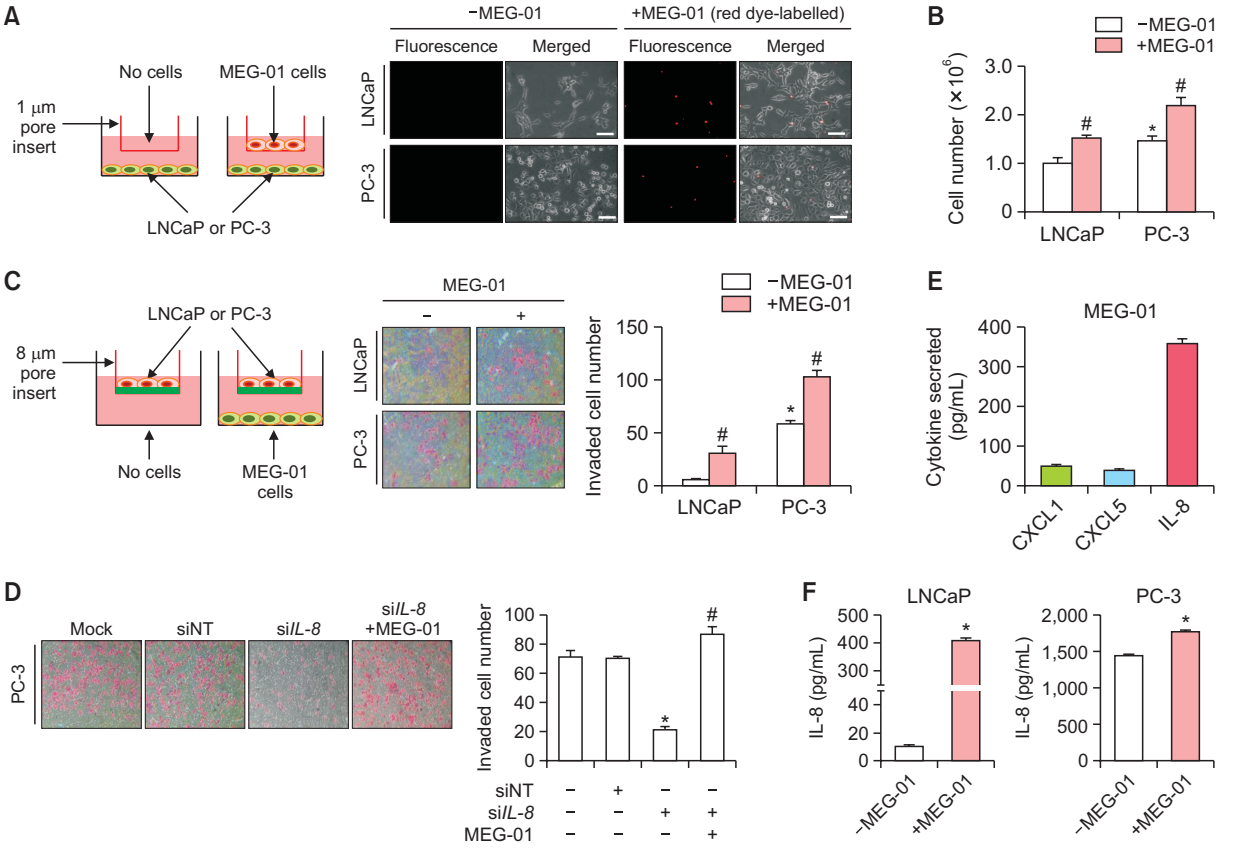

Fig. 3. MEG-01-derived IL-8 promotes proliferation and invasion of prostate cancer cells. (A, B) MEG-01 cells were pre-labeled with CMTPX, a red fluorescent cell tracker prior to their placement in 1 μm pore-sized insert. The insert was mounted on the well with cancer cells (LNCaP or PC-3) and incubated in serum-starved (1%) condition for 24 h. The migrated microparticles of MEG-01 cells were visualized using fluorescence microscopy at 200× magnification (A). The number of cancer cells in the lower well was counted (B). The bar graphs represent the means ± SEM from three independent experiments. **p*<0.05, compared to LNCaP cells without co-culture. #*p*<0.05, compared to MEG-01-untreated group in each cell line. (C) Invasion assay of LNCaP and PC-3 in the absence or presence of MEG-01 cells. Cancer cells were seeded in an 8 μm pore-sized insert and mounted on a well that lacked or contained MEG-01 cells, and incubated for 24 h. The invading cancer cells were stained, photographed, and counted (n=3). **p*<0.05, compared to LNCaP cell without co-culture. #*p*<0.05 compared to MEG-01-untreated group in each cell line. (D) Invasion assay of IL-8-silenced PC-3 cells in the absence and presence of MEG-01 cells for 24 h (n=3). **p*<0.05, compared to siNT. #*p*<0.05, compared to si*IL-8*-treated group. (E, F) CXCL chemokine levels were measured by ELISA (n=3). CXCL chemokines in culture supernatant of MEG-01 cells without cancer cells (E) and with cancer cell co-culture (F). Culture conditions were the same as described in (A). **p*<0.05, compared to MEG-01-untreated group.

proliferation and invasion. However, the signaling molecules mediating each behavior were quite different.

DISCUSSION

The present study demonstrates that IL-8 is the most highly expressed cytokine in ARPC cells, confirming our previous transcriptome analysis of hormone-responsive and hormone-refractory PC cells (Dahal *et al*., 2022). Previously, it was reported that IL-8 is a tumor-intrinsic factor that contributes to PC progression as well as resistance to ADT and immune checkpoint inhibitors (Lopez-Bujanda *et al*., 2021). In the current study, we demonstrate that megakaryocyte-derived IL-8 as an extrinsic factor also contributes to the aggressive behavior of ARPC.

High levels of IL-8 expression are observed in ARPC cells and other cancer cells, including solid tumors and leukemias (Xie, 2001). Clinical studies have shown that high serum IL-8 levels are directly linked to disease progression, and that cancer cell-derived IL-8 facilitates oncogenic signaling, angiogenesis, invasion, and resistance (Long *et al*., 2016). In the present study, we report for the first time the autoregulatory mechanism by which IL-8 upregulates its own expression through CXCR2 signaling. Several TFs are involved in the regulation of IL-8 gene expression. The IL-8 promoter region contains binding sites for activator protein-1 (AP-1), nuclear factor (NF)-IL-6, and NF-kappa B (κB) (Roebuck, 1999). Hypoxia-inducible factor-1 alpha (HIF-1α) modulates IL-8 via the NF-κB pathway (Feng *et al*., 2018). In addition, Snail induces the expression of IL-8 by direct binding to its E3/E4 E-boxes (Hwang *et al*., 2011). In the present study, we observed that Snail, the most highly activated TF in the presence of MEG-01 secreting IL-8, was associated with IL-8 upregulation. These results indicate that the IL-8-induced IL-8 increase in both LNCaP and PC-3 cells may be mediated through Snail.

Lopez-Bujanda *et al*. (2021) demonstrated that AR acts as a transcriptional repressor of the IL-8 gene. However, in the

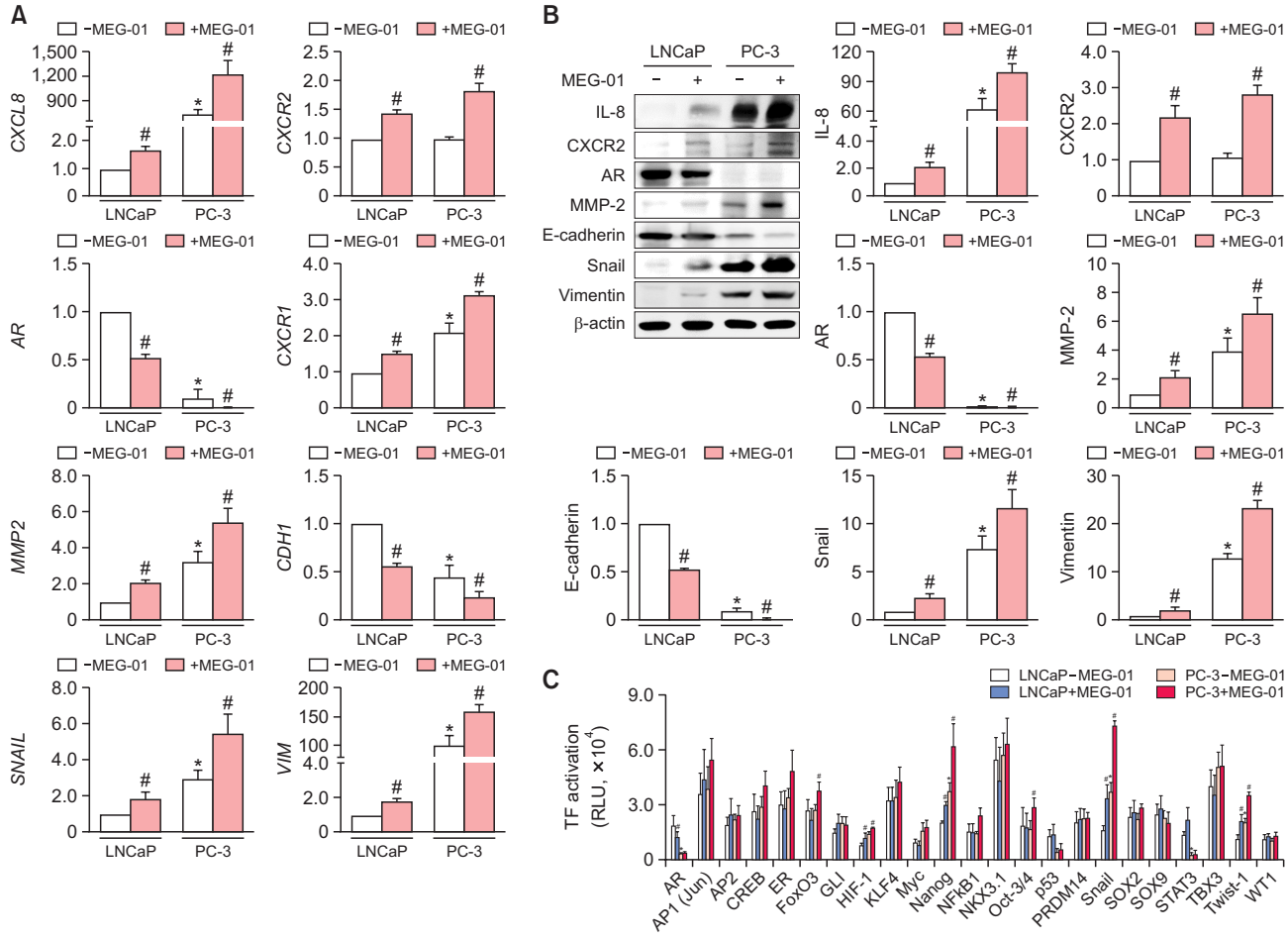

Fig. 4. MEG-01 cell-induced up- and downregulation of genes and transcription factors in prostate cancer cells. Cancer cells were treated with MEG-01 as described in Fig. 3A. (A) mRNA expression was determined by qRT-PCR (n=3). (B) Protein expression was determined by immunoblotting (n=3). **p*<0.05, comparison between LNCaP cells without MEG-01 co-culture. #*p*<0.05, compared to MEG-01-untreated group in each cell line. (C) Activation array of transcription factors of LNCaP and PC-3 cells with or without MEG-01 co-culture for 24 h (n=3). **p*<0.05, compared to LNCaP cells without co-culture. #*p*<0.05, compared to MEG-01-untreated group.

present study autocrine and paracrine IL-8 downregulated AR expression through Gβγ signaling of CXCR2. This result is consistent with previous reports that inflammatory cytokines, including IL-8, decrease AR levels (Culig *et al*., 2002; Araki *et al*., 2007; Chang *et al*., 2014). The collective results suggest that IL-8 and AR expression may be antagonistically regulated via CXCR2 signaling.

Platelets secrete cytokines, including IL-8, in the form of microparticles as well as in a soluble form released from α-granules (Weyrich *et al*., 2003; Denis *et al*., 2005). Although various cells release microparticles, platelet-derived microparticles (PMPs) are the most abundant in the bloodstream, accounting for approximately 70-90% of the microparticles (Horstman and Ahn, 1999; Berckmans *et al*., 2001; Joop *et al*., 2001) The Plasma PMP levels are increased in cancer patients. The levels decrease with cancer treatment, suggesting that PMP is a predictor of cancer aggressiveness and poor clinical outcome (Janowska-Wieczorek *et al*., 2005; Mezouar *et al*., 2014). Apart from the immunomodulatory action in the TME, platelets induce EMT in cancer cells or guide the formation of early metastatic niches (Labelle *et al*., 2014), leading to cancer progression (Germano *et al*., 2008; Turley *et al*., 2015). In the current study, we also showed that co-culture with MEG-01 enhanced the invasion ability of PC cells and even restored the reduced invasion by IL-8 knockdown. In addition, gene expression changes, such as IL-8/CXCR2 upregulation by MEG-01 co-culture, corresponded to that by IL-8 treatment. In conjunction with the much higher level of IL-8 production than that of other chemokines by MEG-01, the current results support the view that megakaryocyte-derived IL-8 acts as a paracrine factor regulating ARPC aggressiveness.

In conclusion, the present study demonstrates that autocrine and paracrine IL-8 upregulate IL-8 and CXCR2 expression, which antagonistically downregulates AR in PC cells. Such antagonistic regulation of IL-8 and AR expression induces ADT resistance and invasiveness in PC cells.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

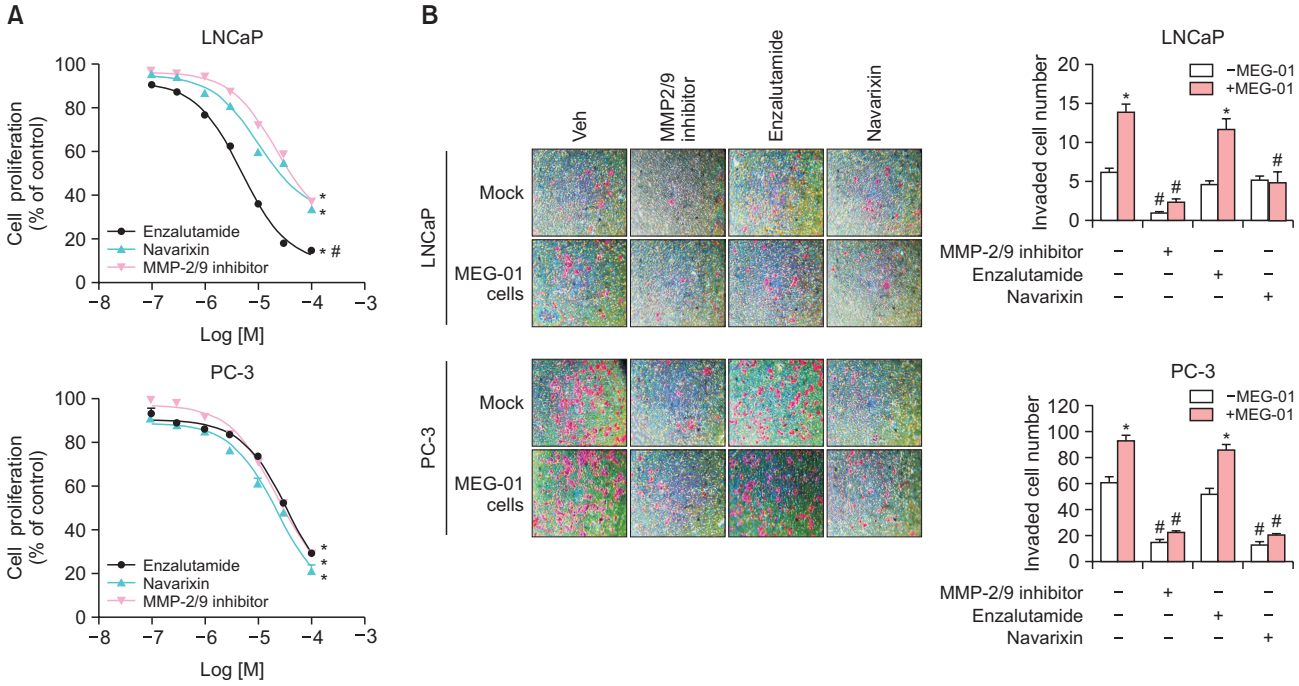

Fig. 5. Effects of inhibitors of MMP-2/9, AR, and CXCR2 on prostate cancer proliferation and MEG-01-induced invasion. (A) Cell proliferation was measured by MTT assay. Each value represents mean ± SEM from three independent experiments with duplicates. **p*<0.05, compared to vehicle-treated group. #*p*<0.05, compared to navarixin- and MMP-2/9 inhibitor-treated group. (B) Invasion assay of LNCaP and PC-3 cells co-cultures with MEG-01 in the absence and presence of inhibitors of MMP-2/9 (5 μM), enzalutamide (5 μM) and navarixin (5 μM) for 24 h (n=3) **p*<0.05, compared to the MOCK-treated group. #*p*<0.05, compared to vehicle-treated group.

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