Residual Prostate Cancer Cells after Docetaxel Therapy Increase the Tumorigenic Potential via Constitutive Signaling of CXCR4, ERK1/2 and c-Myc

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Abstract

Despite an increasing prevalence of patients with docetaxel-refractory prostate cancer, little is known about the tumor biology of the docetaxel-resistant residual tumor cells compared with primary tumor cells. In this study, tumorigenic potential was increased in the docetaxel-resistant residual prostate cancer cell lines (DRD, 1G7 and PC3DR) compared with parental cells (DU145 or PC3). Enhanced tumorigenic potential was conferred by oncogenic c-Myc, which was stabilized by constitutively activated ERK1/2 in DRD, 1G7, and PC3DR cells. Constitutively activated ERK1/2 was maintained by CXCR4, which was upregulated in DRD, 1G7, and PC3DR cells. In docetaxel-treated DU145 cells, transiently activated ERK1/2 induced CXCR4 expression by stabilizing c-Myc. Furthermore, constitutive activation of CXCR4, ERK1/2, and c-Myc signaling was evident in clinical tissue samples from human patients with docetaxel-resistant prostate cancer. In DTX-resistant residual prostate cancer cells, the enhanced tumorigenic potential was reduced by ERK1/2 inhibition, or by AMD3100, a CXCR4 antagonist. Thus, docetaxel treatment constitutively activated the CXCR4, ERK1/2, and c-Myc signaling loop in docetaxel-resistant residual prostate cancer cells.

Implications: Constitutive signaling pathways are viable therapeutic targets for residual prostate tumor cells following acquisition of docetaxel resistance. Mol Cancer Res; 11(9): 1088–100. ©2013 AACR.

Introduction

Prostate cancer is the second leading cause of cancer-related death in males in the United States, and it is a growing problem worldwide (1). Prostate cancer progresses from prostatic intraepithelial neoplasia through locally invasive adenocarcinoma to castration-resistant metastatic carcinoma (2). Although most patients initially respond to androgen ablation, in many cases, castration-resistant metastatic carcinoma (CRPC) develops within a couple of years (3). Chemotherapy is then used to treat patients with CRPC. Among the various chemotherapeutic strategies, docetaxel-based chemotherapy confers survival advantages for patients with CRPC (4, 5). However, the overall survival benefit is only approximately 2 months. Most of the CRPC relapse after docetaxel therapy is due to an increase in docetaxel-resistant residual tumor cells. Although several mechanisms have been proposed to contribute to docetaxel-resistance, such as those mediated by the ABC drug transporter family (6, 7) and clusterin, with its prosurvival functions (8), further studies are needed in this setting because of the increasing prevalence of docetaxel-resistant prostate cancers. Moreover, little is known about the tumor biology of the docetaxel-resistant residual prostate cancer cells compared with primary cancer cells.

Accumulating evidence suggests that residual cancer cells after chemotherapy show a more aggressive phenotype than primary cancer cells (9–14). Furthermore, a recent report showed that residual cancer cells after chemotherapy increased the mammosphere formation efficacy compared with the primary cancer cells in patients with breast cancer (9). Thus, the tumorigenic potential of residual cancer cells may be enhanced by chemotherapeutic agents if the cancer cells were not eradicated by chemotherapy. Therefore, the first aim of this study was to examine whether there was a tumorigenic potential increase in docetaxel-resistant residual prostate cancer cells. If an increase was observed, the second aim was to elucidate the mechanism for maintaining enhanced tumorigenic potential following docetaxel treatment in prostate cancer cells.
It is well known that survival signaling pathways, such as extracellular signal–regulated kinase (ERK1/2), a member of the mitogen-activated protein kinase (MAPK) family, are transiently activated by chemotherapeutic agents, including docetaxel (15–18). It appears that the drug-induced activation of survival pathways could impair the cytotoxic effects of chemotherapeutic drugs. Surprisingly, a recent report showed that the ERK1/2 signaling pathway is constitutively activated in residual breast cancer cells after chemotherapy (14). Among the genes activated by the ERK1/2 signaling pathway, c-Myc plays a major role in prostate tumorigenesis (19, 20). The c-Myc protein level is strictly regulated by ERK1/2 via posttranslational mechanisms (21–25).

Recent reports have also shown that the tumorigenic potential increases in sphere-forming prostate cancer cells (26), and CXCR4, a member of the chemokine receptor family, is upregulated in sphere-forming prostate cancer cells (27, 28). CXCR4 activates survival signaling pathways, including ERK1/2, in the presence of SDF-1, a CXCR4 ligand (29). CXCR4 expression is induced by several cellular stresses, such as hypoxia (30), and c-Myc upregulates CXCR4 promoter activity (31). However, it remains unknown whether the CXCR4 expression is induced by chemotherapy.

Thus, it has been reported that ERK1/2 is upregulated by chemotherapy, and c-Myc and CXCR4 are involved in prostate cancer tumorigenesis. However, it is unclear how proteins such as ERK1/2, c-Myc, and CXCR4 are involved in the increase in the tumorigenic potential of prostate cancer cells following chemotherapy. In this study, we have shown that tumorigenic properties are enhanced in docetaxel-resistant residual prostate cancer cells and reveal the mechanism for the increased tumorigenic potential of docetaxel-resistant prostate cancer cells via the constitutive activation of a CXCR4, ERK1/2, and c-Myc signaling loop.

Materials and Methods
Cell culture and mice

The androgen-independent human prostate cancer cell line DU145 and PC3 were purchased from the American Type Culture Collection. DU145 cells were maintained in RPMI-1640 medium (Nacalai Tesque) and PC3 cells were maintained in Dulbecco’s modified Eagle F12 medium (Nacalai Tesque). All media were supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2. Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice and BALB/c nude mice of ages 5 weeks were purchased from Japan Clea and maintained in a temperature-controlled, pathogen-free room. All animals were handled according to the approved protocols and guidelines of the Animal Committee of Osaka University (Osaka, Japan).

Reagents and antibodies

Docetaxel was obtained from Aventis Pharmaceuticals (Sanofi-Aventis). SDF-1 was purchased from R&D Systems. PD98059, an ERK1/2 inhibitor, and SB203580, a p38 MAPK inhibitor, were purchased from Cell Signaling Technology. AMD3100, a CXCR4 antagonist, was purchased from Sigma-Aldrich. Anti-human phosphorylated ERK1/2 (20G11), anti-human ERK1/2 (137F5), anti-human phosphorylated p38 MAPK (9211), anti-human p38 MAPK (9212), and anti-human c-Myc (D84C12) were purchased from Cell Signaling Technology. Anti-human CXCR4 (ab2074 and ab7199), anti-human ACTB (AC-15), and a rabbit control immunoglobulin G (IgG; ab64650) were purchased from Abcam. Anti-human c-Myc (1472-1) was purchased from Epitomics. Anti-human clusterin (41D) was purchased from Upstate Cell Signaling Solutions.

Establishment of docetaxel-resistant prostate cancer cells

The docetaxel-resistant residual DRD cell line was established from DU145 cells by culturing the DU145 cells in medium containing 0.5 nmol/L docetaxel for 7 weeks. After establishment of the DRD cells, docetaxel-resistant 1G7 cells were cloned from the DRD cells using the limiting-dilution technique. The docetaxel-resistant PC3DR cell line was established from PC3 cells by cloning the PC3 cells after culturing the PC3 cells in medium containing docetaxel for 24 weeks. The concentration of docetaxel in the medium was increased to 0.5, 1, 2, 5, and finally to 10 nmol/L. After the establishment of docetaxel-resistance cell lines, the DRD, 1G7, and PC3DR cells were cultured in medium without docetaxel. All experiments were carried out using passage-matched parental cells.

Sphere culture

Sphere culture was conducted as previously described (26). Briefly, single cells were plated at 1,000 cells/mL on low-attachment dishes. Cells were grown in serum-free epithelial basal medium (Cambrex) supplemented with 4 μg/mL insulin (Sigma-Aldrich), B27, 20 ng/mL EGF, and 20 ng/mL basic fibroblast growth factor (bFGF; Invitrogen) for 14 days. The sphere-forming capacity was assessed by the number of colonies.

Soft agar colony formation assay

Anchorage-independent growth was assayed using the Cytoselect 96-well Cell Transformation Assay (Soft Agar Colony Formation) Kit (Cell Biolabs) as recommended by the manufacturer. Briefly, the cells (1.5 × 105) were mixed with an agar solution and seeded into the wells, and culture medium with or without inhibitors was then added to each well. The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2. Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice and BALB/c nude mice of ages 5 weeks were purchased from Japan Clea and maintained in a temperature-controlled, pathogen-free room. All animals were handled according to the approved protocols and guidelines of the Animal Committee of Osaka University (Osaka, Japan).

MTS assay

CellTiter 96 AQueous One Solution Reagent (Promega) was used to evaluate surviving cells. CellTiter 96 AQueous
One Solution Reagent was added to each well, and the plates were incubated for 1.5 hours at 37°C. The absorbance at 490 nm was then measured in each well using a 96-well plate reader.

Cell-cycle analyses

Cells were harvested after docetaxel treatment and fixed in cold 70% ethanol. Fixed cells were subsequently washed and stained with the PI/RNase staining buffer (BD Biosciences). Flow cytometry analyses were conducted using a BD FACSCanto II flow cytometer (BD Biosciences). Cell-cycle distributions were analyzed using the BD FACSDiva software. Apoptotic populations were measured as the percentage of the total cell population with a sub-G1 DNA content.

CXCR4 cell surface expression

DU145, DRD, and 1G7 cells were incubated with a polyclonal rabbit anti-human CXCR4 antibody (1:50) or rabbit control IgG (isotype control) for 30 minutes on ice, followed by a 30-minute incubation with anti-rabbit Alexa Fluor 488 dye (Invitrogen), and then they were measured by FACSVersr (BD Biosciences).

Real-time quantitative RT-PCR

Total RNA was isolated using the RNeasy RNA Isolation Kit (Qiagen). cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR (qRT-PCR) was conducted using an Applied Biosystems 7900 HT Fast Real-Time PCR system under the following conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Mixtures of probes and primer pairs specific for human c-Myc (Hs00153408_m1), CXCR4 (Hs00976734_m1), clusterin (Hs00165648_m1), ABC2 (Hs00166123_m1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs99999905_m1) and 18S rRNA (Hs99999901_s1) were purchased from Applied Biosystems. The relative expression levels were calculated from a standard curve obtained using log dilutions of cDNA containing the gene of interest, and values were normalized to the expression level of GAPDH or 18S rRNA, which served as the internal control. The human CXCR4 and GAPDH were also amplified by SYBR Premix Ex Taq (Takara Bio). All procedures were carried out according to the manufacturer’s instructions. The primers were as follows: CXCR4: 5’-CAGCGAGCTTAGCAAAGTGA-3’ (forward) and 5’-AGGCGTGTGACACAAAGGAG-3’ (reverse); GAPDH: 5’-ACCAGGCTCCAGCCTACG-3’ (forward) and 5’-TCCACACCCCTGTTGCTTGTA-3’ (reverse).

Western blot analysis

Cells were harvested and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer. Protein samples were separated by SDS-PAGE. The separated proteins were transferred onto polyvinylidene fluoride membranes, and the membranes were then blocked with 5% skimmed milk and incubated overnight at 4°C with anti-phosphorylated p38 MAPK (1:1,000), anti-p38 MAPK (1:1,000), anti-c-Myc (1:500), anti-CXCR4 (1:500), anti-clusterin (1:400), or anti-ACTB (1:2,000) antibodies. The membranes were washed and labeled with a 1:2,000 dilution of a horseradish peroxidase–conjugated secondary antibody (GE Healthcare) at room temperature for approximately 1 hour. Detection by chemiluminescence was conducted according to the enhanced chemiluminescence (ECL) user’s guide (Amersham). Images were captured with an ImageQuant LAS 4000 mini system (GE Healthcare), and the quantification of Western blot signals was conducted by densitometry using the ImageQuant TL software (GE Healthcare).

RNA interference experiments

The following double-strand stealth siRNA oligonucleotides and scrambled siRNA were purchased from Invitrogen. The siRNA oligonucleotide sequences were as follows: c-Myc #1: 5’-CAGCGACUCUGAGGAGAAACAGAA-3’ (sense) and 5’-UUUGUUUCUCCUCAGAGGUGC-UG-3’ (antisense); c-Myc #2: 5’-GAGAAGCGUGCACAAACUUGA-AACACGACUCUGA-3’ (sense) and 5’-UAAGGUUGCUGGACUUCGGAU-3’ (antisense); CXCR4 #1: 5’-AUACGCGCAGAUAAGCCACCA-3’ (sense) and 5’-UGGGUGGCGUUCAUCUCUGCUGUAU-3’ (antisense); CXCR4 #2: 5’-AACAGUGGAAAGAACCCUCGAGG-GCCUC-3’ (sense) and 5’-GAGGCCCUAGCCUUCUCCACUGU-3’ (antisense); and CXCR4 #3: 5’-UGAC-CAGGAUGGACAUCCAUUGCC-3’ (sense) and 5’-GGC-GCAAUGGAUUGCUGAUCUGGAU-3’ (antisense). Transfections were carried out using lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions.

In vivo tumorigenicity experiments

The DU145 and DRD cells were mixed with BD Matrigel Basement Membrane Matrix High Concentration (BD Biosciences), and 100 µL of the Matrigel-mixed cells was subcutaneously injected at varying cell numbers into NOD/SCID mice. The mice were observed for 3 to 5 months for the appearance and development of tumors. The tumor volume was calculated according to the following formula: tumor volume (mm³) = length x (width)²/2.

Docetaxel treatment of prostate cancer cells in a mouse tumor model

The DU145 and DRD cells were subcutaneously injected at 5.0 x 10⁶ cells into BALB/c nude mice. Seven and 14 days after tumor inoculation, docetaxel (22 mg/kg) were intravenously administrated via the tail vein of the mice. The tumor volume was calculated according to the following formula: tumor volume (mm³) = length x (width)²/2.

Microarray analysis

Total RNA was isolated from cell pellets using the RNeasy Kit (Qiagen). Sample preparation for GeneChip analysis was conducted according to a protocol detailed by Affymetrix. Briefly, 250 ng of total RNA was reverse transcribed into cDNA with a T7 oligo d(T) primer (Affymetrix). The cDNA...
synthesis product was then used in an in vitro transcription reaction containing T7 RNA polymerase and biotinylated nucleotide analogs (pseudouridinyl base). The labeled cRNA products were then fragmented, loaded onto a GeneChip(R) Human Genome U133 Plus2.0 array (Affymetrix), and hybridized according to the manufacturer’s protocol. Streptavidin–phycoerythrin (Molecular Probe) was used as a fluorescent conjugate to detect hybridized target sequences. Raw intensity data from the GeneChip array were analyzed using the GeneChip operating software (Affymetrix).

Immunohistochemical analysis

Written informed consent was obtained from all patients for the use of their tissue specimens, and the use of such specimens was approved by the Osaka University Hospital Institutional Review Board (Osaka, Japan). Four patients diagnosed with docetaxel-resistant prostate cancer had undergone biopsy or resection of tumors at Osaka University Hospital. Protein expression was determined by immunohistochemical staining of parafﬁn-embedded tissue sections. Briefly, 6-mm thick sections were deparafﬁnized, rehydrated using xylene and alcohol, and incubated with 0.3% H2O2 to block endogenous peroxidase activity. Before immunostaining, antigen was retrieved by immersing the sections in 10 mmol/L citrate buffer (pH 6.0) and boiling in steam for 20 minutes. Immunohistochemistry was conducted with anti-CXCR4 antibody (1:200), anti-phosphorylated ERK1/2 (1:400), and anti-c-Myc antibody (1:50) using the EnVision Plus Detection System (DAKO) according to the manufacturer’s instructions. Primary antibody was incubated for 60 minutes at room temperature, and the slides were counterstained with or without hematoxylin.

Statistical analyses

The results are reported as the mean ± SE. The two-tailed, unpaired Student t test was used to determine the statistical significance of differences between groups. P < 0.05 was considered statistically signiﬁcant. Statistical analyses were conducted using the JMP9 software program (SAS Institute).

Results

The tumorigenic potential of docetaxel-resistant residual prostate cancer cells is increased

The docetaxel-resistant residual prostate cancer cell line PC3DR was also established from PC3 cells (Supplementary Fig. S3). In docetaxel-resistant residual prostate cancer cells, clusterin and ABC2 expression was upregulated (Supplementary Fig. S4), and it has been reported that docetaxel resistance may depend on clusterin upregulation (8) and/or the ABC family of drug transporters, such as ABC2 (6, 7). We next examined whether the tumorigenic potential increased in docetaxel-resistant residual prostate cancer cells. It has been reported that the tumorigenic potential increases in sphere-forming prostate cancer cells (26). Thus, we examined the sphere-forming capacity of docetaxel-resistant residual prostate cancer cells. The sphere-forming capacity of DRD, 1G7, and PC3DR cells was increased as compared with that of parental DU145 or PC3 cells (Fig. 1A). The sphere-forming capacity of DRD and 1G7 cells was maintained even in the presence of docetaxel (Supplementary Fig. S5). Furthermore, DRD and PC3DR cells have increased clonogenic potential as compared with parental DU145 or PC3 cells when grown under anchorage-independent conditions in soft agar (Fig. 1B), although growth under monolayer conditions was similar to DRD and DU145 cells (Fig. 1C). To test the in vivo tumorigenic potential, DRD and DU145 cells were subcutaneously injected at varying cell numbers into NOD/SCID mice (Fig. 1D). Although the in vivo tumorigenic potential was similar at 1 × 105 cells in DU145 and DRD cells, the DRD cells showed a significantly higher tumorigenic potential than the DU145 cells at 1 × 103 cells or less. Thus, the tumorigenic potential was increased in the docetaxel-resistant residual prostate cancer cells compared with parental prostate cancer cells.

c-Myc expression was increased by ERK1/2 and p38 MAPK activation in docetaxel-resistant residual prostate cancer cells

It has been reported that survival signaling pathways, such as ERK1/2, are transiently activated by docetaxel treatment (15–18). We conﬁrmed that phosphorylated ERK1/2 was induced by docetaxel therapy in DU145 cells (Supplementary Fig. S6). Next, we examined ERK1/2 phosphorylation in DRD and 1G7 cells cultured without docetaxel. Phosphorylated ERK1/2 was increased in DRD and 1G7 cells compared with DU145 cells, even in the absence of docetaxel (Fig. 2A). Phosphorylated ERK1/2 was also increased in docetaxel-resistant PC3DR cells (Fig. 2A). Among the survival signaling pathways, we also found that p38 MAPK was activated in DRD and 1G7 cells (Supplementary Fig. S7A), whereas AKT was not activated in the DRD cells (data not shown). We next examined whether the ERK1/2 signaling pathway is involved in the increase in the tumorigenic potential of the docetaxel-resistant residual prostate cancer cells. The clonogenic potential of DRD, 1G7, and PC3DR cells under anchorage-independent conditions was reduced by PD98059, an ERK1/2 inhibitor (Fig. 2B and C), although the growth of DRD cells under monolayer conditions was not attenuated by PD98059 (Fig. 2D). We also found that the DRD cell clonogenic potential under anchorage-independent conditions was slightly reduced by
reported as the mean and DRD cells were seeded on 96-well culture dishes and incubated for up to 5 days. Cell growth was assessed using an MTS assay at the indicated times and
agar and incubated for 7 days. The clonogenic potential under anchorage-independent growth conditions was examined. Each RFU (relative fluorescent unit)
was assessed by counting the colony numbers. Each relative colony number value is presented as a ratio compared with parental DU145 cells (mean ± SE; n = 10) or PC3 cells (mean ± SE; n = 3). *, P < 0.01. C, cell growth under monolayer conditions. DU145 and DRD cells were seeded on 96-well culture dishes and incubated for up to 5 days. Cell growth was assessed using an MTS assay at the indicated times and reported as the mean ± SE (n – 3) of the fold difference after normalizing to the values at day 1. D, the tumorigenic potential of DU145 and DRD cells were mixed with Matrigel and subcutaneously injected at varying cell numbers into NOD/SCID mice. The tumor volume was calculated according to the following formula: tumor volume (mm3) = length × (width)2/2, and reported as the mean ± SE (n = 6) at the indicated times.

SB203580, a p38 MAPK inhibitor, and strongly reduced by combined treatment with PD98059 and SB203580 (Supplementary Fig. S8A and S8B). Thus, the enhanced tumorigenic potential was reduced by the inhibition of ERK1/2 and p38 MAPK, which were constitutively activated in docetaxel-resistant residual prostate cancer cells.

Among the genes activated by the ERK1/2 and p38 MAPK signaling pathways, we focused on c-Myc because of its involvement in prostate tumorigenesis (19, 20). It has been reported that the c-Myc protein level is regulated by ERK1/2 via posttranslational mechanisms (21–25). It has also been reported that the p38 MAPK signaling pathway regulates c-Myc translation (32). The c-Myc protein level was upregulated in DRD, 1G7, and PC3DR cells as compared with parental DU145 or PC3 cells (Fig. 3A), although the c-Myc mRNA level was similar in resistant and parental cell lines (Fig. 3B). The c-Myc protein level was reduced by PD98059-mediated phosphorylated ERK1/2 inhibition in DRD cells (Fig. 3C), although the c-Myc mRNA level was not attenuated by PD98059 (Fig. 3D). We also found that the c-Myc protein level was reduced by SB203580-mediated p38 MAPK inhibition (Supplementary Fig. S7B). We next examined whether c-Myc was required for the maintenance of the tumorigenic potential of docetaxel-resistant residual prostate cancer cells. The efficacy of c-Myc siRNA was assessed by qRT-PCR and Western blot analysis (Fig. 3E and Supplementary Fig. S9A). The clonogenic potential of DRD, 1G7, and PC3DR cells under anchorage-independent growth conditions was significantly reduced by c-Myc siRNA transfection (Fig. 3F). Thus, c-Myc activation by constitutively activated ERK1/2 and p38 MAPK may increase the tumorigenic potential of docetaxel-resistant residual prostate cancer cells.

**ERK1/2 activation is controlled by the SDF-1/CXCR4 signaling pathway**

We then examined why the ERK1/2 signaling pathway was constitutively activated in docetaxel-resistant residual prostate cancer cells. Among the genes that regulate the ERK1/2 signaling pathway, we focused on CXCR4 because recent reports have shown that CXCR4 is upregulated in sphere-forming prostate cancer cells (26–28). We then conducted a microarray analysis and detected CXCR4 upregulation in DRD and 1G7 cells compared with parental DU145 cells (Supplementary Table S1). We next examined CXCR4 expression using qRT-PCR. CXCR4 was
upregulated in DRD and 1G7 cells compared with parental DU145 cells (Fig. 4A). CXCR4 was also upregulated in PC3DR cells compared with parental PC3 cells (Fig. 4A). The amount of CXCR4 was similarly upregulated in DRD and sphere-forming DU145 cells as compared with wild-type DU145 cells, and much higher in sphere-forming DRD cells (Fig. 4B). Western blot analyses showed that CXCR4 was upregulated in DRD, 1G7, and PC3DR cells compared with parental DU145 or PC3 cells (Fig. 4C). Flow cytometry also showed that the amount of CXCR4-positive cells was much higher in DRD (80.8% ± 2.1%) and 1G7 cells (83.1% ± 2.1%) than parental DU145 cells (32.9% ± 1.6%; Fig. 4D). Thus, CXCR4 was upregulated in docetaxel-resistant residual prostate cancer cells as shown in sphere-forming prostate cancer cells.

It has been reported that CXCR4 activates ERK1/2 in the presence of SDF-1, a CXCR4 ligand (29) that is present in prostate cancer cell culture medium (33). It has also been reported that the p38 MAPK signaling pathway is activated by CXCR4 (34). Next, we confirmed that phosphorylated ERK1/2 was induced by SDF-1, and phosphorylated ERK1/2 induction was inhibited by AMD3100, a CXCR4 antagonist, in DRD cells (Fig. 4E). Phosphorylated p38 MAPK was also slightly induced by SDF-1, and phosphorylated p38 MAPK was inhibited by AMD3100 in DRD cells (Supplementary Fig. S7C). Thus, ERK1/2 activation was controlled by CXCR4, which was upregulated in docetaxel-resistant residual prostate cancer cells.

**c-Myc is involved in enhancing the prostate cancer cell tumorigenicity via CXCR4 activation**

We then examined whether CXCR4 was involved in maintenance of the tumorigenic potential. We first assessed the CXCR4 siRNA efficacy by qRT-PCR and Western blot analyses (Fig. 4F and Supplementary Fig. S9B). ERK1/2 phosphorylation and the c-Myc protein level were reduced in CXCR4 siRNA-transfected DRD cells (Fig. 4F). The c-Myc protein level was also reduced by AMD3100 treatment (Fig. 4G). The clonogenic potential under anchorage-independent growth conditions was significantly reduced by CXCR4 siRNA transfection in DU145, DRD, 1G7, and PC3DR cells (Fig. 5A and B). The clonogenic potential of DRD, 1G7, and PC3DR cells was also reduced by AMD3100 (Fig. 5C and D). Thus, CXCR4 may be involved in the maintenance of the tumorigenic potential via c-Myc protein stabilization by constitutively
activated ERK1/2 in docetaxel-resistant residual prostate cancer cells.

We next focused on CXCR4 expression in wild-type DU145 cells because it has been reported that CXCR4 expression is induced by several cellular stresses, such as hypoxia (50), and that c-Myc upregulates CXCR4 promoter activity (31). We found that CXCR4 expression was induced by docetaxel treatment in DU145 cells (Fig. 6A and B). We then examined the involvement of c-Myc in CXCR4 expression in DU145 cells. Phosphorylated ERK1/2 and the c-Myc protein level were upregulated by docetaxel treatment in scrambled siRNA-transfected DU145 cells, whereas the c-Myc protein level was suppressed in c-Myc siRNA-transfected DU145 cells, despite the activation of ERK1/2 (Fig. 6C). c-Myc knockdown inhibited CXCR4 expression in docetaxel-treated DU145 cells (Fig. 6D). Thus, the ERK1/2

Figure 3. The involvement of c-Myc in maintenance of the tumorigenic potential. A, protein extracts were prepared from DU145, DRD, 1G7, PC3, and PC3DR cells, and the c-Myc expression level was analyzed by Western blot analyses. ACTB was used as a control for protein loading. B, qRT-PCR analyses for c-Myc were conducted using transcripts isolated from DU145, DRD, 1G7, PC3, and PC3DR cells. The c-Myc expression level was normalized by the level of GAPDH in each cell line. The relative expression (mean ± SE; n = 3) represents the expression level compared with DU145 or PC3 cells. C, protein extracts were prepared from DRD cells after incubation with PD98059 (0–50 µmol/L) for 48 hours, and the expression level of phosphorylated ERK1/2 (p-ERK1/2), ERK1/2, and c-Myc was then analyzed by Western blot analysis. The expression of c-Myc relative to ACTB in each lane is presented as the ratio compared with the control (untreated cells). D, qRT-PCR analysis for c-Myc was conducted using transcripts isolated from DRD cells incubated with PD98059 (0–50 µmol/L) for 24 hours. The c-Myc expression level was normalized against the GAPDH expression level in each group. The relative expression (mean ± SE; n = 3) represents the ratio of the expression level in untreated DRD cells. E, DRD, 1G7, and PC3DR cells were transfected with scrambled or c-Myc siRNA and incubated for 48 hours. The RNA interference (RNAi) effect on c-Myc expression was assessed by Western blot analysis. F, DRD, 1G7, and PC3DR cells were transfected with scrambled or c-Myc siRNA, incubated for 48 hours and then reseeded in soft agar and incubated for 7 days. The clonogenic potential under anchorage-independent growth conditions was examined. Each RFU (relative fluorescent unit) value (mean ± SE; n = 3) is presented as a ratio compared with scrambled siRNA-transfected cells. *< P < 0.01.
Figure 4. CXCR4 expression is upregulated in docetaxel-resistant residual prostate cancer cells. 

A, qRT-PCR analyses for CXCR4 were conducted using transcripts isolated from DU145, DRD, 1G7, PC3, and PC3DR cells. The CXCR4 expression level was normalized against the GAPDH level in each cell line. The relative expression (mean ± SE; n = 3) represents the expression level compared with DU145 or PC3 cells. * P < 0.05; ** P < 0.01. B, qRT-PCR analysis for CXCR4 was conducted using transcripts isolated from DU145, DRD, sphere-forming DU145 (DU145 Sp), and sphere-forming DRD (DRD Sp) cells. The relative expression (mean ± SE; n = 3) is presented as described in (A). * P < 0.05; ** P < 0.01. C, protein extracts were prepared from DU145, DRD, 1G7, PC3, and PC3DR cells, and the CXCR4 expression level was analyzed by Western blot analysis. ACTB was used as a control for protein loading. D, CXCR4 surface expression was examined by flow cytometry analysis of DU145, DRD, and 1G7 cells. The amount of CXCR4-positive cells is shown as the mean ± SE; n = 3. CXCR4 (red), isotype control (blue). E, DRD cells were starved for 24 hours in serum-free medium and treated with or without 100 ng/mL SDF-1 for 15 minutes after pretreatment with AMD3100 (0–100 µg/mL) for 1 hour. Western blot analyses were conducted to detect phosphorylated ERK1/2 (p-ERK1/2) and ERK1/2. The expression of p-ERK1/2 relative to ACTB in each lane is presented as the ratio compared with untreated cells. F, DRD cells were transfected with scrambled or CXCR4 siRNA and incubated for 72 hours. Western blot analyses were conducted to detect CXCR4, p-ERK1/2, ERK1/2, and c-Myc. The expression of CXCR4, p-ERK1/2, and c-Myc relative to ACTB in each lane is presented as the ratio of the value of the scrambled siRNA-transfected cells. G, DRD cells were treated with AMD3100 (0–100 µg/mL) for 72 hours. Western blot analyses were conducted to detect CXCR4 and c-Myc. The expression of c-Myc relative to ACTB in each lane is presented as the ratio compared with the control (untreated cells).
activated by docetaxel therapy may induce CXCR4 expression by stabilizing c-Myc.

Finally, we examined whether the CXCR4, ERK1/2, and c-Myc signaling is constitutively activated in clinical cancerous tissue samples from human patients with docetaxel-resistant prostate cancer. The patients diagnosed with docetaxel-resistant prostate cancer had undergone biopsy or resection of tumors, and then, the expression of CXCR4, phosphorylated ERK1/2, and c-Myc was examined by immunohistochemical analysis (Fig. 7 and Supplementary Table S2). The CXCR4, ERK1/2, and c-Myc signaling was constitutively activated in the majority of clinical cancerous
tissue samples from patients with docetaxel-resistant prostate cancer.

Discussion

Although ERK1/2 is known to be transiently activated by docetaxel therapy (15–18), we found that ERK1/2 signaling was constitutively activated in the docetaxel-resistant residual prostate cancer cell lines DRD, 1G7, and PC3DR cells compared with parental DU145 or PC3 cells, even in the absence of docetaxel (Fig. 2). We also showed that the tumorigenic potential increased in docetaxel-resistant residual prostate cancer cells as compared with parental prostate cancer cells because of the constitutively activated ERK1/2 signaling pathway. It has been reported that PD98059, an ERK1/2 inhibitor, can enhance the docetaxel anticancer effects in prostate cancer cells (17). Indeed, ERK1/2 can induce the expression of clusterin (35) and ABC transporters (36). Thus, constitutively activated ERK1/2 may enhance the tumorigenic potential and docetaxel resistance of docetaxel-resistant residual prostate cancer cells.

We showed that c-Myc activation, which was induced by constitutively activated ERK1/2, increased the tumorigenic potential of docetaxel-resistant residual prostate cancer cells (Fig. 3). The c-Myc mRNA level was reported to be elevated in the prostate cancer tissues compared with benign prostatic tissues in the majority of studies (19). Furthermore, the constitutive CXCR4, ERK1/2, and c-Myc signaling activation is shown in clinical cancerous tissue samples from patients with docetaxel-resistant prostate cancer. The patients diagnosed with docetaxel-resistant prostate cancer had undergone biopsy or resection of tumors, and then, the expression of CXCR4 (A and D), phosphorylated ERK1/2 (p-ERK1/2; B and E), and c-Myc (C and F) was examined by immunohistochemical analysis. A representative case is shown. (D–F) are the higher magnification of (A–C).
relative c-Myc copy number gain in patients with prostate cancer was reported to be associated with poor prognosis (37). However, the presence of c-Myc mRNA does not necessarily indicate the presence of c-Myc protein because the c-Myc protein level is strictly regulated by translational and posttranslational mechanisms (19). Activated ERK1/2 is required to stabilize c-Myc by preventing ubiquitination and proteasomal degradation (21–25). Constitutive ERK1/2 activation in prostate cancer cells is associated with an aggressive phenotype, such as a high Gleason score or androgen-independent growth (38, 39). The aggressive phenotype may be induced by c-Myc activation in prostate cancer cells with constitutively activated ERK1/2.

In this study, we focused on CXCR4 as the upstream ERK1/2 signal because sphere-forming prostate cancer cells with a high CXCR4 expression are known to increase the tumorigenic potential (26, 28). Furthermore, CXCR4 upregulation in prostate cancer cells is associated with poor patient prognosis (40). Although it is well known that the activation of the CXCR4 pathway alters the migration and invasion of prostate cancer cells and enhances the bone metastasis of prostate cancer cells (33, 41), we showed that the high CXCR4 expression in docetaxel-resistant residual tumor cells was involved in the increase in the tumorigenic potential of these cell lines via ERK1/2 activation and c-Myc (Figs. 4 and 5).

We first showed that CXCR4 expression was induced by docetaxel therapy in prostate cancer cells. CXCR4 induction by docetaxel may be an important step in the development of highly tumorigenic residual tumor cells. In wild-type DU145 cells, ERK1/2 activation by docetaxel treatment resulted in c-Myc protein upregulation (Fig. 6). In Fig. 6, ERK1/2 activation was maintained even in the absence of docetaxel in c-Myc siRNA-transfected DU145 cells because of the lack of a negative feedback on ERK1/2 by c-Myc (42, 43). CXCR4 upregulation by docetaxel treatment in DU145 cells may then be caused by stabilization of the c-Myc protein by ERK1/2. Without docetaxel treatment, CXCR4 expression was not so much enhanced by c-Myc overexpression in DU145 cells (data not shown), suggesting that stabilization of the c-Myc protein by ERK1/2 is required for robust CXCR4 expression. Taken together, these data show that in response to docetaxel, ERK1/2 becomes activated and stabilizes the c-Myc protein, which then upregulates CXCR4. In addition, the CXCR4/SDF-1 axis enhances ERK1/2 activation further. The constitutive CXCR4, ERK1/2, and c-Myc signaling activation was shown in the majority of clinical cancerous tissue samples from patients with docetaxel-resistant prostate cancer (Fig. 7). Thus, the signaling loop can be constitutively activated following docetaxel treatment, resulting in docetaxel resistance and, finally, an increase in the tumorigenic potential of prostate cancer cells.

Although we showed that ERK1/2 was regulated by CXCR4 in this study, both p38 MAPK and AKT have also been reported to be regulated by the CXCR4 signaling pathway (34). We found that p38 MAPK was constitutively activated in DRD and 1G7 cells, whereas AKT was not activated in DRD cells. AKT activation may be inhibited by PTEN in DU145 cells (44). The p38 MAPK signaling pathway regulates c-Myc translation (32). Thus, constitutive p38 MAPK activation may also contribute to an increase in the tumorigenic potential of DRD and 1G7 cells via an increase in the c-Myc protein (Supplementary Figs. S7 and S8). We also found that the expression of PTEN was maintained in DRD and 1G7 cells, although the expression of PTEN was negative in PC3 and PC3DR cells (data not shown). Although a recent report showed that the loss of PTEN permitted ERK1/2 activation via CXCR4 (45), docetaxel therapy may induce the constitutive CXCR4, ERK1/2, and c-Myc signaling loop activation even in the presence of PTEN.

Our conclusion also indicates that chemotherapy enhances the tumorigenic properties of cancer cells. As cancer cells may alter gene expression patterns following chemotherapy to survive under the stressful conditions, it is difficult for chemotherapy to eradicate all cancer cells. Therefore, residual cancer cells resistant to the chemotherapy have acquired robust tumorigenic properties. In this study, we showed that the cellular sphere-forming capacity was increased in docetaxel-resistant residual prostate cancer cells. Recent reports have shown that prostate cancer cells enriched by sphere formation under anchorage-independent conditions have increased tumorigenic potential in mice (26), and these sphere-forming prostate cancer cells show prostate cancer stem-like cell properties (26, 46). Although we focused on the tumorigenic potential of docetaxel-resistant residual tumor cells in this study, docetaxel treatment may also increase prostate cancer stem-like cell populations. Indeed, CXCR4, whose expression was induced by docetaxel treatment, has been reported to be a marker for prostate cancer stem-like cells (27). On the basis of this idea, chemotherapeutic treatment alone would be undesirable and ineffective if all the cancer cells are not eradicated by the chemotherapy. Our findings also support the hypothesis that multimodal strategies are necessary for cancer therapy. From our results, we recommend that chemotherapy should be combined with the suppression of the CXCR4, ERK1/2, and c-Myc signaling loop. For example, the CXCR4 signaling pathway may become a cancer therapy target because we showed that CXCR4 expression was induced by docetaxel in prostate cancer cells, and CXCR4 was upregulated in docetaxel-resistant residual tumor cells. Furthermore, the expression of SDF-1, the CXCR4 ligand, is also induced in the bone marrow by chemotherapy (47), and SDF-1 induction may result in CXCR4 signaling pathway stimulation in cancer cells. Currently, multiple agents are in development for targeting the CXCR4 pathway in cancer. Of these, AMD3100 is approved for clinical use in patients with leukemia (48). Recently, AMD3100 combined with docetaxel showed anticancer effects in prostate cancer cells in vivo (28, 49). AMD3100 treatment combined with docetaxel may inhibit the increase in the residual cancer cells highly expressing CXCR4.

In summary, we showed the tumorigenic potential increase of docetaxel-resistant residual prostate cancer cells due to CXCR4, ERK1/2, and c-Myc signaling pathway activation. Thus, these signaling pathways may become...
treatment targets for inhibiting aggressive residual tumor cells after chemotherapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: K. Hatano, Y. Kaneda
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Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): K. Hatano, S. Yamaguchi, K. Nimura, K. Fujita, M. Uemura, Y. Nakai, M. Tsuchiya, Y. Kaneda

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Residual Prostate Cancer Cells after Docetaxel Therapy Increase the Tumorigenic Potential via Constitutive Signaling of CXCR4, ERK1/2 and c-Myc

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