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The depletion of Interleukin-8 causes cell cycle arrest and increases the efficacy of docetaxel in breast cancer cells

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ABSTRACT

IL-8 is a multi-functional pro-inflammatory chemokine, which is highly expressed in cancers, such as ER-negative breast cancer. The present study demonstrates the pervasive role of IL-8 in the malignant progression of ER-negative breast cancer. IL-8 siRNA inhibited proliferation and delayed the G1 to S cell cycle progression in MDA-MB-231 and BT549 cells. IL-8 silencing resulted in the upregulation of the CDK inhibitor p27, the downregulation of cyclin D1, and the reduction of phosphorylated-Akt and NF-κB activities. IL-8 depletion also increased the chemosensitivity to docetaxel. These results indicate a role for IL-8 in promoting tumor cell survival and resistance to docetaxel and highlight the potential therapeutic significance of IL-8 depletion in ER-negative breast cancer patients.

1. Introduction

Despite advances in early diagnosis and treatment, breast carcinoma is the most frequent cancer and leading cause of cancer-related deaths for women, with more than a million new cases diagnosed annually, resulting in >450,000 deaths worldwide. Metastasis is the major cause of mortality in human breast cancer. Recent studies have focused on dissecting the role and prognostic value of tumor cell-derived cytokines in human breast cancer. Tumor-derived cytokines exhibit a paracrine function to reprogram the normal stroma into a tumorigenic stroma [1] and work in an autocrine function to promote tumor growth, survival, and the acquisition of metastatic potential [2].

Interleukin-8, a proinflammatory chemokine with a Cysteine-X-Cysteine (CXC) motif, is a soluble mediator secreted from tumor cells that simultaneously exhibits autocrine and paracrine functions within the tumor microenvironment. IL-8 can function not only on leukocyte chemotaxis, inflammatory responses and infectious diseases but also on endothelial cells to promote motility, invasion, and the activation of survival and proliferative pathways in mesenchymal and aggressive tumor cells [3,4]. The upregulation of IL-8 in various tumor types has been attributed to the structure of the IL-8 promoter. The IL-8 promoter binds to NF-κB, AP-1 and other inflammation-related enhancers [5]. Expression of IL-8 in tumor cells has also been associated with the constitutive activation of inflammatory pathways, such as those initiated through the activation of NF-κB, AP-1 and hypoxia-inducible factor-1α in some tumor cells [3,6].

The biological action of IL-8 is mediated through binding to two high-affinity cell surface receptors, CXCR1 (IL-8RA) and CXCR2 (IL-8RB), which are members of the seven transmembrane G-protein-coupled receptors. Accumulating studies suggest that IL-8 plays important and multi-functional roles in malignant tumor progression and metastasis [3,7].

In breast cancer, IL-8 is overexpressed in estrogen receptor (ER)-negative breast cancer tissues and cell lines and has been associated with the invasive potential of these cells [8,9]. We previously reported that IL-8 expression is associated with the ER, vimentin and metastasis status in breast cancer cells [9]. However, the mechanism underlying the growth advantage rendered through constitutive IL-8 production, without overexpression, remains unknown. We hypothesized that IL-8 is a survival factor that not only promotes proliferation pathways but also controls apoptotic pathways through interactions with protein kinase-B (Akt) and NF-κB. Thus, the aim of this study was to further elucidate the biological function of IL-8 in the development and progression of ER-negative breast cancer. Specifically, we characterized the contribution of IL-8 to breast cancer cell-survival, tumor cell invasion, and resistance to chemotherapeutic drugs in two ER-negative breast cancer cell lines, MDA-MB-231 and BT549, using RNA interference.

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2. Materials and methods

2.1. Cell culture and reagents

Human breast cancer cell lines MDA-MB-231 and BT549 were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin in a 5% CO_2 atmosphere at 37 °C. Docetaxel was acquired from spent clinical stocks originally purchased from Aventis Pharmaceuticals (Parsippany, NJ).

2.2. siRNA transfection

Dr. Ruo-Pan Huang (Emory University, USA) provided siRNA targeting human IL-8 (GenBank accession No. NM_000584). Scrambled control siRNA (C siRNA) duplexes were designed and purchased from Ribobio (Guangzhou, China). The sense oligonucleotide sequence for IL-8 was 5′-GAUGCCAGUGAAACUUCAA-3′. MDA-MB-231 (1×10^5) and BT549 (3×10^5) cells were seeded in 6-well plates and cultured overnight for adherence. Both IL-8 and C siRNA were used at the same concentration in all experiments. At the indicated times post transfection, the transfected cells were collected for real-time PCR or Western blot analyses. The culture supernatants were collected to determine the IL-8 concentration through an enzyme linked immunosorbent assay (ELISA).

2.3. Real-time PCR

Total RNA from the cultured cells was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol. The cDNA was synthesized using the PrimeScript RT reagent Kit (TaKaRa Biotechnology) according to the manufacturer's instructions. Real-time PCR was performed using the LightCycler 480 System (Roche Applied Science) with gene-specific primers: IL-8-F 5′-ACACTGCGCCAACACAGAAATTA-3′, IL-8-R 5′-TTTGCTTGAAGTTTCACTGGCATC-3′; integrin β3-F 5′-TTCAATGCCACCTGCCTCAA-3′, integrin β3-R 5′-TTGGCCTCAATGCTGAAGCTC-3′; GAPDH-F 5′-GCACCGTCAAGGCTGAGAAC-3′, GAPDH-R 5′-TGGTGAAGACGCCAGTGGA-3′. The values obtained for the target gene expression were normalized to GAPDH and quantified relative to the expression in the control samples.

2.4. ELISA

The quantification of IL-8 protein was determined using the Human IL-8 ELISA Ready-SET-Go (eBioscience), and the levels were normalized to the cell numbers. The absolute levels of antigen and IL-8 secreted into the culture supernatants were determined using the assay standards provided in the assay kits.

2.5. Cell proliferation and drug sensitivity assay

MDA-MB-231 (2×10^3) and BT549 (3×10^3) cells were seeded in 96-well culture plates and cultured overnight for adherence. IL-8 and C siRNA were added to each well at final concentrations of 10, 30, 50, and 100 nM, and the cells were cultured for 24, 48, and 72 h. The cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8; Dojindo, Japan) according to the manufacturer's protocol. The absorbance was measured at 450 nm using a microplate reader (Tecan).

To determine the additive growth inhibitory effect of anticancer drugs and IL-8 siRNA, MDA-MB-231 (2×10^3) and BT549 (3×10^3) cells were seeded in 96-well culture plates and cultured overnight for adherence. After transfection with 50 nM of siRNA for 24 h, the drugs were added at final concentrations of 5, 10, 15, 20, and 30 nM, and the cells incubated for an additional 48 h. The cell proliferation activity was measured using the Cell Counting Kit-8.

2.6. Cell cycle analysis

The DNA content in the individual cells at 48 h following transfection with C and IL-8 siRNA was determined through flow cytometry. Briefly, the cells were harvested and fixed with 70% cold ethanol on ice for 30 min. The fixed cells were washed twice with phosphate buffered saline (PBS) and resuspended in 100 μl RNase (100 μg/ml, Qiagen) for 5 min at room temperature. Propidium iodide (PI, 400 μl of 50 μg/ml) (Sigma) was subsequently added and incubated at room temperature for 30 min. The DNA content in the cell samples was analyzed through flow cytometry (FACScan Scanford, BD Bioscience, USA), in which 5×10^4 events were collected. The data were analyzed using Cellquest software.

2.7. Migration and invasion assay

The migration assay was performed using BD Falcon™ Cell Culture Inserts (BD Bioscience; pore size, 8-μm) for 24-well plates. The invasive potential of the transfected cells was determined using a BD BioCoat™ Matrigel™ Invasion Chamber (BD Bioscience). Briefly, the cells were harvested at 48 h after transfection with IL-8 or C siRNA using LipofectAMINE 2000 reagent and resuspended at 4×10^4 cells/ml (MDA-MB-231) or 8×10^4 cells/ml (BT549) in serum-free DMEM medium. The cell suspension (0.5 ml) was placed on the top of the chamber. The lower compartment of chamber was filled 750 μl of DMEM containing 10% FBS. The cells were incubated for 24 h at 37 °C, and subsequently fixed in 4% polyoxymethylene for 15 min and stained with 0.05% crystal violet for 15 min. The cells remaining on the upper side of the filters were removed with cotton tipped swabs. The cells on the underside of the filters were examined and counted using a microscope at 200× magnification. For each chamber, the number of invaded cells in five randomly chosen fields was counted. The results were expressed as the mean number of invading cells ± SD per field (200×).

2.8. Western blotting

Whole cells extracts were prepared on ice using Mammalian Protein Extraction Reagent (KenGEN Biotech, Nanjing) according to manufacturer's instructions. Equal amounts of total proteins were separated by 8–12% SDS–PAGE gel and electrophoretically transferred to PVDF membranes (Roche). The blots were probed with primary antibodies overnight at 4 °C, followed by incubation with goat antibody against mouse or rabbit IgG-HRP for 1 h. The secondary antibodies were detected using enhanced chemiluminescence reagents. The following primary antibodies were used for immunoblotting: anti-phosphor-Akt (1:600), anti-Akt (1:2000), anti-$p27^{Kip1}$ (1:800), and anti-Cyclin D1 (1:800) were purchased from Cell Signaling Technology (Beverly, MA) were used for immunoblotting. Anti-integrin β3 (1:200) antibody, purchased from Abcam (Cambridge, MA), were also used for immunoblotting.

2.9. Reporter gene constructs and transfection

pGL4.32/NF-κB Vector (Promega) contains five copies of the NF-κB response element, which drives the transcription of the luciferase reporter gene. pRL-TK vector transfection was used for an internal control reporter. The transfection of siRNA or plasmids was performed using the LipofectAMINE 2000 reagent according to the manufacturer's instructions. After incubation for 6 h, the transfected cells were washed and complete medium was added. After culture for 36 h, the cells were lysed, and the lysates were assayed for

luciferase activity using a Dual-Glo Luciferase Assay System (Promega) and an Ultra Multifunctional Microplate Reader (Tecan).

2.10. Statistical analysis

Student's *t*-test and one-way ANOVA with multiple comparisons using the Newman–Keuls test were used for the statistical analysis. The relationships between the variables were determined using Pearson's correlation coefficient. The statistical analyses were performed using the SPSS 13.0 (SPSS Inc., Chicago, IL) statistical software package and GraphPad Prism 5.0 (GraphPad, San Diego, CA). The data represent the mean ± SD, and *P*-values of 0.05 or less were considered statistically significant.

3. Results

3.1. Effect of siRNA-mediated IL-8 silencing in ER-negative breast cancer cells

MDA-MB-231 and BT549 cells were transfected with 50 nM IL-8 or C siRNA. At 48 h after transfection, a 93% and 85% reduction in IL-8 mRNA levels were observed in MDA-MB-231 and BT549 cells, respectively (Fig. 1A and B). The mean reduction in the secreted IL-8 levels was, respectively, 94% and 86% (Fig. 1C and D).

3.2. Intrinsic IL-8 affects in vitro cell proliferation

The treatment of MDA-MB-231 and BT549 cells for 1–3 days with 10, 30, 50, and 100 nM of IL-8 siRNA resulted in the inhibition of cell proliferation (Fig. 2A and B). The inhibition of cell growth was dose- and time-dependent. In addition, MCF-7 cells, the IL-8 non-secreting and ER-positive breast cancer cell line, were transfected with IL-8 cDNA, and the stable IL-8 overexpression in this cell line (MCF-7/IL-8) induced a more rapid proliferation than observed in cells transfected with empty vector (data not shown). To distinguish between alterations in cell proliferation and cell viability, we used cycle-phase fractionation through flow cytometry, which provides more analytical details of cell cycle distribution among transfected cells.

3.3. IL-8. depletion affects cell cycle distribution

IL-8 siRNA caused an arrest of MDA-MB-231 and BT549 cells at the G0/G1 phase of the cell cycle, and prevented their entry into the S- and G2/M phases (Fig. 2C and D). We next analyzed the levels of key molecules that control the progression of cells from the G1 to the S-phase of the cell cycle. The results showed that IL-8 siRNA upregulated the expression of cyclin-dependent kinase (CDK) inhibitor $p27^{Kip1}$ and downregulated the expression of the CDK regulator cyclin D1 (Fig. 2E).

3.4. Intrinsic IL-8 mediates breast cancer cell migration and invasion

If intrinsic IL-8 is involved in breast cancer cell invasion, IL-8 siRNA will abrogate IL-8 action (reduce cells invading through the Matrigel). Indeed, the transfection of 50 nM of IL-8 siRNA into MDA-MB-231 and BT549 cells reduced the invasiveness of cells approximately 53% and 61%, respectively, compared with C siRNA. Moreover, the potential for the migration of both cell lines was also reduced through IL-8 siRNA (Fig. 3A and B).

3.5. IL-8. siRNA downregulates key factors that control survival and the metastatic pathway

We examined the activities of two key factors involved in survival and the metastatic pathway, protein kinase B (Akt) and

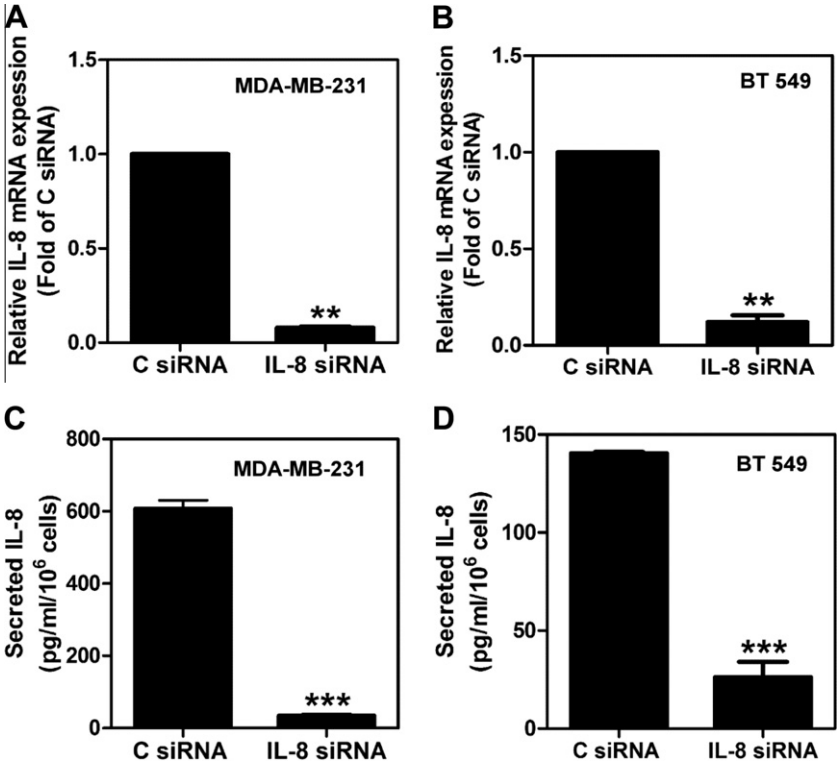

Fig. 1. Depletion of IL-8 in MDA-MB-231 and BT549 cells through siRNA transfection. (A and B) The levels of IL-8 mRNA were analyzed using real-time PCR at 48 h after transfection with 50 nM of IL-8 siRNA. The transcript levels were normalized to the GAPDH expression. (C and D) The levels of IL-8 protein were analyzed using ELISA at 48 h after transfection with 50 nM of IL-8 siRNA. Experiments in A–D were repeated at least 3 times, with similar results. $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$, compared with cells transfected with C siRNA.

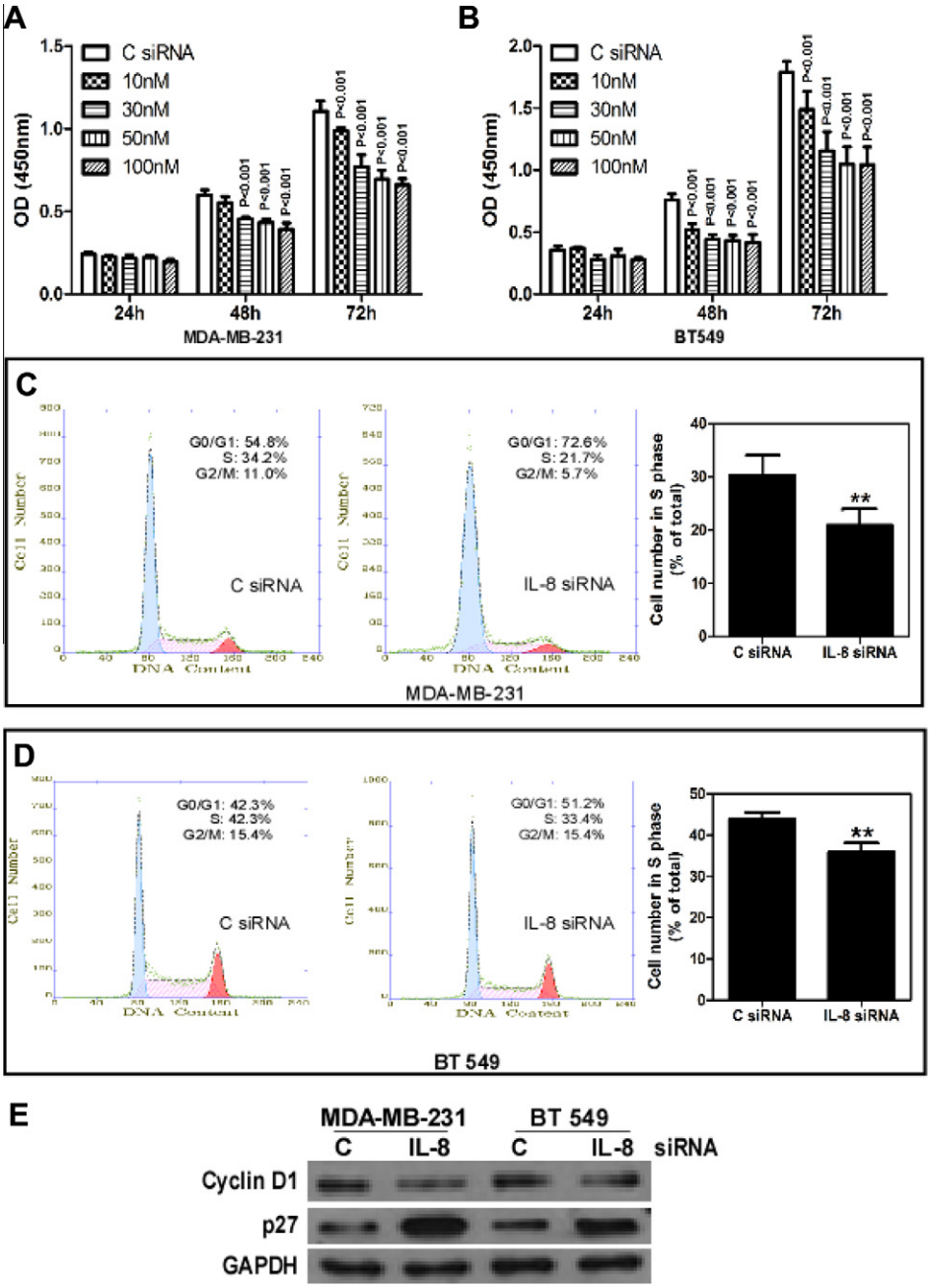

Fig. 2. IL-8 depletion in MDA-MB-231 and BT549 cells reduces cell proliferation and causes cell cycle arrest. (A and B) Inhibitory effects of IL-8 siRNA on the growth of the indicated cells, as determined using a CCK-8 assay. (C and D) Cell cycle arrest at the G1/S boundary. The percentage of cells in each of the three cell cycle phases was determined using flow cytometry at 48 h after siRNA transfection. The results shown are from one transfection experiment. (E) Western blot analysis of Cyclin D1 and p27 expression at 48 h after transfection with 50 nM of IL-8 siRNA. GAPDH was detected as the loading control. The experiments in A–E were repeated at least 3 times, with similar results. $^{**}P \leqslant 0.01$, compared with cells transfected with C siRNA.

NF-κB [10,11]. We observed a significant reduction in the phosphorylation of Akt in IL-8-depleted cells compared with cells transfected with C siRNA alone (Fig. 3C). Furthermore, we observed a significant decrease in the endogenous NF-κB activities in IL-8-depleted cells, using an NF-κB reporter construct (Fig. 3D).

3.6. IL-8. depletion reduces integrin β3 expression

Because IL-8 and integrin β3 have been implicated in increasing the invasiveness and metastatic potential of breast cancer cell lines [12,13], we investigated whether IL-8 depletion reduces the expression of integrin β3. IL-8 depletion through siRNA transfection significantly reduced the mRNA and protein levels of integrin β3 in both MDA-MB-231 and BT549 cells (Fig. 3E and F).

3.7. IL-8. depletion in ER-negative breast cancer cells increases chemosensitivity to docetaxel

The cells transfected with C or IL-8 siRNA for 24 h were exposed to several concentrations of docetaxel for the 48 h. The cell viability was estimated in the untreated control, signal treatment alone, and combined siRNA and drug-exposed cultures using a CCK-8 assay. The combination of docetaxel and IL-8 siRNA transfection significantly enhanced cytotoxicity in MDA-MB-231 and BT549 cells (Fig. 4).

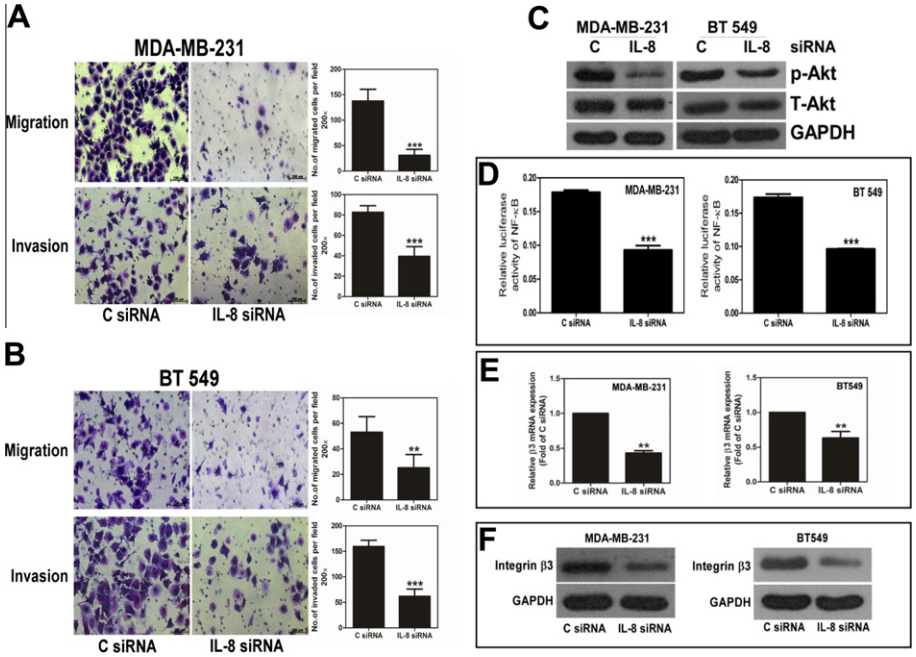

Fig. 3. Reduction in the survival and metastatic factors in IL-8-depleted ER-negative breast cancer cells. (A and B) Reduction in the invasion and migration activity of MDA-MB-231 and BT549 cells transfected with IL-8 siRNA. Representative images and quantification of migrating and invasive cells. Original magnification, 200× (A–B). (C) Western blots of whole cell extracts at 48 h following transfection with C or IL-8 siRNA. GAPDH was detected as the loading control. (D) The luciferase activities driven by NF-κB were suppressed in IL-8 siRNA-transfected MDA-MB-231 and BT549 cells. (E) Changes in the mRNA expression of integrin β3 at 48 h after transfection with 50 nM of IL-8 siRNA were assessed using real-time PCR. The transcript levels were normalized to the GAPDH expression. (F) Western blot analysis of integrin β3 expression at 72 h after transfection with 50 nM of IL-8 siRNA. GAPDH was detected as the loading control. The experiments in A–F were repeated at least 3 times, with similar results. $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$, compared with cells transfected with C siRNA.

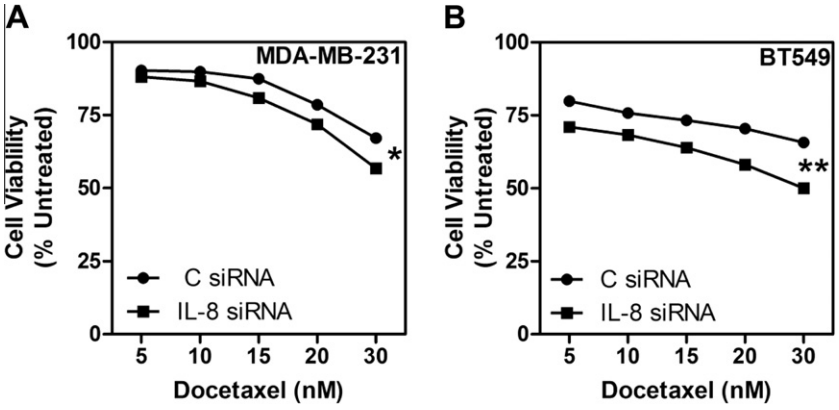

Fig. 4. IL-8 depletion increases the cytotoxicity of docetaxel in ER-negative breast cancer cells. IL-8 or C siRNA-transfected MDA-MB-231 and BT549 cells were exposed to docetaxel at 24 h after transfection. The results shown are from three independent experiments, normalized with respect to the samples with or without drug treatment. The cell viability was evaluated at 72 h post-transfection using a CCK-8 assay. All cytotoxicity data are normalized with respect to the reduction in cell viability due to IL-8 siRNA transfection. We analyzed the cytotoxicity data using non-linear regression and tested the mean survival values using Student's *t*-test.

4. Discussion

This study provides evidence that IL-8 overexpression plays an important role in promoting the tumorigenicity and progression of human ER-negative breast cancer cells. Tumorigenesis is a complex multi-step process, characterized by uncontrolled cell growth and tumor formation, and has largely been associated with the progressive accumulation of genetic and various epigenetic alterations in genes or proteins that regulate cell proliferation [14]. Therefore, the identification of the genes and gene products that lead to tumorigenesis is critical for the development of new diagnostic and prognostic methods and potential therapeutic targets. Mounting evidence has shown that IL-8 plays an important role in tumorigenesis via the regulation of a variety of biological processes, such as proliferation, differentiation, stress responses and apoptosis through the transcriptional induction of the CDK inhibitors, including $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ [15–17]. The inactive cyclin D/CDK4 and $p27^{Kip1}$ ternary complex requires extracellular mitogenic stimuli for the release and degradation of p27 concomitant with an increase in cyclin D levels to affect progression through the restriction point and pRb-dependent entry into the S-phase of the cell cycle [18]. In the present study, we showed that the depletion of endogenous IL-8 expression through siRNA reduced MDA-MB-231 and BT549 cell proliferation and cell cycle

progression. The downregulation of IL-8 induced G1-S phase arrest. Furthermore, we demonstrated that the mechanism of IL-8-mediated proliferation was associated with alternations in the expression of the cell cycle inhibitor $p27^{Kip21}$ and the CDK regulator cyclin D1. Because we observed that IL-8 depletion decreases cell survival, we used an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Bioscience) to determine whether this effect was due to an increase in spontaneous apoptosis following siRNA transfection. However, we observed that the transfection of IL-8 siRNA did not affect cell apoptosis (data not shown).

We observed a significant decrease in the migration and invasiveness of MDA-MB-231 and BT549 cells upon IL-8 depletion. As we previously observed, the neutralization of antibodies against IL-8 specifically blocked IL-8-mediated tumor cell invasion and angiogenesis [9]. Matrigel invasion involves both the proteolytic activity of Type IV collagenase and the chemotactic motility of cells toward the growth factors present in the serum-containing medium in the bottom well of the chemotactic chamber. The well-established role and mechanism of IL-8 as a chemokine is an association between autocrine IL-8 production and an increase in the expression of invasive enzymes and integrins, such as MMP-9 and integrin αvβ3 [3,12,19]. Because the invasive activities were reduced through IL-8 siRNA, IL-8 might be a predominant chemokine in mediating the chemo-invasive potential of ER-negative breast cancer cells.

Integrin β3 plays an important role in a number of physiological and pathological processes such as angiogenesis, tumor invasion and metastasis [13,20]. We observed that the depletion of IL-8 causes a significant reduction in integrin β3 transcription and protein expression. This result was anticipated, as IL-8 increases αvβ3 integrin expression through the PI3K/Akt/AP-1 signal transduction pathway, thereby enhancing the migration of chondrosarcoma cells [21]. Because the depletion of IL-8 expression in ER-negative breast cancer cells reduces the expression of integrin β3 and the invasiveness potential, it is reasonable to infer that endogenous IL-8 might regulate invasion through integrin β3 in human breast cancer cells. The mechanism by which IL-8 regulates integrin β3 expression is currently under investigation in our laboratory.

Akt is a serine/threonine protein kinase that plays key roles in multiple cellular processes, such as cell proliferation, apoptosis, transcription, cell migration and glucose metabolism [11,22]. Activated Akt stimulates phosphorylation and impacts various downstream targets, including NF-κB, p27 and GSK-3β [23,24]. In addition, Akt-dependent phosphorylation regulates the nuclear localization of p27 [25]. In the present study, we demonstrated that the knockdown of endogenous IL-8 expression in ER-negative breast cancer cells reduces the levels of phosphorylated-Akt and upregulates the levels of p27, along with a reduction in NF-κB activities. Accumulating evidence indicates that the transcription of the IL-8 gene requires the activation of either the combination of NF-κB and AP-1 or NF-κB and NF-IL-6, depending on the cell type. However, the activation of NF-κB is indispensable for IL-8 gene activation in any types of cells [5]. The relationship between IL-8 and NF-κB might be critical for ER-negative breast cancer. Based on the data obtained from current studies, additional studies are being considered to dissect the roles of NF-κB in breast cancer progression and to determine the applicability of inhibiting NF-κB either as a primary or adjuvant approach to breast cancer therapy.

Targeted therapy offers a unique opportunity to inhibit the activity of a specific gene critical for growth and metastasis. Notably, the knockdown of IL-8 expression in MDA-MB-231 and BT549 cells, using IL-8 siRNA, significantly enhanced chemotherapy responses as increased cytotoxicity. These observations might provide a new opportunity to enhance the therapeutic efficacy of antitumor drugs, such as docetaxel. The combination of anti-IL-8 and approved chemotherapy protocols might facilitate a reduction in the dose of the drugs and increase drug efficacy.

We provide extensive evidence demonstrating that the IL-8-mediated regulation of complex intracellular molecular signaling leads to aggressive tumor cell behavior and increased survival in response to chemotherapy drug toxicity. The suppression of IL-8, using RNAi or specific cell permeable inhibitors of IL-8 or the IL-8 receptor, might sensitize ER-negative breast cancer to a wide variety of chemotherapeutic agents and increase the survival of patients with end-stage disease.

Acknowledgments

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