Tumor and Stem Cell Biology

Activation of MAPK Pathways due to DUSP4 Loss Promotes Cancer Stem Cell-like Phenotypes in Basal-like Breast Cancer

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Abstract
Basal-like breast cancer (BLBC) is an aggressive disease that lacks a clinically approved targeted therapy. Traditional chemotherapy is effective in BLBC, but it spares the cancer stem cell (CSC)-like population, which is likely to contribute to cancer recurrence after the initial treatment. Dual specificity phosphatase-4 (DUSP4) is a negative regulator of the mitogen-activated protein kinase (MAPK) pathway that is deficient in highly aggressive BLBC treated with chemotherapy, leading to aberrant MAPK activation and resistance to taxane-induced apoptosis. Herein, we investigated how DUSP4 regulates the MAP–ERK kinase (MEK) and c-jun-NH2-kinase (JNK) pathways in modifying CSC-like behavior. DUSP4 loss increased mammosphere formation and the expression of the CSC-promoting cytokines interleukin (IL)-6 and IL-8. These effects were caused in part by loss of control of the MEK and JNK pathways and involved downstream activation of the ETS-1 and c-JUN transcription factors. Enforced expression of DUSP4 reduced the CD44+/CD24− population in multiple BLBC cell lines in a MEK-dependent manner, limiting tumor formation of claudin-low SUM159PT cells in mice. Our findings support the evaluation of MEK and JNK pathway inhibitors as therapeutic agents in BLBC to eliminate the CSC population. Cancer Res; 73(20); 6346–58. ©2013 AACR.

Introduction
Despite the advent of molecularly targeted therapies in the treatment of breast cancer, conventional chemotherapy remains the main treatment of basal-like breast cancer (BLBC). BLBC is a molecular subtype characterized by gene expression patterns and immunohistochemical markers of the basal myoepithelium. It is composed primarily of "triple-negative" tumors that lack detectable estrogen receptor-α, progesterone receptor, and amplification of the HER-2/ERBB2 receptor. BLBC cell lines can be further segregated into two subgroups based on transcriptomic and phenotypic differences: Basal A and Basal B (2–4). Basal B cancer cell lines represent those highly invasive cells that show epithelial-to-mesenchymal (EMT) and cancer stem cell (CSC)-like phenotypes (2, 4). Basal B expression patterns and phenotypes have been identified in human and mouse mammary tumors of the "claudin-low" subtype (5, 6) and are highly enriched for CD44+/CD24− cells (2), which mark the EMT cell population that also contains CSC-like traits. Importantly, many BLBC tumors and cell lines enriched for these characteristics are resistant to conventional chemotherapy (7). Furthermore, drug-resistant cancer cell subpopulations are selected by treatment with chemotherapy, enriching for cells with a more aggressive phenotype (5, 7). In contrast, Basal A tumors lack EMT and CSC markers and express both CD44 and CD24 (2).

Because of the relative poor outcome of patients with BLBC, defining the molecular pathways altered in this cancer subtype remains at the forefront of translational research. It has been established that Ras/MEK/ERK activity can induce EMT (8), a trait of the tumor-initiating or CSC-like population and the claudin-low subtype (8–12). Given that Ras and Raf mutations are rare in breast cancer (13, 14), we hypothesized that alternative mechanisms of mitogen-activated protein kinase activation may play a role in promoting CSC-like traits. We propose that loss of dual specificity phosphatase-4 (DUSP4), a phosphatase against ERK1/2 and c-jun-NH2-kinase (JNK)1/2 (15), is one of those mechanisms. Indeed, we recently identified DUSP4 deficiency as a mediator of docetaxel resistance in triple-negative breast cancer (TNBC), basal-like gene expression, and high MAP–ERK kinase (MEK) activity (16).

Herein, we show that genomic DUSP4 loss is a frequent event in many cancer types, but is more pronounced in aggressive breast cancer subtypes. In BLBC cell lines, DUSP4 modulated...
the expression of CD44+/CD24- markers, mammosphere formation, and tumor initiation. DUSP4 also regulated expression and phosphorylation of cJUN and ETS-1 transcription factors and expression of interleukin (IL)-6 and IL-8. Restoration of DUSP4 expression in BT549 and SUM159PT BLBC cell lines reduced the CD44+/CD24- compartment. CSC-enriched SUM159PT cells with temporally controlled DUSP4 expression showed reduced tumorigenicity. Cells where DUSP4 expression was enforced eventually lost the DUSP4 transgene and restored the CD44+/CD24- population, suggesting that DUSP4 elicits tumor suppressor function. Collectively, these results suggest that DUSP4 is a tumor suppressor that is lost in breast cancer and can influence CSC traits. We propose that in patients with DUSP4-deficient breast cancer, therapeutic inhibition of MEK and JNK may complement chemotherapy in targeting CSCs.

Materials and Methods

Cell culture

ZR75-1, MDA-231, MDA-468, and 293FT cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Gibco) supplemented with 10% FBS (Gibco), BT-549 and HCC1143 cells were maintained in RPMI (Gibco) supplemented with 10% FBS, SUM159PT cells were maintained in DMEM supplemented with 5% FBS and 0.5 μg/mL hydrocortisone. MFM223 luminal androgen receptor (AR; ref. 17) cells were maintained in Minimum Essential Media + 10% FBS, supplemented with 1% FBS and counted after 24 hours and then replated to ultra-low attachment, 6-well or 24-well plates (Corning).

Xenograft studies

MDA-231 xenografts were generated and treated as previously described (16). For the temporally controlled DUSP4 pINDUCER model, athymic nu/nu mice (Harlan Sprague Dawley, Inc.) were primed with doxycycline (2 mg/mL in 5% sucrose, ad libitum) or 5% sucrose (control) for 2 days before injection. SUM159PT/pINDUCER-DUSP4 or parental SUM159PT cells were primed for 4 days with 2 ng/mL doxycycline before injection. Ten thousand cells were injected in Matrigel (BD Biosciences) into the left (pINDUCER cells) or right (parental cells) mammary fat pad. Doxycycline was continued in drinking water for a period of 60 days before sacrifice and examination for tumor formation.

Adenovirus transduction

Transduction and validation of GFP-expressing (AdGFP) adenovirus was conducted as previously reported (18). Adenovirus expressing DUSP4 (AdDUSP4) was purchased from Vector Biolabs.

Reagents and chemicals

Recombinant human IL-6 and IL-8 were purchased from R&D Systems, reconstituted in PBS and used at a final concentration of 10 ng/mL and 100 ng/mL, respectively. Selumetinib, U0126, SP600125, and CI1040 were purchased from Selleckchem, dissolved in dimethyl sulfoxide (DMSO), and used at a final concentration of 1, 10, 10, and 1 μmol/L, respectively. Hydrocortisone and B27 supplement were purchased from Sigma.

Immunoblotting, ELISA, and cytokine arrays

Immunoblotting was conducted as described (18). Antibodies used for immunoblotting were: p-ERK1/2 (p-T202/T204; #9101), calnexin (#2433), p-cJUN (#9165), p-JNK1/2 (#4668), JNK1/2 (#9252; all from Cell Signaling Technology), p-ETS-1 (p-T38; Invitrogen 44-1104G), ETS-1 (Santa Cruz Biotechnology; sc-350), DUSP4 (Cell Signaling Technology; #5419), Anti-HA tag (Santa Cruz Biotechnology; sc-805) and actin (Sigma; A2066). ELISAs (IL-6 and IL-8 Quantikine; R&D Systems), and cytokine arrays (RayBiotech) were conducted according to the manufacturer’s protocol.

siRNA transfection

Cells were reverse transfected in 6-well dishes or 60-mm dishes with 20 nmol/L small interfering RNA (siRNA) using Dharmafect 1 (MDA-231, BT549) or Dharmafect 4 (SUM159PT) reagents according to the manufacturer’s protocol. siRNAs targeting DUSP4 (sequence #9; Thermo Scientific cat# J-003963-09; sequence #72: Invitrogen cat# s4372; sequence #73: Invitrogen cat# s4373), ETS1 (Thermo Scientific cat# M-003887-00-0005), or C/EBP (Thermo Scientific cat# M-003268-03-0005), or nonsilencing control (Thermo Scientific cat# D-001810-10). For mammosphere assays, cells were trypsinized and counted after 24 hours and then replated to ultra-low attachment, 6-well or 24-well plates (Corning).

Flow cytometry

Cells were passed through a 35-μm filter, pelleted, washed in 1× PBS + 1% FBS, and counted. One million cells were suspended in 1× PBS + 1% FBS and stained with anti-CD44-APC conjugate and anti-CD24-PE conjugate (BD Biosciences) for 30 minutes at 4°C. Cells were washed three times and then analyzed by flow cytometry.

Quantitative real-time PCR

RNA was isolated with RNeasy kits (Qiagen) and 1 μg total RNA was used to synthesize cDNA using the iScript Kit (BioRad). Quantitative real-time PCR (qRT-PCR) was conducted on a Bio-Rad IQ thermaclycler. Standard curves were generated to estimate efficiency and the ΔΔCt method was used to quantitate fold change. Primer details are available in Supplementary Methods.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was conducted as previously described (19). MDA-231 cells were treated for 16 hours with selumetinib (1 μmol/L) before harvest; cell lysates were prepared and immunoprecipitated with an ETS-1 antibody (Santa Cruz Biotechnology; sc-350) or rabbit immunoglobulin G (IgG) control. The promoter regions −10,000 to +2000 for IL-6 and IL-8 were evaluated using ChipMapper (20). Two regions containing ETS sites were selected. Primer details are available in Supplementary Methods.

Molecular cloning

The DUSP4 open reading frame (ORF) without a stop codon was obtained from Open Biosystems (Accession no. EU831550) and was recombined into the pINDUCER-22 plasmid (21) using
Figure 1. Low DUSP4 and high MEK activation is associated with tumors and cell lines with CSC-like features. A, CCLE data SNP copy number analysis for the DUSP4 locus across 964 cancer cell lines from various tissue sources (49). Red bars show the median log2 copy number for each tissue origin. B, left, frequency histogram of TCGA (14) SNP copy number analysis for the DUSP4 locus for 443 breast cancers normalized to patient matched control tissue (log2 ratio). Right, DUSP4 log2 copy number ratio plotted by molecular subtype as determined by gene expression using the PAM50 centroids. (Continued on the following page.)
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LR Clonase (Invitrogen), resulting in doxycycline-inducible DUSP4-HA transgene expression. Short-hairpins targeting DUSP4 or nontargeting control (Cat#RHS4348) in pGPZ were purchased from Open BioSys (clone IDs: V3LHS_333999, V3LHS_334001, V3LHS_634002, V2LHS_118839) and cloned into pINDUCER-11 (21) using XhoI and MluI sites and screened for efficacy in 293FT cells. Lentiviral particles were produced by cotransflecting pINDUCER-DUSP4 (short-hairpin or ORF) plasmid with pMD2G and psPAX helper plasmids into 293FT cells. Target cells were transduced in the presence of polybrene and selected for GFP expression by fluorescence-activated cell sorting (FACS). The LacZ gene was also cloned into pINDUCER22 vector as a transgene control in selected experiments.

Mammosphere assays

Mammosphere assays were conducted by plating 10^4 cells in serum-free DMEM/F12 1:1 media (GIBCO) supplemented with EGF (20 ng/mL) and B27 (2%). MDA-231 mammospheres were grown in the absence of B27. Mammospheres were allowed to form for 3 to 7 days and were scanned and visualized by GelCount (Oxford Optronix). Mammospheres more than 100 μm in diameter were counted and average volume was estimated using the software’s (size × density) function. Total mammosphere volume/well was calculated as volume × sphere number.

Microarrays

MDA-231, BT549, and SUM159PT cells were harvested 96 hours after siRNA transfection with siDUSP4 or siCONTROL. siCONTROL cells were also treated with selumetinib (1 mol/L) for 4 or 24 hours before harvest. RNA was isolated with RNasy kits according to the manufacturer's protocol. Microarrays were conducted by the Vanderbilt Genome Sciences Resource. Additional details regarding analysis, including the acquisition and analysis of publicly available datasets are available in Supplementary Methods.

Statistical analysis

Statistical analyses (linear regression, ANOVA, and Student t tests) were conducted in R (http://cran.r-project.org) and GraphPad Prism (GraphPad Software). For two-group analyses, Student t tests were conducted. In 2 or more group analyses, ANOVA was conducted with Tukey post hoc analyses to compare individual groups.

Results

MEK pathway activity coupled with repression of DUSP4 feedback correlates with CSC features

We have reported that methylation of the DUSP4 gene is a frequent event in BLBC. However, the frequency of DUSP4 copy loss, as part of the 8p11-21 region of frequent copy number alterations (22–24), has not been well established. We used the Cancer Cell Line Encyclopedia (CCLE), which integrates genomic data on more than 600 cancer cell lines to determine whether DUSP4 copy loss is a frequent event in breast and other types of cancer cells. DUSP4 copy loss was common across all cancer cell lines, with breast cancer cells showing the lowest median copy number ratio (Fig. 1A). Next, we examined DUSP4 copy number changes in 444 breast cancers and normal breast specimens from The Cancer Genome Atlas (TCGA). Apparent peaks in the frequency distribution histograms showed common hemi- and homozygous deletion events at this locus, which were most frequent in basal-like, HER-2–enriched and luminal B cancers (Fig. 1B), whereas copy number gains were rare. These molecular subtypes represent the most aggressive and/or chemotheraphy-resistant breast cancers. Importantly, DUSP4 copy number ratio across the samples correlated strongly to DUSP4 gene expression (Supplementary Fig. S1).

DUSP4 has phosphatase activity against JNK1/2 and ERK1/2, suggesting that activation of these pathways upon DUSP4 loss drives phenotypes associated with aggressive forms of breast cancer. We used a gene expression signature of MEK activity (16, 25) to determine whether transcriptional output of MEK identifies BLBC cell lines with CSC-like traits, using a ratio of CD44/CD24 mRNA expression as a read out. Expression of CD44 and CD24 is a differentiating factor of luminal, basal A (basal-like, epithelial characteristics), and basal B (EMT and CSC enriched) cell lines (2). The MEK signature score was strongly associated with the CD44/CD24 mRNA ratio (P = 0.00064) in the ICBP50 panel of breast cancer cell lines (Fig. 1C).

Next, we determined whether the MEK signature score was associated with the CSC trait of mammosphere formation (7, 25). The MEK signature score was high in mammospheres derived from primary breast tumors but not in RNA extracted from the matched primary tumors (Fig. 1D and E; P < 0.0001; ref. 7), suggesting that MEK activation is upregulated in the CSC-like population. Furthermore, DUSP4 mRNA expression was negatively associated with the CD44/CD24 mRNA ratio in the ICBP50 panel, specifically in cell lines with a high MEK score (Fig. 1F). This distinction is important, as DUSP4 is an immediate early gene that is upregulated following MEK activation under normal conditions (26). Thus, in cell lines with low MEK activity, DUSP4 expression would also be expected to be low. However, in a MEK-activated cell line, DUSP4 downregulation (via copy loss or methylation) would result in unrestricted pathway activity.

Next, we profiled MEK and JNK pathway activation across a panel of breast cancer cell lines. The majority of BLBC cell lines showed high expression and activation of the ETS-1...
Figure 2. Loss of DUSP4 function upregulates IL-6 and IL-8 and enhances mammosphere growth. A, immunoblot of MDA-231 cells 96 hours after siCONTROL or siDUSP4 transfection in 10% FBS-containing medium or after 24 hours of serum starvation in 0.1% FBS-containing medium. B, mammosphere growth assay in MDA-231 cells, quantitated by GelCount software 6 days after siRNA transfection (5 days after plating to mammosphere culture conditions; *** P < 0.001 for a two-tailed Student t test). (Continued on the following page.)
and cJUN transcription factors, which lie downstream of the DUSP4 targets, JNK1/2 and ERK1/2. These transcription factors were most highly expressed in the Basal B or claudin-low cell lines (MDA-231, SUM159PT, and BT549), which also exhibit CSC-like properties (2, 4, 5). Baseline DUSP4 expression was lower in Basal B cell lines as compared with Basal A and luminal cell lines, including the luminal/AR-expressing MFM223 (17), with the exception of MDA-231 cells. MDA-231 cells, which harbor mutant KRAS<sup>G13D</sup>, had higher DUSP4 expression than the other cell lines tested, consistent with findings in colorectal cancer where KRAS mutations have been shown to upregulate DUSP4 expression to compensate for enhanced MEK pathway activity (27). As this cell line has basallike expression associated with considerable DUSP4 expression and, as such, represents an ideal model to study loss of DUSP4 function.

**Loss of DUSP4 enhances mammosphere formation and MEK- and JNK-dependent IL-6 and IL-8 expression**

In MDA-231 cells, downregulation of DUSP4 by each of three siRNAs resulted in an increase in JNK activity as measured by cJUN phosphorylation and mammosphere volume relative to control siRNA (Fig. 2A and B). siRNA construct #73 produced a more subtle phenotype than the other constructs, despite apparent efficient DUSP4 knockdown. Longer exposures revealed residual DUSP4 expression with this siRNA, which was confirmed by qRT-PCR (data not shown), providing a possible explanation to the variability between effect sizes observed with the siRNAs. An increase in ERK activation could not be observed, possibly due to the high intrinsic activation level of this pathway in MDA-231 cells. However, both cJUN and ETS-1, downstream targets of JNK and ERK, respectively, showed increased levels and/or activation upon loss of DUSP4 in nonadherent conditions (Fig. 2C). To determine whether this phenotype was cell-autonomous, we cultured SUM159PT cells as mammospheres in serum-free media conditioned by MDA231 cells treated with siCONTROL or siDUSP4. Conditioned medium from MDA-231/siDUSP4 cells stimulated SUM159PT mammosphere formation 2- to 3-fold compared with medium from MDA-231/siCONTROL cells, suggesting that loss of DUSP4 resulted in the secretion of mammosphere-stimulating paracrine factors (Fig. 2D). Cytokine arrays of conditioned media showed that IL-6, a cytokine that stimulates CSC expansion (28–31), was upregulated following DUSP4 knockdown (Supplementary Fig. S2) and this effect was primarily transcriptional (Fig. 2E and F). IL-8 was also moderately increased in the conditioned media. DUSP4 knockdown using a doxycycline-inducible DUSP4 shRNA (shDUSP4) resulted in upregulation of both IL-6 and IL-8 when the cells were cultured for longer time periods (>10 days; Supplementary Fig. S3). However, transcriptional upregulation of IL-8 was not observed following transient DUSP4 knockdown using siRNA (data not shown). Transcriptional upregulation of IL-6 was partially blocked by MEK (selumetinib/AZD6244) inhibition, but not by the JNK inhibitor (SP600125), whereas the combination had the most profound effect (Fig. 2G). cJUN phosphorylation and expression were downregulated by both the MEK and JNK inhibitors, with maximal inhibition by the combination (Fig. 2H). This is consistent with previous reports that both ERK and JNK can regulate cJUN (32–34). Combined inhibition of both MEK and JNK abrogated the upregulation of MDA-231 mammosphere growth observed upon knockdown of DUSP4 with siRNA (Fig. 2I).

To determine the specificity of the MEK pathway in tumor self-renewal in MDA-231 cells, we conducted the mammosphere assay in the presence of two additional MEK inhibitors (U0126 and CI-1040) or the dual phosphoinositide 3-kinase (PI3K)/mTOR inhibitor BEZ235 (35). Under basal conditions, only U0126 reduced primary mammosphere growth. However, after the spheres were collected, trypsinized, and replated in the absence of drug, no spheres formed in plates treated with either MEK inhibitor. Spheres treated with DMSO control reformed quickly, whereas BEZ235-treated spheres formed, albeit at a reduced rate. When the secondary spheres and residual cells were collected and plated under adherent conditions in the presence of serum, only the control and BEZ235-treated cells attached and resumed normal proliferation (Supplementary Fig. S4).

**DUSP4 regulates IL-6 and IL-8 expression via ETS-1 and cJUN**

In two large breast cancer datasets, DUSP4 mRNA expression negatively correlated with IL-6 and IL-8 expression, suggesting that DUSP4 regulates the expression of these cytokines in vivo (Fig. 3A). Furthermore, in the TCGA breast cancer dataset, genomic deletion of DUSP4 was associated with high expression of cJUN phosphorylated at Ser73, a known activation site (Supplementary Fig. S5; ref. 36). To determine whether the JNK and MEK pathways regulate IL-6 and IL-8 expression in BLBC cells with low DUSP4 expression, we treated BT549 and SUM159PT cells with the MEK or JNK inhibitor or the combination. IL-6 and IL-8 mRNA expression and respective ligand secretion were inhibited to the greatest degree in both cell lines following treatment with the MEK inhibitor (Fig. 3B and C), whereas the effect of the JNK inhibitor was more variable. Of note, in...
BT549, only the JNK inhibitor downregulated IL-6 transcription, but this downregulation did not translate into reduced IL-6 ligand in the conditioned media. Only the MEK inhibitor downregulated total ETS-1 or T38 P-ETS-1 levels (T38 is phosphorylated by ERK1/2), whereas the MEK and JNK inhibitors additively reduced total cJUN and p-cJUN levels (Fig. 3D). These results support the previously reported cross-talk between the AP-1 (cJUN and cFOS) and ETS-1 transcription factors (37, 38). Finally, in MDA-231 cells, ChIP with an ETS-1 antibody identified a binding region

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Figure 3. DUSP4 regulates ETS-1 and cJUN and downstream IL-6 and IL-8 expression via MEK and JNK. A, correlation of IL-6 mRNA and IL-8 mRNA with DUSP4 mRNA in 2 external microarray datasets of primary breast cancer. B, qRT-PCR analysis of BT549 and SUM159PT cells treated for 16 hours with 10 μmol/L SP600125 (JNKi), 1 μmol/L selumetinib (MEKi), or the combination. C, IL-6 and IL-8 ELISA analyses of conditioned media from BT549 and SUM159PT cells collected after 24 to 48 hours of treatment with the indicated inhibitors. D, immunoblot analysis of lysates from cells shown in B and C, E, immunoblot analysis of BT549 and SUM159PT cells after 16 hours of adenovirus transduction with AdDUSP4 or AdGFP control. F, qRT-PCR analysis of IL-6 and IL-8 mRNAs in cells from G.
of ETS-1 in the IL-8 promoter and ETS-1 was abrogated by treatment with the MEK inhibitor selumetinib (Supplementary Fig. S6).

Adenoviral transduction of DUSP4 (AdDUSP4) recapitulated the effects of the JNK and MEK inhibitors by downregulating p-cJUN, cJUN, p-ETS-1, ETS-1, and p-JNK (Fig. 3E). Interestingly, DUSP4 overexpression did not decrease ERK1/2 phosphorylation. In BT549 and SUM159PT cells, DUSP4 overexpression significantly downregulated IL-6 and IL-8 transcription. siRNA knockdown of ETS-1 or cJUN in SUM159PT cells downregulated IL-6 and IL-8 transcription, suggesting that these transcription factors contribute to expression of this CSC-promoting cytokine (Fig. 3G and H).

MEK regulates mammosphere growth in an IL-6- and/or IL-8–dependent manner in BLBC

Next, we tested whether MEK inhibition would reduce mammosphere growth. Selumetinib treatment inhibited SUM159PT and BT549 mammosphere growth (Fig. 4A and B). Reconstitution with recombinant IL-6 (BT549) or the combination of IL-6 and IL-8 (SUM159PT) restored this phenotype (Fig. 4B). Similar effects were observed with the MEK inhibitors U0126 and CI1040 in SUM159PT cells (Supplementary Fig. S7A). Furthermore, when viable mammospheres were dissociated and replated in the absence of drug, a significant impact on secondary mammosphere formation was also observed (Supplementary Fig. S7B).

**Figure 4.** MEK inhibition decreases mammosphere formation in an IL-6/IL-8 dependent manner. A, representative images of mammospheres derived from SUM159PT and BT549 cells grown in the presence of selumetinib or DMSO. B, quantification of mammospheres derived from SUM159PT and BT549 cells, grown in the presence of DMSO, selumetinib (MEKi), or selumetinib plus either IL-6, IL-8, or both IL-6 and IL-8. P represents the result of an ANOVA with Tukey’s post hoc test to compare individual treatment groups (***, P < 0.001). Bars represent mean of 3 replicates ± SD. C, qRT-PCR analysis of IL-6 and IL-8 mRNAs in MDA-231 xenografts treated for 3 days with docetaxel, selumetinib, or the combination (16). D, MDA-231 xenografts treated for 4 weeks with docetaxel, selumetinib, or the combination (16) were harvested; cells were mechanically and enzymatically dissociated and plated in a mammosphere assay as described in Materials and Methods. E, CD44/CD24 FACS analysis of cells dissociated from MDA-231 xenografts from D.
Taxanes have been shown to spare CSCs (7, 39). To determine whether this is due to drug-induced expression of CSC-promoting cytokines, we used MDA-231 xenografts treated for 4 weeks with docetaxel, selumetinib, or the combination of both drugs (16). Xenografts from docetaxel-treated mice exhibited markedly higher levels IL-6 and IL-8 mRNA levels compared with control-treated tumors (Fig. 4C). Cotreatment with selumetinib partially inhibited this upregulation, suggesting MEK inhibitors may be an effective therapeutic complement to taxanes in BLBC. When tumors were dissociated and plated in a mammosphere assay, cells from selumetinib-treated tumors formed smaller and fewer mammospheres, whereas tumor cells derived from tumors treated with the combination did not form spheres (Fig. 4D). Furthermore, cells dissociated from xenografts that had been treated with the combination of selumetinib and docetaxel contained fewer CD44+/CD24− cells compared with the other treatment groups as analyzed by FACS (Fig. 4E).

Gene expression changes following DUSP4 loss resemble BLBC

Next, we examined global gene expression changes induced by siRNA-mediated DUSP4 loss or treatment with selumetinib for 4 or 24 hours in MDA-231, SUM159PT, and BT549 cells. The genes modulated by DUSP4 siRNA in MDA-231 cells (which have higher expression of DUSP4) tended to oppose those modulated by the MEK inhibitor (Fig. 5A). For example, when comparing siDUSP4 transfection with 24-hour treatment with selumetinib, 24% of genes showed directional concordance consistent with the known biology of DUSP4 (i.e., were upregulated by DUSP4 knockdown and downregulated by the MEK inhibitor). However, 19% of genes showed discordance (i.e., were upregulated by both DUSP4 knockdown and selumetinib).

MDA-231 cells showed the most profound gene expression changes following siDUSP4 transfection. We took the significantly changed genes (up- and downregulated) identified in MDA-231 cells and scored the other cell lines using this
suggestion, siDUSP4 treatment induced similar changes in SUM159PT and BT549 cells, albeit to a lesser extent than in MDA-231 cells (Fig. 5B). Interestingly, treatment with selumetinib for 4 or 24 hours only moderately decreased the DUSP4 loss score, suggesting that loss of DUSP4 also modulates MEK-independent gene expression. These effects of DUSP4 loss may also reflect derepression of the JNK pathway or other yet undiscovered function(s) of DUSP4.

Consistent with our report that DUSP4 loss reduces the chemosensitivity of MDA-231 cells (16), a number of genes associated with drug resistance (another CSC/tumor-initiating trait) were also upregulated following DUSP4 knockdown, including ERCC6, RRM2, and ABCG2 (Fig. 5C). To test how the signature of DUSP4 loss correlates with the molecular subtypes of breast cancer, we plotted the TCGA breast cancer gene expression data (N = 444) and the siDUSP4 score; gene expression patterns induced by loss of DUSP4 most closely resembled those of BLBC (Fig. 5D). Furthermore, DUSP4 loss in MDA231 cells significantly altered genes associated with the claudin-low subtype, a CSC enriched-phenotype (5). In these cells, the claudin-low score was reduced by selumetinib treatment (Fig. 5E). These data suggest that DUSP4 loss transcriptionally activates programs associated with basal-like and claudin-low breast cancer.

**Enforced DUSP4 expression regulates CD44/CD24 and tumor initiation**

To determine whether enforced expression of DUSP4 alters the CD44+/CD24− population in BLBC, we used the pINDUCER rtTA system (21) to conditionally express DUSP4 in SUM159PT and BT549 cells. We detected HA-tagged DUSP4 expression within 24 hours of doxycycline treatment (2 ng/mL; data not shown). Cells were cultured in doxycycline for 0 to 10 days and analyzed by flow cytometry for CD44 and CD24 expression. Both BT549 and SUM159PT are claudin-low/Basal B-cell lines (4, 5) with a high population of tumor-initiating CD44+/CD24− cells. After doxycycline treatment, CD24 expression was markedly increased, shifting the population away from CD44+/CD24−; this effect was maximal at 4 days (Fig. 6A and Supplementary Fig. S8A). Similarly, cells treated for 4 days with selumetinib substantially upregulated the CD24+ population. The JNK inhibitor had only a modest effect (Supplementary Fig. S8B). These findings are supported by the previous microarray data showing upregulation of CD24 mRNAs upon 24 hours of selumetinib. They also imply that MEK activation modulates CD24 expression. Importantly, high CD24 and high CD44 expression is a defining feature of the Basal A subtype (2), suggesting that MEK inhibition may "convert" mesenchymal BLBCs to those with epithelial and thus less aggressive features. Recombinant IL-6 and/or IL-8 coadministered with doxycycline to SUM159PT/pINDUCER-DUSP4 cells did not restore the CD44+/CD24− population, suggesting that enforced DUSP4 expression decreases IL-6 and IL-8 expression as well as alters the CD44+/CD24− population in trans and not in cis (Supplementary Fig. S8C). Doxycycline treatment of SUM159PT/pINDUCER-DUSP4 cells also decreased mammosphere formation in vitro (Fig. 6B). To determine whether enforced DUSP4 expression is sufficient to reduce the tumor-initiating population in vivo, we pretreated SUM159PT/pINDUCER-DUSP4 cells with doxycycline for 2 days and injected 1 x 104 cells into the left mammary fat pad of athymic mice. Parental SUM159PT cells were used to control for the treatment effects of doxycycline and injected in the contralateral mammary fat pad. Following tumor cell inoculation, mice were treated with ± doxycycline for 60 days and monitored for tumor formation (Fig. 6C and D). At 60 days, tumors were apparent in 4 of 5 mice injected with SUM159PT/pINDUCER-DUSP4 cells, whereas no doxycycline-supplemented mice developed obvious tumors. On microscopic inspection of the injection site, one mouse treated with doxycycline showed viable SUM159PT/pINDUCER-DUSP4 cells, suggesting delayed tumor development. This could be partially explained by the observation that after 30 days of DUSP4 induction with doxycycline, SUM159PT cells downregulated the transgene and restored their original CD44+/CD24− population (Supplementary Fig. S9). Collectively, these results suggest that (i) enforced DUSP4 expression reduces the tumor-initiating population in BLBC and (ii) DUSP4 has a tumor suppressor function in BLBC.

**Discussion**

Conventional chemotherapies spare CSC-like tumor cells, leading to the outgrowth of resistant subpopulations that repopulate the tumor (7). An increasing body of literature suggests that the CSC population exhibits plasticity, thus implying that cancer cells can shift between CSC and non-CSC phenotypes (40). Therefore, therapies that target the CSC population in a tumor would be an effective approach to treatment. Herein, we have explored the role of the DUSP4 phosphatase in regulating CSC-like traits in BLBC. We show that DUSP4 is lost in breast cancer at high frequency. Loss-of-function studies in MDA-231 cells showed that DUSP4 suppresses ETS-1 and cJUN activity. Relief from DUSP4-mediated negative feedback upregulates IL-6 and IL-8 in a MEK and JNK-dependent manner, whereas enforced DUSP4 expression downregulates IL-6 and IL-8 expression, decreases the CD44+/CD24− CSC-like population, and reduces tumorigenicity. These effects of DUSP4 are mainly regulated via the MEK pathway, although suppression of JNK signaling contributes as well, possibly via cross-talk between ETS-1 and cJUN.

Our findings linking aberrant MEK activity and CSC-like traits are consistent with concepts proposed by prior studies. In particular, Morel and colleagues showed that immortalized primary breast epithelial cells were primarily CD44+/CD24−, whereas the same cells transformed with activated HRAS (G12V) became CD44+/CD24− (8). These cells, originally generated and characterized by Elenbaas and colleagues (41), underwent EMT and acquired stem cell properties following transduction with HRAS. This result parallels our finding that CD24 expression is acquired in BT549 and SUM159PT BLBC cells following enforced DUSP4 expression.

There are several limitations to these studies. Although mammosphere formation and CD44/CD24 expression are not absolute CSC markers, the use of these assays enriches for CSC-like traits (42, 43). The majority of breast cancer cells grown as mammospheres are CD44+/CD24−, whereas only 10% to 20% of these possess tumor-initiating properties (44). Other
Figure 6. DUSP4 is a tumor suppressor that modulates the CD44+/CD24− stem-enriched population and tumor formation. SUM159PT cells transduced with doxycycline (DOX)-inducible DUSP4-HA were generated. A, flow cytometry analysis of CD44/CD24 expression in cells treated for 1 to 10 days with doxycycline (2 ng/mL). B, SUM159PT/pINDUCER-LACZ cells or SUM159PT/pINDUCER-DUSP4 pretreated for 4 days with doxycycline before plating in a mammosphere assay in the presence or absence of doxycycline as in the pretreatment. C, immunoblot of parental SUM159PT/pINDUCER-LACZ cells or SUM159PT/pINDUCER-DUSP4 treated for 4 days with doxycycline. D, tumor formation of cells from C 60 days after injection the mammary fat pad of athymic mice. Red bars indicate palpable tumors and blue bars indicate tumors identified on histologic examination of the injection site. E, schematic of proposed model based on the presented data: the tumor suppressor DUSP4 negatively regulates ERK and JNK. Upon loss of DUSP4, derepressed ERK activity stimulates ETS1 and cJUN-mediated transcription of IL-6 and IL-8, cytokines that expand the cancer stem-like cell population. Derepressed ERK transcription following DUSP4 loss also suppresses CD24 expression thus increasing the CD44+CD24− compartment, a marker of the CSC population.
DUSP4 Loss Promotes CSC Phenotypes in Basal-like Breast Cancer

attempts have been made to further enrich the CSC population using a variety of markers, including PROCR, EPCAM, and functional aldehyde dehydrogenase activity (ALDEFLUOR assay; refs. 28, 45–47). However, many of these markers are cell line–specific (47), which complicates analysis across multiple cell lines, and are not as well established as CD44/CD24. Second, enforced DUSP4 expression had a more pronounced phenotype in the SUM159PT cell line compared with the BT549 cell line. DUSP4 expression simultaneously downregulated IL-6 and IL-8 expression in SUM159PT cells, whereas only IL-8 was markedly affected in the BT549 line. Likewise, SUM159PT cells escaped enforced DUSP4 expression after 30 days, whereas BT549 cells did not, suggestive of an “acquired” tolerance to DUSP4 expression in SUM159PT cells, whereas an intrinsic resistance was present in BT549 cells. Importantly, BT549 cells are PTEN-null, resulting in deregulated high PI3K activity. This alteration may represent a mechanism for dispensability of MEK and JNK activity during enforced DUSP4 expression. Indeed, other investigators have shown that aberrant PI3K activity can also maintain the claudin-low phenotype (48).

In summary, DUSP4 is frequently lost in breast cancer, representing a mechanism of MEK and JNK activation, which drives CSC-like traits, including mammosphere formation, IL-6 and IL-8 expression, CD44/CD24 expression patterns, and tumor formation. Although these traits are not linearly connected, they seem to predominate from DUSP4-mediated tumor formation. Treatment of DUSP4-deleted populations of tumor cells and thus complement conventional chemotherapy and functional aldehyde dehydrogenase activity (ALDEFLUOR assay; refs. 28, 45–47). However, many of these markers are cell line–specific (47), which complicates analysis across multiple cell lines, and are not as well established as CD44/CD24. Second, enforced DUSP4 expression had a more pronounced phenotype in the SUM159PT cell line compared with the BT549 cell line. DUSP4 expression simultaneously downregulated IL-6 and IL-8 expression in SUM159PT cells, whereas only IL-8 was markedly affected in the BT549 line. Likewise, SUM159PT cells escaped enforced DUSP4 expression after 30 days, whereas BT549 cells did not, suggestive of an “acquired” tolerance to DUSP4 expression in SUM159PT cells, whereas an intrinsic resistance was present in BT549 cells. Importantly, BT549 cells are PTEN-null, resulting in deregulated high PI3K activity. This alteration may represent a mechanism for dispensability of MEK and JNK activity during enforced DUSP4 expression. Indeed, other investigators have shown that aberrant PI3K activity can also maintain the claudin-low phenotype (48).

In summary, DUSP4 is frequently lost in breast cancer, representing a mechanism of MEK and JNK activation, which drives CSC-like traits, including mammosphere formation, IL-6 and IL-8 expression, CD44/CD24 expression patterns, and tumor formation. Although these traits are not linearly connected, they seem to predominate from DUSP4-mediated control over the cJUN and ETS-1 transcription factors. Treatment of DUSP4-deficient BLBC with MEK and potentially JNK inhibitors may improve outcomes by affecting the CSC-like population of tumor cells and thus complement conventional anticancer chemotherapy in this subtype of breast cancer.

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

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References


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