Lactoferrin–Endothelin-1 Axis Contributes to the Development and Invasiveness of Triple-Negative Breast Cancer Phenotypes

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Abstract

Triple-negative breast cancer (TNBC) is characterized by the lack of expression of estrogen receptor-α (ER-α), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER-2). However, pathways responsible for downregulation of therapeutic receptors, as well as subsequent aggressiveness, remain unknown. In this study, we discovered that lactoferrin (Lf) efficiently downregulates levels of ER-α, PR, and HER-2 in a proteasome-dependent manner in breast cancer cells, and it accounts for the loss of responsiveness to ER- or HER-2–targeted therapies. Furthermore, we found that lactoferrin increases migration and invasiveness of both non-TNBC and TNBC cell lines. We discovered that lactoferrin directly stimulates the transcription of endothelin-1 (ET-1), a secreted proinvasive polypeptide that acts through a specific receptor, ET(A)R, leading to secretion of the bioactive ET-1 peptide. Interestingly, a therapeutic ET-1 receptor-antagonist blocked lactoferrin-dependent motility and invasiveness of breast cancer cells. The physiologic significance of this newly discovered Lf–ET-1 axis in the manifestation of TNBC phenotypes is revealed by elevated plasma and tissue lactoferrin and ET-1 levels in patients with TNBC compared with those in ER+ cases. These findings describe the first physiologically relevant polypeptide as a functional determinant in downregulating all three therapeutic receptors in breast cancer, which uses another secreted ET-1 system to confer invasiveness. Results presented in this article provide proof-of-principle evidence in support of the therapeutic effectiveness of ET-1 receptor antagonist to completely block the lactoferrin-induced motility and invasiveness of the TNBC as well as non-TNBC cells, and thus, open a remarkable opportunity to treat TNBC by targeting the Lf–ET-1 axis using an approved developmental drug, Cancer Res; 71(23): 7259–69. ©2011 AACR.

Introduction

Among all breast cancers, approximately 10% to 15% are categorized as triple-negative breast cancer (TNBC; refs. 1–3). TNBC is characterized by the presence of low levels or absence of estrogen receptor-alpha (ER-α), progesterone receptor (PR), and human epidermal growth factor receptor (HER-2), and lack of effective therapies targeting these receptors leads to poor prognosis (4, 5). While reviewing the previously published literature, we noticed evidence of an inverse correlation between the levels of PR or ER-α and lactoferrin (Lf; ref. 6) in endometrial adenocarcinomas or in primary breast tumors, respectively (7–10). Lactoferrin, a member of the transferrin family, was first discovered as an extracellular iron-binding glycoprotein. Since then, lactoferrin has been extensively studied and shown to play a major role in anti-inflammation and bactericidal events. Because lactoferrin is a hormone-responsive gene (11) and its levels are modulated by a variety of signals (12), we hypothesized that elevated levels of lactoferrin may be associated with a decreased expression of ER-α and PR and, perhaps, HER-2 and, therefore, could contribute to the development of TNBC phenotypes. Considering lactoferrin’s abundance in exocrine secretions and its proliferative potential on certain cell types, in this study we aimed to investigate the role of lactoferrin in the development of TNBC, and discovered that lactoferrin efficiently downregulates the levels of ER-α, PR, and HER-2 at a posttranscriptional level in multiple breast cancer cell lines. Furthermore, we found that lactoferrin-induced increased invasion of breast cancer cells and human epidermal growth factor receptor (HER-2; ref. 4),...
mechanistically mediated via transcriptional stimulation of endothelin-1 (ET-1; ref. 13) and could be effectively blocked by therapeutic antagonists of the ET-1 receptor. Although the mechanism of loss of receptors during the development of TNBC is currently understood poorly, our study provides an insight into the molecules/pathways that could be responsible for this progression and, consequently, could contribute to the development of TNBC phenotypes.

Materials and Methods

**Human patient samples**

Dr. Susanne Fuqua (Baylor College of Medicine, Houston, Texas) provided human breast cancer tissue RNA samples. Dr. George C. Prendergast (Lankenau Institute for Medical Research, Wynnewood, Pennsylvania) provided the plasma samples along with the corresponding tissue slides. All the human samples were used in accordance with the Institutional Review Board procedures at the respective institutions.

**Cell lines and cell culture**

The breast cancer cell lines MCF-7, ZR-75, MDA-MB-231, MDA-MB-468, and SK-BR-3 and the colon cancer cell line Caco-2 were obtained from the American Type Culture Collection. All cells were maintained at 37°C in 5% CO₂ in Dulbecco’s Modified Eagle’s Medium (DMEM)/F12-50/50 (Mediatech, Inc.) supplemented with 10% FBS (Atlanta Biologicals) and 1% antibiotics (Gibco, Invitrogen). During serum-starvation, cells were incubated with serum-free DMEM supplemented with 1% antibiotics. The TNBC cell line MDA-MB-231 was selected for generating stable clones. The cells were transfected with the following plasmids: pcDNA 3.1 (Invitrogen; as control), ER, and HER-2. The stable clone cell lines (pcDNA, ER, and HER-2) were maintained in DMEM/F12-50/50 supplemented with 10% FBS, 1% antibiotics, and 0.5 μg/mL of G418 (Sigma-Aldrich).

**Protein analysis**

For lactoferrin treatment, cells were maintained in serum-free DMEM for 48 hours and then treated with 20 μg/mL of lactoferrin (Sigma-Aldrich). Protein samples were separated by SDS-PAGE and then transferred onto a nitrocellulose membrane (Bio-Rad). The primary antibodies used included: anti-ER-α (Bethyl Laboratories), anti-HER-2 (Bethyl Laboratories), anti-PR (Dako), anti-IGF-IRβ (Santa Cruz Biotechnology, Inc.), anti-RXRα (Santa Cruz Biotechnology, Inc.), anti-cyclin D1 (Labvision/Thermo Fisher Scientific), anti-tubulin (Sigma-Aldrich), and anti-vinculin (Sigma-Aldrich).

**Immunohistochemistry and confocal microscopy**

Defaraffinized sections were treated with 0.3% hydrogen peroxide in methanol and subjected to antigen retrieval by boiling the sections in antigen-unmasking solution (Vector Laboratories). The sections were then blocked with 5% skimmed milk in PBS (Mediatech Inc.) and incubated with lactoferrin antibody (Lf-Ab; 1:500) or endothelin antibody (1:100) at 4°C overnight, followed by incubation with Envision (Dako) for 1 hour at room temperature and visualization with liquid DAB+ substrate chromogen system (Dako). Immunostained sections were lightly counterstained with hematoxylin, dehydrated in graded ethanol, cleared in xylene, and mounted with the use of the permount mounting medium.

For confocal imaging, ZR-75 or MCF-7 cells were plated onto cover slips in 6-well plates, starved for 48 hours, and treated with 20 μg/mL of lactoferrin. After a 12-hour treatment, the cells were fixed with 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and blocked in 10% normal goat serum in PBS. The cells were incubated with primary antibodies, washed 3 times in PBS, and then incubated with goat anti-mouse or goat anti-rabbit secondary antibodies conjugated with Alexa-488 or Alexa-555 (Molecular Probes). 4′,6-Diamidino-2-phenylindole (DAPI) was used for nuclear staining. The slides were then examined using a Zeiss LSM 710 confocal microscope and images were acquired with the help of Zen 2009 software. Images were converted to TIFF format using Image J software.

**Northern blotting**

For Northern blotting, MCF-7 as well as MDA-MB-231 and MDA-MB-468 cells were treated with 100 μg/mL lactoferrin for 6 hours. A RNA sample amount of 20 μg was loaded onto a formaldehyde denaturing gel (1.5% agarose, 2.2 mol/L formaldehyde). Subsequently, RNA was transferred to a Hybond-N membrane (GE Healthcare UK Limited) and then immobilized through covalent linkage to the membrane by UV cross-linker. A complimentary RNA ET-1 probe was prepared using Ambion’s In Vitro Transcription Kit (Ambion, Inc.) with radiolabeled UTP. Finally, the membrane was exposed to a phosphor imager and the autoradiogram was developed using the Storm 865 Imager (GE Healthcare UK Limited). For another experiment, MDA-MB-231 cells were plated and then serum-starved for 48 hours. The cells were either treated with 20 μg/mL of cycloheximide (CHX), 100 μg/mL lactoferrin, or both. CHX was administered 1 hour before lactoferrin treatment. After 6 hours of treatment with lactoferrin, RNA was isolated from the cells using TRIzol method, and RNA levels were detected using the Northern blotting protocol as described above.

**Wound-healing assay**

To analyze the effect of lactoferrin with or without tamoxifen on cell migration, MCF-7 cells were plated with 10% fetal calf serum (FCS)–DMEM. Before treatment, each plate received multiple “wounds” with a 200-μL pipette tip. Treatments were carried out with low-serum medium supplemented with 100 μg/mL lactoferrin and 100 nmol/L tamoxifen (Sigma-Aldrich). In another set, the cells were treated with and without lactoferrin in addition to 10 nmol/L of Herceptin (Genentech, Inc.) and in combination. After 24 hours, each plate was examined for the amount of wound closure by measuring the physical separation remaining between the original wound widths using the Olympus DP2-BSW digital camera software (Olympus). Ten separate measurements were made per plate, and each experiment was carried out in triplicate.

**Migration assay**

MCF-7 cells were serum-starved for 48 hours and then resuspended in the presence of 0.1% BSA. Subsequently, 1 ×
10^5 cells/well were loaded onto the upper well of an uncoated Boyden chamber. ET-1 and BQ123 were diluted in plain medium before plating. The lower chamber was supplemented with conditioned medium of NIH-3T3 fibroblasts grown in DMEM/F-12 medium with 0.1% BSA. After fixation with methanol and staining with 0.05% crystal violet, the number of cells that successfully migrated through the filter was counted. Data were analyzed using the Olympus DP2-BSW digital camera software.

**Invasion assay**

Cells were serum-starved for 48 hours and then seeded at a density of 1 × 10^5 cells/well in the upper well of the Matrigel Invasion Chambers (BD Biosciences). At the time of the cell seeding, each well was treated with 100 μg/mL of lactoferrin. For E2 treatment, cells were serum-starved in phenol red–free medium for 48 hours. At 6 hours before trypsinizing for collection, the cells were treated with 100 μg/mL of lactoferrin. After 24- to 48-hour incubation, the number of the invaded cells was counted and the results were expressed as percentages of control. For MDA-MB-231 and MDA-MB-468, the invaded cells at the bottom were fixed with 4% paraformaldehyde and stained for DAPI after 48 hours.

**Microarray analysis of lactoferrin-regulated genes**

MDA-MB-231 and MDA-MB-468 cells were plated in triplicates, serum-starved for 48 hours, and then treated with 100 μg/mL of lactoferrin for 6 hours. RNA was isolated using TRIzol according to the manufacturer’s instructions. CDNA samples were generated and hybridized onto an Affymetrix Human Exon 1.0 ST Array chip. GeneSpring GX was used to process the data and statistical analysis was carried out by means of an unpaired t-test. A fold change of ≥1.5 was used to identify differentially regulated genes and those with a P-value ≤ 0.05 were considered statistically significant. Heat-map analysis of the identified genes for individual arrays was performed using the MultiExperiment viewer version 4.4 (Dana-Farber Cancer Institute).

**Luciferase assay**

MCF-7 as well as MDA-MB-231 and MDA-MB-468 cells were grown to 50% confluence. ET-1 promoter, β-galactosidase, and control plasmids were transfected into cells using FuGENE 6 according to the manufacturer’s instructions (Roche). After lactoferrin treatment, cell lysates were prepared using 200 μL of Tropix Lysis solution (Applied Biosystems). For luciferase promoter activity, 10 μL of lysate was incubated with 100 μL Luciferase Assay Substrate solution (Promega) and measurements were taken using LUMAT LB 9507 luminometer (Bertold Technologies). β-Galactosidase was used as a transfection control.

**Electrophoretic mobility shift assay**

PCR-amplified DNA fragments or oligos containing lactoferrin consensus sites were 5’-end labeled using γ^32p and PNK enzyme. End-labeled fragments were purified using sephadex G-50 spin columns (GE Healthcare UK Limited). Each fragment was incubated with purified holo- or apo-lactoferrin-treated (GenWay Biotech) or lactoferrin-treated as well as untreated nuclear extracts for 15 minutes at room temperature. The reaction samples were resolved on a 5% native PAGE for 2 hours. Gels were removed and dried and exposed to phosphor imager. The final images were developed using the Storm 865 Imager (GE Healthcare UK Limited).

**Plasma protein detection using ELISA**

For detection of lactoferrin, the AssayMax Human Lactoferrin ELISA Kit (AssayPro) was used with a 1:100 dilution of serum samples. ET-1 levels were measured using the Quantiglo Human Endothelin-1 Kit (R&D Systems) by following the manufacturer’s manual of instructions. For ET-1 levels, the 1450 Microbeta Jet Microplate Scintillation and Luminescence Counter (Perkin Elmer Life and Analytical Sciences) were used to measure the luminescence. A dilution of 1:5 for the serum was used for the ET-1 ELISA assay.

**RNA isolation and quantitative real-time PCR**

Total RNA from human breast tumor tissue samples as well as human breast cancer cell lines was isolated using TRIzol (Invitrogen) in accordance with the manufacturer’s protocol. A 2-μg RNA sample was used to synthesize cDNA using Invitrogen’s SuperScript III First-Strand Synthesis SuperMix by following the manufacturer’s instructions. The following primers (synthesized by Sigma-Aldrich) were used for target genes: lactoferrin forward: 5’-ACCCGCACATGAAACTTGT-3’ and lactoferrin reverse: 5’-GGGGAGTCTCTCTTTATGC-3’; ET-1 forward: GCCAAAGGACCTCCAAACACGAG ET-1 reverse: AGCCAGGACCGGCTTGGAC; actin forward: 5’-TCTCTGGA-GAAAGCTTACG-3’; and actin reverse: 5’-GTACTTGGCCT-CAGGAGG-3’. Normalized expression of target genes were calculated relative to endogenous expression of actin (relative expression = 2^(-ΔΔCt)).

**Statistical analysis**

Paired Student t test was used for statistical significance of differences in numerical data. All of the statistical tests were 2-sided. P-value of less than 0.05 was considered to be statistically significant.

**Results**

**Lactoferrin downregulates ER-α, PR, and HER-2 receptor in human breast cancer cells**

Although a large body of studies have firmly established the significance of the loss or reduced expression of ER-α, PR, and HER-2 in TNBC, the nature of the endogenous molecule(s) that might be responsible for the downregulation of these therapeutically receptors and, thus, progression of breast cancer cells to more invasive phenotypes, remains unknown. As a first step to explore this hypothesis, we examined the effect of recombinant iron-saturated lactoferrin on the levels of the 3 receptors in ER-positive ZR-75 breast cancer cells. As illustrated in Fig. 1A, lactoferrin stimulation of breast cancer cells results in a time-dependent downregulation of ER-α, PR, and HER-2 starting 6 hours post treatment. Interestingly, levels of ER-α and HER-2 returned to basal levels in the treated cells after 24 hours. The
noted downregulation of these receptors by lactoferrin was not due to a generalized toxicity as there was no effect of lactoferrin on the levels of the insulin-like growth factor (IGF)-IRβ and retinoid X receptor α (RXRα) receptors, whereas lactoferrin stimulation was accompanied by increased expression of cyclin D1, implying an active cell-cycle progression of lactoferrin-treated cells (Fig. 1A). Furthermore, we did not observe any evidence of an inhibitory effect of lactoferrin on the overall protein synthesis (Supplementary Fig. S1A). The observed downregulation of therapeutic receptors by lactoferrin was not limited to ZR-75 cells because lactoferrin also downregulated the basal levels of ER-α, PR, and HER-2 in ER-positive MCF-7 cells (Supplementary Fig. S1B) as well as in HER-2-positive ER-negative breast cancer SK-BR-3 or colon cancer Caco-2 cells (Supplementary Fig. S1C). Because downregulation of therapeutic receptors by a single dose of lactoferrin was transient in nature, we next assessed whether noted downregulation of receptors could be sustained by repeated lactoferrin dosing or by increasing the dose of lactoferrin. We found that replenishing the cultures with fresh or higher doses of lactoferrin results in a sustained downregulation of ER-α, PR, and HER-2 (Fig. 1B). To validate these findings, we confirmed downregulation of all 3 receptors in ZR-75 (Fig. 1C) or MCF-7 cells (Supplementary Fig. S1D) by immunocytochemical staining and scanning confocal microscopic examination. Together, these findings indicate that persistent stimulation of breast cancer cells by lactoferrin may downregulate therapeutic receptors, and thus, in principle, may contribute to the development of TNBC phenotypes.

Posttranscriptional regulation of therapeutic receptors by lactoferrin

To understand the underlying basis of lactoferrin downregulation of therapeutic receptors, we examined the effect of lactoferrin on the levels of receptor mRNA by Northern hybridization. There is no significant inhibitory effect of lactoferrin treatment on the levels of ER-α, PR, and HER-2 mRNA (Fig. 2A). For subsequent studies, we decided to examine the effect of lactoferrin on ER-α and HER-2 as models of therapeutic receptors in TNBC. In addition, there was no effect of lactoferrin on the half-life of the newly synthesized 35S-labeled ER-α or HER-2 (Supplementary Fig. S1E). However, cotreatment of the cells with a proteasomal inhibitor, MG-132, prevented lactoferrin-induced dose- and time-dependent downregulation of ER-α and HER-2 protein (Fig. 2B), indicating that the lactoferrin-mediated notable reduced expression of ER-α or HER-2 may use mechanisms that are sensitive to inhibitors of proteasomal degradation of proteins. To further validate the posttranscriptional control of ER-α and HER-2 by lactoferrin, we next generated isogenic pooled clones of MDA-MB-231 cells stably expressing ER-α or HER-2 (Fig. 2C). We found that lactoferrin treatment of clones is also accompanied by downregulation of ectopically expressed ER-α and HER-2 (Fig. 2D), which indicates that lactoferrin uses posttranscriptional mechanism(s) to downregulate HER-2 or ER-α in breast cancer cells.

Lactoferrin promotes invasiveness of breast cancer cells

Our observation of lactoferrin stimulation of cyclin D1 expression (Fig. 1A), an endpoint for the G1 to S-phase transition of the cell cycle, is consistent with the lactoferrin-mediated increase in the number of invasive cells (Fig. 1D).

Figure 1. Lactoferrin downregulates ER-α, PR, and HER-2 in human breast cancer cells. A, effect of a single dose of lactoferrin on the levels of indicated proteins in ZR-75 cells. Cells were treated with 20 µg/mL of lactoferrin for indicated times and subjected to Western blot analysis. B, effect of multiple and increasing doses of lactoferrin on expression levels of proteins in ZR-75 cells. C, analysis of ER-α, PR, and HER-2 levels in ZR-75 cells by confocal microscopic examination. Cells were treated with 20 µg/mL of lactoferrin for 12 hours, fixed, and then stained with protein-specific antibodies. Alexa-488 (ER-α and HER-2), Alexa-555 (PR), and DAPI (nucleus). Bar, 5 µm. Cont, control.
progression, indicated that lactoferrin exposure to ZR-75 cells may promote cell-cycle progression. Indeed, we found that lactoferrin treatment of ZR-75 cells leads to an accelerated transition into S-phase compared with untreated control cells (Supplementary Fig. S2A). As lactoferrin stimulation of breast cancer cells was accompanied by downregulation of ER-α, PR, and HER-2 receptor, characteristic of TNBC (Fig. 1A), we hypothesized that lactoferrin stimulation of ER-positive cells may antagonize the responsiveness of breast cancer cells to tamoxifen. In support of this notion, we showed that serum induced cell migration as well as estrogen-mediated invasion of MCF-7 cells (Fig. 3A and C, respectively) or ZR-75 cells (Supplementary Fig. S2B) could be effectively blocked by tamoxifen. However, this action of tamoxifen was compromised when cells were pretreated with lactoferrin, which downregulates ER-α and, consequently, is the target of tamoxifen (Fig. 3A and C; Supplementary Fig. S2B). Similarly, to understand the implication of lactoferrin-mediated downregulation of HER-2 on the ability of cells to respond to HER-2–directed therapies such as Herceptin, we next showed that lactoferrin pretreatment of HER-2–positive breast cancer cells reduced the effectiveness of Herceptin to inhibit serum-stimulated cell migration and heregulin-induced invasion in MCF-7 cells (Fig. 3B and D, respectively) and in ZR-75 cells (Supplementary Fig. S2C). These results indicate that lactoferrin stimulation of ER-α or HER-2–positive breast cancer cells is accompanied by the loss of responses to tamoxifen or Herceptin, presumably due to the loss of its target receptors (Fig. 3; Supplementary Fig. S2C). However, because we did not observe a complete loss of sensitivity of lactoferrin-treated cells to tamoxifen or Herceptin, we hypothesized that lactoferrin could possibly be contributing to the increase in migration and invasion (14) independent of receptor downregulation. Therefore, we presume that lactoferrin is involved in the activation of a gene(s) that could augment migration and invasion of these breast cancer cells. On the basis of these results, as suspected, lactoferrin strongly promoted the migration and invasiveness of TNBC lines such as MDA-MB-231 and MDA-MB-468 as evaluated by the wound-healing or Matrigel invasion assays (Fig. 3E; Supplementary Fig. S2D), indicating the involvement of receptor-independent activation of promigration/proinvasion gene(s).

Lactoferrin stimulates ET-1 transcription

To gain insight into the molecular basis of lactoferrin-induced cell invasiveness of TNBC, we next determined the nature of lactoferrin-regulated genes using microarray analysis of lactoferrin-treated or untreated MDA-MB-231 and MDA-MB-468 cells (Fig. 4A). This analysis led to the identification of 3 lactoferrin-regulated genes (i.e., Et-1, Rgs-2, and Tcbp-1) in both the cell lines (Fig. 4B). Guided by the functions of these genes in the context of our findings in this study, we decided to focus on ET-1 (15), a vasoactive peptide first isolated from vascular endothelial cells (15) that is upregulated in certain human cancers (16,17) including breast cancer (18). We found that, indeed, lactoferrin increased the steady-state levels of ET-1 mRNA in MDA-MB-468, MDA-MB-231, and MCF-7 (Fig. 4C). Lactoferrin-induced increased ET-1 mRNA expression could not be blocked by the inclusion of a protein-synthesis inhibitor, CHX (Fig. 4D), indicating transcriptional regulation of ET-1 by lactoferrin.
lactoferrin. Consistently, lactoferrin also stimulated the transcription driven by the ET-1 promoter-luc in breast cancer cells (Fig. 5A). A close scanning of the ET-1 promoter revealed the presence of 3 lactoferrin-motifs in the ET-1 promoter. Results from the electrophoretic mobility shift assay (EMSA) using oligonucleotides encompassing all 3 potential lactoferrin-interacting DNA regions (Supplementary Fig. S3A) in the ET-1 promoter indicated the formation of specific lactoferrin–DNA complexes with all 3 sites (Fig. 5B). On the basis of this, next we individually mutated lactoferrin consensus motifs 1 to 3 in the full-length ET-1 promoter-luc and found no significant impairment in the ability of lactoferrin to stimulate the transcription of ET-1 with promoter mutant sites 1 and 2 in comparison with consensus motif number 3. This indicates that lactoferrin may preferentially use lactoferrin consensus motif number 3 to stimulate ET-1 transcription (Fig. 5C). To identify the specific base-pair in the functional lactoferrin consensus site 3 that might be responsible for lactoferrin.

Figure 3. Lactoferrin promotes breast cancer cell migration and invasiveness and compromises anti-invasive activity of tamoxifen (Tam) and Herceptin (HCT). A, effect of lactoferrin on cell migration in tamoxifen-treated MCF-7 cells. Right, the quantification of the average difference in wound closure between 0 and 24 hours; left, micrographs show representative images of migrated cells after 24 hours of treatment. B, effect of lactoferrin on cell migration in Herceptin-treated MCF-7 cells. Right, quantification of the average difference in wound closure between 0 and 24 hours; left, micrographs show representative images of migrated cells after 24 hours of treatment. C, effect of lactoferrin on cell invasion in tamoxifen-treated MCF-7 cells. E2 was added to the upper wells to enhance tamoxifen effect. D, effect of lactoferrin on cell invasion in Herceptin-treated MCF-7 cells. Heregulin was added to the upper wells to enhance Herceptin effect. E, effect of lactoferrin on the invasiveness of MDA-MB-231 and MDA-MB-468 cells. Left, micrographs that are representative images of invaded cells; right, quantification of the average number of invaded cells. Error bars indicate SD. Scale bars, 0.5 mm. * P < 0.01. Cont, control.
lactoferrin-binding, we created 3 mutant versions of the lactoferrin consensus site 3 and tested these oligos for lactoferrin-binding using EMSA (Supplementary Fig. S3A). There was a drastic reduction in the binding of lactoferrin to oligo number 3, indicating that lactoferrin may preferentially interact with the GGCACTTGG motif in the ET-1 promoter (Fig. 5D). Furthermore, we observed a progressive increase in the levels of protein–DNA complexes that could be effectively super-shifted by inclusion of Lf-Ab but not isogenic immunoglobulin G (IgG; Fig. 5E, lanes 6 and 7). Furthermore, only recombinant iron-saturated lactoferrin (holo-Lf), but not non-iron saturated Lf (apo-Lf), interacted with the ET-1 promoter oligo (Fig. 5F), and such protein–DNA complexes could be super-shifted by Lf-Ab (Fig. 5E, lanes 9–12). Consistent with these findings, iron-saturated holo-Lf and not apo-Lf downregulated ER-α and HER-2 in ZR-75 cells (Supplementary Fig. S3B). Taken together, these findings establish that ET-1 is a lactoferrin-inducible gene and that only the holo form of lactoferrin is responsible for the observed downregulation as well as increased invasiveness in breast cancer cells. This is particularly interesting because a study by Duarte and colleagues (19) showed that treatment of human breast cancer cell lines, Hs578T and T47D, with bovine apo-Lf increased apoptosis and decreased cell migration. However, as indicated in our present data, we observed an upregulation of ET-1, which is responsible for the lactoferrin-induced invasion and migration, with the holo form of lactoferrin only. Consistent with these results, apo-lactoferrin did not bind to the ET-1 promoter and, subsequently, was unable to drive the transcription of the gene. Therefore, we propose that the increase in invasion, as well as migration, in breast cancer cells is likely to be due to the upregulation of ET-1 after iron-saturated lactoferrin treatment.

**Lf–ET-1 axis promotes TNBC phenotype**

To establish the physiologic relevance of lactoferrin stimulation of ET-1, we next showed that lactoferrin-mediated increase in expression of ET-1 was also accompanied by a significant accumulation of ET-1 in the conditioned media. Interestingly, the levels of baseline ET-1 in TNBC lines such as MDA-MB-231 and MDA-MB-468 were substantially higher than that in MCF-7 cells (Fig. 6A). To assess the significance of secreted ET-1 in the observed lactoferrin-mediated migration, we determined the effect of an antagonist of the ET-1 receptor [ET(Α)R (20) BQ123 (21–23)], on the action of lactoferrin. We found that inclusion of BQ123 in the cultures abrogated the ability of lactoferrin as well as of recombinant ET-1 to promote migration and invasion of MCF-7 cells in the Boyden chamber (Fig. 6B) as well as in the Matrigel invasion (Fig. 6C) assays, respectively.

To assess the significance of these observations in a physiologic setting, we determined the status of lactoferrin in human breast tumors from patients with TNBC or ER+/PR+ by quantitative real-time PCR (qRT-PCR). We found that the levels of lactoferrin (Fig. 7A) as well as ET-1 (Fig. 7B) were significantly increased in TNBC specimens compared with those in the control ER+/PR+ breast tumors. Because both lactoferrin and ET-1 are secreted polypeptides, we next determined the levels of lactoferrin and ET-1 in the plasma samples...
from TNBC as well as from ER/PR/HER-2–positive patients. We observed an elevated level of ET-1 as well as lactoferrin in TNBC when compared with the ER/PR/HER-2–positive samples (Fig. 7C). Consistent with these findings, we also found strong and medium matching staining of lactoferrin and ET-1 in TNBC samples when compared with ER-α, PR, and HER-2–positive tissue samples as determined by immunohistochemistry. Data in Fig. 7D illustrate representative examples of increased levels of lactoferrin and ET-1 staining in the tumor specimens from patients with TNBC as compared with the ER/PR/HER-2–positive tumors, providing a proof-of-principle evidence of a correlative nature of lactoferrin and ET-1 in tumor samples. Collectively, these findings revealed that lactoferrin-mediated increase in invasiveness of breast cancer cells is mediated via increased expression and secretion of ET-1, at least in part, and these changes might account for the generally noted aggressive behavior of TNBC (Fig. 7E).

**Discussion**

Findings presented in this article show for the first time that lactoferrin, a ubiquitous secretory protein, can down-regulate all 3 therapeutic receptors, ER-α, PR, and HER-2, in breast cancer cells. This observation is particularly interesting as it raises the possibility that increased expression of lactoferrin could contribute to the development of TNBC phenotypes due to the loss or downregulation of these receptors. Because we have also observed an increased expression of lactoferrin in tumor specimens as well as in the levels of circulating lactoferrin in plasma samples from the patients with TNBC as compared with those in samples from ER+ patients, we hypothesize a potential contributing role of lactoferrin in supporting the TNBC phenotype. In this context, it is noteworthy that an earlier correlative report, dating back to the 1990s, has shown an inverse relationship
between the levels of ER-\(\alpha\) and lactoferrin (7). In addition, there are immunocytochemical studies that show changes in the synthesis and secretion of lactoferrin in breast cancer (24); however, in endocervical adenocarcinoma, lactoferrin has been identified as a useful predictive marker (25).

Furthermore, while our studies were being conducted, Schulz and colleagues have also noticed an increased expression of lactoferrin in samples of patients with TNBC (26).

Our finding that lactoferrin stimulation of both non-TNBC and TNBC cells was accompanied by an increased invasiveness is of particular importance as it indicates the possibility that the loss of the 3 receptors by lactoferrin is not only associated with an expected insensitivity to antiestrogen or Herceptin but also contributes to invasiveness of breast cancer cells—both central features of TNBC.

While exploring the molecular basis of lactoferrin-induced invasion of breast cancer, we identified lactoferrin-regulated genes using a microarray approach. These and other molecular studies presented in this article led to the identification of ET-1, another secreted protein, as the newest direct target of lactoferrin. This is particularly exciting as ET-1 has been previously shown to promote breast cancer progression, and increased amounts of ET-1 participates in the process of invasion and metastasis, and ET-1 levels correlate well with a diminished disease-free and overall survival rate (18). ET-1 exerts its biologic effects through an autocrine and/or paracrine manner through specific receptors and supports tumor cell proliferation, invasion, angiogenesis, and neovascularization (27–33). The relevance of these mechanistic studies to a physiologic setting was recognized by the noted co-overexpression of lactoferrin and ET-1 in tumors as well as elevated circulating levels in serum from TNBC as compared with samples from ER-, PR-, and HER-2–positive breast tumors.

Because the ET-1 pathway is being actively targeted via specific pharmacologic inhibitors to its receptor and Food and Drug Administration (FDA)-approved ET-1 receptor antagonists are moving forward in clinical trials, these studies raise a
tangible opportunity to use such ET-1 inhibitors for slowing down the progression and invasiveness of TNBC. Results presented in this article provide proof-of-principle evidence supporting therapeutic effectiveness of ET-1 receptor antagonists to completely block the lactoferrin-induced motility and invasiveness of the TNBC cells, and thus, open a remarkable

Figure 7. Lf–ET-1 axis promotes TNBC phenotypes. A, quantitative PCR analysis of lactoferrin mRNA levels in tumor samples from patients with or without TNBC. B, qRT-PCR analysis of ET-1 mRNAs in TNBC or non-TNBC breast tumors. mRNA experiments were repeated 3 times, with each point in triplicate. C, concentration of lactoferrin and ET-1 in plasma samples from patients with or without TNBC by ELISA. D, immunohistochemical evaluation of lactoferrin and ET-1 expression in breast tumors from the same patients used in (B). Table depicts TNBC and non-TNBC tissue samples with scoring of lactoferrin and ET-1 staining intensity: +++ (strong); ++ (medium). E, working model for lactoferrin-mediated downregulation of receptors and cell migration/invasion in TNBC cells. Error bars indicate SD.
opportunity to treat TNBC by targeting the LF-ET-1 axis using an approved developmental drug. As expected, similar to many other new discoveries, the present studies have not only provided several significant leads that can be followed, but they have also posed a number of new questions to the scientific community such as nature of factors that might promote persistent elevation of lactoferrin.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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