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

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Running title: Lactoferrin-Endothelin axis in TNBC phenotypes

The Editor-in-Chief of Cancer Research is an author of this article. In keeping with the AACR’s Editorial Policy, the paper was peer reviewed and a member of the AACR’s Publications Committee rendered the decision concerning acceptability.
ABSTRACT

Triple-negative breast cancer (TNBC) is characterized by the lack of expression of ERα, PR and HER-2 receptors and the pathway(s) responsible for this downregulation and thus, aggressiveness remains unknown. Here we discovered that lactoferrin (Lf) efficiently downregulates the levels of ERα, PR and HER-2 receptors in a proteasome-dependent manner in breast cancer cells, and accounts for the loss of responsiveness to ER- or HER-2- targeted therapies. Further we found that Lf increases migration and invasiveness of both non-TNBC and TNBC cell lines. We discovered that Lf directly stimulates the transcription of endothelin-1 (ET-1), a secreted pro-invasive polypeptide that acts through a specific receptor ET(A)R, leading to secretion of bioactive ET-1 peptide. Interestingly, a therapeutic ET-1 receptor antagonist drug completely blocked Lf-dependent motility and invasiveness of breast cancer cells. Physiologic significance of this newly discovered Lf-ET-1 axis in the manifestation of TNBC phenotypes is revealed by elevated plasma and tissue Lf and ET-1 levels in TNBC patients as compared to those in ER+ cases. These findings describe the first physiologically relevant polypeptide as a functional determinant of downregulating all three therapeutic receptors in breast cancer which utilizes another secreted ET-1 system to confer invasiveness. Results presented here provide proof-of-principle evidence in support of therapeutic effectiveness of ET-1 receptor antagonist to completely block the Lf-induced motility and invasiveness of the TNBC as well as non-TBNC cells, and thus, opening a remarkable opportunity to treat TNBC by targeting the Lf-ET-1 axis using an approved developmental drug.
INTRODUCTION

Among all breast cancers, approximately 10-15% is categorized as triple-negative breast cancer (TNBC) (1-3). TNBC is characterized by the presence of low or no levels of estrogen receptor-alpha (ERα), progesterone receptor (PR) and human epidermal growth factor receptor (HER-2) (4) and lack of effective therapies targeting these receptors leads to poor prognosis (4,5). While reviewing the earlier literature, we noticed evidence of an inverse correlation between the levels of PR or ERα and lactoferrin (Lf) (6) in endometrial adenocarcinomas or in primary breast tumors, respectively (7-10). Lactoferrin (Lf), a member of the transferrin family, was first discovered as an extracellular iron binding glycoprotein. Since then, Lf has been extensively studied and shown to play a major role in anti-inflammation and bactericidal events. Since Lf is a hormone responsive gene (11) and its levels are modulated by a variety of signals (12), we hypothesized that elevated levels of Lf may be associated with a reduced expression of ERα 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, here we set to investigate the role of Lf in the development of TNBC and discovered that lactoferrin efficiently downregulates the levels of ERα, PR and HER-2 receptors at a post-transcriptional level in multiple breast cancer cell lines. We also found that Lf-induced increased invasion of breast cancer cells mechanistically mediated via transcriptional stimulation of endothelin-1(13) and could be effectively blocked by therapeutic antagonists of endothelin-1 receptor. While the mechanism of loss of receptors during the development of...
TNBC is currently poorly understood, 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) provided human breast cancer tissue RNA samples. Dr. George C. Prendergast (Lankenau Institute for Medical Research) provided the plasma samples along with the corresponding tissue slides. All the human samples used were in accordance with the IRB 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 colon cancer cell line Caco-2 were obtained from ATCC. All cells were maintained at 37°C and 5% CO₂ in DMEM/F12-50/50 (Mediatech, Inc.) supplemented with 10% FBS (Atlanta Biologics) and 1% antibiotics (Gibco®, Invitrogen). During serum-starvation, cells were incubated with serum-free DMEM supplemented with 1% antibiotics.

The triple-negative breast cancer (TNBC) cell line MDA-MB-231 was chosen for generating stable clones. The cells were transfected with the following plasmids: pcDNA 3.1 (Invitrogen) (control), ERα and HER-2. The stable clone cell line (pcDNA, ER and HER-2) were maintained in DMEM/F12-50/50, supplemented with 10% FBS, 1% antibiotics and 0.5µg/ml of G418 (Sigma Aldrich).

**Protein Analysis**

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For lactoferrin treatment, cells were maintained in serum-free DMEM for 48 hrs and then treated with 20µg/ml of lactoferrin (Sigma Aldrich). Protein samples were separated by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane (Bio-Rad). The following primary antibodies were used: anti-ERα (Bethyl Laboratories), anti-HER-2 (Bethyl Laboratories), anti-PR (Dako), anti-IGF-IRβ (Santa Cruz Biotechnology, Inc.), anti-RXRα (Santa Cruz Biotechnology, Inc.), anti-cyclinD1 (Labvision/Thermo Fisher Scientific), anti-tubulin (Sigma-Aldrich) and anti-vinculin (Sigma-Aldrich).

**Immunohistochemistry and Confocal Microscopy**

Deparaffinized 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 (1: 500) or endothelin antibody (1: 100) at 4°C overnight, followed by incubation with EnVision (Dako) for 1 hr 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 and MCF-7 cells were plated onto cover slips in six-well plates, starved for 48 hrs and treated with 20µg/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 three 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, dihydrochloride (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 transferred 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 100µg/ml of lactoferrin for 6 hrs. A RNA sample amount of 20µg was loaded onto a formaldehyde denaturing gel (1.5% agarose, 2.2M 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 radio-labeled 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 hrs. The cells were either treated with 20µg/ml of Cycloheximide (CHX) or 100µg/ml Lactoferrin (Lf) or both. CHX was administered an hour before Lf treatment. After 6 hrs of treatment with Lf, 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**

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To analyze the effect of lactoferrin with or without tamoxifen on cell migration, MCF-7 cells were plated with 10% fetal calf serum-DMEM. Before treatment, each plate received multiple “wounds” with a 200µl pipette tip. Treatments were done with low-serum medium supplemented with 100µg/ml lactoferrin and 10⁻⁷ tamoxifen (Sigma Aldrich). In another set, the cells were treated with and without lactoferrin in addition to 10nM of Herceptin (Genentech, Inc.) and in combination. After 24 hrs, 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 performed in triplicates.

**Migration assay**

ZR-75 cells were serum-starved for 48 hrs and then resuspended in the presence of 0.1% BSA. Subsequently, 1 x 10⁵/well were loaded onto the upper well of an uncoated Boyden chamber. ET-1 and BQ-123 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 × 10⁵ 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

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cells were treated with 100 µg/ml of lactoferrin. After 24 to 48 hrs 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 hrs.

**Microarray Analysis of Lactoferrin-Regulated Genes**

MDA-MB-231 and MDA-MB-468 cells were plated in triplicates, serum-starved for 48 hrs and then treated with 100µg/ml of lactoferrin for 6 hrs. RNA was isolated using TRIzol® following the manufacturer’s protocol. 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 employed to identify differentially regulated genes and those with a p-value of ≤ 0.05 were considered statistically significant. Heat map analysis of the identified genes for individual arrays was performed using 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% confluency. ET-1 promoter, β-galactosidase and control plasmids were transfected into cells using FuGENE® 6 according to the manufacturer’s protocol (Roche). After lactoferrin treatment, cell lysates were prepared using 200µl of Tropix® Lysis solution (Applied Biosystems). For luciferase promoter activity, 10µl of lysate was incubated with 100µl Luciferase Assay Substrate solution (Promega) and measurements were taken using LUMAT LB 9507 luminometer (Berthold Technologies). β-galactosidase was used as a transfection control.

**Electrophoretic Mobility Shift Assay**

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PCR amplified DNA fragments or oligos containing lactoferrin consensus sites were 5’-end labeled using $\gamma^{32}$p 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 (GenWay Biotech) or lactoferrin-treated and untreated nuclear extracts for 15 min at room temperature. The reaction samples were resolved on a 5% native PAGE for 2 hrs. 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) following the manufacturer’s manual. 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 Real-time Quantitative PCR**

Total RNA from human breast tumor tissue samples as well as human breast cancer cell lines was isolated using TRIzol® (Invitrogen) according to the manufacturer’s protocol. A 2µg RNA sample was used to synthesize cDNA using Invitrogen’s SuperScript® III First-Strand Synthesis SuperMix following the manufacturer’s manual. The following primers (synthesized by Sigma Aldrich) were used for target genes: Lf Forward: 5’-ACCAGCACATGAACTTG-3’ and Lf

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Reverse: 5'-GGGGGAGTCTCTCTTTATGC-3'  ET-1  Forward: GCCAAGGAGCTCCAGAAACAGCAG ET-1 Reverse: AGCAGGAGCAGCGCTTTGGAC; Actin  Forward: 5' -TCCCTGGAGAAGAGCTACGA 3' and Actin Reverse: 5'-GTACTTTGCGCTCAGGAGGAG-3'. Normalized expression of target genes were calculated relative to endogenous expression of actin (Relative expression= $2^{-\Delta \Delta C(t)}$).

**Statistical analysis**

Paired Student’s t test was used for statistical significance of differences in numerical data. All the statistical tests were two sided. P-value of less than 0.05 was considered to be statistically significant.

**RESULTS**

**Lactoferrin downregulates ERα, PR, and HER-2 in Human Breast Cancer Cells**

While a large body of studies have firmly established the significance of the loss or reduced expression of ERα, PR and HER-2 receptors in TNBC, the nature of the endogenous molecule(s) that might be responsible for the downregulation of these therapeutic 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 (Lf) on the levels of the three receptors in ER-positive ZR-75 breast cancer cells. As illustrated in Figure 1A, Lf stimulation of breast cancer cells results in a time-dependent downregulation of ERα, PR and HER-2 starting 6 hrs post treatment. Interestingly, levels of ERα and HER-2 returned back to the basal levels in the treated cells after 24 hrs. The noted downregulation of these receptors by Lf was not due to a generalized toxicity as there was no effect of Lf on the
levels of IGF-IRβ and RXRα receptors while Lf stimulation was accompanied by increased expression of cyclin D1, implying an active cell cycle progression of Lf-treated cells (Fig. 1A). Further, we did not observe any evidence of an inhibitory effect of Lf on the overall protein synthesis (Supplementary Fig. 1A). The observed downregulation of therapeutic receptors by Lf was not limited to ZR-75 cells as Lf also downregulated the basal levels of ERα, PR and HER-2 in ER-positive MCF-7 cells (Supplementary Fig. 1B) as well as in HER-2-positive ER-negative SK-BR-3 breast or colon cancer Caco-2 cells (Supplementary Fig. 1C). Since downregulation of therapeutic receptors by a single dose of Lf was transient in nature, we next assessed whether noted downregulation of receptors could be sustained by repeated Lf-dosing or by increasing the dose of Lf. We found that replenishing the cultures with fresh or higher doses of Lf results in a sustained downregulation of ERα, PR and HER-2 (Fig. 1B). To validate these findings, we confirmed downregulation of all three receptors in ZR-75 cells (Fig. 1C) or MCF-7 cells (Supplementary Fig. 1D) by immunocytochemical staining and scanning confocal microscopy. Together, these findings suggest that persistent stimulation of breast cancer cells by Lf may downregulate therapeutic receptors, and thus, in-principle, may contribute, to the development of TNBC phenotypes.

Post-transcriptional Regulation of Therapeutic Receptors by Lf

To understand the underlying basis of Lf downregulation of therapeutic receptors, we examined the effect of Lf on the levels of receptor mRNA by Northern hybridization. There is no significant inhibitory effect of Lf treatment on the levels of ERα, PR and HER-2 mRNA (Fig. 2A). For subsequent studies, we chose to examine the effect of Lf on ERα and HER-2 as models.
of therapeutic receptors in TNBC. There was also no effect of Lf on the half-life of the newly synthesized $^{35}$S-labeled ER$\alpha$ or HER-2 (Supplementary Fig. 1E). However, co-treatment of the cells with a proteasomal inhibitor, MG-132, prevented Lf-induced dose- and time-dependent downregulation of ER$\alpha$ and HER-2 protein (Fig. 2B), suggesting that Lf-mediated noted reduced expression of ER$\alpha$ or HER-2 may utilize mechanisms that are sensitive to inhibitors of proteasomal degradation of proteins. To further validate the post-transcriptional control of ER$\alpha$ and HER-2 by Lf, we next generated isogenic pooled clones of MDA-MB-231 cells stably expressing ER$\alpha$ and HER-2 (Fig. 2C). We found that Lf treatment of clones is also accompanied by downregulation of ectopically expressed ER$\alpha$ and HER-2 (Fig. 2D), implying that Lf utilizes post-transcriptional mechanism(s) to downregulate HER2 or ER in breast cancer cells.

**Lf Promotes Invasiveness of Breast Cancer Cells**

Our observation of Lf stimulation of cyclin D1 expression (Fig. 1A), an endpoint for the G1-to-S phase progression, suggested that Lf exposure to ZR-75 cells may promote cell cycle progression. Indeed, we found that Lf treatment of ZR-75 cells leads to an accelerated transition into S-phase as compared to untreated control cells (Supplementary Fig. 2A). As Lf stimulation of breast cancer cells was accompanied by a downregulation of ER$\alpha$, PR, and HER-2, characteristic of TNBC (Fig. 1A), we hypothesized that Lf stimulation of ER-positive cells may antagonize the responsiveness of breast cancer cells to tamoxifen. In support of this notion, we showed that serum increased cell migration as well as estrogen-mediated invasion of MCF-7 cells (Fig. 3A and 3B, respectively) or ZR-75 cells (Supplementary Figure. 2B) could be
effectively blocked by tamoxifen. However, this was compromised when cells were pre-treated with Lf which downregulates ERα and consequently, is the target of tamoxifen (Figs. 3A, 3B and Supplementary Figure. 2B). Similarly, to understand the implication of Lf-mediated downregulation of HER-2 on the ability of cells to respond to HER-2-directed therapies such as Herceptin, we next showed that Lf pre-treatment 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. 3C and 3D, respectively) and in ZR-75 cells (Supplementary Fig. 2C). These results suggest that Lf stimulation of ER- 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 (Figs. 3C, 3D and in Supplementary Fig. 2C). However, since we did not observe a complete loss of sensitivity of lactoferrin-treated cells to tamoxifen or Herceptin, we hypothesize 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) which could augment migration and invasion of these breast cancer cells. Based on these results, as suspected, Lf strongly promoted the migration and invasiveness of TNBC lines such as MDA-MB-231, MDA-MB-468 as evaluated by the Wound-healing or Matrigel™ invasion assays (Supplementary Fig. 2D and Fig. 3C) suggesting the involvement of receptor-independent activation of pro-migration/pro-invasion gene(s).

**Lf Stimulates Endothelin-1 Transcription**

To gain insight into the molecular basis of Lf-induced cell invasiveness of TNBC, we next determined the nature of Lf-regulated genes using microarray analysis of Lf-treated or untreated cells.
MDA-MB-231 and MDA-MB-468 cells (Fig. 4A). This analysis led to the identification of three Lf-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 here, we decided to focus on endothelin-1 (ET-1) (15), a vasoactive peptide first isolated from vascular endothelial cells (15) which is upregulated in certain human cancers (16,17) including breast (18). We found that indeed, Lf increased the steady-state levels of ET-1 mRNA in MDA-MB-468, MDA-MB-231 and MCF-7 (Fig. 4C). Lf-induced increased ET-1 mRNA could not be blocked by the inclusion of protein synthesis inhibitor, cycloheximide (CHX) (Fig. 4D), suggesting transcriptional regulation of ET-1 by Lf. Consistently, Lf 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 three Lf-motifs in the ET-1 promoter. Results from the EMSA assay using oligonucleotides encompassing all three potential Lf-interacting DNA regions (Supplementary Fig. 3A) in the ET-1 promoter indicated the formation of specific Lf-DNA complexes with all three sites (Fig. 5B). Based on this, next we individually mutated Lf-consensus motifs # 1-3 in the full-length ET-1 promoter-luc and found no significant impairment in the ability of Lf to stimulate the transcription of ET-1 with promoter mutant sites 1 and 2 in comparison to consensus motif number 3. This suggests that Lf may preferentially utilize Lf-consensus motif number 3 to stimulate ET-1 transcription (Fig. 5C). To identify the specific base-pair in the functional Lf-consensus site 3 that might be responsible for Lf-binding, we created three mutant versions of the Lf-consensus site 3 and tested these oligos for Lf-binding using EMSA. There was a drastic reduction in the binding of Lf to oligo number 3, suggesting that Lf may preferentially interact with the GGCACTTGG motif in the ET-1 promoter (Fig. 5D). We also observed a progressive

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increase in the levels of protein-DNA complexes which could be effectively super-shifted by inclusion of Lf-Ab but not isogenic IgG (Fig. 5E, lanes 6, 7). Further, only recombinant iron-saturated Lf (holo-lactoferrin) but not non-iron saturated Lf (apo-lactoferrin) 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, 11 and 10 respectively). Consistent with these findings, iron-saturated hololactoferrin and not apolactoferrin downregulated the three therapeutic receptors in ZR-75 cells (Supplementary Fig. 3B). Taken together, these findings establish that ET-1 is an Lf-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 et al. (19) showed that treatment of human breast cancer cell lines, Hs578T and T47D with bovine apo-lactoferrin increased apoptosis and decreased cell migration. However, as indicated in our present data, we observed an upregulation of endothelin-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 endothelin-1 promoter and therefore 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 are likely to be due to the upregulation of endothelin-1 upon iron-saturated lactoferrin treatment.

Lf-ET-1 axis Promotes TNBC Phenotype

To establish the physiological relevance of Lf stimulation of ET-1, we next demonstrated that Lf-mediated increased expression of ET-1 was also accompanied by a significant accumulation of ET-1 in the conditioned media. Interestingly, the levels of base-line ET-1 in TNBC lines such...
as MDA-MB-231 and MDA-MB-468 were substantially higher than that of MCF-7 cells (Fig. 6A). To assess the significance of secreted ET-1 in the observed Lf-mediated migration, we determined the effect of a pharmacological antagonist of the ET-1 receptor (ET(A)R) (20), BQ123 (21-23), on the action of Lf. We found that inclusion of BQ123 in the cultures abrogated the ability of Lf 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 physiological setting, we determined the status of Lf in human breast tumors from TNBC or ER+/PR+ patients by quantitative-RT-PCR (q-PCR). We found that the levels of Lf (Fig. 7A) as well as ET-1 (Fig. 7B) were significantly increased in TNBC specimens as compared to those in the control ER+/PR+ breast tumors. Since both Lf and ET-1 are secreted polypeptides, we next determined the levels of Lf 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 Lf in TNBC when compared to the ER/PR/HER-2 positive samples (Fig. 7C). Consistent with these findings, we also found strong and medium co-staining of lactoferrin and ET-1 in TNBC samples when compared to ERα, PR and HER-2 positive tissue samples as determined by IHC. Data in Figure 7D illustrate representative examples of increased levels of Lf and ET-1 co-staining in the tumor specimens from TNBC patients as compared to the ER/PR/HER-2 positive tumors, providing a proof of principle evidence of a correlative nature of Lf and ET-1 in tumor samples. Collectively, these findings revealed that Lf mediated increased invasiveness of breast cancer cells is mediated via

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increased expression and secreted of ET-1, at least in-part, and these changes might account for the generally noted aggressive behavior of TNBC.

**DISCUSSION**

Findings presented here have shown for the first time that lactoferrin, a ubiquitous secretory protein, can downregulate all three therapeutic receptors ER\(\alpha\), PR and HER-2 in breast cancer cells. This observation is particularly interesting as it raises the possibility that increased expression of Lf could contribute to the development of TNBC phenotypes due to the loss or downregulation of these receptors. Since we have also observed an increased expression of Lf in tumor specimens as well as in the levels of circulating Lf in plasma from the TNBC patients as compared to those in ER+ patients, we suggest a potential contributing role of lactoferrin in supporting the TNBC phenotype. In this context, it is noteworthy that an earlier correlative report dated back to the 1990s has shown an inverse relationship with the level of estrogen receptor and Lf (7). There are also immunocytochemical studies which show changes in the synthesis and secretion of Lf in breast cancer (24), while in endocervical adenocarcinoma, lactoferrin has been identified as a useful predictive marker (25). Further, while our studies were in progress, Schulz DM et al. have also noticed an increased expression of Lf in TNBC patient samples (26).

Our finding that Lf stimulation of both non-TNBC and TNBC cells was accompanied by an increased invasiveness is of particular importance as it points to the possibility that the loss of the three receptors by Lf is not only associated with an expected insensitivity to anti-estrogen or Herceptin but also contributes to invasiveness of breast cancer cells – both central features of TNBC.

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

Since the ET-1 pathway is being actively targeted via specific pharmacological inhibitors to its receptor and 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 here provide proof-of-principle evidence in support of therapeutic effectiveness of ET-1 receptor antagonist to completely block the Lf-induced motility and invasiveness of the TNBC cells, and thus, opening a remarkable opportunity to treat TNBC by targeting the Lf-ET-1 axis using an approved developmental drug. As expected, like many other new discoveries, the present studies have not only provided several significant leads to follow, but they have also poised a number of new questions to the scientific community such as nature of factors which might promote persistent elevation of Lf.
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FIGURE LEGENDS

Figure 1. Lactoferrin downregulates ERα, PR, and HER-2 in human breast cancer cells.
(A) Effect of a single dose of Lf on the levels of indicated proteins in ZR-75 cells. Cells were treated with 20 µg/ml of Lf for indicated times and subjected to Western blot analysis (B) Effect of multiple and increasing doses of Lf on expression levels of proteins in ZR-75 cells. (C) Analysis of ERα, PR and HER-2 levels in ZR-75 cells by confocal microscopy. Cells were treated with 20µg/ml of Lf for 12 hrs, fixed and then stained with protein-specific antibodies. Alexa-488 (ERα and HER-2), Alexa-555 (PR) and DAPI (nucleus). Bar = 5µm.

Figure 2. Lactoferrin downregulates ERα, PR, and HER-2 at a transcriptional level
(A) Northern blot analysis of ERα, PR and HER-2 mRNAs in MCF-7 cells treated with or without 100 µg/ml of Lf for indicated times. (B) MCF-7 cells were treated with or without Lf for indicated time points or with different concentrations of Lf in the presence or absence of proteasomal inhibitor, MG-132. Vinculin was used as a loading control. (C) MDA-MB-231 stable clones expressing ERα or HER-2 were generated as described in the materials and methods. Vinculin was used as a loading control. (D) Effect of Lf on the levels of ERα and HER-2 receptors in MDA-MB-231 pooled clones stably expressing ERα or HER-2.

Figure 3. Lf promotes breast cancer cell migration and invasiveness and compromises anti-invasive activity of tamoxifen and Herceptin.
(A) Effect of Lf on cell migration in tamoxifen-treated MCF-7 cells. On the right is the quantification of the average difference in wound closure between 0 and 24 hrs. Left

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micrographs show representative images of migrated cells after 24 hrs of treatment. (B) Effect of Lf on cell migration in Herceptin-treated MCF-7 cells. On the right is the quantification of the average difference in wound closure between 0 and 24 hrs. Left micrographs show representative images of migrated cells after 24 hrs of treatment. (C) Effect of Lf on cell invasion in tamoxifen-treated MCF-7 cells. E2 was added to the upper wells to enhance tamoxifen effect. (D) Effect of Lf on cell invasion in Herceptin-treated MCF-7 cells. Heregulin was added to the upper wells to enhance Herceptin effect. (E) Effect of Lf on the invasiveness of MDA-MB-231 and MDA-MB-468 cells. Left micrographs are representative images of invaded cells Right is the quantification of the average number of invaded cells. Error bars indicate standard deviation. Scale bars = 0.5mm. *, P < 0.05; **, P < 0.01; ***, P < 0.001

Figure 4. Endothelin-1 is a transcriptional target of Lf.

(A & B) Analysis of differentially regulated genes after Lf treatment in MDA-MB-231 and MDA-MB-468 cells by Affymetrix Human Exon 1.0 ST microarrays using Gene-Spring GX 10.0.2. (C) Effect of Lf on ET-1 mRNA levels in breast cancer cells. Actin was used as a loading control. (D) Effect of Lf in the presence or absence of cycloheximide on the induction of ET-1 mRNA in MDA-MB-231 cells.

Figure 5. Lf stimulates Endothelin-1 transcription.

(A) Effect of Lf on ET-1 promoter activity in breast cancer cells. Cells were treated with 100 μg/ml of Lf for 36 hrs. (B) EMSA analysis of Lf binding to all three Lf-consensus DNA sequences in the ET-1 promoter using the nuclear extracts from MCF-7 cells treated with or

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without Lf for 6 hrs. (C) Effect of Lf on the ET-1 promoter with wild-type or mutant Lf-binding sequences in MDA-MB-468 cells. (D) EMSA analysis of Lf binding to oligonucleotides encompassing the wild-type Lf-consensus DNA sequence 3 or its three mutant versions using the nuclear extracts from MCF-7 cells treated with or without Lf for 6 hrs. (E) EMSA analysis of Lf-stimulated nuclear extracts from MCF-7 cells in the presence of antibody to Lf. Antibody to rabbit-IgG was used as a negative control (F) Effects of holo- or apo-Lf on the ET-1 promoter luc activity in MDA-MB-468 cells. Error bars indicate standard deviation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

**Figure 6. Endothelin-1 receptor antagonist blocks lactoferrin-dependent invasiveness.**

(A) Concentration of ET-1 in the conditioned media from breast cancer cells treated with or without 100 µg/ml of Lf for 24 hrs as measured by ELISA. (B) Effect of ET-1-receptor inhibitor BQ123 on cell migration of MCF-7 cells treated with or without Lf or ET-1, using Boyden chambers. (C) Effect of ET-1-receptor inhibitor BQ123 on cell invasion of MCF-7 cells treated with or without Lf or ET-1 using Matrigel™ invasion chambers. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

**Figure 7. Lf-ET-1 axis promotes TNBC phenotypes.**

(A) Quantitative PCR analysis of Lf mRNA levels in tumor samples from TNBC and non-TNBC breast cancer patients. (B) Q-PCR analysis of ET-1 mRNAs in TNBC or non-TNBC breast tumors. mRNA experiments were repeated three times, with each point in triplicate. (C) Concentration of Lf and ET-1 in plasma samples from TNBC or non-TNBC patients by ELISA.

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(D) IHC evaluation of Lf and ET-1 expression in breast tumors from the same patients used in panel B. Table depicts TNBC and non-TNBC tissue samples with scoring of Lf and ET-1 staining intensity: +++ (strong), ++ (medium). (E) Working model for Lf-mediated downregulation of receptors and cell migration/invasion in triple negative breast cancer cells.
Figure 1
Figure 2

(A) Western blots for ERα, PR, HER-2, and Actin in control (Cont) and Lf-treated samples over 3 and 6 hours.

(B) Western blots for ERα, HER-2, and Vinculin with DMSO and MG-132 treatment at various concentrations.

(C) Western blots showing the expression of ERα, HER-2, and Vinculin with UntranspcD, pcDNA#1, pcDNA#2, ERα#1, and HER2#1#2 treatments.

(D) Western blots for ERα, HER-2, and Vinculin with ERα#1 and ERα#2 treatments.

Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

### Table

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(p-values: 0.0028, 0.00437, 0.000293, 0.000101)
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Cancer Res  Published OnlineFirst October 17, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-1143

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2011/10/17/0008-5472.CAN-11-1143.DC1

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