The Role of Lactoferrin in Breast Cancer Progression

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Dedication

This dissertation is dedicated to my parents, Hà Minh Châu and Nguyễn Thị Yến and my sister and best friend, Hà Ngọc-Trâm. Con xin kính tặng bài luận án này cho Ba và Mẹ kính yêu và em thương mến của con.

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Abstract of Dissertation

The Role of Lactoferrin in Breast Cancer Progression

Triple-negative breast cancer is characterized by loss of ERα, PR and HER-2 receptors and hence, lack of responsiveness to therapies targeting these receptors. However, the nature of endogenous molecules/pathways that might be responsible for the loss of three therapeutic receptor targets and thus, progression of breast cancer remains unknown. Clinical studies have shown that there is an increased risk for basal-like breast cancer in young women at first full term pregnancy. Since lactoferrin is a major milk protein, we explored the relationship between the levels of these receptors and the lactoferrin, an iron-binding ubiquitous protein with multiple functions. Here we discovered that lactoferrin efficiently downregulates the levels of ERα, PR and HER-2 receptors in a proteosome-dependent manner in multiple ER+ or HER-2+ breast cancer cells, and leads to the loss of responsiveness to HER-2- or ER-targeted therapies and increased invasiveness of lactoferrin-stimulated cells; in contrast, TNBC cell lines were inherently invasive in response to lactoferrin.

To understand the mechanism underlying of lactoferrin-mediated invasiveness of breast cancer cells, we used microarray chip hybridization approach to identify lactoferrin-regulated genes, and identified endothelin-1 (ET-1), a secreted pro-invasive polypeptide that acts through a specific receptor ET(A)R, as lactoferrin-regulated gene in multiple breast cancer cell lines. We found that

lactoferrin directly binds to specific iron-binding consensus motifs in the ET-1 promoter and stimulates its transcription, leading secretion of bioactive endothelin-1 peptide. Interestingly, a therapeutic ET-1 receptor antagonist drug completely blocked lactoferrin-dependent motility and invasiveness of non-TNBC and TNBC cells. To establish the physiologic significance of this newly discovered lactoferrin-ET-1 axis in the development and manifestation of TNBC phenotypes, we found evidence of elevated serum levels of lactoferrin and ET-1 as well as co-upregulation of lactoferrin and ET-1 in tumor tissues from TNBC patients as compared to the levels in ER+ breast cancer patients, suggesting a potential causal association of lactoferrin with TNBC phenotypes and associated invasiveness, presumably, due to transcriptional stimulation of ET-1. These findings describe the first physiologically relevant polypeptide as a determinant of downregulating all three therapeutic receptors in breast cancer which utilizes another secreted ET-1 system to confer invasive TNBC phenotypes. Since a number of approved drugs targeting ET-1 system are in non-cancer clinical trials, these studies raise the possibility of targeting lactoferrin-ET-1 axis to delay and/or reverse the progression of clinical features of TNBC patients.

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List of Symbols / Abbreviations

1.	ATCC	American Type Cell Culture
2.	BRCA1	Breast Cancer 1, early onset
3.	BRCA2	Breast Cancer 2, early onset
4.	BSA	Bovine Serum Antigen
5.	CHX	Cycloheximide
6.	DAB	3,3' Diaminobenzidine
7.	DAPI	4’6-diamidino-2-phenylindole, dihydrochloride
8.	DMEM	Dulbecco’s Modified Eagle Medium
9.	DMEM/F21/50/50	Dulbecco's Modification of Eagle's Medium/Ham's F-12 50/50 Mix
10.	DMSO	Dimethyl Sulfoxide
11.	ECE	Endothelin-Converting Enzyme
12.	ECM	Extracellular Matrix
13.	EGF	Epidermal Growth Factor
14.	EGFR	Epidermal Growth Factor Receptor
15.	ELISA	Enzyme-Linked Immunosorbent Assay
16.	ERα	Estrogen Receptor alpha
17.	ET(A)R	Endothelin-A Receptor
18.	ET(B)R	Endothelin-B Receptor
19.	ET-1	Endothelin-1

20.	FBS	Fetal Bovine Serum
21.	GPCR	G Protein-Coupled Receptor
22.	GTP	Guanosine 5'-Triphosphate
23.	HER-2	Epidermal Growth Factor Receptor-2
24.	HER-3	Epidermal Growth Factor Receptor-3
25.	HER-4	Epidermal Growth Factor Receptor-4
26.	hrs	Hours
27.	MMP	Matrix Metalloproteinases
28.	NADPH	Nicotinamide Adenine Dinucleotide Phosphate
29.	Pak1	p21-activated kinase 1
30.	PBS	Phosphate-Buffered Saline
31.	PBS-T	Phosphate-Buffered Saline Tween 20
32.	PDGFR	Platelet-Derived Growth Factor Receptor
33.	PI	Propidium Iodide
34.	PR	Progesterone Receptor
35.	qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
36.	RIPA	Radio Immuno Precipitation Assay
37.	SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
38.	SERM	Selective Estrogen Receptor Modulator
39.	TNBC	Triple-Negative Breast Cancer

Chapter 1: Introduction

1.1 Breast Cancer

1.1.1 Statistics

According to the statistics published by the American Cancer Society (American Cancer Society, 2011), breast cancer is the most common cancer among all types of carcinomas in women. Accounting for 30% of all female tumor-related cases, breast cancer continues to be the most frequently diagnosed cancer among women in 2011. While mammary carcinoma has the highest incident rate with approximately 290,000 new cases in 2011 (230,000 invasive breast cancers and 60,000 in situ breast tumors), its mortality rate is significantly lower with an estimated 40,000 deaths annually (American Cancer Society, 2011). The discrepancy between the rate of incidence and mortality is in part due to the advancement of not only earlier, but also more improved screening such as more sensitive mammograms. In addition, technological advances have allowed for better prognostication of breast cancers and therapy management. Such examples include Mammaprint and Oncotype DX are FDA-approved genomics tests that were developed to predict distant recurrence and the suitability of combining tamoxifen and cytotoxic chemotherapy, etc. (van't Veer et al., 2002; Kaklamani, 2006). Furthermore, a better understanding of the basic biology of breast carcinomas as well as the introduction of targeted therapies such as tamoxifen and Herceptin has contributed to the slight decline of deaths associated with breast cancer.

However, while there is a big gap between the rate of incidence and death, breast cancer unfortunately still remains the second leading cause of death in women, behind lung cancer (American Cancer Society, 2011). Even with the advances made in terms of early diagnosis and better detection, breast cancer is and continues to be a fatal disease. Therefore, ongoing research aims to reduce the breast-cancer fatalities by further developing targeted therapies. While the general etiology of breast cancer is known, there are still many knowledge gaps to be filled. Studies on the etiology of breast cancer have concluded that this type of carcinoma is not a single disease owing to its heterogeneity at the pathohistochemical, molecular and genomics level (Sorlie et al., 2001; Weigelt et al., 2005; Reis-Filho and Pusztai, 2011; Russnes et al., 2011) therefore making it very difficult to narrow down to a single culprit. Like most cancers, it is considered a complex disease with multifactorial influences which contribute to a poorly understood etiology and subsequently add to a lack of personalized treatment options.

1.1.2 Etiology

Broadly speaking, breast cancer is a disease that is greatly influenced by genetic as well as environmental factors. Therefore, there are many different research areas concentrating on the various aspects that contribute to breast cancer varying from cancer-susceptible genes to environmental causes such as life style. The knowledge obtained over decades of studying the etiology of breast

cancer has greatly facilitated earlier and improved detection technologies as well as the development of targeted therapy; however there still exists the need for better understanding on the mechanism and pathways involved in breast cancer.

Because breast cancer is considered to be a cancer of the reproductive system, many studies have focused on understanding how the endocrine system plays a role in breast carcinoma. With all the research conducted in the past decades, it is now known that most breast cancers are hormone-dependent which require estrogen, progesterone and other growth stimulating factors for cell proliferation (McPherson et al., 2000; Bernstein, 2002; Basu and Rowan, 2005). While normal cells also need hormones and other signals for growth and development, their malignant counterparts have developed mechanisms and pathways through which they continuously obtain growth signals leading to proliferation. Therefore, the difference between normal and breast cancer cells lies in the fact that the malignant cells have adapted to loss of self-control and subsequently they proliferate aberrantly. However, the exact mechanisms through which the cells lose self-growth control and the specific signals and molecules that contribute to hormone-dependent growth still remain to be elucidated.

Since it is known that hormones greatly contribute to uncontrolled growth, steroid receptors such as the estrogen receptor (ER) and progesterone receptor (PR) are some of the most widely studied molecular markers in breast tissue. (Althuis et al., 2004; Basu and Rowan, 2005). Extensive research on these receptors has allowed for the development of targeted therapies such as tamoxifen

or aromatase inhibitors which have been proven to be effective treatment options in hormone-dependent breast cancers (McPherson et al., 2000; Sledge and Miller, 2003). Furthermore, over the past decade, numerous independent studies have shown that an overexpression of human epidermal growth factor receptor 2 (HER-2) correlates with more aggressive breast cancer (Olayioye et al., 2000) which led to the development of a monoclonal antibody against HER-2 by Genentech (St. Francisco, CA). This monoclonal antibody, named Herceptin (trastuzumab) was shown to be successful in reducing the proliferation of breast cells and is used today as the first line of therapy for breast cancers with overexpressed HER-2 (Hudis, 2007). While there have been huge improvements on targeted breast cancer treatments, there are still many subtypes of breast carcinomas that currently lack treatment mainly because the etiology and their heterogeneity is less well understood.

In addition to hormones, there are also other risk factors that contribute to the incidence of breast cancer. Similar to most cancers, age is a common risk factor for the development of mammary carcinomas (McPherson et al., 2000; Bernstein, 2002). In general, the risk increases with an increase in age as most women will be diagnosed with breast cancer later in life (Trichopoulos et al., 2008). Because hormones are a risk factor, it has been shown that prolonged hormonal exposure throughout a woman's fertile years increases her chances of developing breast cancer (McPherson et al., 2000; Bernstein, 2002). Repeated menstrual cycles that are under the strict regulation of hormones are correlative with the risk for breast

cancer as well (Bernstein, 2002). Age and hormone exposure therefore, have a positive correlation with the incidences of breast carcinoma (Guzman et al., 1999). Interestingly, this noted observation does not apply to all types of breast cancers since studies have shown that the triple negative breast cancer, a subtype defined by no or low levels of ERα, PR and HER-2, affects younger women and the exact reasons for this phenomenon are currently still unknown (Carey et al., 2010; Shinde et al., 2010).

Because breast cancer development has been linked to exposure to hormones, many epidemiological studies have shown that there is also a correlation between pregnancy and the incidence of mammary carcinomas (Bernstein, 2002; Woo et al., 2003). It is thought that a delay in parity or nulliparity increases the risk for breast cancer as it has been observed that pregnancy exhibits protective effects against the development of breast carcinoma (McPherson et al., 2000; Trichopoulos et al., 2008). The mechanisms for this observation are still not well understood, however a few theories have been developed: Russo and Russo have hypothesized that protection during pregnancy occurs due to the differentiation of breast lobules most susceptible to carcinogenesis. Using a rodent model as well as human tissues to study this hypothesis, they were able to identify certain epithelial cells within the breast tissue that were more susceptible to chemical-induced carcinogenesis. Prior to pregnancy, these epithelial cells which make up lobules type 1 called terminal ductal lobular units, are undifferentiated and highly proliferative making them vulnerable to mutations and therefore contribute to

neoplastic transformation. Upon pregnancy, the breast epithelium undergoes irreversible terminal differentiation and consequently protects the lobules from tumorigenesis. To model this, the investigators showed that pregnant mice were less vulnerable to chemically induced mammary tumorigenesis (Russo and Russo, 1996; Russo et al., 2006) indicating a protective mechanism by pregnancy against carcinogenesis. While these data give a plausible explanation for pregnancy-induced protection against the development of breast cancer, it does not account for all cases of breast cancer that are observed in later stages of life, those occurring in pregnant women and the ones affecting women following parity.

Although most studies show that early pregnancies could possibly protect against breast cancer in comparison to late child birth, certain subtypes, such as triple negative breast cancers, have a positive correlation between parity and the incidence of cancer (Bonnier et al., 1997; Woo et al., 2003). The hypothesis for the latter case is that breast cells which are undergoing remodeling during pregnancy are exposed to higher levels of female hormones and therefore are more likely to develop TNBC over the other subtypes. Whether neoplastic transformation had occurred prior or during pregnancy remains unknown, yet there is a higher incidence of receptor-negative breast cancer among pregnant patients who have been diagnosed with breast carcinoma (Ishida et al., 1992; Bonnier et al., 1997; Agrawal et al., 2007; Shinde et al., 2010). While it is still unclear how pregnancy can also predispose a woman to mammary carcinoma, multiple studies have shown that women with breast cancer during or after pregnancy have a worse

diagnosis compared to age-matched nulliparous women (Petrek, 1994; Woo et al., 2003; Lyons et al., 2011). A few theories have been postulated to explain the phenomenon of pregnancy-related incidences of aggressive breast carcinoma. One explanation could be the delay in the detection of breast cancers in new mothers due to pregnancy-associated swelling of the breast. The increase in breast tissue density could make it more difficult to detect any signs of abnormal growth, therefore delaying diagnosis and leading to increased tumor size and late stage cancer at the time of detection (Petrek et al., 1991). In addition, among lactating women there is an increased incidence of breast-feeding-induced infections leading to misdiagnosis of mastitis instead of breast carcinoma (Petrek, 1994). These and other reasons could explain for the noted aggressiveness in pregnancy-related mammary tumors.

Furthermore, it is postulated that gestational hormones throughout pregnancy and breastfeeding could influence tumor growth and aggressiveness. Because pregnancy-related hormones are composed of high levels of estrogen, progesterone and other growth-stimulating factors, it is hypothesized that these could contribute to the poor prognosis seen in pregnant women and new mothers. Research studies on xenograft mouse models as well as in cell culture have demonstrated that rise in gestational hormones lead to increased breast cancer cell proliferation and tumor size (Thorne and Lee, 2003) and that this aggressiveness could be blocked with anti-hormone therapy (Gottardis et al.,

1989). Therefore, it seems plausible to hypothesize an increase of breast cancer incidence following exposure to gestational hormones.

In addition, it is known that following pregnancy and lactation, the breast tissue undergoes a process called mammary gland involution which causes the cells within the breast to regress to their pre-pregnancy state (Hurley, 1989). This process involves a myriad of pathways and factors, including tissue-remodeling programs and inflammatory processes (Schedin, 2006). It is hypothesized that during this process, breast cells are exposed to many factors that can lead to tumorigenesis or cancer progression (Bemis and Schedin, 2000; McDaniel et al., 2006; Lyons et al., 2011). As part of the tissue remodeling process, the basement membrane has to undergo extracellular matrix degradation that involves an upregulation of factors such as matrix metalloproteinases (MMP) to ensure the loss of cell adhesion to the basement membrane. Therefore it is postulated that during the local disruption of the basement membrane, the existing tumor cells are able to start infiltrating into the surrounding stroma which includes the extracellular matrix (ECM). Following that, the tumor cells come into contact with the surrounding stroma and the secreted factors stimulate them to be become more invasive. It is thought the ECM in the mammary microenvironment has pro-proliferative and tumorigenic effects on breast cancer cells (Wiseman and Werb, 2002; Schedin et al., 2004; McDaniel et al., 2006; Lyons et al., 2011). Several studies have shown that ECM isolated from pregnant rats was able to stimulate cell proliferation *in vitro* in comparison to the ECM of nulliparous rats, which

inhibited tumor progression (Wiseman and Werb, 2002; Schedin et al., 2004). The hypothesis therefore is that if a woman has already undergone undetected neoplastic transformation, pregnancy followed by mammary gland involution could further contribute to the aggressive behavior of breast cancer cells leading to a worse prognosis. While these studies provide a plausible explanation, further extensive research needs to be conducted to better understand the processes and pathways involved in pregnancy-related incidences of aggressive breast cancer progression.

Although hormonal influences play a major role on tumor progression, other genetic risk factors have been shown to contribute to the development of breast cancer. Research in the past decade has been able to identify certain genes that have a high correlation with breast cancer. Such genes include tumor suppressor genes BRCA1 and BRCA2, which are tumor suppressor genes mostly involved in DNA damage repair. It has been shown that women with germline mutations in BRCA1 as well as in BRCA2 have a greatly increased risk for developing hereditary breast tumors (Martin and Weber, 2000; Fackenthal and Olopade, 2007; Trichopoulos et al., 2008). Statistical analyses of mutations in these genes have shown that the risk is up to 80% for the development of mammary carcinomas compared to those without genetic mutations in these genes (Easton et al., 1995; Martin and Weber, 2000). However, the exact mechanisms how these mutations predispose women to breast cancer is still unclear. Since BRCA1 and BRCA2 are genes involved in DNA damage repair it is hypothesized that a loss-of-

function mutation can render the cells unable to protect against DNA damage and thus increase the risk of breast cancer (Deng and Wang, 2003). Interestingly, this predisposed risk is also seen in other reproductive cancers such as ovarian carcinoma indicating that genetic variations can lead to a higher risk for cancer overall, especially those with hormone dependency (Easton et al., 1995; Martin and Weber, 2000). Therefore, in conjunction with family history, mutations in BRCA1 and BRCA2 greatly predispose the affected patient to developing hereditary breast cancer.

In addition to genetic risk factors, lifestyle and other environmental causes can greatly contribute to the development of breast cancer (Bernstein, 2002; Trichopoulos et al., 2008). These include smoking, diet, weight, alcohol consumption and hormone replacement therapies (McPherson et al., 2000). In comparison to genetic predispositions, these are factors that can be controlled and therefore can decrease the risk for breast cancer if managed well. Therefore, these factors do not pose a high risk compared to those that are not controllable.

In conclusion, while all the pathways that lead to breast cancer are currently unknown, major contributions to the malignant transformation of breast epithelial cells is the exposure to hormones and other growth factors. Research in the last decades in addition to ongoing efforts in understanding breast cancer have shown some of the pathways through which hormones and hormone-related factors can influence breast cancer progression. Because of some of the reasons described in this section, many studies are focused on genes and pathways related to hormone

signaling in the hope to find areas that could benefit from targeted therapy. While many other factors not discussed here can contribute to the development of breast cancer (for example see (Martin and Weber, 2000; Althuis et al., 2004; Trichopoulos et al., 2008), the focus in this dissertation will be on hormonal influences on breast cancer and how they can promote aggressiveness.

1.1.3 Types of breast cancer

Breast cancer, as the name implies, is a malignant disease that originates in the breast tissue. Generally speaking, breast cancer can occur in any cell within the tissue, however, it most commonly takes place within the ducts or lobules of the breast (Fig 1). Due to their origin of carcinogenesis, these cancers are referred to as ductal and lobular carcinomas. The majority of breast cancer incidences fall into the former category while a smaller percentage belongs to the latter (Li et al., 2003).

Clinically and biologically speaking, breast carcinomas can be further classified beyond their tissue/place of origin (Li et al., 2005). When identifying breast cancers with regards to their receptor statuses, they can be categorized into three different subgroups (Sorlie et al., 2001; Deng and Wang, 2003; Vargo-Gogola and Rosen, 2007; Reis-Filho and Pusztai, 2011):

1) Luminal subtype: As mentioned, most types of breast cancer are hormone-dependent tumors implying that they are estrogen and progesterone receptor-positive cancers with a normal expression of HER-2. This subtype is named

luminal subgroup due to the expression of genes and proteins that are similar to those of luminal epithelial cells (Sorlie et al., 2001). The majority of breast cancers are considered to be luminal and because these cancers are the most common subtype, they are well studied and can benefit from targeted therapies such as tamoxifen or aromatase inhibitors. Due to their genetic nature, this subtype of breast cancer is slower growing compared to the other subtypes and therefore has a higher survival rate (Sorlie et al., 2001; Dunnwald et al., 2007; Leong and Zhuang, 2011).

2) HER-2 subtype: There is a small percentage of breast cancer that has an overexpression of the HER-2/neu gene that translates to an overproduction of the HER-2 receptor. These receptors naturally bind growth factors and therefore contribute to normal cell proliferation and development. When overexpressed, however, they can be overstimulated by hormones and growth factors that in turn promote tumorigenesis. For reasons currently unknown, this subtype of breast cancer is more aggressive and its survival rate is much lower in comparison to the luminal subgroup (Sorlie et al., 2001). One of the possibilities relates to the enormous signaling pathways within the EGFR family. HER-2 signaling occurs when the receptor homodimerizes or when it dimerizes with another receptor within the family, such as EGFR, HER-3 or HER-4. Depending on the dimerization, the subsequent signaling pathways differ leading to a diversity of cellular activities such as cell proliferation, migration, invasion and inhibition of apoptosis (Olayioye et al., 2000). Fortunately, a majority of patients affected with HER-2++ (HER-2

over-expressed) breast cancers can benefit from a HER-2 targeted therapy that has a high success rate (Leong and Zhuang, 2011).

3) Basal-like/Triple negative subtype: Among all breast cancer incidences, approximately 10-15% is categorized as triple negative breast cancer (TNBC). This subtype is referred to as triple negative because it is immunohistochemically defined by low or absent levels of ERα, PR and HER-2 (Dent et al., 2007; Carey et al., 2010). Additionally, it is coined basal-like because it has a high similarity to normal basal epithelial cells by staining positive for basal cell-specific cytokeratins 5/6 and 17 (Rakha et al., 2008). Furthermore, TNBC can be distinguished from different types of breast cancers by its unique molecular profile, relative aggressive behavior, distinct patterns of metastasis, and lack of targeted therapies (Rochefort et al., 2003; Carey et al., 2010). While, the exact mechanisms of endogenous molecules/pathways that are thought to be responsible for the loss of three therapeutic receptor targets remain unknown, it has been shown that TNBC affects primarily pre-menopausal women, African-Americans and those with a BRCA1 mutation (Dent et al., 2007). Furthermore, clinical studies indicate an increased risk for TNBC in young women at first full term pregnancy (Bonnier et al., 1997; Agrawal et al., 2007). Because of the lack of ERα, PR and HER-2, the conventional hormone- or hormone receptor-targeted therapies are ineffective, rendering chemotherapy and/or surgery as the only options in the therapy of breast cancer. In an effort to provide patients diagnosed with TNBC with less

invasive forms of treatment, current research aims to find other potential targets for therapy (Rochefort et al., 2003).

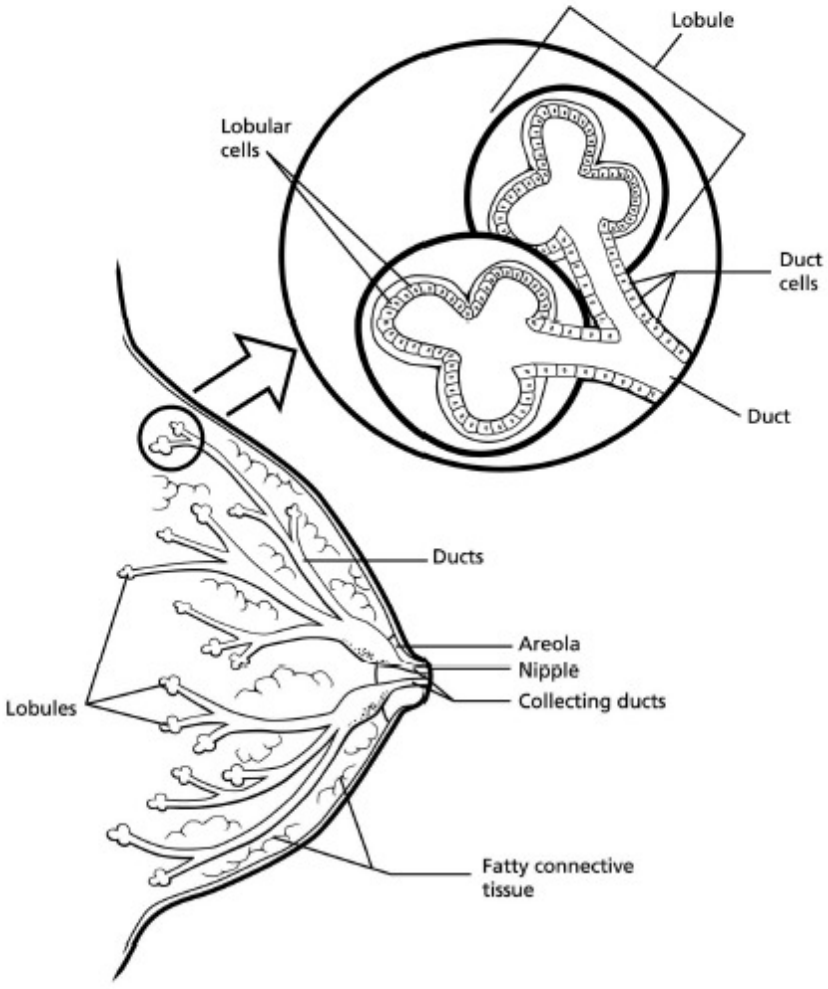

Figure 1. Diagram of areas in the breast tissue in which cancer occurs (Adapted from the American Cancer Society)

1.1.4 Treatment

As already eluded to in the previous section, treatment options for breast cancer can range from conventional cytotoxic chemotherapy to targeted treatment depending on the tumor subtype. Even though research on breast cancer has allowed for the development of a few targeted therapies (Sledge and Miller, 2003), the most conventional treatment for breast cancer remains surgery along with radiation and adjuvant cytotoxic chemotherapy.

Depending on the stage of the cancer at diagnosis, surgery is considered the best option for those affected by breast cancer. While it is not a pharmacological treatment, it is the physical removal of cancerous cells from the breast. This leads to a great reduction of tumor cells within the tissue allowing subsequent adjuvant treatment options to be more successful.

In addition to surgery, the first and foremost pharmacological therapy against breast cancer still remains cytotoxic chemotherapy. Chemotherapeutic agents are not considered targeted treatment because they employ the conventional method of killing cells: they interfere with the replication of cells that proliferate frequently. By intercalating into the DNA, the class of cytotoxic agents known as anticancer antibiotics disrupts cell replication rendering the cells to undergo cellular death. While these agents are successful in killing rapidly growing tumor cells, they are not specific enough to distinguish between cancer cells and normal fast proliferating cells (such as hair follicles, gastrointestinal, bone marrow). Therefore, side effects of chemotherapy include alopecia, immune- and myelosuppression

and nausea and vomiting. Since hair follicles, bone marrow cells as well as digestive cells naturally have a high proliferative index, they are also affected by chemotherapeutic agents leading to these adverse effects. When patients are given adjuvant chemotherapy after surgical resection of the tumor, the success rate can be fairly high, depending on the type and stage of the tumor (Shapiro and Recht, 2001).

Due to the extensive side effects and the narrow therapeutic window of cytotoxic chemotherapeutic agents, many studies have focused on discovering better targets for personalized treatment. Not only will this allow a tailored regimen for breast cancer patients leading to better outcome, it will also potentially reduce the side effects that are common with cytotoxic chemotherapy. Since a majority of breast cancer belongs to the luminal subtype, the etiology as well as pathways involved are better understood and therefore this subtype benefits from better therapies. In addition to conventional cytotoxic chemotherapy and surgery, luminal breast cancers can profit from targeted treatment such as tamoxifen or aromatase inhibitors (Muss, 1992).

Tamoxifen was developed in 1966 as an estrogen receptor antagonist (Harper and Walpole, 1966) and it belongs to the family of selective estrogen receptor modulators (SERM). It works by competitively binding to the estrogen receptor and therefore inhibiting estrogen interaction with its receptor leading to a block of any growth-stimulating signals. This subsequently leads to a decrease in cell proliferation and migration. In most cases, tamoxifen is given along with cytotoxic

chemotherapy to increase the chances of successful outcome (Osborne, 1998). Tamoxifen is effective in treating luminal breast cancers, and is therefore considered to be advancement with regards to personalized therapy. However, a small percentage of tumors unfortunately become resistant to this treatment (Ring and Dowsett, 2004; Riggins et al., 2007; Prat and Baselga, 2008; Musgrove and Sutherland, 2009; Rosano et al., 2010).

Aromatase inhibitors are also considered standard therapy options for the treatment of hormone-dependent breast carcinomas in post-menopausal women. While it is not considered a receptor-targeted therapy, it is still a successful treatment by inhibiting the conversion of androgens to estrogen, a hormone important in breast cancers. Because aromatase inhibitors block the production of estrogen, it is also given for the treatment of ovarian cancers (Smith and Dowsett, 2003).

For HER-2 overexpressed cancers, recent discoveries have led to the development of Herceptin (trastuzumab), a HER-2-specific monoclonal antibody. Analogous to tamoxifen, it works by binding to the HER-2 receptor and therefore inhibiting the binding of growth factors to the receptor. As discussed, an overexpression of HER-2 leads to excessive growth stimulation and therefore contributing to an increase in proliferation and aggressiveness. By having a monoclonal antibody such as Herceptin bind to the HER-2 receptor, the cells undergo cell cycle arrest in G1 phase thus leading to a decrease in cell proliferation, migration and invasion. Since the discovery of this personalized

treatment by Genentech, Herceptin is considered the standard therapy for breast cancers that overexpress HER-2. When given in combination with cytotoxic chemotherapy, it has a very high success rate (Hudis, 2007).

While these treatment options have proven to be successful advancement for the treatment of breast cancers, many other subtypes still rely on conventional chemotherapy and surgery. Thus far the best outcomes have been achieved when breast cancers are treated with targeted therapy after surgery and along with chemotherapeutic agents. Unfortunately, subtypes such as triple negative breast cancers, which do not express ERα, PR and HER-2, currently do not have any available targeted therapy regimens and therefore they primarily rely on chemotherapy and/or surgery (Cleator et al., 2007). While our knowledge base on the etiology of breast cancer is improving and continues to progress as a consequence of more sophisticated tools, technologies and computational approaches, there is still an urgent need to develop additional targeted therapies.

1.2 Current Gaps

While it is known that genetics as well as environmental factors play an important role in the development of breast cancer, there are still many knowledge gaps to be filled on the pathogenesis of neoplastic transformation of the breast epithelium. Owing to the heterogeneity of the disease, specific pathways leading to cancer still need to be better delineated to gain a better understanding towards the etiology of various subtypes of breast cancer. As discussed earlier, breast cancer

subtypes can be categorized based on receptor status: luminal A ($ER^+/PR^+/HER\text{-}2^+$), luminal B ($ER^+/PR^+/HER\text{-}2^+$), HER2 overexpressed ($ER^-/PR^-/HER\text{-}2^{++}$) and triple-negative ($ER^-/PR^-/HER\text{-}2^-$) (Sorlie et al., 2001). Receptor status in breast cancer usually indicates a successful therapy outcome as most conventional drugs target the hormone receptors in these cells. These targeted therapies include tamoxifen or Herceptin which are used for the treatment of ER-positive or HER-2 overexpressed breast cancers, respectively. TNBC is a subtype of breast tumors in which there are low or no levels of ERα, PR and HER-2 (Carey et al., 2010) and is characterized by a more relative aggressive behavior and lower survival rates. Due to the absence of the therapy-targetable receptors, there is currently a lack of personalized treatment for TNBC. Consequently, this subtype of breast cancer is in urgent need for the development of targeted treatment regimens.

Furthermore, the mechanism of how the receptors are downregulated in TNBC is currently unknown making this breast cancer subtype intriguing to investigate. Currently no receptor-targeted therapy is available for TNBC due to the lack of expression of ERα, PR and HER-2 and consequently, recurrence rates continue to be higher (Dent et al., 2007). While many hypotheses have been formed to understand the etiology of breast cancer and how hormones could possibly influence tumorigenesis, the exact mechanisms are still unknown.

Breast cancer is multifactorial, making it difficult to narrow down pathways involved in this complex disease. Because it is known that hormones and possibly pregnancy are major contributors to breast cancer progression, we set out to

analyze one of the hormonally-regulated genes, lactoferrin. This protein has been shown to be estrogen-responsive and therefore it is highly expressed during the menstrual cycle and pregnancy (Green and Pastewka, 1978; James et al., 1988; Teng, 2002). The aim of this dissertation therefore is to examine the contribution of lactoferrin in breast cancer and how it may influence mammary carcinoma progression. In addition, since lactoferrin is known to activate gene transcription by acting as a transcription factor, we also aimed to analyze genes regulated by lactoferrin. By understanding its involvement and contribution to breast cancer progression, we hope to get a better insight on how lactoferrin and its regulated gene(s) influence mammary carcinoma.

1.3 Lactoferrin

Lactoferrin, as the name suggests, is an iron-binding protein that was first isolated from bovine milk in 1939 (Sorenson and Sorenson, 1939). Because of its similarity in function and size, it is considered a member of the transferrin family which also binds and sequesters iron. While lactoferrin was first thought to only function in milk, it has subsequently been shown to have similar actions in exocrine secretions, including bile and pancreatic juice, as well as tears and small intestinal fluid, underlying its diversity of actions (Mulder and Morris, 2010). Since its first discovery, lactoferrin has been extensively studied and its functions have extended beyond its original bacteriostatic, iron-binding action (Sanchez et al., 1992; Garcia-Montoya et al., 2011).

Structurally, lactoferrin exists in two conformations: holo- and apolactoferrin, the first being iron-saturated and the latter referring to its iron-deficient form. This basic, positively charged 703-amino acid protein exists as a single polypeptide chain with two globular lobes for the binding of iron (Levay and Viljoen, 1995). Upon binding of iron, lactoferrin undergoes conformational changes and therefore, studies have suggested that the degree of iron binding is an important aspect to lactoferrin's physiological functions (Byrd and Horwitz, 1991). While it has been shown that lactoferrin has the highest affinity for iron, it can also bind other metals such as copper, magnesium, zinc and gallium (Levay and Viljoen, 1995).

Because lactoferrin was discovered as a protein capable of binding iron in milk and other secretions, the first biological role described for it was bacteriostatic function (Farnaud and Evans, 2003; Mulder and Morris, 2010). By binding and sequestering iron, it diminishes the iron availability to bacteria and other microbes which need it for proliferation and ultimately survival. In addition to bacteriostatic actions, studies have also shown that lactoferrin could possibly have bactericidal functions by damaging the outer membrane of bacteria (Farnaud and Evans, 2003). Therefore, lactoferrin has shown to play an important role in maintaining the host defense mechanism.

Since lactoferrin has a high isoelectric point and it is positively charged, studies on identifying tissue-specific lactoferrin receptors have been difficult. Due to these characteristics, lactoferrin is able to bind non-specifically to many molecules and proteins. This has made the discovery of all lactoferrin receptors very complex and

therefore only a few bona fide lactoferrin receptors have been identified. These include tissue-specific lactoferrin receptors in the intestine, brain, and on macrophages and neutrophils (Suzuki et al., 2005). Unfortunately, lactoferrin-specific receptors for the mammary gland have yet to be discovered.

Lactoferrin was first identified in neutrophils but later studies suggested that it is also produced in mucosal epithelial cells (Mulder and Morris, 2010). Being a secreted protein, lactoferrin is most highly expressed in plasma, however it is also found in other secreted fluids. Human as well as rodent studies have shown that lactoferrin can be regulated via hormonal signals. Promoter studies have identified an imperfect estrogen response element on the lactoferrin promoter indicating its ability to be transcriptionally regulated by estrogen (Teng, 2002). Furthermore, other studies have pointed towards prolactin-responsive regulation that could explain the presence of lactoferrin during pregnancy (Green and Pastewka, 1978; Nakajima et al., 2008). Throughout gestation, the levels of lactoferrin increase drastically, mainly via hormonal regulation. It is found at its highest level during the third trimester and upon lactation, the levels of lactoferrin decrease. The primary role for lactoferrin during pregnancy is not currently known but since it is has been shown to respond to prolactin (Green and Pastewka, 1978) its role could be important in the production of milk. Because lactoferrin exhibits anti-microbial actions, it is also thought that lactoferrin protects the breast tissue from mastitis during breast-feeding (Farnaud and Evans, 2003). Interestingly, several studies showed that the expression of lactoferrin is increased during mammary gland

involution after pregnancy and upon cessation of lactation (Welty et al., 1976; Levay and Viljoen, 1995). However, the role of lactoferrin during this process is not clearly understood.

One of the interesting characteristics regarding lactoferrin is its ability to regulate gene transcription. He et al. have described its DNA binding capability and they were also able to show the specific binding sequences for lactoferrin (He and Furmanski, 1995). This indicates that lactoferrin has the ability to enter the nucleus via a mechanism that is currently unknown and that it can regulate gene transcription by binding to promoter region of target genes. Therefore, it can be said that lactoferrin's additional role in cellular regulation is acting a transcription factor or transcriptional regulator. It is interesting to note that even though this discovery was made almost a decade ago, there are not many studies investigating the genes that lactoferrin regulates or any other consequences resulting from binding to DNA.

In conclusion, there are a number of functions that can be attributed to lactoferrin. While lactoferrin has clearly been shown to play a significant role in the immune response, particularly in fighting against bacterial infection, its other functions are less well studied and understood. Being a secreted protein that is ubiquitously found in body fluids (Mason and Taylor, 1978), it can be hypothesized that lactoferrin may carry out other functions that are currently unknown. Since the focus of this dissertation is on breast cancer, we wanted to study its role in mammary carcinoma.

1.3.1 Lactoferrin and Cancer

Since discovering and describing lactoferrin's primary function, extensive studies have shown it to be involved in many other aspects of human physiology and pathophysiology. As discussed in the previous section, lactoferrin's role in iron binding and immune response is fairly well studied. However, not much is known about lactoferrin in the context of cancer.

Since lactoferrin was discovered to be a hormone-regulated gene, many studies have focused on its expression in endocrine tissues such as the breast and uterus (Teng, 2002). Early immunohistochemical investigations showed it to be expressed in normal as well as mammary and endometrial tumors (Tuccari and Barresi, 2011). A particular study by Campbell et al. (Campbell et al., 1992) obtained a cDNA clone from human breast carcinoma metastasis mRNA in an effort to identify possible epitopes that could give insight into the tumor progression. Screening the cDNA library for a clone against the human milk fat globule membrane, a well-established source for breast glycoproteins, they isolated lactoferrin as the highest immunoreactive protein. Upon isolation, the authors used lactoferrin to investigate its expression in normal and tumorigenic breast tissues. While lactoferrin immunoreactivity was found in normal and lactating breast tissue, they observed it to be expressed in 56% of mammary carcinoma samples. Interestingly, the investigators noted an inverse correlation between the immunoreactivity of lactoferrin with the presence of ER in the carcinoma tissue samples (Campbell et al., 1992).

Similarly, a genome wide expression profiling study on endometrioid endometrial adenocarcinoma samples from Hong Kong Chinese women cohort identified lactoferrin to be almost five-fold more highly expressed in the cancerous tissue in comparison to normal matched controls (Wong et al., 2007). In addition, Schulz et al. (Schulz et al., 2009) showed a similar trend when examining TNBC in comparison to HER-2 positive breast tumors. Using 2-D difference gel electrophoresis followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, they identified lactoferrin to be more highly expressed in TNBC cases compared to HER-2 tumors.

Because endometrial carcinoma as well as breast cancer progression is dependent on estrogen signaling and other hormonal factors, the observation of high lactoferrin gene and protein expression is not surprising. However, the mechanistic role of lactoferrin in hormone-dependent endometrial or mammary carcinoma still needs to be determined. Identification of a pathway through which lactoferrin could possibly be involved in tumor progression could give a better explanation for the high expression of lactoferrin in carcinoma samples.

Furthermore, some studies have shown lactoferrin to be involved in cell proliferation and differentiation (Sanchez et al., 1992; Yanaihara et al., 2000; Lee et al., 2009), especially in osteoblasts and fibroblasts. Having pro-proliferative properties lactoferrin could possibly be linked to tumorigenesis and/or cancer progression. The existing data on lactoferrin and carcinogenesis however is conflicting as there are studies that have observed anti-tumorigenic effects while

others have reported it to play a role in promoting cell proliferation (Yanaihara et al., 2000). A study led by Duarte et al. (Duarte et al., 2011) showed that treatment of breast cancer cell lines with bovine apolactoferrin induced a decrease in cell proliferation and an increase in apoptosis. In contrast, Albright and Kaufman (Albright and Kaufman, 2001) demonstrated lactoferrin-dependent increase in cell proliferation of endometrial carcinoma cell lines. Interestingly, the same study suggested that a stable expression of lactoferrin in endometrial carcinoma cells could play a role in tamoxifen resistance. The differences in results between these studies could be the source from which lactoferrin was obtained (human versus bovine milk), the concentration of lactoferrin used, or the differences in cell as well as cancer types. Because lactoferrin's primary function is anti-inflammatory and anti-bacterial, many studies have focused on lactoferrin's potential anti-tumorigenic effect. A literature search on lactoferrin in regards to cancer, however yields conflicting results with no known mechanisms on lactoferrin's functional involvement in cancer. Therefore, it can be postulated that lactoferrin could possibly be involved in both stimulating tumor progression and protection against carcinogenesis using mechanisms that are currently not well understood.

While no mechanisms for lactoferrin with regards to cancer progression have been found, there were interesting observations from immunohistochemical studies on tumor tissues. As already eluded to earlier, there were a few studies that demonstrated an inverse correlation between steroid receptors and lactoferrin expression (Giuffre et al., 2006). In addition to the studies already discussed,

significant results from investigations conducted in the 1990s also noted lactoferrin expression in receptor-negative tissues (Campbell et al., 1992; Das et al., 1998). The immunohistochemical data from this particular study revealed overexpression of lactoferrin mRNA and protein in endometrial adenocarcinoma that lack progesterone receptors (Walmer et al., 1995).

In conclusion, while there is a lack of a definitive mechanistic role linking lactoferrin to cancer initiation and/or progression, many immunohistochemical data point to a possible contribution. Furthermore, it is interesting to note that a few studies have observed higher lactoferrin expression in cells that are stained negative for ER or PR. Since TNBCs do not express ERα, PR or HER-2 it would be of interest to understand lactoferrin's functional role in such tumors.

1.4. Endothelin-1

Endothelin-1 (ET-1) is part of a family of peptide hormones known as endothelins, which is involved in blood pressure control (Kawanabe and Nauli, 2011). While there are two additional members of endothelins, ET-1 is the most widely studied and in the context of this dissertation, only the role of ET-1 will be discussed. ET-1 was first described as a vasoconstrictive peptide isolated from porcine aortic endothelial cells (Yanagisawa et al., 1988) and because of this discovery is has been linked to cardiovascular diseases. Since its discovery, ET-1 has been well studied with specific emphasis on its role in cardiovascular

physiology and pathophysiology. As a consequence of its known functions within the vascular system, its involvement in vasoconstriction and related signaling is well understood.

The endothelin-1 gene is located on chromosome 6 and this 21 amino acid peptide is synthesized as preproendothelin that is converted to its active form by endothelin-converting enzyme (ECE). Once activated, ET-1 is secreted and exerts its functions in a paracrine and endocrine manner. Because of its involvement in vascular physiology, factors such as thrombin, angiotensin II, insulin, etc can activate the production of ET-1 (Kawanabe and Nauli, 2011). ET-1 is primarily produced by endothelial cells and therefore its major role is in the vascular system. Since ET-1 is a secreted peptide, extensive research have identified two ET-1 receptors, ET(A)R and ET(B)R. ET-1 receptors are part of the G protein-coupled receptor gene superfamily, and ET(A)R and ET(B)R have been shown to elicit different functional responses upon activation by ET-1. In the cardiovascular system, ET(A)R has been demonstrated to be the dominant receptor. It was shown that ET-1 upon binding to the A receptor isoform causes vasoconstriction of smooth muscle cells while binding to ET(B)R could induce vasodilation. Since ET-1 is linked to cardiovascular disease, an ET(A)R antagonist, BQ123, was developed that has specific binding to the A isoform only. By blocking ET-1 binding, vasoconstriction is inhibited and therefore has been proven helpful in studying cardiac failure and hypertension (Kawanabe and Nauli, 2011).

1.4.1 Endothelin-1 and Cancer

While most studies on ET-1 have focused on its role in hypertension and other cardiovascular diseases, recently, it has also been shown ET-1 to play a role in tumorigenesis (Bagnato and Rosano, 2008). The first study of ET-1 possibly being involved in cancer was reported by Kusuhara et al. (Kusuhara et al., 1990) demonstrating ET-1 production by several tumor cell lines including prostate, glioblastoma and breast. This opened up an avenue to study the function role of ET-1 in cancer progression. Since then, it has been reported that ET-1 may control cell proliferation, migration, apoptosis and tumor invasion (Nelson et al., 2003; Bagnato et al., 2011).

In addition to ET-1 production by cancer cell lines, it has been demonstrated that patients with solid tumors have elevated plasma levels of ET-1 with higher expression being found in those with metastatic cancers (Grant et al., 2003). *In vivo* studies have also shown high immunoreactivity of ET-1 in liver, ovarian and breast cancers indicating a potential role of ET-1 in tumor progression (Yamashita et al., 1991; Suzuki et al., 1998; Bagnato et al., 1999).

While there has been an observation of higher ET-1 expression in cancer cell lines as well as tumor tissues, it has also been demonstrated that there is an elevated expression of ET(A)R on carcinoma cells (Wulfing et al., 2003; Lalich et al., 2007). Since ET-1 primarily binds to its A receptor isoform it is not surprising to note a higher production of ET(A)R as well. Interestingly, ET(B)R was found to be more highly expressed in normal tissues compared to cancer samples.

Furthermore, within a cancer context, ET-1 binding to ET(A)R has been shown to increase invasion and migration, leading to a more relative aggressive phenotype (Bagnato et al., 1997).

The functional role hypothesized for ET-1 with regards to tumor progression includes an increase in cell proliferation, migration and invasion. Rosano et al. (Rosano et al., 2010) as well as Bagnato et al. (Bagnato et al., 1995) have identified ET-1 to be an autocrine growth factor in ovarian carcinoma cells. Furthermore, another study led by Schrey et al. (Schrey et al., 1992) revealed an increased production of ET-1 in human breast cancer cells stimulated by bombesin and glucocorticoids. These studies therefore further verify ET-1 to have mitogenic functions that can be involved in tumor progression.

In addition to stimulating cell growth, there is increasing evidence that ET-1 is also involved in migration and invasion leading to aggressive cancer progression. It has been shown that by upregulating the expression of matrix metalloproteinases, ET-1 can increase invasion in human ovarian cancer cell lines (Rosano et al., 2010).

In conclusion, there is extensive evidence implicating ET-1 in the progression of various solid tumors, including breast cancer (Grimshaw, 2005; Hagemann et al., 2005). Not only has it been demonstrated to be more highly expressed in cancer cell lines as well as tumor tissues, the functional role of ET-1 has also been elucidated. Having mitogenic, migratory and invasive properties allows ET-1 to be

involved in cancer progression by mechanisms that still need to be further elucidated.

1.5 Pak1

p21-activated kinase 1 (Pak1) is a molecule that was first discovered as a 65kD protein in the rat brain by Manser et al. (Manser et al., 1994). Because it was identified as a new target for GTP-binding proteins (or small G-proteins) Cdc42 and Rac1 which are small p21 proteins, it named p21-activated kinase (Manser et al., 1994). GTPases are known to play a role in a variety of cellular functions, both under normal and pathological conditions. Upon binding to their effectors, they can elicit several downstream signaling pathways leading to many cellular events. Since Pak1 was identified as a target of these small p21 proteins, it also plays a role in cellular communication that modulate a variety of biological activities in the cell (Knaus and Bokoch, 1998).

Pak1 is the most studied member of the Pak family. While the other six members are structurally similar, their functions are different. Because of this, the Pak family can be divided into two general categories that are based on their structural and sequence homology: group I comprises of Pak1-3 and Pak4-6 belonging to group II (Jaffer and Chernoff, 2002; Ong et al., 2011).

Since Pak1 is an effector protein for Cdc42 and Rac1, its activity becomes enhanced upon binding of the GTP-bound form of these small proteins. Once Pak1 becomes autophosphorylated, it is freed from Cdc42 and Rac1 leading to

prolonged activation of its kinase activity (Bokoch, 2003). Upon kinase activity stimulation, Pak1 can then elicit a myriad of cellular activities that are involved in the maintenance as well as changes of the cell. Because Pak1 was first discovered in the rat brain and was thought to be primarily expressed in neuronal cells, Pak1's original perceived roles were limited to brain development and function. However, since the regulatory functions include differentiation and growth, it has since been studied in a variety of normal and disease states.

Pak1 has been shown to be activated through receptor tyrosine kinase signaling such as EGF binding through EGFR. Via this binding, Pak1 is activated through the Erk1/2 pathway leading to downstream changes in cell motility (Takeuchi and Ito, 2009). This indicates that Pak1 is involved in regulation of growth in addition to cytoskeleton remodeling (Parrini et al., 2005).

Furthermore, Pak1 has been demonstrated to be activated via other receptors such as G protein-couple receptors (GPCR). Knaus et al. (Knaus et al., 1995) were the first to show that activation of GCPR were able to regulate Pak1 function via binding of GTP-bound Cdc42 and Rac1. While they demonstrated that this activation of Pak1 might be involved in the NADPH activity, studies since have shown that its functions extend to many other cellular events. Pak1 stimulation via growth factor receptor activation has been demonstrated to lead to cellular differentiation and growth. Even though Pak1 is involved in maintenance of normal cellular functions, overexpression or increased stimulation of Pak1 kinase activity

has also been shown to be play an important role in tumor initiation and progression.

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1.5.1 Pak1 and Cancer

In recent years, the Pak family, especially Pak1 has been found to play a significant role in cancer signaling. It is well-documented that an increase in Pak1 expression as well as an upregulation of Pak1 activity is involved in many cancers, including breast tumors (Kumar et al., 2006; Wang et al., 2006; Dummler et al., 2009). Studies in our laboratory and others have shown that an increase in Pak1 activity, stimulated by various signals, activates numerous signaling pathways leading to the progression of cancer phenotypes in cancer cells (Wang et al., 2002; Kumar et al., 2006). Furthermore, it has been established that G protein-coupled receptor-dependent mechanisms can activate Pak1 activity and therefore can drive cancer cells to increase cell motility, survival and proliferation (Dummler et al., 2009).

One of the mechanisms through which Pak1 kinase activation can lead to tumor progression is through its involvement in cytoskeletal dynamics (Bokoch, 2003). Pak1 has been shown to colocalize with actin structures upon activation of various growth factor receptors such as EGFR, PDGFR, insulin receptor, etc. Once stimulated, Pak1 relocalizes from the cytosol to the leading edge of motile cells thus driving cell motility. In addition, Pak1 was demonstrated to localize to vinculin-containing focal adhesion points (Molli et al., 2009).

Furthermore, Pak1 overexpression has been observed in several tumors, including mammary carcinoma. Increased Pak1 protein expression has been correlated with advanced stage tumors and therefore is correlated with relative aggressiveness. *In vitro* data have demonstrated that overexpression of Pak1 leads to an increase in invasion of cancer cells, recapitulating the high expression of Pak1 in more aggressive tumors (Wang et al., 2006). While an upregulation of Pak1 protein expression and activity could be the result of gene amplification, an increase in Pak1 kinase activity can also lead to tumor progression. Such increase in activity could be due to an upregulation of small GTPases or an increase in extracellular stimulation of Pak1-activating receptors.

Pak1 overexpression or an increase in its kinase activity has been well documented in breast cancer. *In vitro* as well as *in vivo* data have confirmed that Pak1 deregulation leads to tumorigenesis and/or contributes to a more aggressive tumor phenotype. Studies in our laboratory have shown that constitutively active Pak1 leads to increased cell proliferation, mitotic defects and anchorage-independent growth in breast cancer cells (Vadlamudi et al., 2000). In comparison, kinase-dead Pak1 was able to inhibit the observed aggressiveness. Furthermore, we have demonstrated that increased growth-factor stimulation of Pak1 results in relocalization of Pak1 to the leading edge, therefore promoting invasiveness of breast carcinoma cells. Using a receptor-specific antagonist against HER-2, Pak1-dependent invasiveness was blocked (Adam et al., 1998).

In conclusion, the role of Pak1 in normal as well as cancer cells has been extensively studied and its involvement in breast cancer is fairly well understood. Interestingly, it has been shown that G protein-coupled receptors are able to stimulate Pak1 kinase activity and it would be of significance to further study the role of certain G-protein coupled receptors in breast cancer progression.

1.6. Rationale, Hypothesis and Aims

The focus of this research is to understand the role of lactoferrin, an iron-binding secreted protein found in body fluids including milk with roles in anti-inflammation and bactericidal events (Mulder and Morris, 2010), in breast cancer cells and how it could possibly contribute to mammary carcinoma progression. Based on the knowledge of breast cancer and lactoferrin, our overarching hypothesis is that lactoferrin is involved in the downregulation of ERα, PR and HER-2 and that it can therefore contribute to an aggressive triple-negative phenotype. During the course of our preliminary work the following questions were addressed:

1) Can lactoferrin treatment downregulate ERα, PR and HER-2 in human breast cancer cells?

2) Acting as a potential transcription factor, does lactoferrin regulate genes that can contribute to breast cancer progression and relative aggressiveness?

Based on this rationale, we have discovered that lactoferrin downregulates the levels of ER, PR, and HER-2. In addition, we found that lactoferrin also promotes cell motility as well as stimulates p21-activated kinase 1 (PAK1) activity, a molecule known to promote motility and invasion by well-defined pathways (Kumar et al., 2006; Dummler et al., 2009). The third interesting observation includes lactoferrin stimulation of the transcription and secretion of bioactive endothelin-1 (ET-1), a molecule aberrantly over-expressed in cancer cells and promotes motility and invasion using ill-defined pathways (Grant et al., 2003; Nelson et al., 2003).

Based on these novel findings, the testable hypothesis of this dissertation is that lactoferrin, a secretory protein, may play a role in the development of a triple-negative breast cancer phenotype by downregulating ERα, PR and HER-2. Additionally, lactoferrin may regulate breast cancer cells' motility and invasion by the ET(A)R-Pak1 pathway via acting as a transcriptional factor for *endothelin-1*. We propose to elucidate the mechanism by which lactoferrin affects the biology of breast cancer cells with a special emphasis on the mechanisms of lactoferrin downregulation of ERα, PR and HER-2, lactoferrin stimulation of ET-1 and PAK1 activity, and also connect these molecular effects with the phenotypic changes such as motility and invasion of breast cancer cells. We will achieve these goals by investigating the following specific aims (Fig. 2):

Aim 1. To test the hypothesis that lactoferrin may downregulate ERα, PR and HER-2 and thus, contribute to the development of TN-phenotype in ER+/HER2+ cells. To identify the role of lactoferrin in triple negative breast cancer we will perform the following experiments:

Sub aim 1a) To investigate if lactoferrin can contribute to a more relative aggressive phenotype we will determine its role in cell invasion and migration.

Sub aim 1b) To validate the phenotypic changes using a stable clone overexpression lactoferrin.

Sub aim 1c) Further validation of this hypothesis will be done via immunohistochemistry and ELISA in human breast cancer samples.

Aim 2. To investigate the mechanism of receptor downregulation by lactoferrin. Receptor downregulation can occur at a transcriptional or post-translational level and to identify via which pathway lactoferrin downregulates ERα, PR and HER-2.

Sub aim 2a) To determine the mechanism at the transcriptional level

Sub aim 2b) To determine the mechanism at a post-translational level.

Aim 3. To define the nature of lactoferrin-regulated genes that could contribute to the relative aggressiveness of breast cancer cells including triple negative breast cancer. We hypothesize that lactoferrin can contribute to a relative aggressive breast cancer phenotype. To identify genes that could play a role we will perform the following experiments:

Sub aim 3a) To perform a microarray analysis using lactoferrin-treated breast cancer cells.

Sub aim 3b) To further study selected gene(s) using biological assays.

Sub aim 3c) To validate findings in human breast cancer samples.

Aim 4. To explore the role of lactoferrin in the induction of PAK1 activity.

Sub aim 4a) To define the basis and significance of lactoferrin-mediated stimulation of PAK1 activity in breast cancer cells via Pak1 kinase assay.

Sub aim 4b) To explore the biological endpoints of lactoferrin-dependent activation of Pak1 kinase activity.

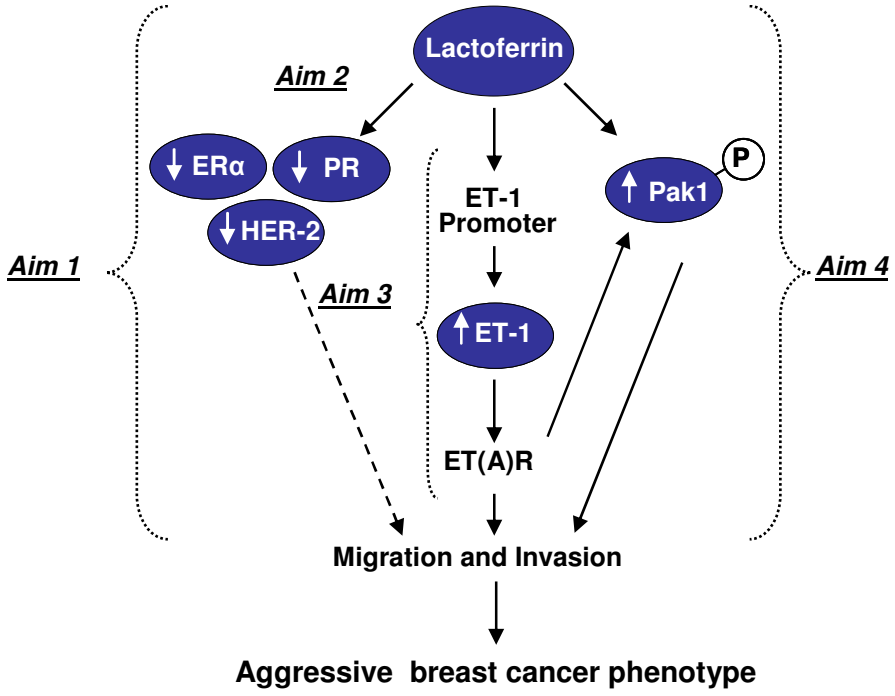


Figure 2. Proposed model of lactoferrin-ET-1 axis

Chapter 2: Materials and Methods

Patients, Materials and Methods

Human Patient Samples

Dr. Suzanne Fuqua (Baylor College of Medicine, Houston, TX) provided human breast cancer tissue RNA samples. Dr. George C. Prendergast (Lankenau Institute for Medical Research, Hershey, PA) 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 American Type Culture Collection (Manassas, VA). All cells were maintained at 37℃ and 5% CO_2 in Dulbecco's Modification of Eagle's Medium/Ham's F-12 50/50 Mix (DMEM/F12-50/50) (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologics, Lawrenceville, GA) and 1% antibiotics (Gibco®, Invitrogen, Carlsbad, CA). During serum-starvation, cells were incubated with serum-free or low-serum (0.2% FBS) DMEM supplemented with 1% antibiotics. The stable clone cell lines, MDA-MB-231 (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, St. Louis, MO).

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® according to the manufacturer's protocol. A 2 µg RNA sample was used to synthesize cDNA using Invitrogen's SuperScript® III First-Strand Synthesis SuperMix and following manufacturer's manual. For amplification of the samples a 10 µl master mixture of the following reagents was used per reaction: 5 µl of iQ^{TM} SYBR® Green Supermix (Bio-Rad, Hercules, CA), 1 µl of 10µM forward primer, 1 µl of 10 µM reverse primer and 3 µl of H_2O. The following primers (synthesized by Sigma, St. Louis, MO) were used for target genes: Lf Forward: 5''-ACCGCAGACA-TGAAACTTGT-3' and Lf Reverse: 5'-GGGGGAGTCTCTCTTTATGC-3'; ET-1 Forward: GCCAAGGAGCTCCAGAA-ACAGCAG ET-1 Reverse: AGCAGGAGCAGCGCTTGGAC; Actin Forward: 5' – TCCCTGGAGAAGAGCTACGA 3' and Actin Reverse: 5'-GTACTTGCGCTCAGGAGGAG-3'. Amplification of target genes was performed using Bio-Rad's CFX96^{TM} Real-Time System (Bio-Rad, Hercules, CA) with reaction conditions of 3 minutes at 95°C, followed by 45 cycles of 95°C for 10 seconds, 59°C for 10 seconds and 72°C for 30 seconds, and then 10 seconds at 95°C. Normalized expression of target genes were calculated relative to endogenous expression of actin (Relative expression= $2^{(-\Delta C\,(t))}$).

Downregulation of Receptors upon Lactoferrin treatment

Immunoblot Analysis

For lactoferrin treatment, cells were plated at 40-50% confluency in 60mm dishes and maintained in serum-free DMEM for 48 hrs. After a 48-hr starvation, cells were treated with 20 µg/ml and 100µg/ml of lactoferrin from human milk (~90% iron saturated; L3770, Sigma Aldrich, St. Louis, MO) for indicated time points. At the end of treatment, cell lysates were prepared in RIPA-modified lysis buffer (50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, containing 0.5% 1 x protease inhibitor cocktail (Roche, Indianapolis, IN). Protein samples of 50-100µg were then separated by 8% sodium dodecyl sulfate- polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) and blocked with 5% non-fat dry milk for 1 hr. The following primary antibodies were used for overnight incubation at 4℃: anti-ERα (Bethyl Laboratories, Montgomery, TX, 1:5000), anti-HER2 (Bethyl Laboratories, Montgomery, TX 1:5000), anti-PR (Dako, Carpinteria, CA, 1:4000), anti-IGF-IRβ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA 1:1000), anti-RXRα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, 1:1000), anti-cyclinD1 (Labvision/Thermo Fisher Scientific, Fremont, CA), anti-tubulin (Sigma-Aldrich, St. Louis, MO, 1:4000), anti-vinculin (Sigma-Aldrich, St. Louis, MO 1:20,000). After secondary incubation (GE Healthcare UK Limited, Amersham Place, UK) at room temperature for 1 hour and several PBS-T (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4, 2 ml of tween-20)

washes, the signals were visualized using the enhanced chemiluminescence system as described by the manufacturer (GE Healthcare UK Limited, Amersham Place, UK)

Immunohistochemistry and Confocal Microscopy

Deparaffinized sections were treated with 0.3% hydrogen peroxide in methanol for 30 min and subjected to antigen retrieval by boiling the sections in antigen unmasking solution (Vector Laboratories, Burlingame, CA) for 20 min. The sections were then blocked with 5% skimmed milk in PBS (Mediatech Inc., Manassas, VA) for 1 hr and incubated with lactoferrin antibody (1: 500 dilution) or endothelin antibody (1: 100 dilution) at 4℃ overnight, followed by incubation with EnVision (Dako, Carpinteria, CA) for 1 hr at room temperature and visualization with liquid DAB+ substrate chromogen system (Dako, Carpinteria, CA). Immunostained sections were lightly counterstained with hematoxylin, dehydrated in graded ethanol, cleared in xylene, and mounted with the use of the peramount mounting medium.

To visualize downregulation of the three receptors via confocal imaging, ZR-75 and MCF-7 cells were plated onto cover slips in six-well plates, starved for 48 hours in serum-free DMEM and treated with 20 μg/ml of lactoferrin. After a 12-hr treatment, the cells were fixed with 4% paraformaldehyde and then incubated with anti-ERα, anti-HER2 and anti-PR antibodies for 1 hr at room temperature. The secondary antibody incubation was done for 40 min using Alexa 488 (ERα and

HER-2) and Alexa 555 (PR) (Invitrogen Eugene, Oregon). 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes, Eugene, Oregon) was used for nuclear staining. The coverslips were mounted onto glass slides with Prolong Gold Antifade reagent (Invitrogen, Molecular Probes, Eugene, Oregon). 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.

Plasma Protein Detection using ELISA

Lactoferrin and ET-1 levels in human breast cancer serum samples were measured using protein-specific ELISA kits according to the manufacturer's protocol. For detection of lactoferrin, the AssayMax Human Lactoferrin ELISA Kit (AssayPro, St. Charles, MO) was used with 1:100 dilution of serum samples. The absorbance was read at 450nm using the ELISA plate reader. ET-1 levels were measured using the QuantiGlo Human Endothelin-1 Kit (R&D Systems, Minneapolis, MN) following the manufacturer's manual. For ET-1 levels, the 1450 Microbeta Jet Microplate Scintillation and Luminescence Counter (Perkin Elmer Life and Analytical Sciences, Waltham, MA) were used to measure the luminescence. A dilution of 1:5 for the serum was used for the assay.

Preparation of conditioned medium

ELISA was carried out with the conditioned medium from various cell lines (MCF-7, MDA-MB-231 and MDA-MB-468). Conditioned medium was prepared by growing each cell line in complete medium until they reached full confluency. Then the cells were rinsed with PBS and grown for an additional 24 hrs in serum free medium. This medium was collected and spun at high speed in 50 ml falcon tubes at 4ºC. The medium was transferred to fresh tubes and aliquoted. Subsequently, this conditioned medium was used for the ET-1 ELISA. The dilutions of conditioned medium (done with calibrator RD-13 from kit) used for the assay was 1:10 and 1:25 for MCF-7 and MDA-MB-231 and MDA-MB-468 respectively.

Wound-Healing Assay

To analyze the effect of lactoferrin with or without estrogen ($E_{2)}$ and tamoxifen on cell migration, MCF-7 and ZR-75 cells were plated in 60 mm dishes with 10% fetal calf serum-DMEM. When cells were 80-90% confluent, they were rinsed with PBS and cultured in 1% DCC in phenol red-free medium for 24 hrs. Before treatment, each plate received multiple “wounds” by scratching the cells with a 200 µl pipette tip. Treatments were done with medium supplemented with 100 µg/ml lactoferrin, 10^{-8} M estradiol (Sigma, St. Louis, MO), 10^{-7} tamoxifen (Sigma, St.Louis, MO) or in various combinations. In another set, the cells were treated with and without lactoferrin in addition to 20 ng/ml of recombinant heregulin (Neomarkers, Fremont, CA, USA), 10nM of *Herceptin* (Genentech, Inc., South San Francisco, CA) and in

combination. In this set, 1% DMEM was used when the cells were 80-90% confluent. For the wound healing assay for +/- Lf and +/- ET-1 antagonist and ET-1 or in combination, 500 ng/ml of ET-1 (Sigma Aldrich, St. Louis, MO) and 600nM of the ET-1 antagonist, BQ-123 (Peptides International, Louisville, KY) were used. Here, MCF-7 cells were grown to 80% confluency in complete medium and then rinsed with PBS followed by 48 hrs of serum starvation. Treatments were done after wounding the dishes at multiple places across the plate. After an additional 24 hrs, each plate was examined by phase contrast microscopy 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, Center Valley, PA). Ten separate measurements were made per plate, and each experiment was performed in triplicates. Data represent the mean of three experiments.

Migration assay using uncoated Boyden Chambers

To measure the cell migration potential, ZR-75 cells were serum-starved for 48 hrs. The cells were trypsinized for collection, washed in PBS, and then resuspended in medium in the presence of 0.1% BSA and loaded onto the upper well of an uncoated Boyden chamber at 10,000 cells/well. The agents ET-1 and BQ-123 were diluted in plain medium before plating. The lower chamber of the separating filter was supplemented with conditioned medium of NIH-3T3 fibroblasts grown in DMEM/F-12 medium with 0.1% BSA. The number of cells that

successfully migrated through the filter was counted after fixation with methanol and was stained with 0.05% crystal violet in methanol. Experiments were performed in triplicates, and results are expressed as arbitrary units of the mean values (SD) of the migrated cells relative to untreated controls. Data were analyzed using the Olympus DP2-BSW digital camera software.

Invasion Assay Using Matrigel™ Invasion Chambers

Cells were grown in complete medium until they were semi-confluent. Cells in serum-free media were then seeded at a density of 100,000 cells/well in the upper well of the Matrigel™ Invasion Chambers (BD Biosciences). The lower chambers contained conditioned media from NIH-3T3 cells. At the time of cell seeding, each well was treated with 100 µg/ml of lactoferrin. After 24 hrs, the invaded cells at the bottom were fixed with 4% paraformaldehyde and stained for DAPI. The number of invaded cells was counted under a microscope and average number of cells among replicates was plotted.

Lactoferrin Effect on ET-1 Promoter

Northern Blotting

For Northern Blotting, MCF-7 as well as MDA-MB-231 and MDA-MB-468 cells were plated in 60 mm dishes and treated with 100 µg/ml of lactoferrin for 6 hrs. RNA isolation was performed using TRIzol® according to the directions of the manufacturer. After measuring the RNA concentration via Nanodrop

(Thermoscientific, Wilmington, DE), 20 µg of RNA was loaded onto a formaldehyde denaturing gel (1.5% agarose, 2.2M formaldehyde). Subsequently, RNA was transferred to a Hybond^{TM}-N membrane (GE Healthcare UK Limited, Amersham Place, UK) via capillary diffusion 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., Austin, TX) with radio-labeled UTP. Hybridization and washings were done according the Ambion's Northern blotting protocol. Finally, the membrane was exposed to a phosphor imager and the autoradiogram was developed using the Storm^{TM} 865 Imager (GE Healthcare UK Limited, Amersham Place, UK). For another experiment, MDA-MB-231 cells were plated in four 60 mm plates. After the cells were approximately 60% confluent, they were serum-starved for 48 hrs. After 48 hrs, after adding fresh media, three plates were either treated with 20 µg/ml of Cycloheximide (CHX) or 100 µg/ml lactoferrin or both. CHX was administered an hour before lactoferrin treatment. The control plate only contained serum-free media. Six hours post-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.

Luciferase Assay

MCF-7 as well as MDA-MB-231 and MDA-MB-468 cells were grown in 60 mm plates to 50% confluency. ET-1 promoter, β-galactosidase and control plasmids

were then transfected into cells using FUGENE® 6 according to the manufacturer's protocol (Roche, Indianapolis, IN). After lactoferrin treatment, cell lysates were prepared using 200 µl of Tropix® Lysis solution (Applied Biosystems, Carlsbad, CA). For luciferase promoter activity, 1 0µl of lysate was incubated with 100 µl Luciferase Assay Substrate solution (Promega, Madison, WI) and measurements were taken using LUMAT LB 9507 luminometer (Berthold Technologies, Oak Ridge, TN). To check for β-galactosidase, 20 µl of lysate were incubated with 60 µl of Tropix® Galacto Reaction Buffer Diluent and (Applied Biosystems, Carlsbad, CA) for 60 min and then with the addition of 100 µl of Tropix® Accelerator II (Applied Biosystems, Carlsbad, CA), the activity of β-galactosidase was measured using the same luminometer.

Electrophoretic Mobility Shift Assay

PCR amplified DNA fragments or oligos containing lactoferrin consensus sites were 5'-end labeled using γ^{32}p and PNK enzyme. End labeled fragments were purified using sephadex G-50 spin columns (GE Healthcare UK Limited, Amersham Place, UK). Each fragment was incubated with purified holo- or apo-lactoferrin (GenWay Biotech, San Diego, CA) 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, Amersham Place, UK).

SDS-Page Gel and Protein Staining

Lactoferrin-treated ZR-75 cell lysates (50 µg) were loaded onto an 8% SDS-Page gel and run at 130V for 1.5 hours. The gel was then carefully removed and stained with GelCode® Blue Stain Reagent (Thermo Fisher Scientific) for 30 min and then destained using distilled water overnight. Images were captured using a Kodak Image Station 2000MM (Kodak).

Cell Cycle Analysis using Fluorescent Assorted Cell Sorting

ZR-75 and MCF-7 cells were plated in triplicates in 60 mm plates, starved in serum-free DMEM for 48 hours and then treated with 20 and 100µg/ml of lactoferrin for 12 and 24 hours. At the end of treatment, cells were washed once with 2 ml of PBS and then 0.5ml of PBS was added and cells were removed using a cell scraper. Cells were collected into pre-chilled 15 ml tubes and an additional 1ml of PBS was used to wash the rest of the plate. After the final collection, cells were centrifuged at 1000 rpm for 15 min at 4℃. At the end of centrifugation, the supernatant was aspirated leaving only the cell pellet. The cells were then vortexed in 0.5 ml of PBS and while vortexing, 1.5 ml of ice-cold 100% ethanol was added drop-wise and collected by centrifugation at 3000 rpm for 3 min. The supernatant was removed and 1ml of PBS/PI buffer (200 µg/ml RNase (Sigma

Aldrich) 20µg/ml propidium iodide (Sigma, St. Louis, MO), 0.1% Triton-X-100 (Sigma Aldrich) per 1ml of PBS) was added to cells. After vortexing, cells were incubated at 37℃ for 15 min. Finally, cells were transferred to Falcon tubes and sorted using the FACSCalibur (Becton Dickinson) and data was analyzed by the Cell Quest Pro software (BD BioSciences).

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.

Chapter 3: Results Part I- Lactoferrin and its Contribution to an Aggressive Breast Cancer Phenotype

3.1 Downregulation of Receptors

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-targetable receptors and thus, progression of breast cancer cells to more invasive phenotypes, remains unknown. Based on the literature reviews and other factors indicating lactoferrin's potential role in breast cancer progression, we hypothesize that lactoferrin could possibly be involved in downregulating the therapeutic receptors and therefore contribute to a more relative aggressive breast cancer phenotype.

As a first step to explore this hypothesis, we examined the effect of recombinant iron-saturated holo-lactoferrin on the levels of the three receptors in ER/PR/HER-2-positive ZR-75 breast cancer cells (*Note: HER-2 positive from here on refers to normal HER-2 expression*). The cells were treated with lactoferrin for 3, 6, 12 and 24 hours and then the cell lysates were subjected to western blotting. Subsequently, the membranes were immunoblotted with receptor-specific antibodies to assess protein levels of each receptor. As illustrated in **Figure 3**, lactoferrin stimulation of breast cancer cells resulted in a time-dependent downregulation of ERα, PR and HER-2 starting 6 hrs post-treatment at the protein level. Interestingly, levels of the three receptors returned back to the basal levels in

the treated cells after 24 hrs indicating that a one time treatment of lactoferrin may transiently downregulate the therapeutic-targetable receptors in ER/PR/HER-2-positive breast cancer cells.

Since this observation was made in ER/PR/HER-2-positive ZR-75 cells, we wanted to assess whether this phenomenon holds true in another receptor-positive breast cancer cell line. To check this, we used MCF-7 cells that are ER/PR/HER-2-positive adenocarcinoma breast cancer cells. Similar to ZR-75 cells, we observed the same time-dependent downregulation of receptors in the MCF-7 breast cancer cell line which indicates that this noted downregulation is not cell line specific (**Fig. 3B**) and could possibly be observed in other breast cancer cells that express the receptors.

Thus far, the data indicate that the downregulation of therapeutic receptors by a single dose of lactoferrin was transient in nature and therefore we next assessed whether noted downregulation of receptors could be sustained by repeated lactoferrin dosing or by increasing the dose of lactoferrin. We treated ZR-75 cells with indicated doses of lactoferrin and found that replenishing the cultures with fresh or higher doses of lactoferrin results in a sustained downregulation of ERα, PR and HER-2 (**Fig. 4A**) indicative of a time- and dose-dependent regulation.

While the data indicate that lactoferrin-dependent receptor downregulation is specific to ERα, PR and HER-2, we wanted to experimentally investigate this. To test for specificity, we treated ZR-75 cells with a single dose of lactoferrin over 3, 6, 12 and 24 hrs and checked for protein levels of IGF-IRβ and RXRα with antibodies

specific to the two receptors. These two receptors are steroid receptors and therefore would allow checking for receptor specificity. As suspected, the noted downregulation of ERα, PR and HER-2 receptors by lactoferrin was not due to a generalized toxicity as there was no effect of lactoferrin on the levels of IGF-IRβ and RXRα receptors (**Fig. 3A**). Additionally, to further validate the specificity of lactoferrin to ERα, PR and HER-2, we subjected lactoferrin-treated ZR-75 whole cell lysates to SDS-PAGE analysis with subsequent staining of the gel with Coomassie blue to determine the protein levels. As suspected, we did not observe any evidence of an inhibitory effect of lactoferrin on the overall protein synthesis (**Fig. 4B**) which further validates that lactoferrin-dependent downregulation is specific to ERα, PR and HER-2 only.

Furthermore, we wanted to visualize the downregulation of the receptors by lactoferrin using immunocytochemical staining and scanning confocal microscopy. ZR-75 as well as MCF-7 cells were treated with 100 µg/ml of lactoferrin for 6 hrs, fixed and then stained with receptor-specific antibodies. As demonstrated in **Figure 5**, upon lactoferrin treatment there was downregulation of all three receptors in both ZR-75 and MCF-7.

Since the observed downregulation of therapeutic receptors by lactoferrin was noted in two breast cancer cell lines that express ERα, PR and HER-2, we wanted to investigate whether this downregulation was tissue specific or dependent on the co-expression of all three receptors. SK-BR-3 cells are ERα-negative but overexpress HER-2 making it a good cell line to test if downregulation of HER-2 is

dependent of expression of ERα receptor. Similarly, Caco-2 is a colorectal adenocarcinoma cell line that expresses ERα, PR and HER-2 and thus, using this cell line allows examining tissue specificity. Therefore, to test this, we treated SK-BR-3 cells as well as Caco-2 cells with lactoferrin and then analyzed HER-2 protein expression via western blotting (**Fig. 6**). HER-2 expression was downregulated upon lactoferrin treatment in both cell lines, indicating that this phenomenon may not be tissue specific nor is the expression of ERα needed for lactoferrin-dependent HER-2 downregulation.

Taken together, these findings thus far suggest that persistent stimulation of breast cancer cells by lactoferrin may downregulate ERα, PR and HER-2 and that this downregulation is specific to the three receptors only. Furthermore, we demonstrated that downregulation of the receptors is not tissue-specific and that HER-2 downregulation is not dependent on the expression of the ERα. Overall, the data suggest that downregulation of ER, PR and HER-2 could possibly be the first step in the development of a more relative aggressive breast cancer phenotype in receptor-positive breast cancer cells.

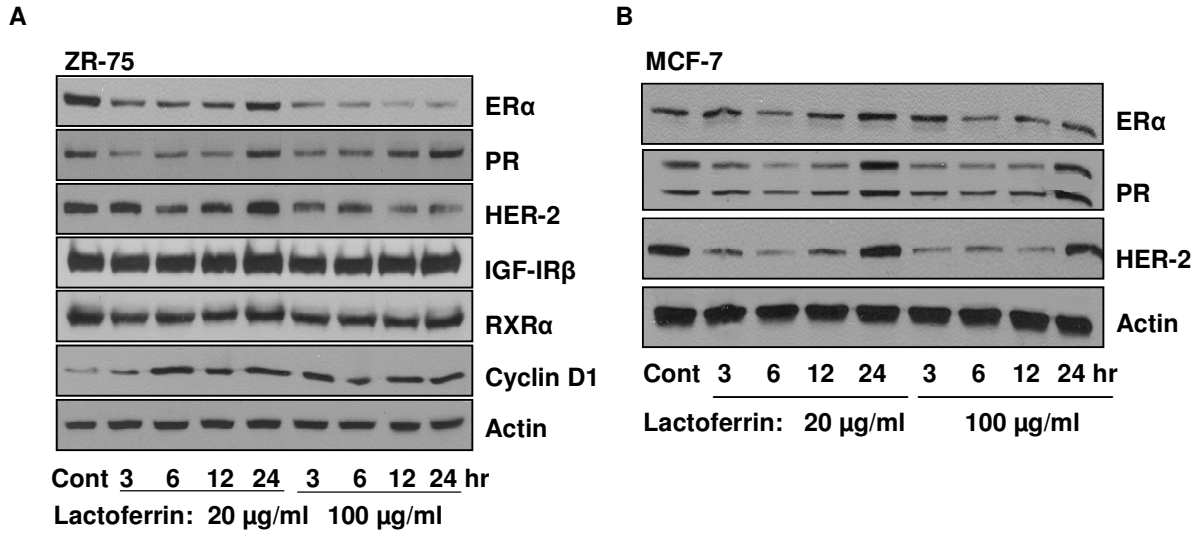


Figure 3. Lactoferrin downregulates ERα, PR and HER-2 in human breast cancer cells.

(A+B) Effect of a single dose of lactoferrin on the levels of indicated proteins in (A) ZR-75 and (B) MCF-7 cells. Cells were treated with 20 µg/ml and 100 µg/ml of lactoferrin for indicated times and subjected to Western blot analysis.

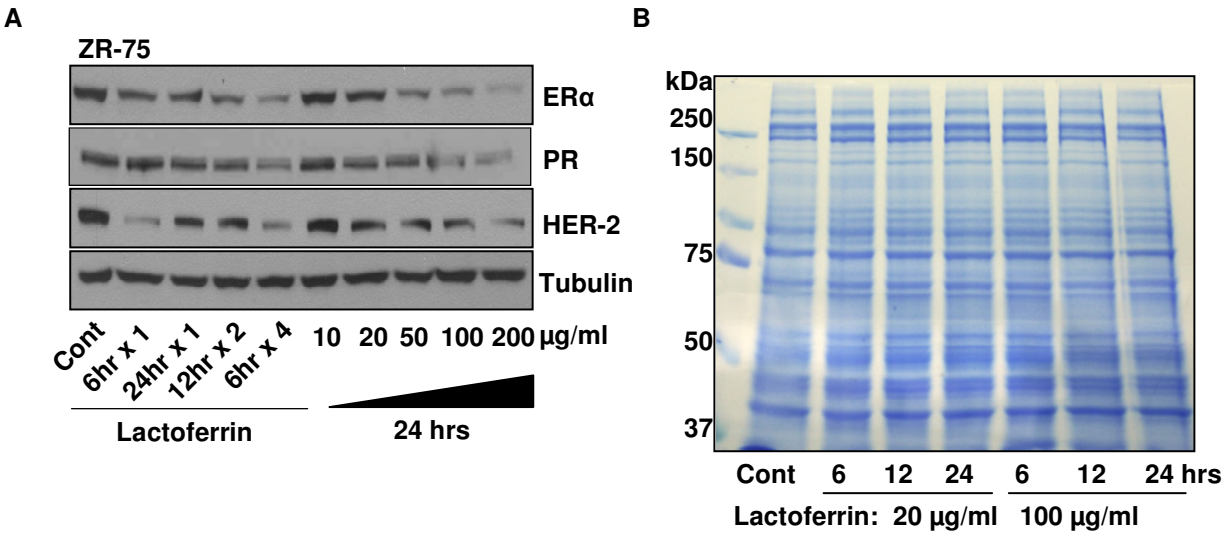


Figure 4. Lactoferrin downregulates ERα, PR and HER-2 in human breast cancer cells.

(A) Effect of multiple and increasing doses of lactoferrin on expression levels of receptor proteins in ZR-75 cells.

(B) Effect of lactoferrin on overall protein synthesis in ZR-75 cells. Cells were treated with or without different concentrations of lactoferrin for indicated time points. Cell lysates were prepared and subjected to SDS-PAGE analysis and the gel was stained with Coomassie blue to determine the protein levels.

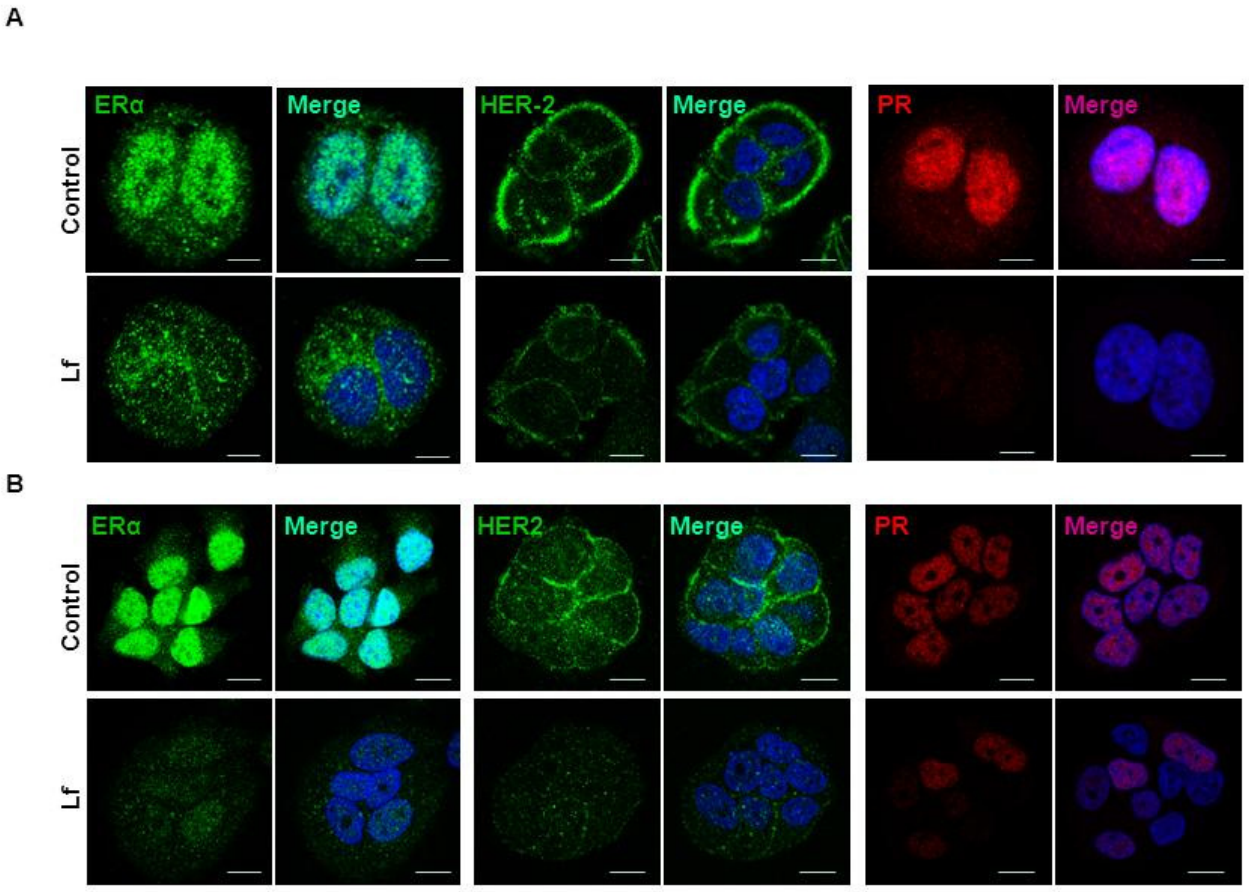


Figure 5. Lactoferrin downregulates ERα, PR and HER-2 in human breast cancer cells.

(A+B) Analysis of ERα, PR and HER-2 levels in (A) ZR-75 and (B) MCF-7 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.

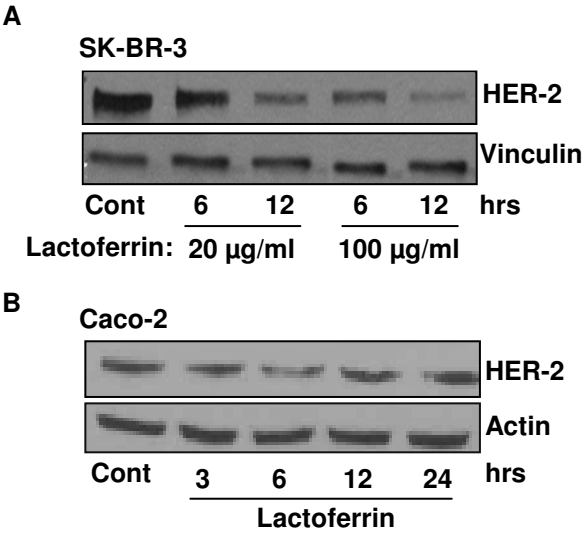


Figure 6. Lactoferrin downregulates HER-2 in HER-2-overexpressed and non-breast cancer cell lines

Effect of a single dose of lactoferrin treatment on the levels of HER-2 in (A) SK-BR-3 and (B) Caco-2 cells. SK-BR-3 cells were treated with 20 and 100 µg/ml of lactoferrin for 6 and 12 hrs, and Caco-2 cells were treated with 100 µg/ml for 3, 6, 12 and 24 hrs. Whole cell lysates were prepared and immunoblot analysis was carried out using anti-HER-2 antibody. Vinculin and actin were used for SK-BR-3 and Caco-2 as loading controls, respectively.

3.1.1 Post-transcriptional Regulation of Therapeutic Receptors by Lactoferrin

Our preliminary data thus far suggest a role of lactoferrin in downregulating ERα, PR and HER-2. To understand the underlying basis of this observation, we set out to investigate the mechanism(s) by which lactoferrin could possibly downregulate these receptors. Lactoferrin has been shown to act as a transcription factor (He & Furmanski, 1995) and therefore we suspect that the downregulation of the receptors could occur at the transcription level. To test this hypothesis, MCF-7 cells were treated with lactoferrin for 3 and 6 hrs, followed by RNA isolation and then mRNA analysis was performed via Northern blot hybridization. At 6 hrs, a time point that was sufficient for receptor downregulation at the protein level, there was no significant inhibitory effect of lactoferrin treatment on the steady state levels of ERα, PR and HER-2 mRNA (**Fig. 7**), indicating that lactoferrin most likely does not affect the transcription or mRNA stability of the three receptors.

Since receptor mRNA steady state levels were not affected by lactoferrin, we wanted to investigate its role on post-translational regulation next. Newly synthesized proteins could undergo post-translational modifications such as degradation by proteasomes. Therefore, we wanted to test whether lactoferrin could induce proteasomal degradation of ERα, PR and HER-2. To examine this, MCF-7 cells were treated with lactoferrin in the presence or absence of MG-132, a proteasomal inhibitor. As seen in **Figures 8A and 8B**, co-treatment of the cells

with MG-132 prevented lactoferrin-induced time- and dose-dependent downregulation of ERα and HER-2 protein, suggesting that lactoferrin-mediated noted reduced expression of ERα or HER-2 may utilize mechanisms that are sensitive to inhibitors of proteasomal degradation of proteins.

To further corroborate the post-transcriptional control of ERα and HER-2 by lactoferrin, we generated isogenic pooled clones of MDA-MB-231 (TNBC) cells stably expressing ERα or HER-2 (**Fig. 9A**). We found that lactoferrin treatment of stable clones is also accompanied by downregulation of ectopically expressed ERα and HER-2 (**Fig. 9B**), implying that lactoferrin utilizes post-transcriptional mechanism(s) to downregulate ERα and HER-2 in breast cancer cells. As noted earlier in our experiment with SK-BR-3 cells (**Fig. 6**), HER-2 downregulation was independent of ERα expression and therefore downregulation of the ectopically expressed receptors further validates that lactoferrin-dependent downregulation most likely undergoes post-translational regulation independent of co-expression of the other receptors.

Taken together, our data so far have demonstrated that lactoferrin-induced downregulation of ERα, PR and HER-2 is not transcriptionally regulated nor does it affect steady state levels of mRNA. Using a proteasomal inhibitor, we showed that the possible mechanism for downregulation is via proteasomal degradation.

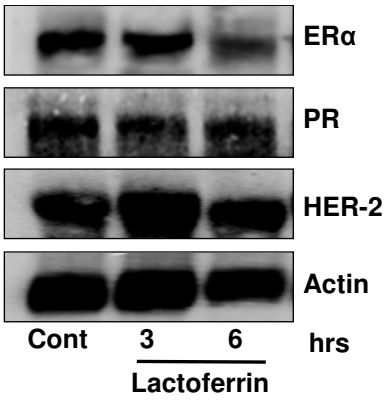


Figure 7. Effect of lactoferrin on receptor mRNA level.
Northern blot analysis of ERα, PR and HER-2 mRNAs in MCF-7 cells treated with or without 100 μg/ml of lactoferrin for indicated times.

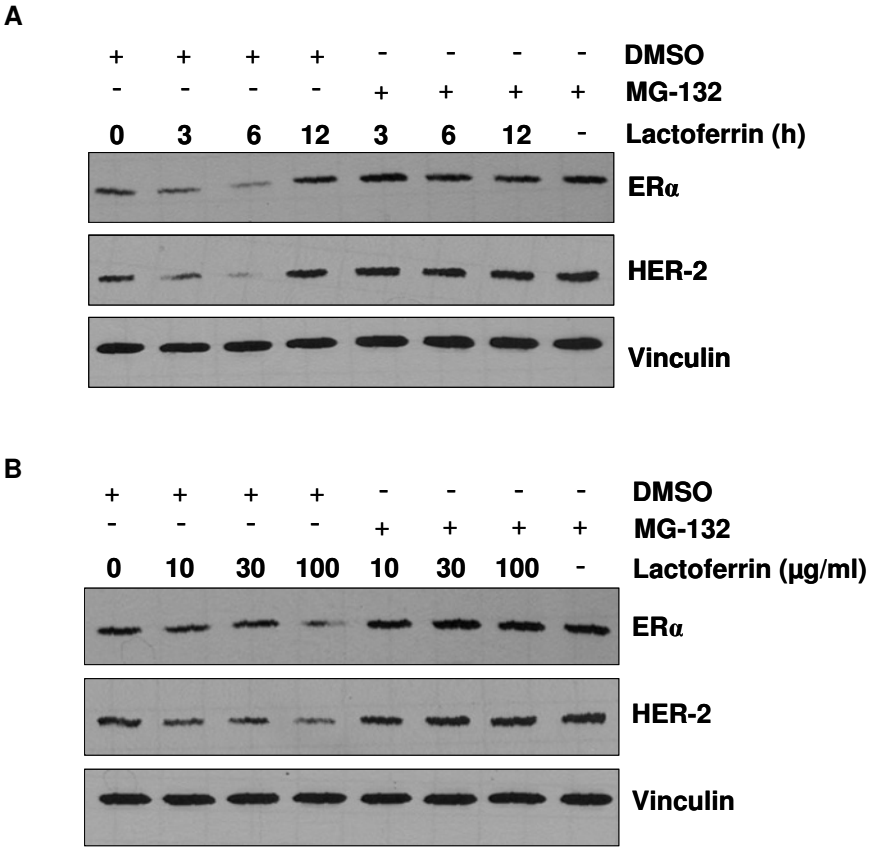


Figure 8. Lactoferrin-dependent receptor downregulation is post-transcriptionally regulated

MCF-7 cells were treated with or without lactoferrin for indicated time points, or with different concentrations of lactoferrin in the presence, or absence of proteasomal inhibitor MG-132. Vinculin was used as a loading control.

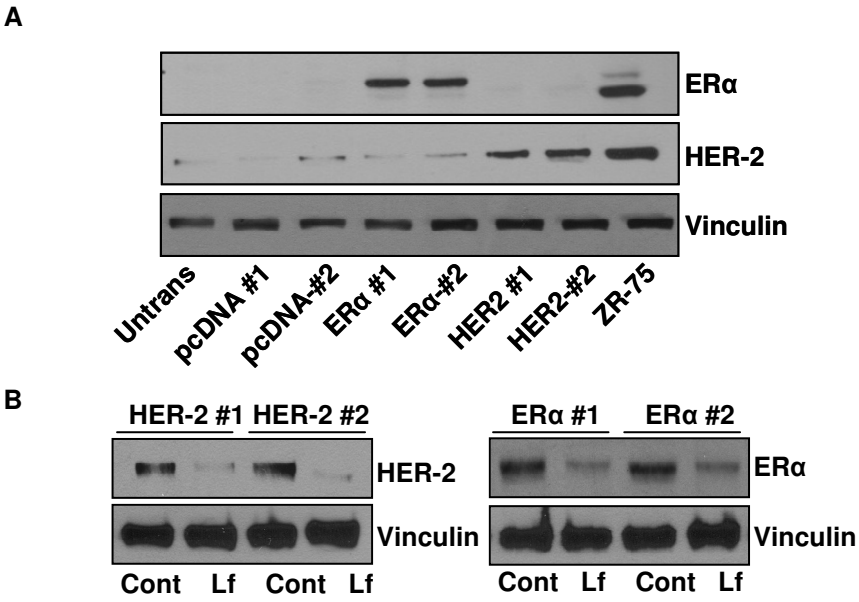


Figure 9. Lactoferrin does not downregulate ERα, PR, and HER-2 at the transcriptional level

(A) 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. (B) Effect of lactoferrin on the levels of ERα and HER-2 receptors in MDA-MB-231 pooled clones stably expressing ERα or HER-2.

3.1.2 Lactoferrin and its Effect on Receptor-targeted Therapy

Because we observed early on that lactoferrin stimulation of breast cancer cells was accompanied by downregulation of ERα, PR, and HER-2 (**Fig. 3**), we hypothesized that lactoferrin stimulation of ERα-, HER-2-positive cells may abrogate the responsiveness of breast cancer cells to 4-hydroxytamoxifen as well as *Herceptin*. In support of this notion, we employed the migration and invasion assays to examine lactoferrin's role on these targeted therapies and how lactoferrin-dependent downregulation of receptors could affect the cells' ability to respond to receptor antagonists. Since migration is biological endpoint studies to test for sensitivity to targeted therapies, we first treated control MCF-7 cells with 4-hydroxytamoxifen and subjected the cells to the Boyden chamber assay. As expected, serum-induced cell migration could be effectively blocked by 4-hydroxytamoxifen (**Fig. 10A**). However, this observed inhibition was compromised when cells were pretreated with lactoferrin that downregulates ERα and consequently, is no longer targetable by 4-hydroxytamoxifen (**Fig. 10A**). Similarly, to understand the implication of lactoferrin-mediated downregulation of HER-2 on the ability of cells to respond to HER-2-directed therapies, we repeated the experiments with *Herceptin*. As shown in **Figure 10B**, treatment of control MCF-7 cells with *Herceptin* blocked serum-mediated cell migration. Conversely, lactoferrin pretreatment of HER-2-positive breast cancer cells reduced the effectiveness of *Herceptin* to inhibit serum-stimulated cell migration.

While the migration assay suggests that lactoferrin-treated cells lost their sensitivity to the targeted therapies, we wanted to further analyze this hypothesis using another biological endpoint. Invasion is a good indication of the aggressiveness of cancer cells, and therefore we wanted to check if lactoferrin-treated MCF-7 cells could lose their sensitivity to 4-hydroxytamoxifen and *Herceptin* as measured by the Matrigel™ invasion assay. To test this, we treated the cells with 4-hydroxytamoxifen alone and measured their invasive properties using Matrigel-coated chambers. Comparable to the migration assay, MCF-7 cells' invasive potential was inhibited upon 4-hydroxytamoxifen treatment (**Fig. 11A**). However, once the cells were pre-treated with lactoferrin followed by 4-hydroxytamoxifen, the observed inhibition was lost and MCF-7 cells were invasive (**Fig. 11A**). Similarly, when the cells were treated with *Herceptin*, as expected, the MCF-7 cells became less invasive (**Fig. 11B**). However, pre-treatment of the cells with lactoferrin rendered them insensitive to Herceptin (**Fig. 11B**).

These results agree with the findings that lactoferrin is involved in downregulating ERα, PR and HER-2, as presented earlier. The data suggest that cells treated with lactoferrin become insensitive to targeted therapies against these receptors mainly due to the fact that lactoferrin treatment is accompanied by the loss of its targetable receptors.

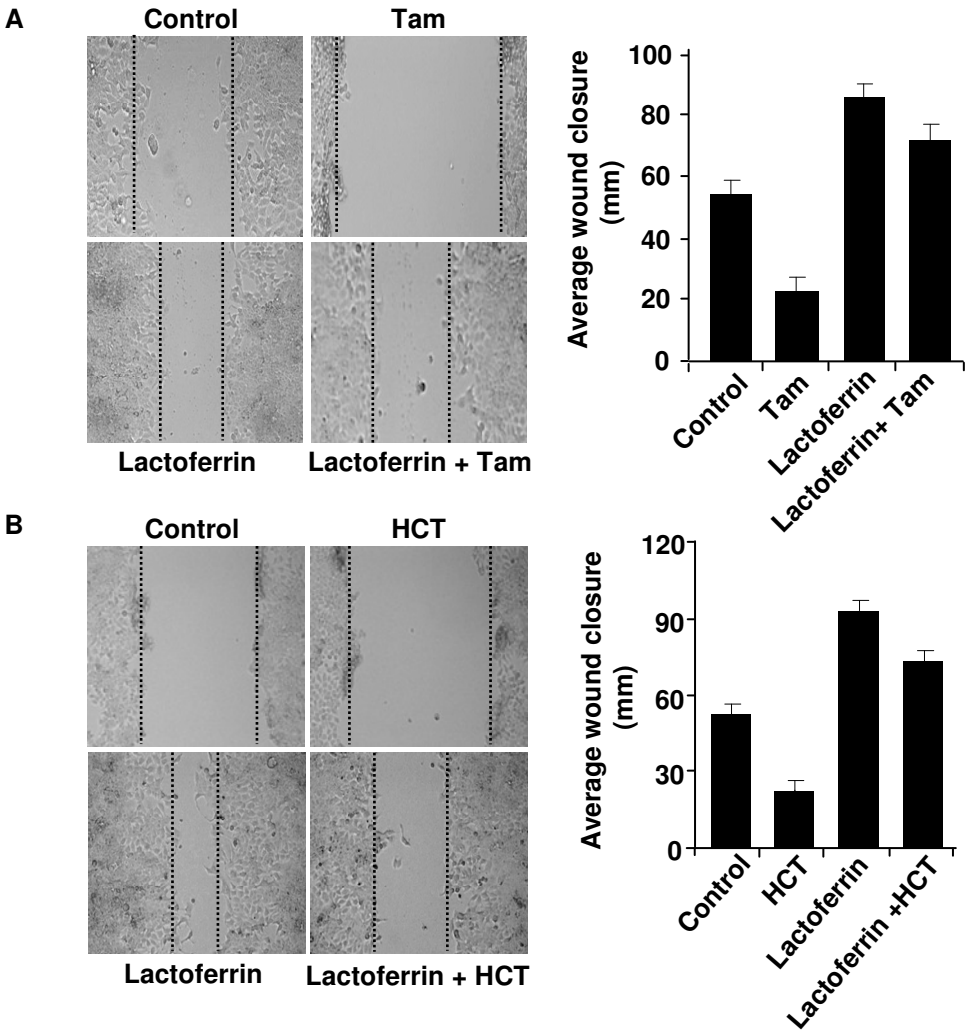


Figure 10. Lactoferrin desensitizes breast cancer cells to tamoxifen and Herceptin as measured by the Wound-healing assay.

(A) Effect of lactoferrin 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 micrographs show representative images of migrated cells after 24 hrs of treatment. (B) Effect of lactoferrin 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.

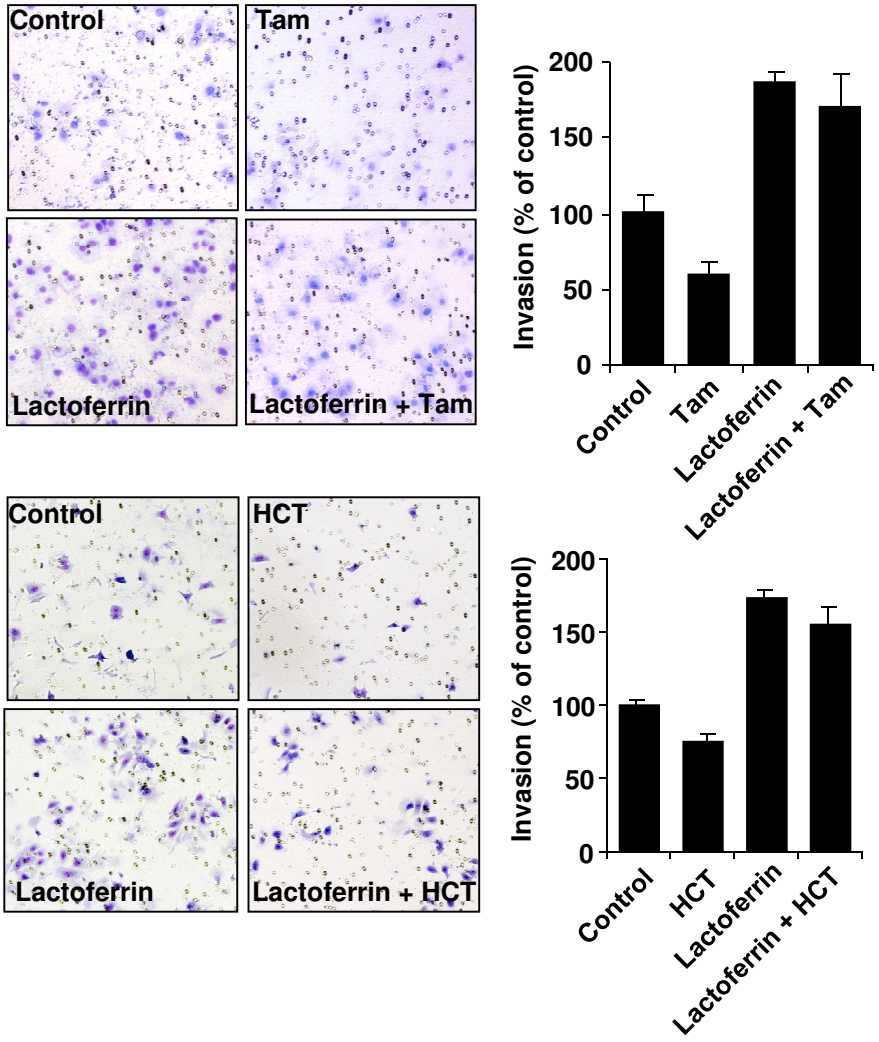


Figure 11. Lactoferrin desensitizes breast cancer cells to tamoxifen and Herceptin as measured by Matrigel invasion assay.

(A) Effect of lactoferrin on cell invasion in tamoxifen-treated MCF-7 cells. E2 was added to the upper wells to enhance tamoxifen effect. (B) Effect of lactoferrin on cell invasion in *Herceptin*-treated MCF-7 cells. Heregulin was added to the upper wells to enhance *Herceptin* effect.

3.2 Lactoferrin Promotes Invasiveness of Breast Cancer Cells

The data we have presented thus far have shown that lactoferrin is able to downregulate ERα, PR and HER-2 in a time- and dose-dependent manner and that this downregulation is possibly via proteasomal degradation. Furthermore, we demonstrated that lactoferrin treatment of ER/PR/HER-2-positive breast cancer cells renders the cells insensitive to receptor-targeted therapies most likely due to the downregulation of ERα and HER-2. However, since we do not know if the observed downregulation of the receptors has any biological consequences on the cells, we set out to measure biological endpoints with lactoferrin-treated cells.

Because we hypothesize that lactoferrin could contribute to a more aggressive breast cancer phenotype, possibly through downregulation of ERα, PR and HER-2, we wanted to examine its role in cell cycle progression and cell proliferation. Our early observation of lactoferrin stimulation of cyclin D1 expression (**Fig. 3A** and **Fig. 12A**), an endpoint for the G1-to-S phase progression, suggested that lactoferrin exposure to ZR-75 cells may promote cell cycle progression. To test this hypothesis, we treated ZR-75 cells with lactoferrin for 12 and 24 hrs, fixed and stained the cells with propidium iodide (PI) and subjected the cells to Fluorescence-activated cell sorting (FACS) analysis. Indeed, we found that lactoferrin treatment of ZR-75 cells leads to an accelerated transition into S-phase as compared to untreated control cells (**Fig. 12B**).

Given that a faster progression through the cell cycle is mostly indicative of an increase in cell proliferation, we next wanted to analyze the effect of lactoferrin on

cell growth. ZR-75 as well as MCF-7 cells were treated with lactoferrin daily for three days and then counted using a Z1 Beckman's Coulter Counter. As suspected, lactoferrin treatment increased cell proliferation in comparison to control cells (**Fig. 13**). Furthermore, we observed that lactoferrin-dependent increase in cell proliferation was independent of serum inclusion in the media.

To further test the hypothesis that lactoferrin could have an effect on increased aggressiveness in breast cancer cells, we wanted to analyze lactoferrin's contribution to cell migration and cell invasion. Increased migration as well as invasion are characteristic properties of malignant cancer cells and therefore are fundamental biological endpoint studies to pursue. To examine the functional significance of lactoferrin on its effect on cell migration, we employed the wound healing assay to measure cell migration. Briefly, ZR-75 and MCF-7 cells were plated in 6-well plates and received multiple wounds before treatment with lactoferrin. After 24 hrs of Lf treatment, the wounds were measured for migration of cells. Both cell lines had an increase in cell migration upon lactoferrin treatment (**Fig. 14A**).

Since these observations were made in non-aggressive breast cancer cell lines, we wanted to examine the effect of lactoferrin on a cell line known to exhibit a more aggressive and invasive phenotype. For this we used the triple-negative breast cancer (TNBC) cell line, MDA-MB-231 cells which have been shown to be more aggressive in nature than other breast cancer cell lines such as MCF-7 cells (Price et al., 1990). Interestingly, lactoferrin treatment also stimulated an increase

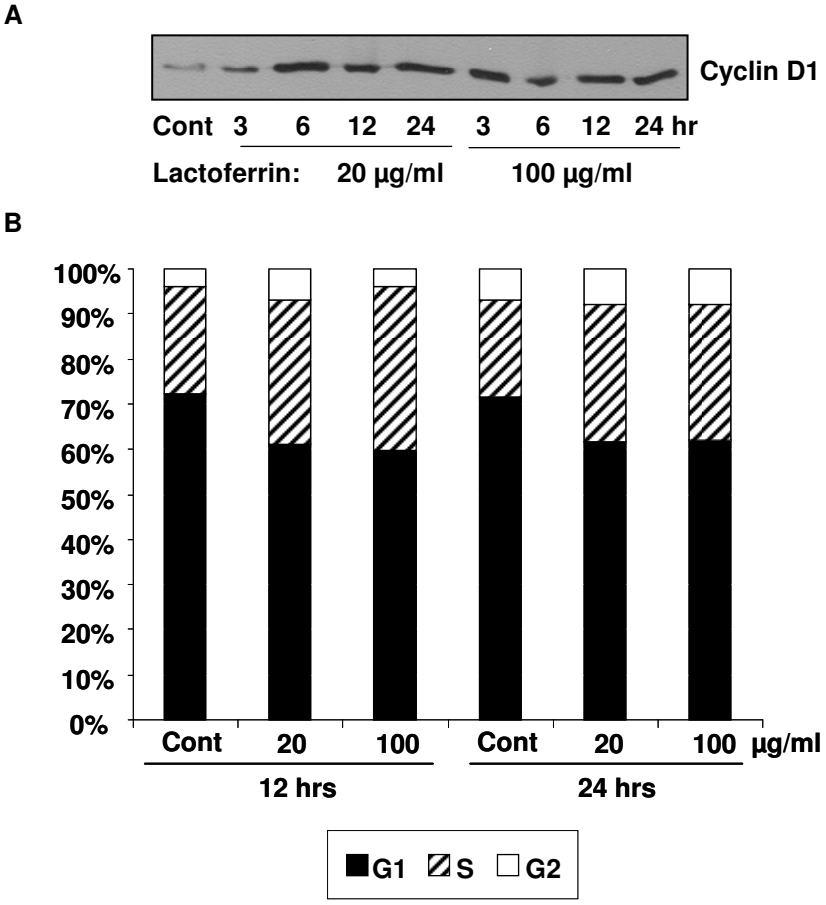


Figure 12. Lactoferrin stimulates cell cycle progression

(A) Effect of lactoferrin on protein levels of cyclinD1.

(B) Effect of lactoferrin on cell cycle in ZR-75 cells. Cells were treated with or without different concentrations of lactoferrin for indicated time points. Cells were harvested and were subjected to flow cytometry analysis for determining the number of cells in each stage of cell cycle.

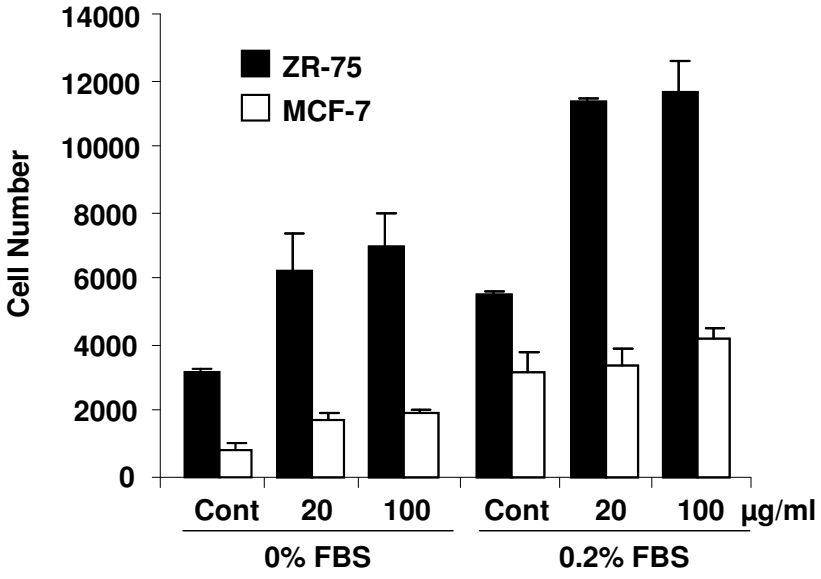


Figure 13. Lactoferrin increases cell proliferation of breast cancer cells.

Effect of lactoferrin on cell proliferation in ZR-75 and MCF-7 cells. Cells were treated with or without different concentrations of lactoferrin daily for 3 days.

in cell migration in MDA-MB-231 cells dose-dependently (**Fig. 14B**). To further validate the contribution of lactoferrin to the aggressive phenotype in breast cancer, we examined the invasive properties of TNBC cell lines. In addition to MDA-MB-231 cells, we included another TNBC cell line, MDA-MB-468- both of which are highly invasive. To study the invasivness of these cell lines upon lactoferrin treatment, we used Matrigel^{TM} invasion chambers. At the end of the 48-hr incubation period, the cells that have successfully invaded through the matrigel were fixed, stained, counted and plotted against control treated cells (**Fig. 15**). As suspected, lactoferrin increased the invasive properties of MDA-MB-231 and MDA-MB-468 cells suggesting that this increase in aggressiveness is independent of receptor downregulation.

Overall, the data so far demonstrated that lactoferrin not only downregulates ERα, PR and HER-2 but this peptide also promotes cell migration and invasion in receptor-positive breast cancer cells. Interestingly, we also observed the same increase in migratory and invasive properties in TNBC cells indicating that the noted aggressiveness is not dependent on the downregulation of ERα, PR and HER-2 per se. Based on the observations thus far, we therefore hypothesize that lactoferrin could be involved in the activation of a gene(s) which could augment migration and invasion of these breast cancer cells.

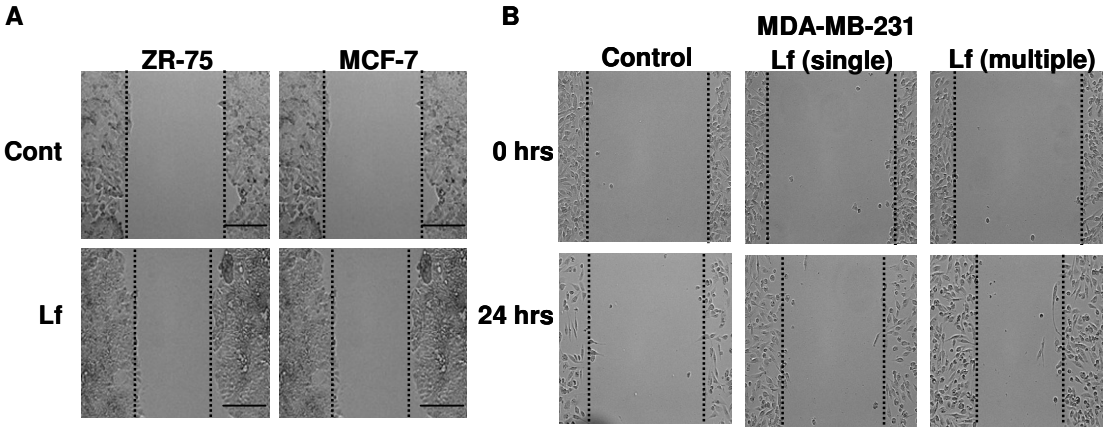


Figure 14. Lactoferrin stimulates cell migration
Effect of Lf on wound-healing in (A) ZR-75, MCF-7 and (B) MDA-MB-231 cells. (A) ZR-75 and MCF-7 cells were treated with a one-time dose of Lf for 24 hrs. (B) Multiple wounds were made and MDA-MB-231 cells were treated as followed: Lf (s)- single 100µg/ml of Lf; Lf (m)- four doses of 100µg/ml of Lf. Measurements were taken after 24 hrs of treatment.

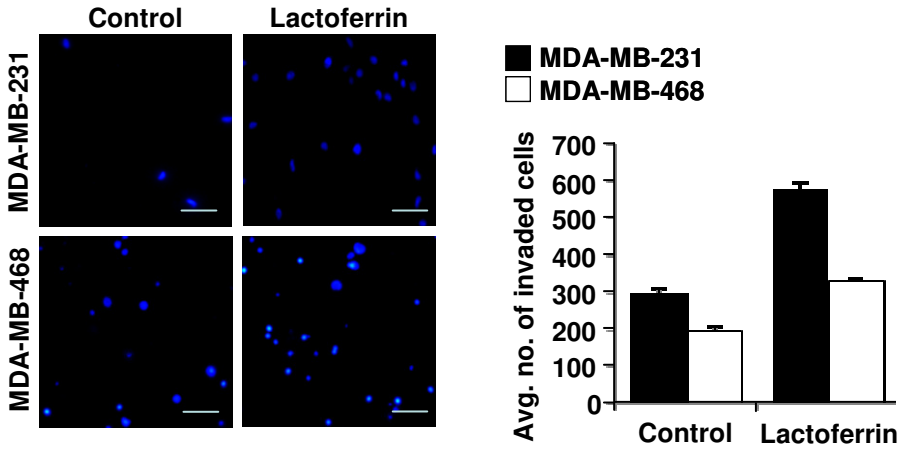


Figure 15. Lactoferrin increases cell invasion in breast cancer cells.

Effect of lactoferrin 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.

3.2.1 Lactoferrin-overexpressing Stable Clones

The data obtained thus far indicate that lactoferrin is possibly involved in contributing to a more aggressive breast cancer phenotype. Since we have shown the implication of lactoferrin on receptor downregulation as well as possible contribution to aggressiveness in a transient manner only, we wanted to study the involvement of lactoferrin in a long-term effect. In the studies presented here, the cell lines were treated with exogenous recombinant lactoferrin and therefore we set out to generate lactoferrin-expressing stable breast cancer cells. The long-term goal of this project beyond the scope of this thesis is to use these established clones for studying the effect of lactoferrin *in vivo*. Next we wanted generated a stable cell line overexpressing lactoferrin in a tetracycline-regulatable cell line. Because our previous studies were done in MCF-7 cells, we chose this cell line for the generation of such stable clone. MCF-7 Tet-off cells were purchased from Clontech and then used to further transfect with a lactoferrin-containing plasmid. Under the selection of Zeocin^{TM}/zeomycin, pooled as well as single stable clones were generated. To test for positive stable clones, pooled and single clones were treated with or without 1µg of doxycycline (a derivative of tetracycline) for 24 hours. Since the lactoferrin plasmid included a myc tag, we were able to check for the expression of lactoferrin under the induction of doxycycline via western blotting (**Fig. 16A**). The majority of the clones generated had a high expression of lactoferrin indicated by high levels of myc (**Fig. 16A**).

Furthermore, since we hypothesize lactoferrin in the downregulation of ERα, PR and HER-2, our preliminarily examination of the expression of these receptors upon doxycycline-dependent induction of lactoferrin indicated an easily detectable downregulation of ERα and HER-2 upon a 24 hr induction with doxycycline (**Fig. 16B**).

To test for the overexpression of lactoferrin *in vivo*, we wanted to test biological endpoints such as cell proliferation and migration of these inducible stable clones. To examine cell proliferation upon induction of lactoferrin, we treated the cells with or without 1 mg/ml of doxycycline for two days and then measured the cell numbers using a Z1 Beckman's Coulter Counter. As seen in **Figure 17A**, there was no significant difference between the control and treated cells. Furthermore, to assess the migratory behavior of these stable clones, we performed a Boyden chamber assay. The cells were treated with or without doxycycline for 24 hrs and then the migrated cells were fixed and stained. There was no difference in migration found between control and treated cells (**Fig. 17B**).

In brief, the successful generation of these lactoferrin-inducible stable clones will enable us to further study the role of lactoferrin in the contribution of an aggressive breast cancer phenotype. While we were able to generate successful lactoferrin-inducible stable clones, additional future studies are needed to fully define the contribution of regulatable expression of lactoferrin as the invasiveness of breast cancer beyond growth and migration.

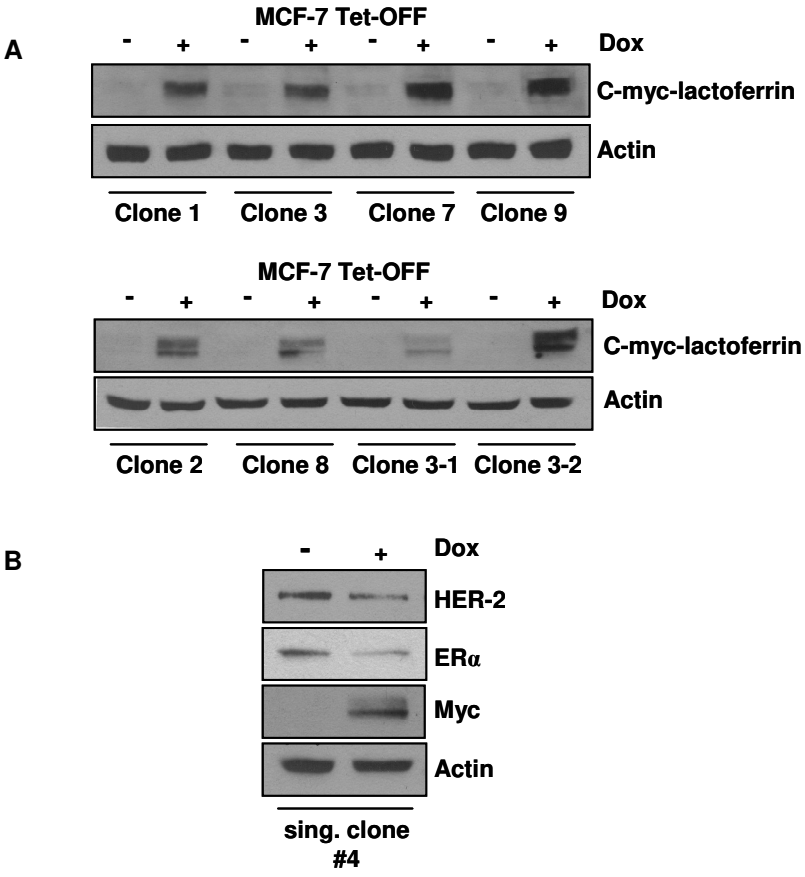
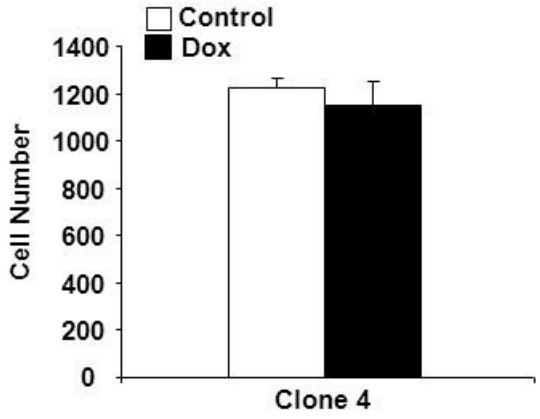


Figure 16. Establishment of lactoferrin-overexpression stable clones

(A) Successful establishment of pooled MCF-7 stable clones expressing c-myc-lactoferrin

(B) Downregulation of ER and HER-2 upon treatment with doxycycline in single MCF-7 clone

A



B

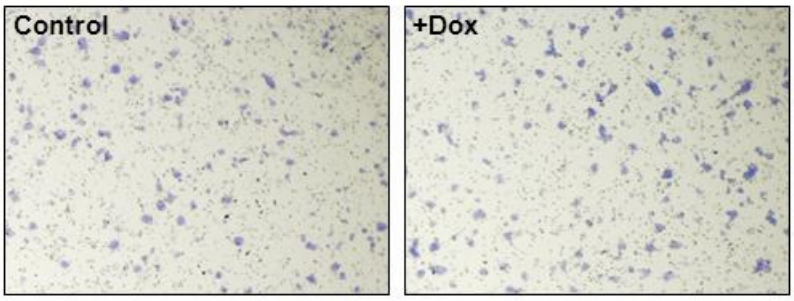


Figure 17. Establishment of lactoferrin-overexpression stable clones

(A) Cell proliferation of lactoferrin-inducible stable clones upon doxycycline treatment.

(B) Boyden chamber migration of lactoferrin-inducible stable clones upon doxycycline treatment.

Chapter 4: Results Part II- The Lactoferrin-Endothelin-1 Axis in Breast Cancer

4.1 Lactoferrin Stimulates Endothelin-1 Transcription

Since we observed an increase in cell migration and invasion with lactoferrin treatment in TNBC cells, we hypothesize that lactoferrin can induce a gene/protein involved in these aggressive phenotypes. Lactoferrin has been previously shown to be able to bind to DNA sequences and for that reason it has been suggested to act as a transcription factor (He & Furmanski, 1995**)**. Therefore, to gain insight into the molecular basis of lactoferrin-induced cell invasiveness of breast cancer, we first wanted to determine the nature of lactoferrin-regulated genes using microarray analysis of lactoferrin-treated and untreated MDA-MB-231 and MDA-MB-468 cells (**Fig. 18**). To test this hypothesis, both cell lines were treated with 100 µg/ml of lactoferrin, and the isolated RNA samples were hybridized onto an Affymetrix Human Exon 1.0 ST Array chip followed by analysis with GeneSpring GX. This analysis led to the identification of three lactoferrin-regulated genes (i.e. *Et-1, Rgs-2 and Tcbp-1*) in both the cell lines (**Fig. 18**). Guided by the functions of these genes in the context of our findings here, we decided to focus on endothelin-1 (ET-1), a vasoactive peptide first isolated from vascular endothelial cells (Yanagisawa et al., 1988) which is found to be upregulated in certain human cancers (Nelson et al., 2003) including breast (Wulfing et al., 2003). Therefore, to verify the microarray analysis, we performed a Northern blot with control and lactoferrin-treated samples and found that indeed, lactoferrin increased the steady-state

levels of ET-1 mRNA in MDA-MB-468, MDA-MB-231 as well as MCF-7 cells (**Fig. 19A**).

The data so far indicate that lactoferrin could upregulate the expression of ET-1 mRNA. Since ET-1 has been shown to play a role in tumor progression and metastasis the finding here could explain the increase in invasion and migration that we observed upon lactoferrin treatment.

To better understand the regulation of ET-1 mRNA by lactoferrin, we set out to do biochemical experiments that can give an insight into the mechanism of upregulation and to examine the biological consequences of such upregulation. Since we observed an increase in mRNA level of ET-1 after lactoferrin treatment, we first wanted to further verify the regulation of ET-1 by lactoferrin. An increase in mRNA could indicate transcriptional regulation of ET-1 or an increase of protein synthesis and therefore, treating the cells with cycloheximide (CHX), a protein synthesis inhibitor, allows checking the level of regulation of ET-1 by lactoferrin. To test for this, we treated MDA-MB-231 cells with or without lactoferrin in the presence or absence of cycloheximide. As seen in **Fig. 19B**, lactoferrin-induced increased ET-1 mRNA could not be blocked by the inclusion of cycloheximide, suggesting transcriptional regulation of ET-1 by lactoferrin.

Because the data suggest transcriptional regulation of *endothelin-1* by lactoferrin, we wanted to examine if lactoferrin can directly bind to the promoter region and act as a transcription factor. In earlier studies, it has been shown that lactoferrin has the ability to enter the nucleus and bind to DNA (He & Furmanski,

1995) and therefore, we hypothesize that lactoferrin binds to the promoter region of ET-1 and transcriptionally activates it. To verify this, we used the Alggen Promoter program to examine the promoter region for any possible binding sites for lactoferrin. A close scanning of the ET-1 promoter revealed the presence of three imperfect lactoferrin motifs in the ET-1 promoter (**Fig. 20**).

As a first step to verify direct transcriptional regulation of ET-1 by lactoferrin, we performed a luciferase assay. MCF-7, MDA-MB-231 and MDA-MB-468 cells were transfected with the ET-1 promoter-luc plasmid followed by treatment of lactoferrin and then the isolated cell lysates were measured for luciferase promoter activity. Consistently, lactoferrin stimulated the transcription driven by the ET-1 promoter-luc in all three breast cancer cell lines (**Fig. 21**) indicating direct transcriptional regulation by lactoferrin.

To further verify the involvement of lactoferrin in the activation of ET-1 transcription, we set out to examine the potential binding sites of lactoferrin on the ET-1 promoter. Since scanning of the promoter program suggested three possible binding sites, we performed an electrophoretic mobility shift assay (EMSA) to confirm binding of lactoferrin to the ET-1 promoter region. Briefly, PCR-amplified, radio-labeled DNA fragments containing each of the three lactoferrin consensus sites were incubated with or without lactoferrin and then run on a Native gel. Results from the EMSA assay using oligonucleotides encompassing all three potential lactoferrin-interacting DNA regions in the ET-1 promoter indicated the formation of specific lactoferrin-DNA complexes with all three sites (**Fig. 22**).

Since we observed binding with all three sites, we wanted to next examine which of the three binding motifs is the bona fide consensus site. To test this, we individually deleted lactoferrin-consensus motifs # 1-3 in the full-length ET-1 promoter-luc, transfected MDA-MB-468 cells with each deletion construct along with the wildtype plasmid, treated the cells with lactoferrin and then subjected the cell lysates to the luciferase promoter assay. As depicted in **Figure 23**, we 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 to consensus motif number 3. These data therefore suggest that lactoferrin may preferentially utilize lactoferrin-consensus motif number 3 to stimulate ET-1 transcription.

To further identify the specific base-pairs in the functional lactoferrin consensus site 3 that might be responsible for lactoferrin binding, we created three mutant versions of the lactoferrin consensus site 3 (**Fig. 20**) and tested these oligos for lactoferrin binding using EMSA. There was a drastic reduction in the binding of lactoferrin to oligo number 3 (**Fig. 24**), suggesting that lactoferrin may preferentially interact with the GGCACTTGG motif (**Fig. 20**) in the ET-1 promoter.

Finally, we wanted to test for nuclear extract protein binding to site #3 in the ET-1 promoter region using lactoferrin-treated nuclear extracts. The data presented thus far showed lactoferrin complexing with the ET-1 promoter based on incubation with a purified holo-lactoferrin peptide, however we wanted to verify the findings using treated nuclear extracts. Employing EMSA again, we observed a progressive increase in the levels of protein-DNA complexes which could be

effectively super-shifted by inclusion of lactoferrin antibody but not isogenic IgG (**Fig. 25**, lanes 6, 7).

Furthermore, we set out to examine if binding of lactoferrin to the ET-1 promoter was dependent on the level of iron saturation of lactoferrin. As mentioned before, lactoferrin exists in two forms: apo-lactoferrin and holo-lactoferrin, with the latter form being iron-saturated. In an early study, which originally identified the DNA consensus binding motifs for lactoferrin, it was shown that holo-lactoferrin demonstrated more avid binding to DNA in comparison to the apo-form of lactoferrin. Therefore, to test this, we treated the cells with both the holo- as well as the apo-form of lactoferrin and observed that only recombinant iron-saturated lactoferrin (holo-lactoferrin) but not non-iron saturated lactoferrin (apo-lactoferrin) interacted with the ET-1 promoter oligo and such protein-DNA complexes could be super-shifted by the lactoferrin antibody (**Fig. 25**, lanes 9, 11 and 10 respectively). Consistent with these findings iron-saturated holo-lactoferrin and not apo-lactoferrin upregulated ET-1 promoter-luc activity in MDA-MB-468 cells (**Fig. 26A**). Furthermore, we also observed that holo-lactoferrin but not apo-lactoferrin was able to downregulate the three therapeutic receptors in ZR-75 cells (**Fig. 26B**).

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 et al. (Duarte et al., 2011) 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 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 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 is likely to be due to the upregulation of ET-1 upon iron-saturated lactoferrin treatment.

Overall, the data presented thus far indicate that lactoferrin indeed could be involved in the aggressiveness of breast cancer cells by upregulating ET-1. We further showed that it does so by directly binding to the GGCACTTGG consensus motif on the ET-1 promoter region and therefore can transcriptionally upregulate it. Additionally, we demonstrated that iron-saturation is an important factor in lactoferrin's ability to bind to the ET-1 promoter as well as downregulate ERα, PR and HER-2.

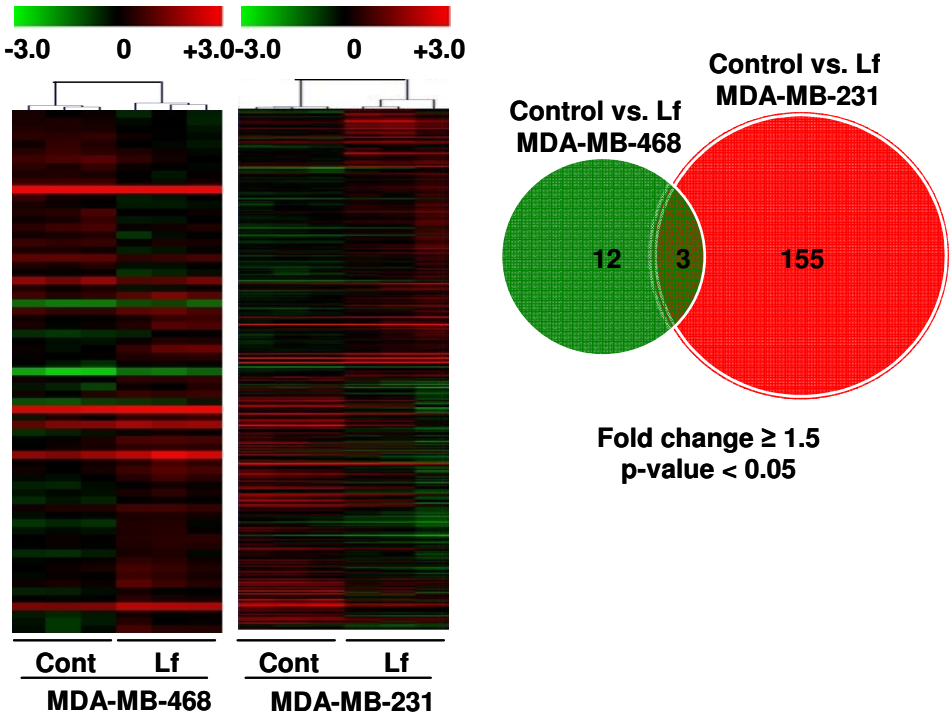


Figure 18. Lactoferrin-regulated genes
Analysis of differentially regulated genes after lactoferrin treatment in MDA-MB-231 and MDA-MB-468 cells by Affymetrix Human Exon 1.0 ST microarrays using Gene-Spring GX 10.0.2.

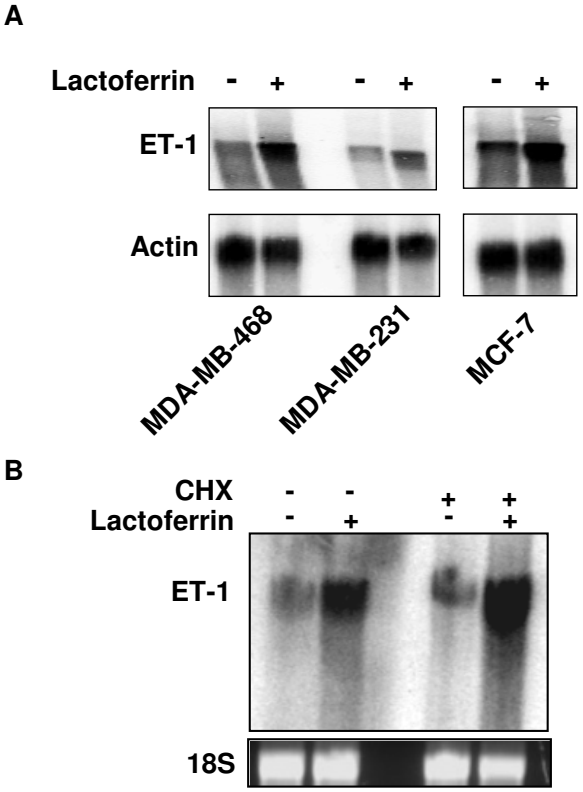


Figure 19. Endothelin-1 is a transcriptional target of lactoferrin.

(A) Effect of lactoferrin on ET-1 mRNA levels in breast cancer cells. Actin was used as a loading control.

(B) Effect of lactoferrin in the presence or absence of cycloheximide on the induction of ET-1 mRNA in MDA-MB-231 cells.

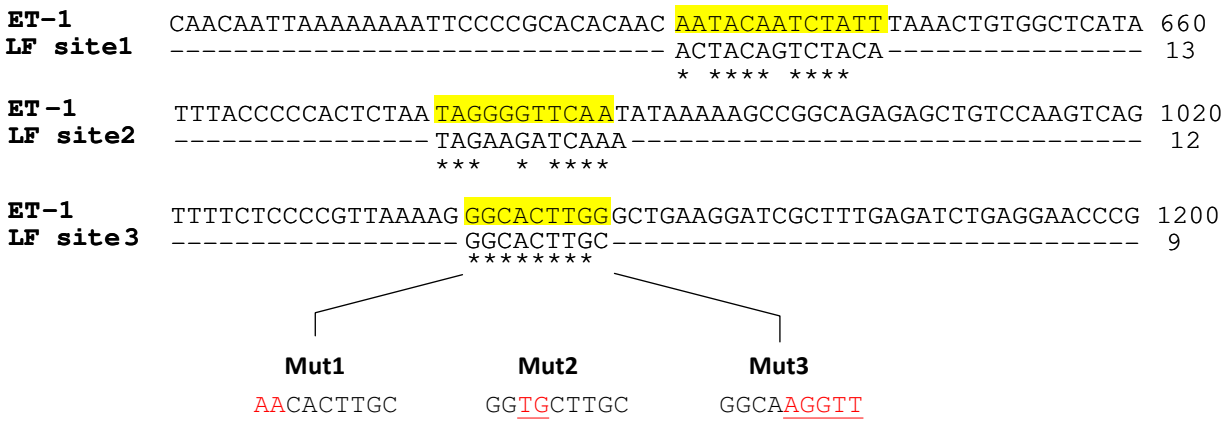


Figure 20. Endothelin-1 is a transcriptional target of lactoferrin. Lactoferrin binding sites in ET-1 Promoter and Lf site 3 mutant oligos used for EMSA.

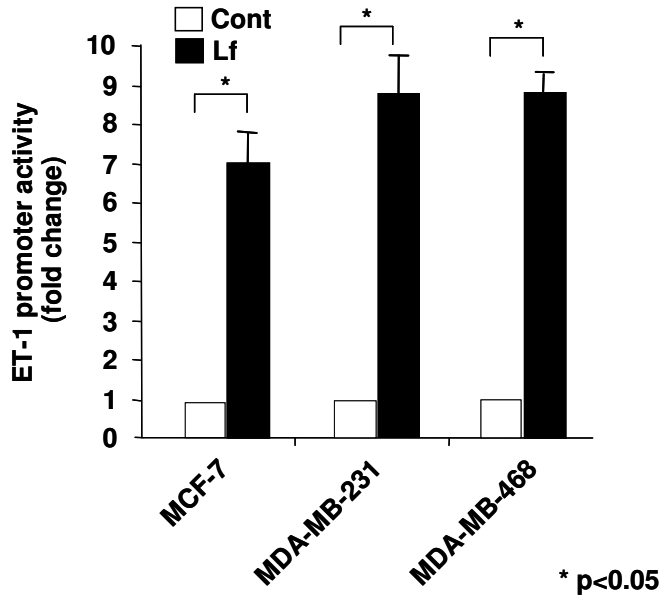


Figure 21. Endothelin-1 is a transcriptional target of Lf.
Effect of Lf on ET-1 promoter activity in breast cancer cells. Cells were treated with 100 µg/ml of Lf for 36 hrs.

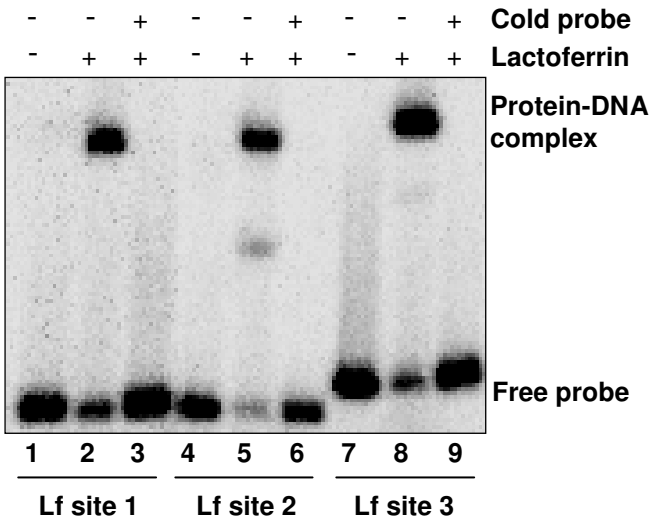


Figure 22. Endothelin-1 is a transcriptional target of lactoferrin. EMSA analysis of lactoferrin binding to all three Lf-consensus DNA sequences in the ET-1 promoter using the nuclear extracts from MCF-7 cells treated with or without lactoferrin for 6 hrs.

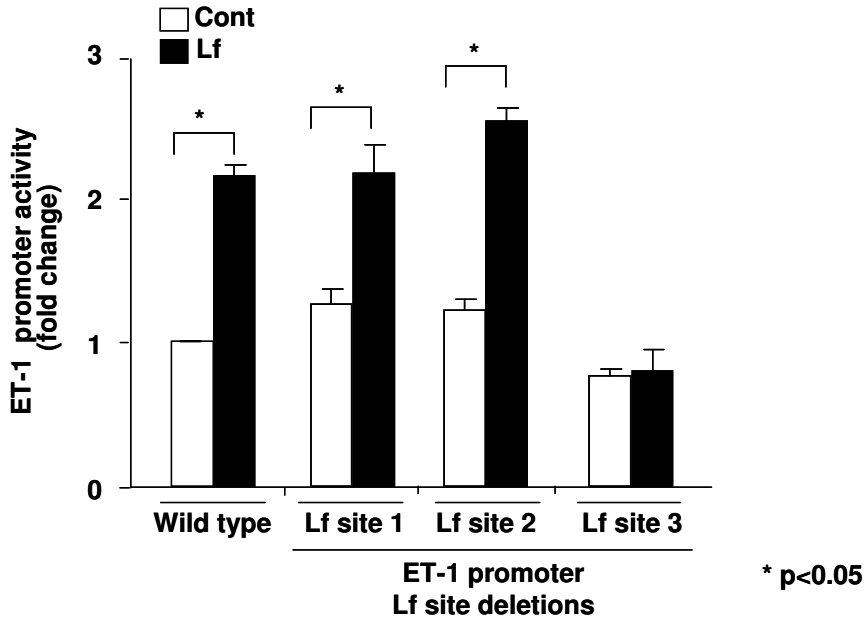


Figure 23. Endothelin-1 is a transcriptional target of Lf.
Effect of Lf on the ET-1 promoter with wild-type or mutant Lf-binding sequences in MDA-MB-468 cells.

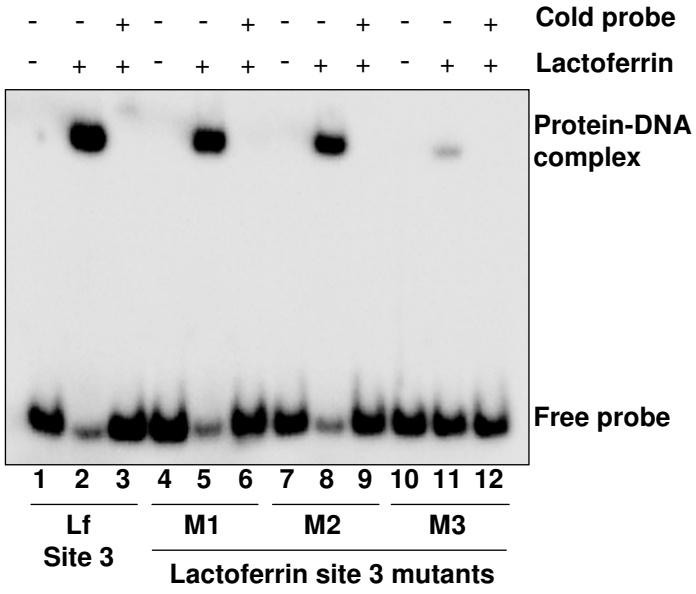


Figure 24. Endothelin-1 is a transcriptional target of lactoferrin. EMSA analysis of lactoferrin binding to oligonucleotides encompassing the wild-type lactoferrin-consensus DNA sequence 3 or its three mutant versions using the nuclear extracts from MCF-7 cells treated with or without lactoferrin for 6 hrs.

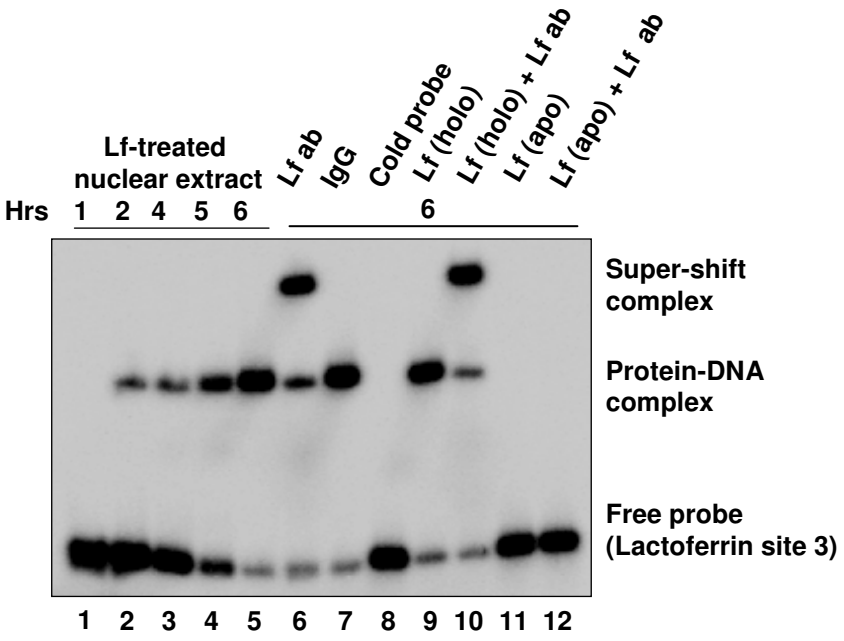


Figure 25. Endothelin-1 is a transcriptional target of lactoferrin.
EMSA analysis of lactoferrin-stimulated nuclear extracts from MCF-7 cells in the presence of antibody to lactoferrin. Antibody to rabbit-IgG was used as a negative control

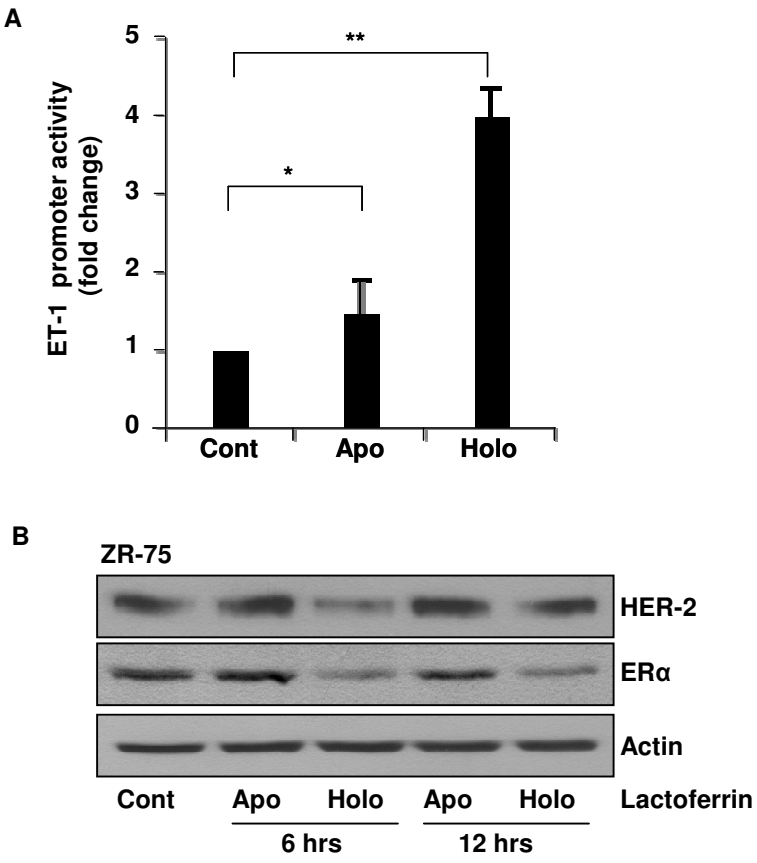


Figure 26. Effect of Apo- and Holo-lactoferrin

(A) Effects of holo- or apo-lactoferrin on the ET-1 promoter luc activity in MDA-MB-468 cells. *, *P > 0.05, ** , P>0.01*

(B) Effect of apo- and holo-lactoferrin on the downregulation of ERα and HER-2.

4.2 Lactoferrin- Endothelin-1 Axis Promotes TNBC Phenotype

Since we have established that lactoferrin can bind to the promoter region of ET-1 and upregulate its transcription, we wanted to next investigate the biological involvement of such regulation. Overexpression of ET-1 has been shown to play a role in cancer (Kusuhara et al., 1990; Grant et al., 2003) and therefore we wanted to examine its function in breast cancer with respect to lactoferrin. We hypothesize that lactoferrin contributes to the noted aggressiveness in breast cancer cells by upregulation the transcription of ET-1 which then can exert its migratory and invasive actions. To test this hypothesis, we set out to conduct biological endpoint studies as well as examine the expression of ET-1 in human breast cancer samples. Because ET-1 is known to be a secreted polypeptide, we first wanted to investigate the physiological relevance of lactoferrin stimulation of ET-1 in lactoferrin-treated cells. We treated MCF-7, MDA-MB-231 as well as MDA-MB-468 cells with lactoferrin and then collected the conditioned media. Using an ET-1-specific ELISA kit, we measured the concentration of ET-1 in the conditioned media of treated and non-treated cells and observed that lactoferrin-mediated increased expression of ET-1 was also accompanied by a significant accumulation of ET-1 in the collected media (**Fig. 27**). 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. 27**). This observation therefore indicates that not only does lactoferrin transcriptionally upregulate ET-1 but that the consequence of such activation is the secretion of ET-1.

As previously mentioned, upregulation of ET-1 has already been shown to be pro-mitogenic as well as pro-invasive (Rosanò et al., 2010) and therefore we wanted to assess whether lactoferrin-mediated upregulation of ET-1 has an effect on biological endpoints, such as cell migration and invasion. To test this, we employed the uncoated Boyden chamber and the Matrigel™ invasion assay. We treated MCF-7 cells with or without lactoferrin in the presence or absence of the endothelin-1 peptide. In addition to lactoferrin and endothelin-1, we also included BQ123 (21-23), which is a pharmacological inhibitor of the endothelin-1 A receptor (ET(A)R). It is known that ET-1 preferentially binds to ET(A)R isoform to exerts its actions. Since we hypothesize that ET-1 contributes to cell migration and invasion via binding to its own receptor, we included BQ123 to test if the hypothesis holds true. As depicted in **Figure 28**, we observed an increase in migration of MCF-7 cells in the presence of ET-1 as expected. Similarly, when the cells were treated with lactoferrin alone, there was an increase in the number of migrated cells which we have shown previously. The lactoferrin-dependent observed migration, however, was diminished in the presence of BQ123 pretreatment, indicating that the ET(A)R receptor is important in the signaling of ET-1. This observation suggests, as hypothesized, that the lactoferrin-ET-1 axis might indeed play a role in the development of a more aggressive breast cancer phenotype.

Since invasion is one of the hallmarks of cancer aggressiveness, we wanted to examine the invasive properties of MCF-7 cells in the presence of lactoferrin, endothelin-1 peptide and BQ123. For this, we employed the Matrigel™ invasion

chambers with the same treatments as the cell migration assay. Again, we observed an increase in invasion in the presence of lactoferrin and ET-1 alone, and this invasion was abrogated with the pretreatment of BQ123 (**Fig. 29**).

The data therefore indicate that lactoferrin-dependent upregulation of ET-1 promotes cell migration and invasion in breast cancer cell lines and that this observed invasion is abrogated with the inclusion of BQ123, a ET(A)R specific antagonist. This demonstrates that the lactoferrin-ET-1 axis is involved in the contribution to an aggressive breast cancer phenotype.

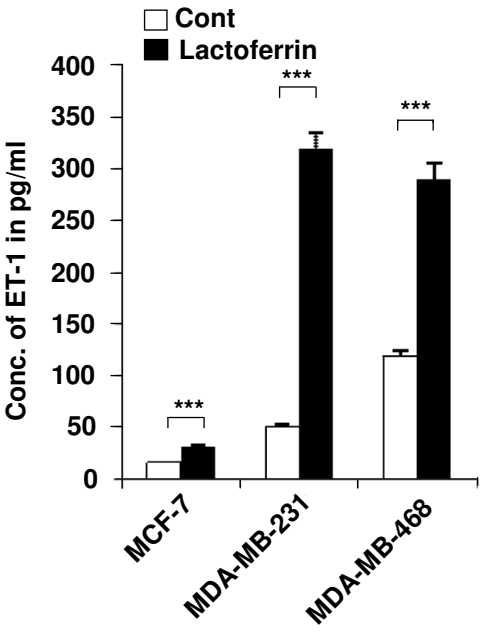


Figure 27. Concentration of ET-1 in the conditioned media from breast cancer cells treated with or without 100 µg/ml of lactoferrin for 24 hrs as measured by ELISA. ****, P> 0.001*

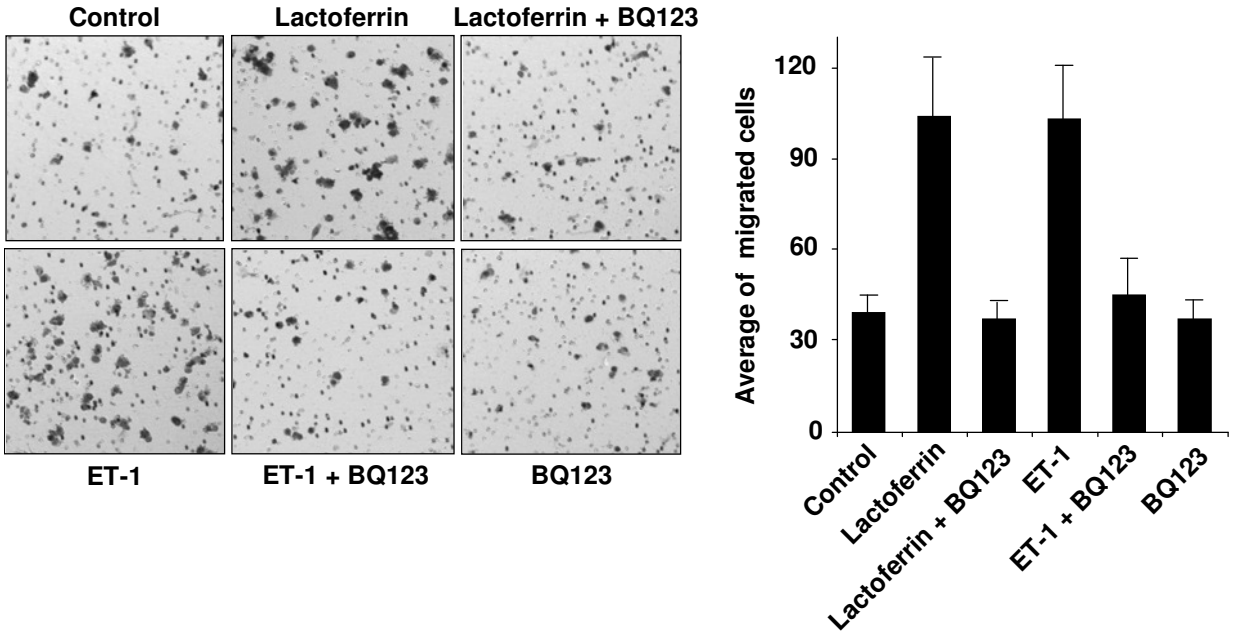


Figure 28. Effect of ET-1-receptor inhibitor BQ123 on cell migration of MCF-7 cells treated with or without lactoferrin or ET-1, using Boyden chambers.

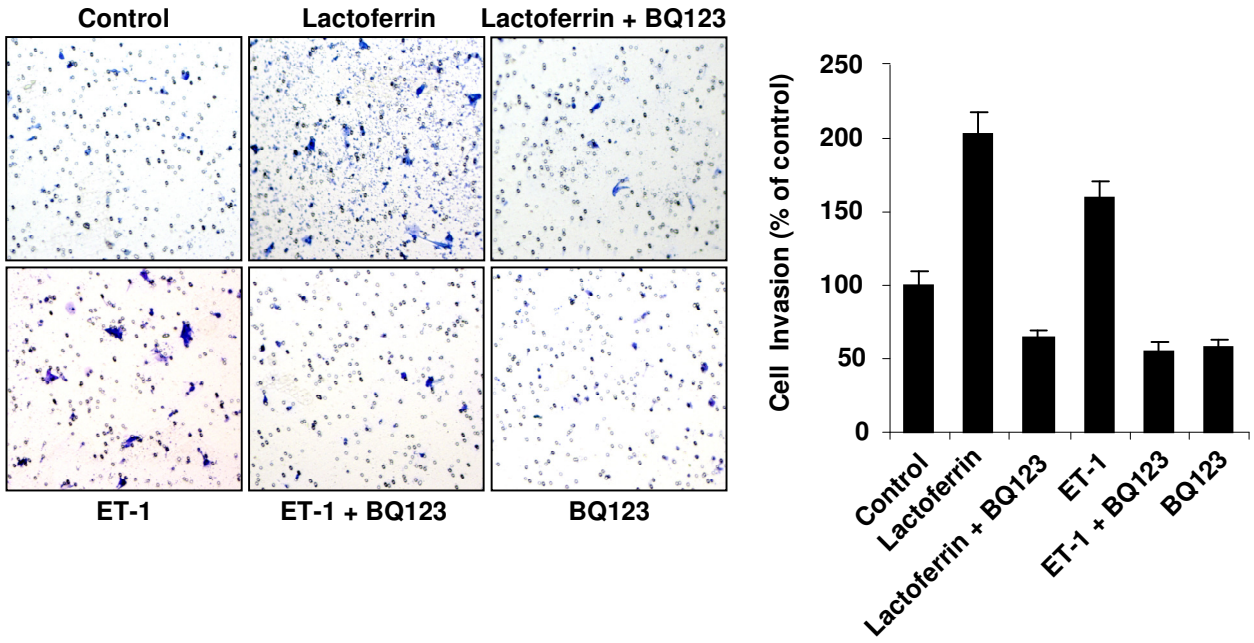


Figure 29. Effect of ET-1-receptor inhibitor BQ123 on cell invasion of MCF-7 cells treated with or without lactoferrin or ET-1 using Matrigel™ invasion chambers

4.2.1 Lactoferrin- Endothelin-1 Axis in Human Breast Cancer Samples

Because our data so far have indicated a potential role for the lactoferrin-ET-1 axis in the progression of breast cancer, we next wanted to assess the significance of these observations in a physiological setting. TNBC is a more aggressive subtype of breast cancer in comparison to ER/PR/HER-2-positive breast tumors and since we hypothesize that lactoferrin and ET-1 could play a role in breast cancer progression from ER/PR/HER-positive to a TNBC phenotype, we determined the status of lactoferrin in human breast tumors from TNBC and ER+/PR+ patients by quantitative-RT-PCR (q-PCR). We found that the levels of lactoferrin (**Fig. 30**, left) as well as ET-1 (**Fig. 30**, right) were significantly increased in TNBC specimens as compared to those in the control ER+/PR+ breast tumors. These data indicate that lactoferrin as well as ET-1 could both be more highly expressed in more aggressive types of breast cancer.

Since both lactoferrin and ET-1 are secreted polypeptides, we next wanted to determine the levels of lactoferrin and ET-1 in the plasma samples from TNBC as well as from ER/PR/HER-2-positive patients. In our earlier data, we observed a higher level of ET-1 in the conditioned media of lactoferrin-treated breast cancer cell lines (**Fig. 27**) indicating that lactoferrin treatment results in an increase in ET-1 secretion. Using a lactoferrin- and ET-1-specific ELISA kit, we analyzed the levels of these two proteins in human breast cancer serum samples and observed an elevated level of ET-1 as well as lactoferrin in TNBC when compared to the ER/PR/HER-2 positive samples (**Fig. 31**).

Furthermore, we wanted to analyze human breast cancer tissue samples obtained from the same patients as the serum samples. Staining these tissue slides with lactoferrin- and ET-1-specific antibodies, we also found strong and medium co-staining of lactoferrin and ET-1 in TNBC samples when compared to ER/PR/HER-2 positive tissue samples as determined by immunohistochemistry (IHC). This is consistent with the data we obtained from the serum samples and therefore further validating our hypothesis. Data in **Figure 32** illustrate representative examples of increased levels of lactoferrin and ET-1 co-staining in the tumor specimens from TNBC patients as compared to the ER/PR/HER-2-positive tumors. We analyzed a total of 10 TNBC and 5 non-TNBC tissue slides and found 80% with strong co-staining for lactoferrin and ET-1 in TNBC samples versus 40% with strong co-staining in non-TNBC tissue slides (**Table 1**). These data indicate that there is a correlation between the expression of lactoferrin and ET-1 and that this expression is higher in aggressive breast cancers as hypothesized.

Collectively, these findings revealed that lactoferrin-stimulated increased invasiveness of breast cancer cells is mediated via increased expression and secretion of ET-1 and that these changes might account for the generally noted aggressive behavior of TNBC. Furthermore, the human data provided a confirmation of the findings we have obtained in the breast cancer cell lines, a possible explanation on how the lactoferrin-ET-1 axis is contributing to breast cancer progression.

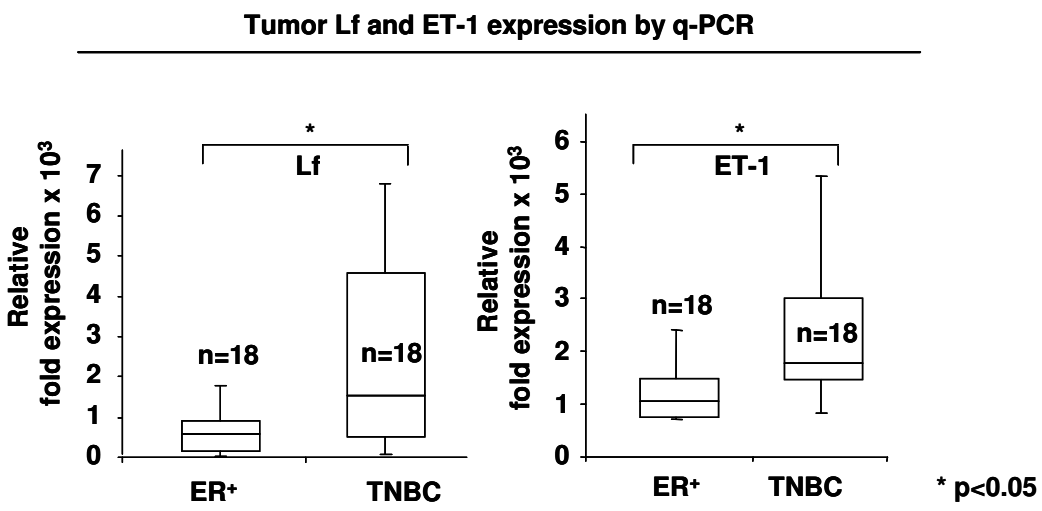


Figure 30. Lactoferrin and endothelin-1 expression in human breast cancer samples

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.

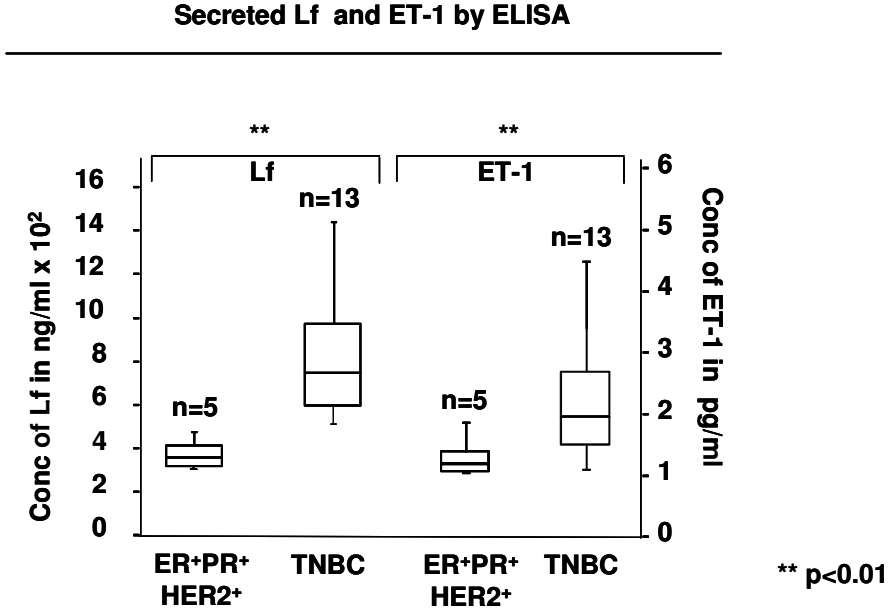


Figure 31. Lactoferrin and endothelin-1 expression in human breast cancer samples

Concentration of Lf and ET-1 in plasma samples from TNBC or non-TNBC patients by ELISA.

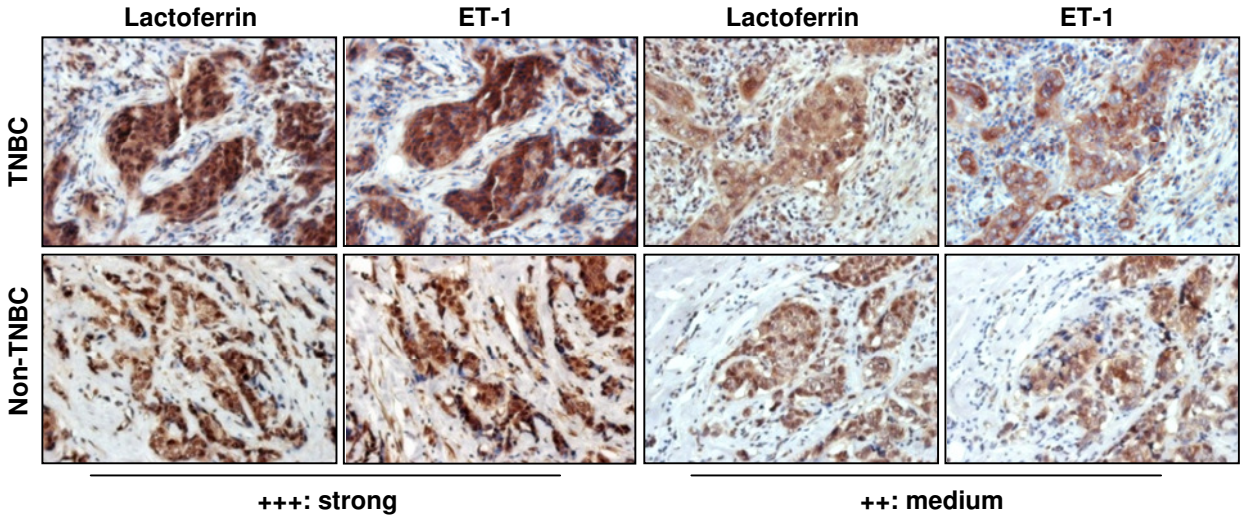


Figure 32. Lactoferrin and endothelin-1 expression in human breast cancer samples
IHC evaluation of lactoferrin and ET-1 expression in breast tumors from the patients used in Fig. 33.

Type	Slide No.	Lf staining		ET-1 staining	Match
TNBC	1	+++		+++	Yes
	2	+++		+++	Yes
	3	+++		+++	Yes
	4	+++	n = 8	+++	Yes
	5	+++	(80%)	+++	Yes
	6	+++		+++	Yes
	7	+++		+++	Yes
	8	+++		++	No
	9	++	n = 2	++	Yes
	10	++	(20%)	++	Yes
Non-TNBC	1	+++	n = 2	+++	Yes
	2	+++	(40%)	+++	Yes
	3	++		++	Yes
	4	++	n = 3	++	Yes
	5	++	(60%)	++	Yes

Table 1. Lactoferrin and endothelin-1 expression in human breast cancer samples

Table depicts TNBC and non-TNBC tissue samples with scoring of lactoferrin and ET-1

Chapter 5: Results Part III- Lactoferrin Stimulation of Pak1 Kinase Activity

Thus far, we have presented data that suggest that lactoferrin is involved in downregulating ERα, PR and HER-2 in a proteasomal-dependent manner in receptor-positive breast cancer cells. Furthermore, we demonstrated that lactoferrin is able to directly bind to the ET-1 promoter region and therefore transcriptionally upregulate ET-1, leading to an increase in ET-1 production and secretion. In concordance with known data about ET-1, we showed that the lactoferrin-dependent upregulation of ET-1 leads to an increase in aggressiveness of breast cancer cells as demonstrated by migration and invasion assays. To further study the pathway by which lactoferrin-dependent upregulation of ET-1 could possibly be involved in migration of breast cancer cells, we wanted to analyze its contribution to Pak1 activation.

It is known that an activation or overexpression of Pak1 plays a role in cancer via a variety of mechanisms. One of the mechanisms by which Pak1 increases cell motility is through G protein-coupled receptor (GPCR) signaling. Hormonal signaling peptides upon binding to their GPCR can activate downstream signaling molecules that will activate Pak1 kinase activity leading to cytoskeleton changes (Parrini et al., 2005) and an increase in cell motility. Since the ET(A)R receptor is a GPCR and can transduce downstream signals via binding of ET-1, we hypothesize that lactoferrin-dependent upregulation of ET-1 can activate Pak1 kinase activity. To examine if lactoferrin plays a role in Pak1 kinase stimulation, we performed a kinase assay using MCF-7, ZR-75 and MDA-MB-468 breast cancer cells. The cells

were treated with lactoferrin for 6 hours, a time point at which upregulation of ET-1 was observed, and then the cell lysates were immunoprecipitated with Pak1 and subsequently subjected to a kinase assay. To measure Pak1 kinase activity, a specific Pak1 substrate, myelin basic protein (MBP) was used. We observed lactoferrin-dependent stimulation of Pak1 in MCF-7 as well as ZR-75 cells as evidenced by an increase in radio-labeled phospho-MBP (^{32}P-MBP) (**Fig. 33**). No noticeable upregulation of Pak1 kinase activity was observed for MDA-MB-468 cells. The preliminary data therefore suggest that treatment of breast cancer cells with lactoferrin can activate Pak1 kinase activity presumably via upregulation of ET-1.

Since we hypothesize that the activation of Pak1 kinase activity is regulated by ET-1 binding to its ET(A)R receptor, we wanted to further study the involvement of ET(A)R. To test this hypothesis, we decided to use MCF-7 cells for further experiments because they exhibited the highest lactoferrin-dependent Pak1 kinase activity (**Fig. 33**). The cells were treated in the presence of lactoferrin as well as with and without BQ123, a specific antagonist for ET(A)R. Additionally, we wanted to verify the time-dependent stimulation of Pak1 upon lactoferrin treatment and therefore we treated the cells for 15', 30' and 1, 3 and 6 hrs. As expected, we observed an increase in Pak1 kinase activity at 3 and 6 hrs (**Fig. 34**, left panel). Furthermore, lactoferrin-dependent stimulation of Pak1 was decreased upon BQ123 pretreatment indicating the involvement of ET(A)R in this upregulation (**Fig. 34**, right panel). The data therefore demonstrates that lactoferrin-dependent

stimulation of Pak1 kinase activity is not a direct effect as we only observed an increase in ^{32}P-MBP during the longer treatment times. In addition, since we were able to moderately block Pak1 kinase activity with BQ123 pretreatment, we can presume that lactoferrin-dependent stimulation occurs in part via the ET-1-ET(A)R pathway. This further confirms our hypothesis that lactoferrin-dependent upregulation of ET-1 in turn stimulates Pak1 kinase activity.

The data thus far indicate that lactoferrin can indirectly activate Pak1 kinase activity and we wanted to further examine the consequences of such stimulation. It has been shown that GPCR-dependent stimulation of Pak1 results in cytoskeletal changes and motility. To check for cytoskeletal remodeling and membrane ruffling that are indicative of motile cells, we used confocal microscopy. The cells were treated with or without lactoferrin, endothelin-1 and EGF. Since it is known that EGF can stimulate Pak1 kinase activity, we added EGF treatment as a positive control for membrane ruffling and cytoskeletal changes. After treatment, the cells were fixed, blocked and then stained with the following antibodies: actin- to visualize cytoskeletal changes in the cell; vinculin- to observe focal points which are indicative in motile cells (Molli et al., 2009); total Pak1 which should localize to the leading edge and 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) for nuclear visualization. Upon lactoferrin, endothelin-1 as well as EGF treatment we observed membrane changes compared to control cells (**Fig. 35**). Furthermore, we saw a slight increase in Pak1 plasma membrane/leading edge localization in

comparison to nuclear membrane Pak1 staining in control cells that is typical of Pak1-dependent motile cells (Molli et al., 2009).

Since we hypothesize and demonstrated that the lactoferrin-dependent Pak1 activation involves the upregulation of ET-1 which in turn binds to its specific ET(A)R receptor, we wanted to show that treatment with BQ123 will abrogate the cytoskeletal changes we observed earlier. To examine this, we treated MCF-7 with lactoferrin in the presence and absence of BQ123, the ET(A)R specific antagonist. Upon staining for actin, vinculin and total Pak1, we observed the same membrane ruffling with lactoferrin treatment as shown earlier. However, after BQ123 pretreatment, we saw an inhibition of cytoskeletal remodeling indicating that ET(A)R is involved in such changes (**Fig. 36**). In addition, we saw less Pak1 staining at the leading edge with BQ123 treatment that was not seen in lactoferrin inclusion alone.

The data presented here demonstrated that lactoferrin can indirectly activate Pak1 kinase activity, an event that is increased in more aggressive cancers (Kumar et al., 2006). We showed that the hypothesized pathway through which lactoferrin stimulates Pak1 kinase activity is via the transcriptional upregulation of ET-1. Increased ET-1 then binds to its specific ET(A)R receptor to activate Pak1 in a GPCR-dependent manner. In our confocal microscopy data, we observed that such lactoferrin-dependent activation leads to membrane ruffling which is frequently observed in Pak1-dependent increase in motility of cancer cells.

In conclusion, we demonstrate that lactoferrin can transcriptionally activate ET-1 which in turn binds to its ET(A)R receptor and as a consequence of such binding, we demonstrated the activation of Pak1 kinase activity. A possible pathway through which lactoferrin increases cell motility could be via the ET-1-ET(A)R-Pak1 axis.

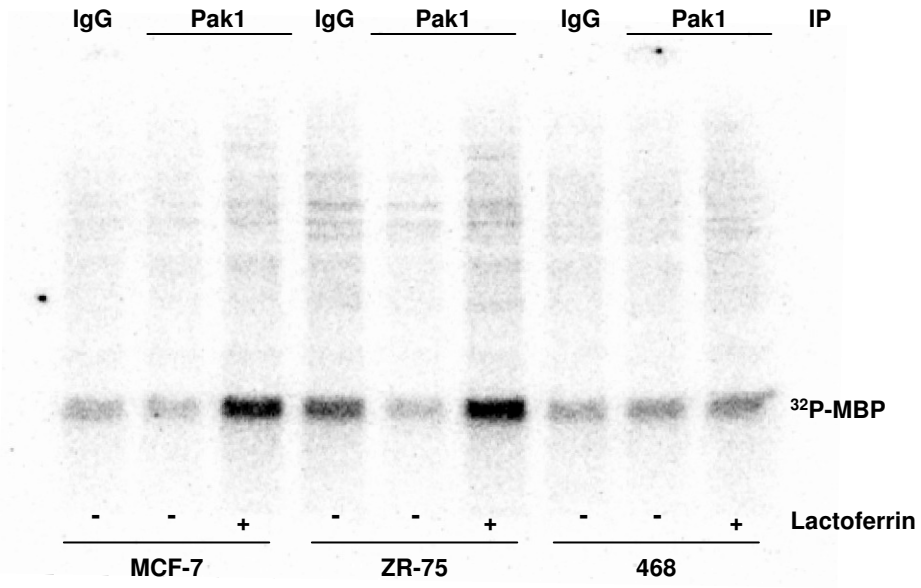


Figure 33. Lactoferrin effect on Pak1 kinase activity
Pak1 kinase assay in three lactoferrin-treated breast cancer cell lines

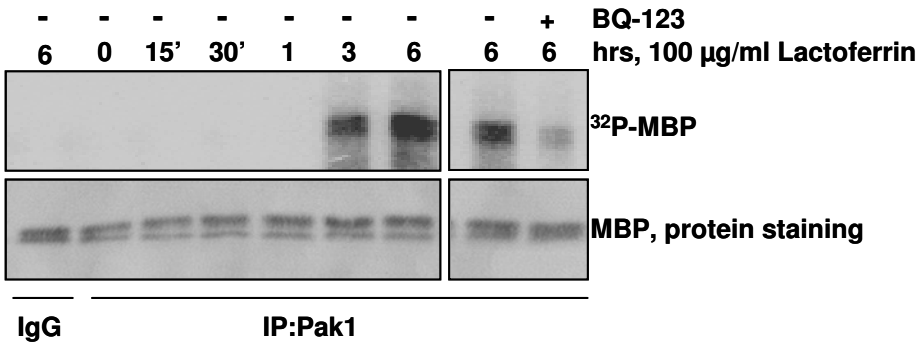


Figure 34. Lactoferrin effect on Pak1 kinase activity
Time-dependent activation of Pak1 upon lactoferrin treatment. Right panel, BQ123 inhibits lactoferrin-induced Pak1 kinase activation

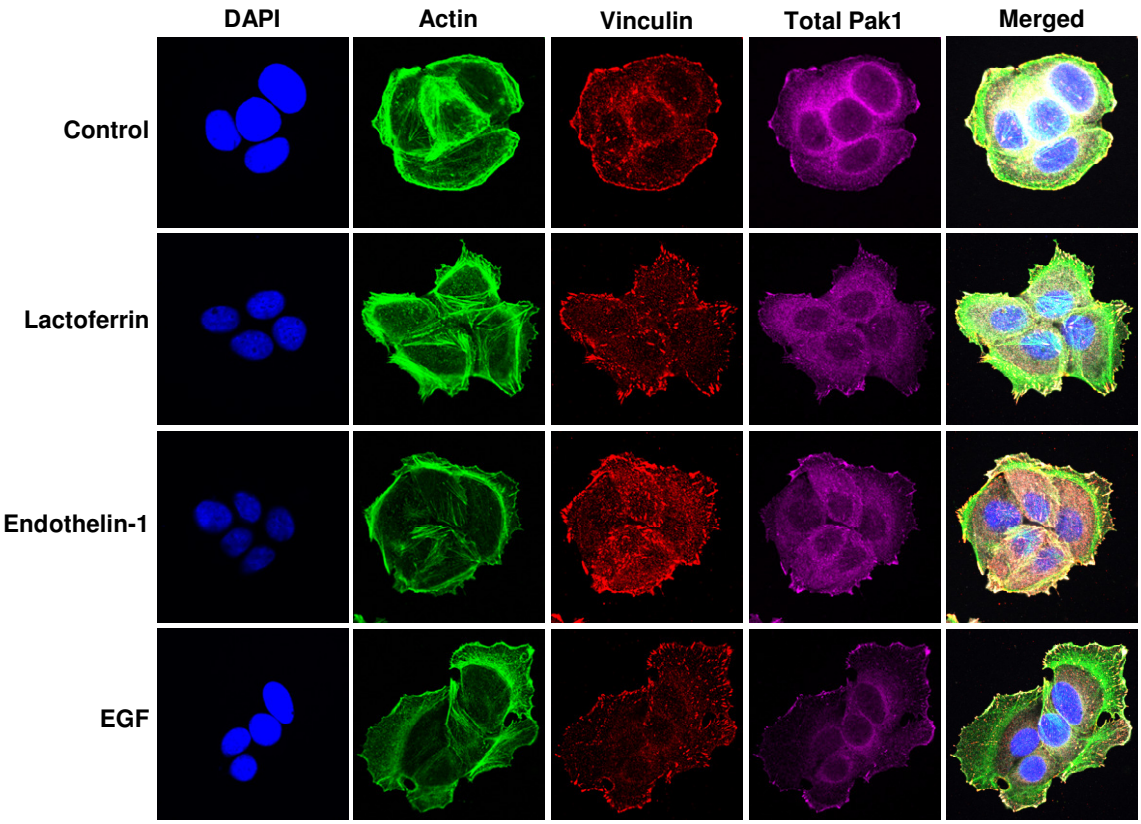


Figure 35. Lactoferrin effect on cytoskeleten remodelling
Lactoferrin induces membrane ruffling in MCF-7 cells as measured by confocal microscopy. EGF was used as a positive control.

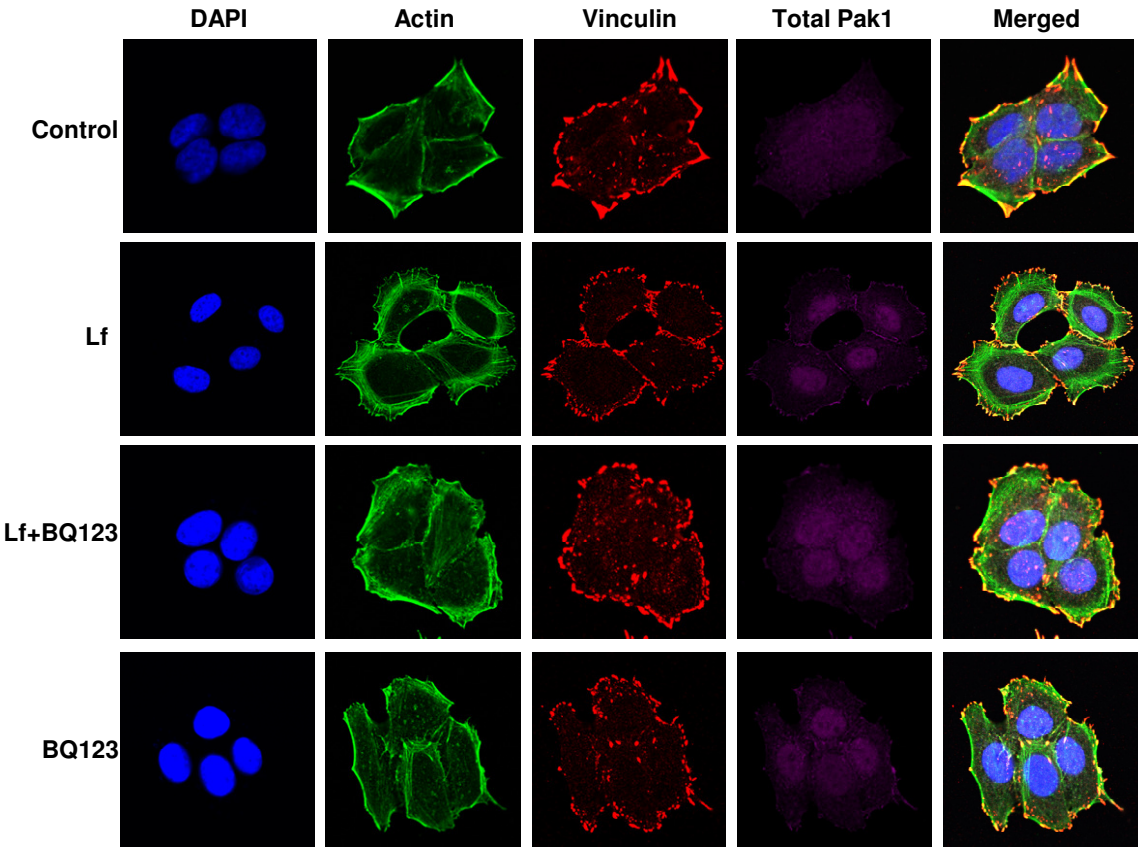


Figure 36. Lactoferrin effect on cytoskeleten remodelling
ET-1 inhibits lactoferrin-dependent induction of membrane ruffling in MCF-7 cells as measured by confocal microscopy.

Chapter 6: Discussion

6.1 Lactoferrin's Contribution to Relative Aggressiveness in Breast Cancer Cells

The findings that we have presented here demonstrate for the first time that lactoferrin, a ubiquitous secretory protein, can downregulate all three therapeutic-targetable receptors ERα, PR and HER-2 in breast cancer cells. We were able to demonstrate the downregulation of these three receptors by treatment of MCF-7 as well as ZR-75 cells with exogenous recombinant iron-saturated lactoferrin. Using western blotting as well as immunocytochemistry with confocal microscopy analyses, we confirmed the downregulation of the receptors at the protein level. To investigate the possible mechanisms of receptor downregulation by lactoferrin we experimentally showed that the regulation is conceivably due to proteasomal degradation. Upon treatment with the proteasomal inhibitor MG132, the lactoferrin-dependent downregulation of ERα, PR and HER-2 was abrogated. Therefore, these data strongly implicate lactoferrin in the downregulation of these therapeutic-targetable receptors leading to a triple negative breast cancer (TNBC) phenotype.

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 or any other related mechanism. In this context, it is noteworthy to mention that an earlier correlative report dated back to the 1990s has shown an inverse correlation with the level of ERα and lactoferrin in malignant mammary tissue (Campbell et al.,

1992). This study evaluated human breast cancer samples and observed a significant inverse correlation between the expression of ERα and lactoferrin. Furthermore, using immunohistochemistry, several other studies have observed a differential expression of lactoferrin in normal versus tumorigenic breast as well as endometrial tissues. Walmer et al. (Walmer et al., 1995) observed an inverse correlation between the expression of lactoferrin and PR in endometrial adenocarcinoma tissue samples. These studies therefore indicate a potential inverse expression of lactoferrin and steroid receptors such as ERα and PR. However, these data were not able to implicate lactoferrin to be a biomarker for neoplastic transformation or tumor progression, because expression was also found in normal samples. Even though these findings were interesting and provided potential leads to follow, no studies since then have been forthcoming to provide a scientific and mechanistic explanation for this phenomenon. Our study therefore is the first one to report a possible mechanism for lactoferrin-dependent downregulation of ERα, PR and HER-2. The results we obtained correlate well with these earlier studies and consequently we can hypothesize that the overexpression of lactoferrin is involved in the downregulation of ERα, PR and HER-2.

Since TNBC is a more aggressive subtype of breast cancer in comparison to other types, we wanted to investigate if lactoferrin could possibly contribute to an increase in invasion and migration, both of which are biological endpoints of tumor progression. As demonstrated, treatment of exogenous lactoferrin in non-TNBC as

well as TNBC cell lines stimulated an increase in cell proliferation, migration and invasion, indicating that not only does lactoferrin downregulate ER, PR and HER-2 but also causes a more aggressive phenotype. Interestingly, our finding that lactoferrin 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 observed aggressiveness of breast cancer cells appears not to be strictly associated with lactoferrin-dependent receptor downregulation. Contrary to our initial hypothesis, the observed increase in migration as well as invasion was independent of lactoferrin-dependent degradation of receptors.

Furthermore, it was interesting to observe that the downregulation of these receptors was not specific to breast cancer cells. We were able to demonstrate the same phenomenon in human epithelial colon cancer cells as well. These data could indicate that lactoferrin has similar effects on ERα and HER-2 regardless of tissue type. This finding is not unexpected, because we showed that lactoferrin downregulation of these receptors occurred in a proteasomal-dependent manner. If lactoferrin signaling indeed induces ER and HER-2 to undergo proteasomal degradation, tissue origin is of less importance since lactoferrin is specific to these receptors.

In addition, we showed that the downregulation of each receptor was not dependent on the expression of the other receptors. Using TNBC cell lines that were stably transfected with HER-2 and therefore only expressed this receptor, we were able to demonstrate downregulation of HER-2 upon lactoferrin treatment

without the co-expression of ERα or PR. Similarly, when we exogenously expressed ERα in a TNBC cell line, lactoferrin treatment downregulated the receptor in spite of the absence of HER-2 or PR. This indicates that the downregulation of each receptor occurs through a mechanism(s) that is independent of the expression of other receptors.

Lactoferrin is a secreted protein and is therefore found highly expressed in fluids such as breast milk and circulating plasma. Regulation of lactoferrin expression is transcriptionally dependent on signals such as estrogen or prolactin (Green and Pastewka, 1978; Teng, 2002; Nakajima et al., 2008), however it can also be secreted from neutrophils during inflammatory processes (Van Snick et al., 1974). Since breast cancer has inflammatory components during tumor progression, an increase in lactoferrin could be due to neutrophil secretion during inflammation. Additionally, it has been shown that epithelial cells could also express lactoferrin (Mulder and Morris, 2010) and therefore it is possible that lactoferrin could be produced by the tumor cells themselves (Luzzi et al., 2001). When analyzing human plasma samples we observed an increased expression of lactoferrin in tumor specimens as well as circulating levels of lactoferrin in plasma from the TNBC patients as compared to ER-positive patients. While the exact mechanism(s) responsible for the elevated levels of lactoferrin in TNBC versus ER-positive samples is not known, we hypothesize that this phenomenon contributes and/or supports the TNBC phenotype. Because the observations were made using clinical samples, we cannot identify the exact source or mechanism by

which lactoferrin was upregulated. However since lactoferrin was highly expressed in the more aggressive breast cancer subtype, we can hypothesize that it may play a role in tumor progression. Interestingly, while our studies were in progress, Schulz et al. (Schulz et al., 2009) reported an increased expression of lactoferrin in TNBC patient samples. Using mass spectrometry to look at protein expression differences in TNBC versus HER-2-positive breast cancers, they identified lactoferrin to be more highly expressed in TNBC patient samples. Therefore, this study correlates well with our findings here and could implicate lactoferrin as being involved in contributing to a more aggressive phenotype. Taken together, the data presented here give a plausible explanation for the high expression of lactoferrin found in the TNBC samples in comparison to their less aggressive counterpart.

In our study, we treated the breast cancer cells with lactoferrin that was isolated from human milk and therefore we hypothesize that lactoferrin that is produced within the mammary gland is needed for receptor downregulation. While we did not test any other source of lactoferrin in our experiments, the rationale of milk lactoferrin is plausible. The finding that human milk lactoferrin could possibly be involved in the downregulation of ERα, PR and HER-2 is particularly interesting: it has been shown that most breast cancers observed during or after pregnancy have the tendency to be triple-negative mammary carcinomas. Furthermore, pregnancy-related breast cancers also tend to be more aggressive in nature for reasons currently unknown. Lactoferrin expression is highly regulated by hormones such as estrogen and prolactin and therefore it is increased during

pregnancy and lactation. While it has been shown that both pregnancy and lactation can be protective against mammary tumorigenesis (Russo and Russo, 1996), other studies have also demonstrated that pregnancy-related breast cancers have worse prognosis and survival rates (Lyons et al., 2011). Furthermore, there are studies indicating that tumorigenesis or cancer progression could occur upon cessation of lactation, during so-called mammary gland involution (Schedin et al., 2004). Lactoferrin has been shown to be highly expressed during this remodeling process (Hurley, 1989) and therefore it is plausible that it is involved in breast cancer progression. Based on the results we have obtained here, we hypothesize that lactoferrin could have a role in downregulating therapeutic-targetable receptors as well as increasing aggressiveness of breast cancer cells. Furthermore, we hypothesize that such downregulation accompanied by increased aggressiveness could occur during pregnancy-related mammary tumors since lactoferrin is highly expressed during gestation. Additionally, since we only show the downregulation and aggressiveness in cancer cells, we hypothesize that lactoferrin has these effects in tumorigenic cells and not normal cells. This could explain the various beneficial functions that lactoferrin exhibits, such as anti-bacterial and anti-viral actions (Farnaud and Evans, 2003; Mulder and Morris, 2010). Lactoferrin has been shown to play a role in maintaining the host defense mechanism and therefore, it is possible that lactoferrin has adverse effects in a tumor microenvironment and not

during normal physiological conditions. While these rationales are theoretical in nature, it would be of interest to further examine such possibilities.

To investigate whether lactoferrin had lasting effects, we were able to show that the downregulation of these receptors could be sustained using increasing concentrations as well as multiple treatments of lactoferrin. Based on the initial findings that replenishing the cell culture media with repeated treatments of lactoferrin induced persistent receptor downregulation, we set out to examine long-term effects of lactoferrin. In order to investigate the long-term effects of lactoferrin on receptor downregulation as well as its contribution to tumor aggressiveness, we established a doxycycline-dependent inducible lactoferrin stable cell line using MCF-7 cells. With this cell line, we wanted to examine if an overexpression of endogenous lactoferrin could sustain the downregulation we observed with exogenous lactoferrin treatment. Furthermore, we wanted to study whether an upregulation of lactoferrin could induce the observed aggressiveness. While one of the stable clones showed downregulation of ERα and HER-2, we were not able to see an increase in migration and cell proliferation upon doxycycline treatment. These results could be due to a number of reasons: lactoferrin function has been shown to be possibly dependent on degree of saturation as iron binding has been demonstrated to induce conformational changes in lactoferrin structure (Byrd and Horwitz, 1991). The lactoferrin-induced increased migration upon exogenous treatment was observed with iron-saturated lactoferrin. Because the cells were treated with lactoferrin that was more than 90% saturated with iron, we

hypothesize that lactoferrin-dependent aggressiveness depends on the holo-form of lactoferrin. The stable clones that we have established were shown to overexpress lactoferrin, as measured by an increase in myc-levels (i.e., lactoferrin tagged with myc epitope); however since this upregulation of lactoferrin is endogenous using cell culture, the level of iron saturation could be low to none. If we hypothesize that iron-saturation is necessary for lactoferrin function, then the lack of iron could explain the negative data we observed. Furthermore, the lactoferrin plasmid we used for stably transfecting the breast cancer cells, included a tag for myc-protein. There is the possibility that tagging lactoferrin could interfere with the conformational structure of this secreted protein and therefore inhibiting it from optimal function. Since we showed that lactoferrin-dependent increase in invasion and migration was due to the upregulation of ET-1, we could hypothesize that myc-tagging may induce structural changes that would inhibit lactoferrin from optimally binding to the ET-1 promoter region. Given that these plausible explanations have yet to be empirically proven at this juncture, future experiments will be needed to shed light on the nature of our negative data.

With regards to therapy, we were able to demonstrate that lactoferrin-dependent downregulation of ERα and HER-2 led to a desensitization of breast cancer cells to 4-hydroxytamoxifen and Herceptin. This observation is very interesting as it points to the possibility that lactoferrin overexpression could lead to insensitivity to targeted therapies commonly used in breast cancer treatment. By downregulating ERα and HER-2, lactoferrin indirectly contributed to decreased

effectiveness of 4-hydroxytamoxifen as well as Herceptin. These are relevant and germane findings as lactoferrin levels in patients could potentially serve as a marker for predicting the success of targeted therapy. It is not known from our preliminary data if overexpression of lactoferrin could induce permanent downregulation of ERα and HER-2 and therefore there is a possibility that the observed downregulation of these targetable receptors is transient in nature. If this possibility was to hold true it would translate to potential cyclic or short-term downregulation of ER and HER-2 in patients with lactoferrin overexpression and therefore treatment with tamoxifen and Herceptin would only be partially effective leading to less successful therapeutic outcome. Consequently, if levels of lactoferrin could be correlated with receptor expression, it would be of importance to assess lactoferrin levels before tamoxifen or Herceptin treatment. Taken together, these data on lactoferrin-dependent decreased sensitivity to tamoxifen and Herceptin could provide remarkable implications for breast cancer treatment.

Altogether, the findings presented here indicate that an overexpression of lactoferrin can downregulate ERα, PR and HER-2 leading to a decrease in sensitivity to targeted therapies, such as tamoxifen and Herceptin. Furthermore, we showed that lactoferrin treatment also increased migratory and invasive properties of breast cancer cells. These data give a plausible explanation and potential mechanism for earlier observed data of higher expression of lactoferrin in more aggressive cancers as well as an inverse correlation of this protein with ERα and PR. While the exact mechanism of how lactoferrin contributes to this remains

unclear, we hypothesize that an increase in lactoferrin possibly resulting from pregnancy or lactation, could promote a more aggressive breast cancer phenotype. Since lactoferrin is hormonally regulated, and therefore is upregulated upon a surge of estrogen and other signals, it seems highly plausible that lactoferrin plays a role during pregnancy-related breast cancers which tend to be TNBC and therefore more aggressive in nature.

6.2 Lactoferrin-Endothelin-1 Axis

While exploring the molecular basis of lactoferrin-induced migration and invasion of breast cancer, we set out to identify lactoferrin-regulated genes using a DNA microarray approach. Since we observed that an increase in migration as well as invasion was independent of lactoferrin-induced downregulation of ERα, PR and HER-2, we wanted to identify genes that could be involved in contributing to such aggressiveness. Furthermore, an interesting earlier study has reported on lactoferrin's ability to regulate transcription by binding to DNA sequences (He and Furmanski, 1995). Based on this and the results obtained earlier, we aimed to investigate lactoferrin-regulated genes that could explain the observed increase in migration and invasion.

The results from the microarray gene expression analysis of both breast cancer cell lines led to the identification of three lactoferrin-regulated genes (*Rgs2, Tbcp-1 and Et-1*) from which we chose one to study further, namely ET-1. We decided to examine ET-1 as a direct target of lactoferrin because this gene/protein has been

previously shown to promote breast cancer progression, and increased ET-1 was demonstrated to participate in the process of invasion and metastasis. Additionally, earlier studies have reported that the levels of ET-1 correlate well with a diminished disease-free and overall survival (Wulfing et al., 2003). Furthermore, 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 (Pedram et al., 1997; Salani et al., 2000; Carducci et al., 2003).

Based on this prior knowledge about ET-1, we set out to investigate its role in lactoferrin-induced aggressiveness. First, to confirm the DNA microarray analysis, we were able to identify the bona fide binding sites for lactoferrin and showed physical interaction of lactoferrin to the ET-1 promoter using EMSA. Furthermore, to show that such interaction was of physiological importance, we demonstrated that lactoferrin was also able to transcriptionally upregulate the expression of ET-1 using the luciferase-reporter assay. The discovery that lactoferrin can bind to the promoter region of ET-1 and therefore transcriptionally upregulate its expression is particularly exciting as it gives an insight into the possible mechanism of lactoferrin-induced aggressiveness. Even though lactoferrin has been shown to possibly act as a transcription factor by binding to DNA, only a few studies have looked at lactoferrin's potential targets. One particular study by Oh et al. (Oh et al., 2001) demonstrated lactoferrin-dependent upregulation of MMP-1; however, the investigators did not directly implicate such upregulation with cancer progression. In our study, not only did we show upregulation of ET-1 upon lactoferrin treatment,

we were also able to demonstrate that the overexpression of ET-1 led to an increase in invasion as well as migration. To further examine the lactoferrin-ET-1 axis in breast cancer progression, we showed that the observed aggressiveness was abrogated using a specific inhibitor of ET(A)R, the receptor to which ET-1 primarily binds. Therefore, to our knowledge, this is the first study to show a lactoferrin-regulated gene with a direct role in tumor progression.

Furthermore, it was interesting to note that lactoferrin binding to the *endothelin-1* promoter depended on holo-lactoferrin, the iron-saturated form of this protein. As discussed earlier, lactoferrin function has been shown to be dependent on iron saturation as such binding results in conformational changes in lactoferrin structure. Treatment with apo-lactoferrin revealed no protein-DNA complex in the EMSA assay indicating that the binding and subsequent transcriptional activation of ET-1 relies on iron saturation of lactoferrin. 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 of ERα, PR and HER-2 as well as increased invasiveness in breast cancer cells. This is particularly interesting because a study by Duarte et al. (Duarte et al., 2011) 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 is likely to be due to the upregulation of endothelin-1 upon iron-saturated lactoferrin treatment.

In addition to the biochemical data, we demonstrated the lactoferrin-ET-1 axis to possibly be involved in human breast cancer samples as well. The relevance of these mechanistic studies to the physiologic setting is supported by the noted co-overexpression of lactoferrin 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 ET-1 is a secreted protein, it is plausible to observe higher levels of ET-1 in these serum samples. Interestingly, the expression of both lactoferrin as well as ET-1 was observed to be higher in TNBC samples. In agreement with our findings, other studies have also shown that an increase in ET-1 is correlated with more aggressive tumors (Grant et al., 2003). Since we observed co-overexpression of lactoferrin and ET-1 in human breast cancer serum as well as cancer specimens from patients, it can be hypothesized that the lactoferrin-ET-1 axis could play a role in the observed tumor aggressiveness. Since these observations were made in clinical samples only, we cannot definitively show that the increase of ET-1 was due to a high expression of lactoferrin. Therefore, these correlative data can only be considered as proof of principle for the biochemical studies that were obtained earlier. Nonetheless, it was interesting to observe an increased expression of both proteins of interest in the

more aggressive TNBC samples in comparison to their less invasive receptor-positive counterparts.

Lastly, the finding that lactoferrin-dependent increase in ET-1 was able to stimulate Pak1 kinase activity is particularly interesting. While an overexpression of Pak1 as well as an increase in its activity has already been shown to play a role in tumor progression, we were able to identify a protein downstream of ET(A)R that could explain the observed increase in migration. While it has already been demonstrated that ET-1 can activate Pak1 kinase activity in endothelial cells, no studies thus far have implicated such activation in cancer progression. Based on the known functions of Pak1, implicating it to be involved in cell migration is an important aspect for understanding the mechanism of lactoferrin-dependent migration. Albeit we only demonstrated an increase in membrane ruffling upon lactoferrin treatment, this change in the cytoskeleton is usually indicative of motile cells. Interestingly, pre-treatment of the cells with BQ123, the ET(A)R antagonist, reduced lactoferrin-dependent membrane ruffling indicating the involvement of ET(A)R signaling in this process. Furthermore, since we hypothesize lactoferrin-dependent stimulation of Pak1, the delayed increase in this activation (3-6 hours) could possibly indicate the need for endothelin-1 transcription. Taken together, establishing this lactoferrin-ET-1-Pak1 axis gives a better insight into the possible mechanism through which lactoferrin contributes to a more aggressive breast cancer phenotype.

6.3 Conclusion

In conclusion, the data presented here in this dissertation have elucidated a new function for lactoferrin and its possible involvement in breast cancer progression. We demonstrated that lactoferrin treatment of breast cancer cells resulted in a downregulation of ERα, PR and HER-2 leading to the development of a TNBC-like phenotype. Furthermore, we showed that lactoferrin was able to induce cell proliferation, migration and invasion independent of receptor downregulation. To account for lactoferrin-dependent aggressiveness, we identified ET-1 as a transcriptional target for lactoferrin. By binding to the ET-1 promoter and therefore transcriptionally upregulating ET-1 mRNA expression, we demonstrate the lactoferrin-ET-1 axis to be involved in the increased migration as well as invasion of breast cancer cells. Using a pharmacological inhibitor to ET(A)R, the primary receptor for ET-1, we were able to abrograte lactoferrin-dependent invasion as well as migration. Furthermore, while studying the downstream effects of the ET-1 upregulation, we discovered Pak1 to be a potential protein that is involved in the observed migration. Lactoferrin treatment of breast cancer cells resulted in an increase of Pak1 kinase activity leading to membrane ruffling that is indicative of motile cells. Furthermore, we were able to inhibit lactoferrin-dependent stimulation of Pak1 kinase activity via the ET(A)R antagonist indicating that the increase in activity is ET-1-ET(A)R dependent.

In conclusion, the results presented in this study are based on an *in vitro* model to understand the involvement of lactoferrin and ET-1 in breast cancer progression

and how these molecules can contribute to the relative aggressiveness of mammary carcinoma. The data could serve as surrogate markers for relative aggressiveness, such as invasion and migration, however future studies are needed to ascertain their relevance in metastasis. As expected, like many other new discoveries, the present study has not only provided several significant leads to follow, 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.

6.4 Implication and future studies

The data presented in this dissertation have elucidated a new mechanism and function for lactoferrin, a protein previously shown to be involved in iron sequestration and immune system function.

In future studies, it would be interesting to further investigate the exact mechanisms of lactoferrin-dependent downregulation of ERα, PR and HER-2. Since we showed proteasomal-dependent downregulation of the receptors, it would be of interest to identify the proteins involved in this process. For example, estrogen (E2)-dependent proteasomal degradation of ERα has been elucidated in several studies (Nawaz et al., 1999; Lonard et al., 2000) and others have revealed potential mechanisms during the absence of receptor-specific ligands (Tateishi et al., 2004). These studies include hypoxia-dependent degradation of ERα (Stoner et al., 2002). Additionally, HER-2 receptor downregulation has been demonstrated

to occur via inhibiting heat shock factor 90 and therefore inducing the proteasomal degradation of the receptor (Lerdrup et al., 2006). Understanding the exact mechanism of how these receptors are downregulated by lactoferrin is important as it could provide leads for the development of targeted drugs against possible proteasomes involved in the downregulation. If lactoferrin-induced downregulation of ER, PR and HER-2 could be reversed in breast cancer, targeted therapies such as tamoxifen and Herceptin would be more effective.

Furthermore, since the ET-1 pathway is being actively targeted via the development of specific pharmacological inhibitors to its receptor, and FDA-approved ET-1 receptor antagonists are moving forward in clinical trials (Lalich et al., 2007), such 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 the therapeutic effectiveness of ET-1 receptor antagonist to block lactoferrin-induced motility and invasiveness of the TNBC cells, and thus, opening a remarkable opportunity to treat TNBC by targeting the lactoferrin-ET-1 axis using an approved developmental drug (i.e. repurposing of FDA-approved drugs).

Finally, future work is needed for a complete elucidation of the significance of Pak1 pathway by lactoferrin in breast cancer cells and biologic significance of such a regulatory circuit.

Altogether, the data presented here provide many potentials leads to be followed. Understanding the complete mechanism as well as the role of the

lactoferrin-ET-1-Pak1 axis would be important because it would allow for the development of potential targets.

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