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Introduction

NGAL project was initiated based on the genome-wide transcript analysis using RNA samples of 318 breast cancer patients diagnosed in MD Anderson Cancer Center. We found that the transcript of NGAL from the tumor mass correlates with advanced tumor stage, metastatic status and other poor clinical indexes. NGAL (neutrophil gelatinase–associated lipocalin) is a glycosylated secreted protein and it also can be detected at high concentration in the plasma of various types of cancer patients including breast cancer patients.

We tested the function of NGAL in breast tumorigenesis using a spontaneous mammary tumor mouse model. Our data on LCN2 function in promoting mouse mammary tumor in vivo was published in Cancer Research 2009 Nov 15;69(22):8579-84 (See Appendix I), which confirmed our hypothesis that NGAL is a driving factor for mammary tumor formation and metastasis. Our results are largely supported the later data from other labs. As requested by Journal of Cell physiology, we reviewed the progress in NGAL studies in breast cancer (J Cell Physiol. 2011 Feb 226(2):309-14) (See Appendix II).

The goals of this DOD funding are to: 1) develop methods to block NGAL function for producing a clinically applicable drug; and 2) dissect the molecular mechanisms of how NGAL promote breast tumorigenesis. We have designed high throughput screen methods to identify small inhibitory chemical compounds blocking the interaction of NGAL and matrix metalloproteinase 9 (MMP-9) or blocking NGAL entry into the cells. We constructed luciferase-expressing human breast tumor cell lines for future testing the NGAL inhibitors in vivo. For the second goal, we found that mouse mammary tumor lacking NGAL expression retained CK-18 (a good prognostic marker) expression. Reducing mouse NGAL expression greatly attenuated the invasive potential of 4T1 cells. High-molecular weight structures containing gelatinase activities are present in the plasma of mice expressing mouse NGAL, suggesting its role in stabilizing/enhancing gelatinase activity and in facilitating tumor cell migration.

Body

Background: Examining the role of NGAL in mammary tumor formation using mouse models

In light of its significance in the patient samples, we focused on analyzing the roles of NGAL in mammary tumorigenesis using mouse or human mammary tumor cell lines, in mouse xenograft tumor models, or in mouse models that generate spontaneous mammary tumors in collaboration with Dr. Yun Wu. The conclusions from these studies suggest that elevated NGAL expression promotes the invasive potential of breast tumor cells and mammary tumor formation. In detail, we found that HER2-positive human breast cancer cell line SKBr3 expresses high levels of NGAL. Knocking down NGAL expression by shRNA in SKBr3 reduced the migration and invasion ability of the cells. Using the xenograft mouse model, we detected a more significant numbers of invasive events in the tumors arising from MDA-MB-468 cells that contain high endogenous NGAL expression compared to the MDA-MB-468 cells with lowered NGAL levels (1). On the other hand, over-expression of NGAL in MCF-7 greatly increased the cell motility (data not shown, see Appendix I).
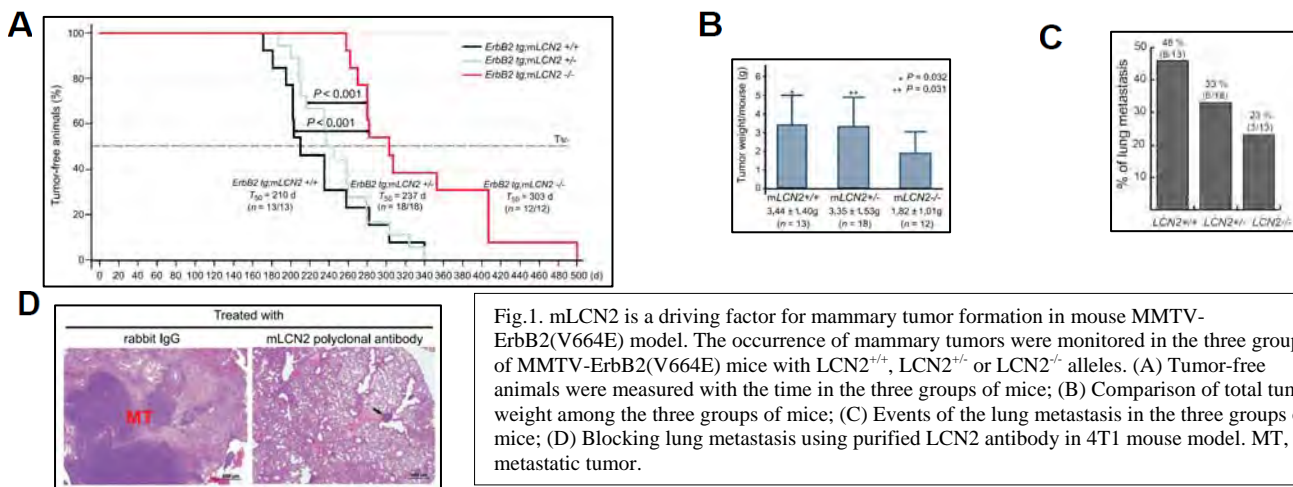
Fig.1. mLCN2 is a driving factor for mammary tumor formation in mouse MMTV-ErbB2(V664E) model. The occurrence of mammary tumors were monitored in the three groups of MMTV-ErbB2(V664E) mice with LCN2$^{+/+}$, LCN2$^{+/-}$ or LCN2$^{-/-}$ alleles. (A) Tumor-free animals were measured with the time in the three groups of mice; (B) Comparison of total tumor weight among the three groups of mice; (C) Events of the lung metastasis in the three groups of mice; (D) Blocking lung metastasis using purified LCN2 antibody in 4T1 mouse model. MT, metastatic tumor.

We used an *in vivo* spontaneous mammary tumor mouse model to establish that NGAL is critical factor that drives breast tumorigenesis. The mouse homologue of NGAL is called mLCN2 (mouse Lipocalin 2). We examined the kinetics of the mammary tumor formation and the involvement of lung metastasis in MMTV-ErbB2 (V664E) transgenic mice in LCN2$^{+/+}$, LCN2$^{+/-}$ or LCN2$^{-/-}$ backgrounds (1). We found that the initiation timing of the mammary tumor in mice lacking LCN2 expression is greatly prolonged (T_{50}=303days) compared to the mice with either one copy of LCN2 allele (T_{50}=237days) or two copies of LCN2 alleles (T_{50}=210days) (Fig.1A). Moreover, tumor burden measured by the weight and the number of the tumors per mouse are significantly less in the LCN2$^{-/-}$ mice group compared to the other two groups (Fig.1B). We also found a significant reduction in the number of mice in the LCN2$^{-/-}$ group showing lung metastasis (Fig.1C).
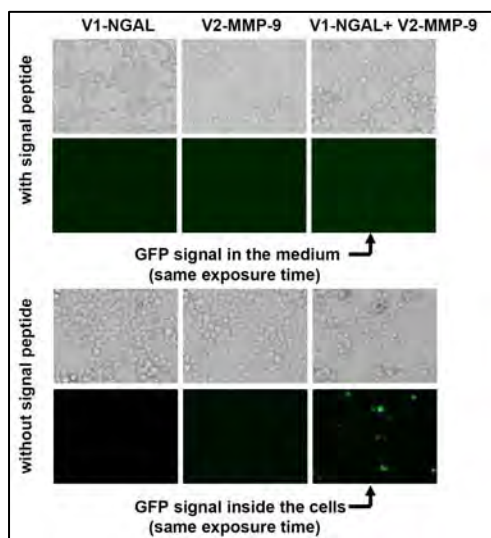
The mouse mammary tumor cell line 4T1 has been used in several studies to examine the role of LCN2 in mammary tumor formation. The 4T1 cell line is an aggressive cell line isolated by Dr. Miller in a series of mouse mammary tumor cell lines that are syngenic to Balb/C mice (2). We found that increased endogenous LCN2 expression correlates with the aggressiveness in this series of the cell lines. Lowering the expression of LCN2 in the 4T1 cells by shRNA inhibits its invasive ability (1). The high endogenous expression of LCN2 in the original 4T1 cells greatly promotes its ability for cell migration and invasion (3). Moreover, mice implanted with 4T1 cells ectopically expressing LCN2 generates a significant more number of lung metastatic nodules compared to that implanted with the unmodified 4T1 cells (3). Consistent with these results, we found that the lung metastasis causing by 4T1 cells can

be blocked by injection of a polyclonal antibody against LCN2 (1), suggesting that strategies to inhibit LCN2 function could block breast cancer metastasis (Fig.1D).

Aim1: To identify small molecules that block NGAL function and evaluate the effects of these new NGAL inhibitors in mouse breast cancer models (Yr 1-3)

1.1 High throughput screen for inhibitory molecules that block NGAL/MMP-9 interaction and cellular internalization of NGAL

We used BiFC system (Bimolecular Fluorescence Complementation), in which two known binding proteins are expressed as fusion proteins with either N- or C-part of GFP. When binding occurs inside the cells, GFP parts complement each other and form active flurophore to emit green signals thereby setting up a high throughput screen for small molecules that block NGAL function. In addition to the original proposal of identification of the inhibitors that disrupt the NGAL/MMP-9 complex formation, we added another strategy to block NGAL function by screening for the inhibitors that block NGAL entry into the targeted cells. For screening the inhibitors that disrupt the NGAL/MMP-9 complex, we have constructed expression vectors that expression NGAL and MMP-9 with two different parts of GFP (Venus system, Dr. Wichnick lab), either with signal peptide (secrete out of the cells) or without signal peptide (stay inside of the cells). When co-transfected into 293T cells, we observed an increased GFP signal in the medium in cells co-expressed NGAL and MMP-9 with signal peptide and GFP-positive cells when co-transfected NGAL and MMP-9 without signal peptide (Fig.2).

Fig. 2. Testing the NGAL/MMP-9 complex formation in Venus system. NGAL and MMP-9 was expressed in 293T cells with different parts of GFP molecule. The GFP signal is observed when there is a complex formation of NGAL and MMP-9 either in the medium (with signal peptide) or inside the cells (without signal peptide).
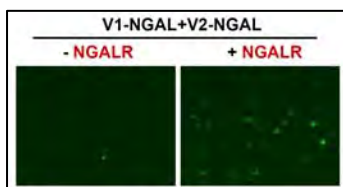

Fig. 3. Internalization of NGAL by NGAL receptor (NGALR). 293T cells were co-expressed V1-NGAL and V2-NGAL, with or without NGAL receptor. GFP signal emits when the imported NGAL form homodimer inside the cells.
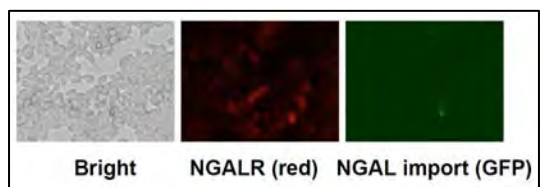

Fig.4. Establishing a stable 293T cell line expressing NGALR that intakes NGAL. 293T cells expressing NGALR (sorted with co-expression marker RFP) was incubated with conditioned medium containing both NGAL-V1 and NGAL-V2. GFP signal emits when the imported NGAL form homodimer inside the cells.

The receptor for NGAL was identified by Devireddy et al. (4) and they found that the insensitivity of Bcr-Abl leukemia cells to mouse homologue of NGAL is due to the lack of receptor for NGAL (4). In addition, they conclude that the apoptosis effect caused by mouse NGAL is due to its import to the targeted cells through its receptor for depletion of the intracellular iron source (4). Therefore, blocking NGAL import by disrupting its binding to its receptor should greatly reduce the apoptosis effect in the normal tissues caused by the increased NGAL level from the tumor cells. For this purpose, we have 1) tested whether NGAL receptor (NGALR) is a bona fide NGAL receptor in an artificial 293T system (Fig.3); 2) established a stable 293T cell line expressing the NGALR that will be used for the inhibitor screen. We constructed 293T cell with co-expression of RFP as a sorting marker (Fig.4). The idea is to set-up a high throughput screen, in which the 293T-NGALR cells incubated with the conditioned medium containing both NGAL-V1 and NGAL-V2 (NGAL can form homodimer and

therefore emit GFP signals when imported inside the cells, see Fig.9), and for individual drug compounds that inhibit NGAL entry into the cells, the GFP intensity level inside the cells will be decreased and be used as a measurement for screening the inhibitors that block LCN2 import (Fig.5).

We collaborated with Dr. William Bornmann, an organic chemist who is in charge of the Translational Chemistry Core Facility (TCCF) in our institution for large-scale screen. Dr. Bornmann agreed to send us a small library for screen purpose and gave the suggestion to obtain a larger pool of compounds using GCC libraries (Gulf Coast Consortium). The schematic outline of our screen is illustrated in Fig. 5. To increase the chance for a successful screen, we are collaborating with TCCF stuff members to utilize the structural information on NGAL to bias the selection of compounds for screening. Models of the binding site in NGAL will be created based on the interactions with carboxymycobactin along with information obtained from modeling software that can identify key interactions in the binding site. We have finished the settings of the screen and we are in the process of performing a large-scale screen for NGAL inhibitors.
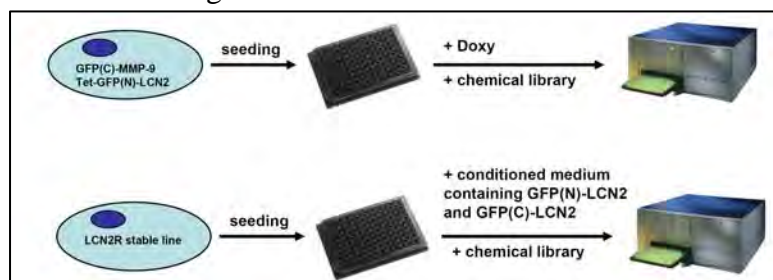

Fig.5. Schematic outline of the screen of the inhibitory molecule against NGAL. (top) represents the scheme for molecules that disrupt NGAL/MMP-9 interaction; (bottom) is for molecules that block NGAL entry inside the cells. The GFP signal will be read using BioTek Synergy HT Multidetection Microplate Reader.

1.2 Testing NGAL inhibitors using mouse model.
We constructed luciferase-expressing human breast tumor cell lines (MDA-231) for future testing of NGAL inhibitors in vivo.

Aim2: To examine the molecular mechanism underlying NGAL function in breast tumorigenesis

1.1 NGAL in regulating tumor differentiation
By analyzing the mammary tumors from the three groups of MMTV-ErbB2(V664E) mice with mLCN2$^{+/+}$, mLCN2$^{+/-}$ or mLCN2$^{-/-}$ alleles, we found a difference in CK18 expression. CK18 is a good prognostic marker in breast cancer. A higher CK18 expression means a more differentiated and a lower grade tumor. We found that CK18 expression only retained in the mammary tumors lacking of mLCN2 expression, suggesting that higher levels of NGAL can lead to a much higher aggressive tumor type (Fig.6).
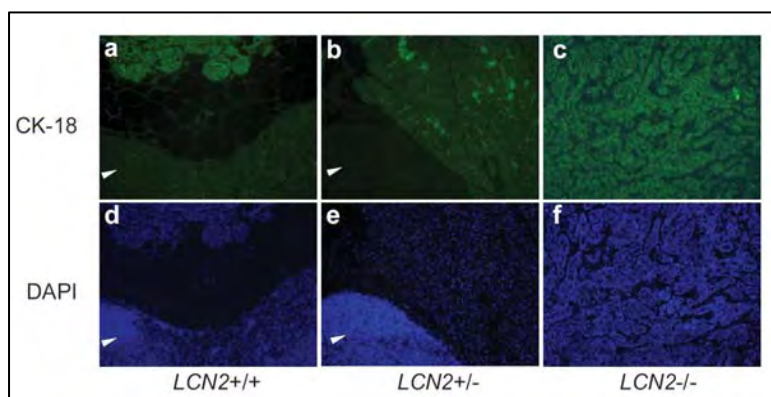

Fig.6. CK18 expression is only retained in the tumor sample lacking LCN2 expression. Immunohistochemistry using FITC-CK18 antibody was performed using tumor samples from the three groups of MMTV-ErbB2(V664E) mice with LCN2$^{+/+}$, LCN2$^{+/-}$ or LCN2$^{-/-}$ alleles. White arrow indicates the highly proliferated tumors that lost CK18 expression in LCN2$^{+/+}$ and LCN2$^{+/-}$ background.

1.2 NGAL in regulating MMP-9 activity

Many studies have documented the role of MMPs, in particular, MMP-9 in tumorigenesis and metastasis (5). Studies have suggested that the binding of NGAL to MMP-9 can slightly accelerate MMP-9 activation (6) or can block its autodegradation (7). We tested this using the NGAL homologue of mouse LCN2 ermed 24p3. We found that knocking down LCN2 level in 4T1 cells decreased MMP-9/LCN2 complex formation and correspondingly lowered its invasion ability (Fig.7). We found that LCN2 level is very high in the plasma of the mammary tumor-bearing mice expressing LCN2. Furthermore, we found that the gelatinase activities are enhanced in the blood from the tumor-bearing MMTV-ErbB (V664E) mice with LCN2 expression compared to that from the group of mice without LCN2 expression (1) (Fig.8, right). Similar observations were reported using the MMTV-PyMT tumor-bearing mice with different numbers of LCN2 alleles (8). These data suggest a possible role of LCN2 in enhancing gelatinase activity, which can facilitate the tumor growth by promoting its invasion of neighboring tissues or metastasis to distal sites.
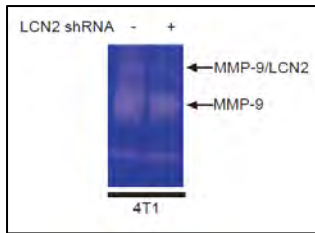

Fig.7. Knocking-down LCN2 in 4T1 diminishes MMP-9/LCN2 complex gelatinase activity and invasion ability.
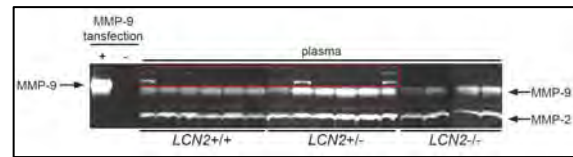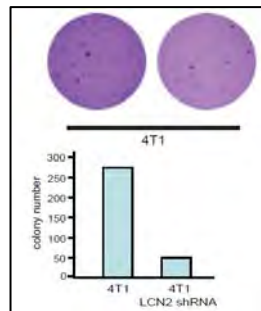

Fig.8. LCN2 level and gelatinase activity in the plasma of the three groups of MMTV-ErbB2(V664E) mice with LCN2$^{+/+}$, LCN2$^{+/-}$ or LCN2$^{-/-}$ alleles.

Conclusions:

Our studies conclude that NGAL is a key factor that drives the mammary tumorigenesis (1, 9). Elevated NGAL indicates poor survival in human breast cancers. To translate these findings into clinical settings, we further designed and constructed a fluorescence-based high throughput screening method to block NGAL function with a goal of developing a small chemical inhibitor for breast cancer therapy. Understanding the underlying mechanisms of the action of NGAL holds the key for targeting NGAL for future clinical application. Our current results indicate that the presence of NGAL greatly influences the tumor differentiation, grade and metastasis. We will further study the molecular mechanisms of NGAL to gain better understanding at the genome level of how NGAL affects the host-tumor microenvironment for breast tumor growth and metastasis.

Key Research Accomplishments

- Published a research paper regarding the role of NGAL in promoting mammary tumorigenesis (Leng et al., Can.Res. Nov 2009)(1);
- Finished the establishment of two high throughput screen method for NGAL inhibitors, one for blocking the complex formation of NGAL and MMP-9 and one for blocking NGAL entry in the targeted cells;
- Published an invited review on the role of NGAL in mammary tumorigenesis (Leng et al., J of Cell. Physiol. 2011 Feb;226(2):309-14)(9).

Bibliography of Publications

- Inhibition of lipocalin 2 impairs breast tumorigenesis and metastasis.
  Leng X, Ding T, Lin H, Wang Y, Hu L, Hu J, Feig B, Zhang W, Pusztai L, Symmans WF, Wu Y, Arlinghaus RB.
  Cancer Res. 2009 Nov 15;69(22):8579-84.PMID: 19887608
- Relationships of lipocalin 2 with breast tumorigenesis and metastasis.
  Leng X, Wu Y, Arlinghaus RB.
  J Cell Physiol. 2011 Feb;226(2):309-14. PMID: 20857428

Meeting Attendance and Abstract

AACR 101st Annual Meeting
April 17-21, 2010
Washington, DC.

6th Era of Hope Conference
August 2-5, 2011
Orlando, Florida.

BC076078-3256
THE FUNCTION OF NGAL IN MAMMARY GLAND TUMORIGENESIS AND ITS CLINICAL IMPLICATIONS
Xiaohong H. Leng and Ralph Arlinghaus
NGAL (also called LCN2) is a secreted protein whose level is found to be elevated in leukemia and breast cancer patients. In breast cancer, its high expression is associated with negative ER/PR status, HER2 positivity, high tumor grade, lymph node metastasis, and decreased disease-free survival. Despite these observations, its precise role in both types of cancers is not known. To address these questions, we carried out genetic experiments to delineate the role of NGAL in mammary carcinoma mouse models. We found that the timing of mammary tumor formation in MMTV-ErbB2 (V664E) transgenic mice lacking mLCN2 expression is greatly prolonged (T50 = 303 days) as compared to similar mice with either one copy or two copies of mLCN2 alleles (T50 = 210 days or 237 days, respectively). Moreover, tumor burden measured by the weight and number of the tumors per mouse are significantly less in the mLCN2-/- mice group than the other two groups. We also found a significant reduction in the number of mice in the mLCN2-/- group showing lung metastasis. These data suggest that mLCN2 serves as a promoting factor in mammary tumor formation. Importantly, we found that the occurrence of lung metastasis caused by implanted mouse mammary tumor 4T1 cells can be greatly inhibited by injecting a polyclonal antibody against mLCN2 [1], suggesting the future application of an inhibitory antibody or small molecules against NGAL for breast cancer therapy. One possible mechanism involved in LCN2-driven mammary tumorigenesis is through its association and stabilization of MMP-9. Therefore, we set up a MMP-9/NGAL-binding assay based on BiFC (bi-molecule fluorescent complementation measuring GFP formation) for screening small molecules that can disrupt this interaction. In this assay, we observed the formation of the heterodimer of NGAL and MMP-9, the homodimer of NGAL, and the homodimer of MMP-9. We are using a chemical library to screen for the small molecules that interfere with the binding of NGAL and MMP-9 as manifested by decreasing the GFP signals. Furthermore, we are investigating the mechanisms of the role of NGAL in promoting breast tumorigenesis by measuring changes in the epithelial-mesenchymal transition markers and the NGAL receptor in normal- and breast cancer-patient samples.

Salaried Personnel
Dr. Ralph B. Arlinghaus
Dr. Xiaohong Leng

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9. Leng X, Wu Y, Arlinghaus RB. Relationships of lipocalin 2 with breast tumorigenesis and metastasis. J Cell Physiol.

Appendices

(I) <u>Inhibition of lipocalin 2 impairs breast tumorigenesis and metastasis.</u>
    Leng X, Ding T, Lin H, Wang Y, Hu L, Hu J, Feig B, Zhang W, Pusztai L, Symmans WF, Wu Y, Arlinghaus RB.
    Cancer Res. 2009 Nov 15;69(22):8579-84. Epub 2009 Nov 3.PMID: 19887608

(II) <u>Relationships of lipocalin 2 with breast tumorigenesis and metastasis.</u>
    Leng X, Wu Y, Arlinghaus RB.
    J Cell Physiol. 2011 Feb;226(2):309-14)(9). PMID: 20857428