

AD_____

AWARD NUMBER: W81XWH-08-1-0597

TITLE: Interfering Breast Cancer Metastases by Blocking NGAL Function

PRINCIPAL INVESTIGATOR: Ralph B. Arlinghaus, Ph.D.

CONTRACTING ORGANIZATION: University of Texas MD Anderson Cancer Center
Houston, TX 77025

REPORT DATE: September 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 1 September 2009		2. REPORT TYPE Annual		3. DATES COVERED 1 Sep 2008 – 31 Aug 2009	
4. TITLE AND SUBTITLE Interfering Breast Cancer Metastases by Blocking NGAL Function				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-08-1-0597	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Ralph B. Arlinghaus, Ph.D. E-Mail: rarlingh@mdanderson.org				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Texas MD Anderson Cancer Center Houston, TX 77025				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The first aim of this proposal is to identify small molecules that block NGAL function, a key molecule that our preliminary data suggested to be important in breast cancer and metastases. A high throughput screen was proposed to address this aim and currently we have constructed the essential reagents. The second goal is to examine the molecular mechanisms underlying NGAL function in breast tumorigenesis. In this regard, we have finished the mouse experiment depicting the role of NGAL in mammary tumor formation and the paper is in press in Cancer Research. In this publication, we found that ablation of mouse homologue of NGAL (mLCN2) significantly delayed the formation of mammary tumors in MMTV-ErbB2 (V664E) transgenic mice. We observed a much reduced tumor weight and delayed lung metastases in mLCN2 knockout background. Importantly, an affinity purified antibody against mLCN2 largely blocks the lung metastasis in the established 4T1 induced mammary tumors, suggesting the possibility of using a small molecule to inhibit NGAL function for breast cancer treatment.					
15. SUBJECT TERMS NGAL, NMP-9, breast cancer, metastasis, small molecule inhibitors					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 22	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	Page
Introduction -----	4
Key research accomplishments -----	5
Reportable outcomes -----	6
Conclusions -----	16
References -----	16
Bibliography -----	18
List of Personnel -----	19
Appendices (Acceptance letter for publication in Cancer Research) -----	20

Introduction

The first aim of this proposal is to identify small molecules that block NGAL function, a key molecule that our preliminary data suggested to be important in breast cancer and metastases. A high throughput screen was proposed to address this aim and currently we have constructed the essential reagents. The second goal is to examine the molecular mechanisms underlying NGAL function in breast tumorigenesis. In this regard, we have finished the mouse experiment depicting the role of NGAL in mammary tumor formation and the paper is in press in Cancer Research. In this publication, we found that ablation of mouse homologue of NGAL (mLCN2) significantly delayed the formation of mammary tumors in MMTV-ErbB2 (V664E) transgenic mice. We observed a much reduced tumor weight and delayed lung metastases in mLCN2 knockout background. Importantly, an affinity purified antibody against mLCN2 largely blocks the lung metastasis in the established 4T1 induced mammary tumors, suggesting the possibility of using a small molecule to inhibit NGAL function for breast cancer treatment.

Key research accomplishments

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).

We propose to use BiFC system (Bimolecular Fluorescence Complementation) to set up the high-throughput screen for small molecules that block NGAL function by disrupting the NGAL/MMP-9 complex formation. We have obtained the Venus system (similar to the original BiFC system but with much easier cloning sites) from Dr. Wichnick lab in University of Montreal. We have inserted NGAL into all four vectors in the system and tested its homodimerization in 293T cells (Figure 1). We also have constructed human MMP-9 in these vectors and the testing for the NGAL/MMP-9 complex formation is underway. As the Venus system is a transient transfection system, we have obtained Tet-regulated lentiviral vector (pTRIPz) from OPEN Biosystem. Once the formation of NGAL/MMP-9 complex is confirmed in the transient transfection experiment, we will clone the corresponding fragments into pTRIPz vector followed by virus production and establishment of stable cell lines for screening usage.

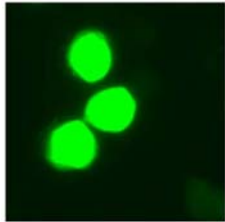


Figure 1: Testing the Venus system. (left) Co-transfection of NGAL(-SP)-Venus(1) and NGAL(-SP)-Venus (2) into 293T cells; homodimerized NGAL complexes resulted GFP signal. No GFP signals were detected in cells transfected with single plasmid alone (data not shown). (right) Untransfected 293T cells with the same exposure time.

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

Two essential tasks have been done in the first funding period. One is the preliminary data presented in the proposal have been finalized and the paper has been accepted to be published in Cancer Research (See Appendix for the paper).

Second, in the original proposal, we propose to dissect the gene expression profile using microarray to analyze the primary mammary tumor samples derived from the three mice groups: LCN2(+/+), LCN2 (-/+) and LCN2 (-/-). We mentioned that these samples were derived from two genetic backgrounds (C57/B6 and FVB/NJ) and the possible confusions might exist in our conclusions due to genetic heterogeneity. With this concern in mind, we have backcrossed the LCN2(-/-) mice (C57B) seven-times with FVB/NJ wild type mice and obtained mLCN2 knock-out mice in FVB/NJ background. We have already crossed these mice with MMTV-ErbB2 mice. The idea is to do microarray analysis on the tumors solely containing the FVB background to identify the changes in gene signatures that depend on LCN2 expression. Although this new approach will cause a bit delay, we think that it is definitely worth the waiting as the data shall be much more conclusive compared the original approach.

Reportable outcomes (Taken from in press paper in Cancer Research)

Inhibition of Lipocalin 2 Impairs Breast Tumorigenesis and Metastasis

Xiaohong Leng^{1,6}, Tian Ding^{1,6}, Hui Lin¹, Yan Wang¹, Limei Hu², Jianhua Hu³, Barry Feig⁴, Wei Zhang², Lajos Pusztai⁵, W Fraser Symmans², Yun Wu^{2,7}, and Ralph B. Arlinghaus^{1,7}

¹ Department of Molecular Pathology; ² Department of Pathology; ³ Department of Biostatistics; ⁴ Department of Surgical Oncology; ⁵ Department of Breast Medical Oncology. The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030

⁶ These authors contributed equally to this work

⁷ Co-corresponding authors

Keywords: lipocalin 2, NGAL, breast cancer, metastasis

Contact: Ralph B. Arlinghaus, Ph.D.
Department of Molecular Pathology
University of Texas M. D. Anderson Cancer Center
7435 Fannin St., Unit 951
Houston, Texas 77054
Email: rarlingh@mdanderson.org
Phone: (713) 792-8995
Fax: (713) 834-6082

Yun Wu, M.D., Ph.D
Department of Pathology
University of Texas M. D. Anderson Cancer Center
1515 Holcombe Blvd., Unit 085
Houston, TX 77030
Email: YunWu@mdanderson.org
Phone: (713) 563-1847
Fax: (713) 563-1848

Running title: Lipocalin 2 enhances breast cancer formation and metastasis

ABSTRACT

Lipocalin 2 (LCN2, also known as NGAL) is a secreted glycoprotein and its elevated expression has been observed in breast cancers. However, the importance of LCN2 in breast tumorigenesis is unclear. Here, we employed a spontaneous mammary tumor mouse model showing that MMTV-*ErbB2*(V664E) mice lacking of mLCN2 had significantly delayed mammary tumor formation and metastasis with reduced MMP-9 activity in the blood. LCN2 expression is up-regulated by HER2/PI-3 kinase/AKT/NF- κ B pathway. Decreasing LCN2 expression significantly reduced the invasion and migration ability of HER2⁺ breast cancer cells. Furthermore, injecting an anti-mLCN2 antibody into mice bearing established murine breast tumors resulted in significant blockage of lung metastasis. Our findings indicate that LCN2 is a critical factor in enhancing breast tumor formation and progression, possibly in part by stabilizing MMP-9. Our results suggest that inhibition of LCN2 function by an inhibitory

monoclonal antibody has potential for breast cancer therapy, in particular by interfering with metastasis in aggressive types of breast cancer.

INTRODUCTION

Recent studies have implicated LCN2, a member of the lipocalin family, in leukemia (1-3) and other solid tumors (4). The lipocalin family is a large group of small secreted glycoproteins involved in binding and transportation of small lipophilic molecules (5). The physiological ligand for LCN2 is not known. Upon bacterial infection, LCN2 tightly binds to bacterial iron-siderophores and deprives the iron source for their growth (6), which is shown by the delayed clearance of bacteria infection in LCN2 knock-out mice (7, 8).

It was observed that increased LCN2 levels were present in the plasma of leukemia patients with CML (1-3). We found that mouse LCN2 is required for BCR-ABL-induced leukemia in a mouse bone marrow transplant model and BCR-ABL⁺ mouse leukemic cells utilize the secreted LCN2 to reduce hematopoietic cells in normal bone marrow and spleen by inducing apoptosis of normal hematopoietic cells for leukemia cell expansion (1, 3). The requirement of LCN2 for leukemia cell expansion is consistent with the previously identified role of LCN2 in inducing apoptosis of mouse hematopoietic cells upon withdrawal of the cytokine growth factor IL-3 (9). The ability of LCN2 to mediate apoptosis appears to be due to its ability to export a still unknown iron-form from the target cells (10). Importantly, we found that LCN2 is also essential for solid tumor formation, as BCR-ABL-expressing leukemia cells lacking LCN2 expression failed to form subcutaneous allograft tumor nodules (3).

In breast cancer, a high molecular weight of complex consisting of LCN2 and MMP-9 was observed in the patient urine samples (11). Elevated LCN2 expression was shown to associate with decreased survival in breast cancer patients (12). Increased LCN2 expression was found to associate with increased tumor cell motility (13, 14). LCN2 was shown to stabilize MMP-9, a critical enzymatic activity required for extracellular matrix remodeling during tumor growth and metastasis (15, 16) and LCN2 may promote breast cancer progression by regulating epithelial to mesenchymal transition (14). However, the significance and how LCN2 is involved in breast carcinogenesis remain elusive.

We tested the role of LCN2 in breast tumorigenesis using a spontaneous MMTV-*ErbB2*(V664E) transgenic mammary tumor mouse model crossed with LCN2^{-/-} mice. Our findings presented here illustrate the importance of LCN2 in breast cancer tumor formation and metastasis and the possible underlying molecular mechanisms promoted by LCN2 expression in breast cancer, indicating that inhibition of LCN2 has potential in breast cancer therapy.

Materials and Methods

Tissue culture and mice

Cell lines were grown in DMEM with 10% FBS and Pen/Strep at 37 °C in humidified incubator with 5% CO₂. LCN2 knockout (LCN2^{-/-}) mice were a gift from Dr. Alan Aderem (8). MMTV-*ErbB2*(V664E) transgenic mice (17) and athymic nude mice (8-12 wks) were purchased from The Jackson Laboratory and Harlan, respectively. Mice experiments were performed in MDACC animal facility with approved IACUC protocols.

Antibodies and constructs

Purified polyclonal antibodies against mouse or human LCN2 were generated in our lab using recombinant proteins purified from *E. coli*, a gift from Dr. Roland Strong (6). Antibodies against

AKT, pAKT, HER2, pY-HER2, and β -actin were purchased from Abcam. Dominant negative and constitutive active AKT constructs are gifts from Dr. Zhou Songyang.

shRNA experiments and lentiviral infection

shRNA lentiviral constructs (Sigma-Aldrich) containing shRNAs for *NGAL* (clone D: TRCN0000060290 and clone E: TRCN0000060290) and *mLCN2* (TRCN0000055328) were used. Puromycin was used to select cells containing shRNA after lentiviral infection.

RT-PCR

Total RNA extracted from cells was used to synthesize the cDNA as PCR template. RT-PCR primers for *mLCN2*: 5'-AGC CAG ACT TCC GGA GCG ATC-3'; and 5'-ACT TGG CAA AGC GGG TGA AAC G-3'; for mouse *actin*: 5'-GCT GGA AGG TGG ACA GTG AG-3' and 5'-ATG GAT GAC GAT ATC GCT GC-3'.

Two-chamber migration and invasion assays

Cell migration and invasion assays were performed using the two-chamber migration assay (8 μ m pore size, BD Biosciences). For SKBr3 cells, 1×10^5 cells were seeded in serum-free medium in the upper chamber and migrated toward 10% FCS in the lower chamber for 18 hrs followed by fixation and staining with crystal violet 0.2%/methanol 20%. Quantification was performed using unpaired *t*-test.

Western blotting and zymogram of plasma samples

1-2 μ l of plasma was separated on 20% SDS-PAGE followed by transferring and blotting with purified anti-mLCN2 polyclonal antibody (1:3000). 2-5 μ l of plasma were analyzed using 10% zymographic gel (Invitrogen).

Imaging, histology, H&E staining and immunohistochemistry

Freshly collected lung tissues were imaged using XENGEN IVIS 200 Imaging System for GFP signals. For histological analysis, tissues were post-fixed with 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μ m, and stained with H&E.

Statistical data analysis

The Kaplan-Meier method (the long-rank test) was used to determine the survival curves of tumor formation in MMTV-*ErbB2*(V664E) mice with various alleles of *mLCN2*. The log-rank test was used in the intergroup comparisons. The Wilcoxon rank-sum test was used to analyze the fluorescence imaging results of the in vivo antibody studies.

RESULTS

Up-regulation of LCN2 (NGAL) through HER2/neu signaling pathway in human breast cancer. We observed a significant clinical correlation of higher LCN2 expression with HER2 positivity among 318 newly diagnosed breast cancer patients (unpublished), implying that LCN2 might be a downstream target of the HER2 signaling pathway. When blocking HER2 function with Herceptin in HER2⁺ SKBr3 cells, we found that LCN2 expression was inhibited (Figure 1A). LCN2 expression in SKBr3 cells was reduced in a dose-dependent manner by the PI-3K inhibitor LY294002 and by Bay 11-7082, which specifically blocks I κ B phosphorylation needed for NF- κ B activity (Figure 1B-C). Secretion of LCN2 into conditioned medium was also effectively inhibited under these conditions (Figure S1). In addition, overexpression of dominant negative AKT led to reduced expression of LCN2, while overexpression of constitutive active AKT increased LCN2 levels (Figure 1D). These results indicate a role of the HER2/PI-3K/AKT/NF- κ B signal cascade in inducing LCN2 expression in human breast cancer, consistent with a recent study showing that LCN2 expression was largely dependent on the NF- κ B pathway in thyroid neoplastic cells (18).

Mice lacking mLCN2 have impaired ErbB2(V664E)-induced mammary tumor formation.

We tested mouse LCN2 (mLCN2) effects in a spontaneous mouse mammary tumor model. Transgenic mice carrying the mutant form of *ErbB2*(V664E) driven by the mammary specific promoter MMTV develop multiple primary breast tumors and lung metastases (17). By using MMTV-*ErbB2*(V664E)^{tg/tg} mice (FVB) and *mLCN2*^{-/-} mice (C57B/6) (8), we generated three groups of mice expressing ErbB2(V664E) with variations in mLCN2 alleles (*mLCN2*^{+/+}, *mLCN2*^{+/-}, and *mLCN2*^{-/-}) (Figure S2A-C).

In this genetic study for the effects of mLCN2 on *ErbB2*(V664E)-induced breast tumors, we observed striking differences in the timing of tumor formation, as well as the number and the size of primary tumors among the three groups. Groups carrying either one or two alleles of mLCN2 started to develop multiple large (>1 cm) mammary tumors around 170 days after birth. In contrast, *mLCN2*^{-/-} mice did not form similar size of tumors until approximately 260 days, a time when >60% mice in the groups expressing mLCN2 were already terminated due to excessive tumor burden (Figure 2A). The overall tumor occurrence was significantly delayed in the *mLCN2*^{-/-} group (260 – 500 days with T₅₀ = 303 days) compared to the *mLCN2*^{+/+} mice (170-340 days with T₅₀ = 210 days) (Figure 2A). Although the *mLCN2*^{+/-} group showed a slightly delayed course of tumor occurrence compared to the *mLCN2*^{+/+} group, no significance was observed regarding the tumor occurrence and the tumor volume between these two groups (Figure 2A-B), indicating that one allele mLCN2 deficiency was not sufficient to interfere with the formation of ErbB2(V664E)-induced breast tumors. We observed greater numbers and larger volumes of tumors per mouse in the groups expressing mLCN2 compared to the *mLCN2*^{-/-} group (Figure 2B and Figure S2D). Notably, the lung metastases in *mLCN2*^{-/-} mice were significantly delayed compared to the *mLCN2*^{+/+} mice ($p < 0.05$) (Figure 2C). By Kaplan-Meier analysis, the T₅₀ for lung metastasis in the *mLCN2*^{+/+} group is approximately 260 days. In contrast, the T₅₀ value was not reached in the *mLCN2*^{+/-} and the *mLCN2*^{-/-} groups, suggesting that deficient mLCN2 expression also impairs lung metastasis in this model.

Unlike the healthy WT mice with minimal levels of mLCN2 in the plasma (Figure 2D, top panel, last three lanes), we detected a dramatically increased mLCN2 level in the plasma of tumor-bearing MMTV-*ErbB2*(V664E) mice (Figure 2D, top panel). Interestingly, we observed elevated MMP-9 gelatinase activity and the presence of higher molecular weight gelatinase

(HMW MMP) activity in the plasma of tumor-bearing mice expressing mLCN2 compared to mLCN2^{-/-} group (Figure 2D, bottom panel, outlined with red box). We also noticed the relatively weak MMP-9 gelatinase activity in the plasma of tumor-bearing mLCN2^{-/-} mice (Figure 2D, bottom panel), and these samples displayed distinct gelatinase bands with MW lower than the MMP-9 band, suggesting the beneficial effects of mLCN2 in maintaining MMP-9 activity potentially though stabilizing MMP-9, as implied by the previous studies (15, 16).

LCN2 expression in HER2⁺ breast tumor cells stimulates cell invasion *in vitro* and their metastatic potential in mouse xenograft model. Using HER2⁺ SKBr3 cells (high LCN2 expression), we observed a significant reduction in cell migration and invasion upon knocking down LCN2 expression compared to either parental cells or cells expressing non-specific shRNA (Figure 3A-B). We used MDA-MB-468 (high LCN2 expression) to test LCN2 effects in mouse xenograft model. Note, we did not observe any change in cell growth rate in culture upon lowering LCN2 expression by shRNA in either SKBr3 or MDA-MB-468 cells (data not shown and Figure S3A). We injected mammary fat pads of nude mice with one million of either parental MDA-MB-468 cells, or its derivatives expressing either the non-targeted shRNA or the shRNA for LCN2. We analyzed the primary tumor and the surrounding tissues 42 days after implantation. No significant differences in the primary tumor size/weight were found among the three groups (Figure S3B). However, the tumor cells' capacity for invasion and metastasis, as measured by the events of lymphovascular invasion (Figure 3C, panel b), intramammary lymph node metastasis (Figure 3C, panel c) and chest/abdominal wall invasion (Figure 3C, panel d) were significantly reduced in the group injected of MDA-MB-468 cells with the LCN2 shRNA knockdown (Figure 3D).

mLCN2 expression correlates with aggressive tumor formation in murine mammary tumor cell lines. The association of LCN2 expression with aggressive human breast cancer types was further demonstrated when using a series of mouse breast tumor cell lines (67NR, 168FARN, 4T07, and 4T1), which were derived from the mammary tumors of the same mouse but with distinct metastatic potentials (19). 4T07 and 4T1 cells are the most aggressive and develop lung metastases. We found that only 4T07 and 4T1 cells have mLCN2 transcripts and secrete mLCN2, with the most aggressive 4T1 cells having the highest levels of mLCN2 (Figure 4A). Knocking down mLCN2 by shRNA in 4T1 cells (Figure S4A, left panel) reduced MMP-9 activity (Figure S4A, right panel). Reduction of mLCN2 expression greatly decreased 4T1 invasive ability (Figure S4B) and colony formation in soft agar (Figure S4C). Similar to our findings in the plasma from *ErbB2*-induced breast tumor-bearing mice, we detected high levels of mLCN2 in the plasma of breast tumor-bearing mice implanted with 4T1 cells and increased MMP-9 activity in the plasma compared to normal healthy mice (Figure S4D).

Intravenous administration of anti-mLCN2 antibody to mice with established mammary tumors reduces lung metastases. To explore the possibility that inhibition of the secreted LCN2 might block distant metastasis, we intravenously injected an affinity-purified rabbit polyclonal antibody against mLCN2 (anti-mLCN2) into nude mice seven days after implantation with 5000 GFP-labeled 4T1 cells, when visible breast tumors (~2 mm) were formed. Antibody injection (~100 µg) was done once per week for four times, with purified rabbit IgG as control. We observed a dramatic decrease in lung metastases in the anti-mLCN2 antibody treated group as compared to the control IgG-treated group, measured by GFP signal intensities in freshly

collected lung tissues ($p=0.028$) (Figure 4B-C and Figure S5A). Histological examination confirmed significant differences in lung metastases between the two groups (Figure 4D). We also found that the levels of circulating 4T1 tumor cells were reduced in the blood of anti-mLCN2 antibody-treated mice (Figure S5B), suggesting that the antibody was able to block the emergence of disseminating tumor cells from the primary tumor sites. Taken together, our results strongly suggest that LCN2 could serve as a new therapeutic target for treating breast cancer metastasis.

DISCUSSION

The results presented here provide strong pre-clinical and experimental evidence that LCN2 is a critical factor that facilitates breast tumorigenesis and metastasis, and it is a potential therapeutic target for aggressive forms of breast cancer. We were the first to use a spontaneous mouse breast cancer model to study the function of LCN2 in breast tumorigenesis. Mice that lack mLCN2 expression had a significant delay in the formation of breast tumors in the MMTV-*ErbB2*(V664E)^{tg} background, and when formed, these tumor displayed reduced tumor numbers and size compared to that from the mLCN2^{+/+} and mLCN2^{+/-} mice. The timing of lung metastases was correspondingly delayed in the mLCN2^{-/-} mice compared to the mLCN2^{+/+} mice. This result indicates that although breast tumor formation and metastasis may not absolutely require LCN2 expression, LCN2 plays an essential role in enhancing the aggressiveness of breast cancer formation and metastasis. Our studies using human breast cancer cell lines and murine mammary tumor cells indicate that LCN2 expression led to more aggressive behavior including lung metastasis, tumor cell migration and invasion, and anchorage independent growth *in vitro*.

How LCN2 is involved in solid tumor formation and progression is not fully understood. We observed an overall increased MMP-9 gelatinase activity, formation of higher molecular weight MMP complexes (20), and increased MMP-9 stability in tumor-bearing mice expressing LCN2. In contrast, in tumor-bearing mice with ablated LCN2 expression, an overall decrease in higher MW MMP activities and MMP-9 activity was observed, consistent with the previous findings of the protective role of LCN2 on MMP-9 (16). The importance of these higher MMP activities in tumorigenesis is not known. The effects of loss of LCN2 expression on these MMP-related events are not yet understood and need further investigation.

Most importantly, we provided evidence that i.v. injection of an affinity-purified antibody made against mLCN2 strongly interfered with lung metastasis in an aggressive mouse 4T1-induced mammary tumor model. The primary breast tumors were not prevented from continued growth by i.v. injection of the mLCN2 antibody, which may be due to the insufficient levels of the mLCN2 antibody in reaching an already-established tumor microenvironment. However, we did observe that anti-mLCN2 antibody-treated mice had slightly smaller primary tumors (10% reduction on average, data not shown). We found less 4T1 cells in the blood of anti-mLCN2 antibody-treated mice. Possible mechanisms include that the anti-mLCN2 destabilizes LCN2/MMP-9 complex, thereby reducing the exit of 4T1 cells from the primary tumor mass and decreasing distant tumor metastasis. More studies are needed to clarify the mechanism of how anti-LCN2 reduces metastases. Our findings suggest that blocking LCN2 in blood circulation using a neutralizing monoclonal antibody against LCN2 might be an effective therapeutic option to interfere with breast cancer metastasis either alone or in combination with current therapeutic approaches.

Figure

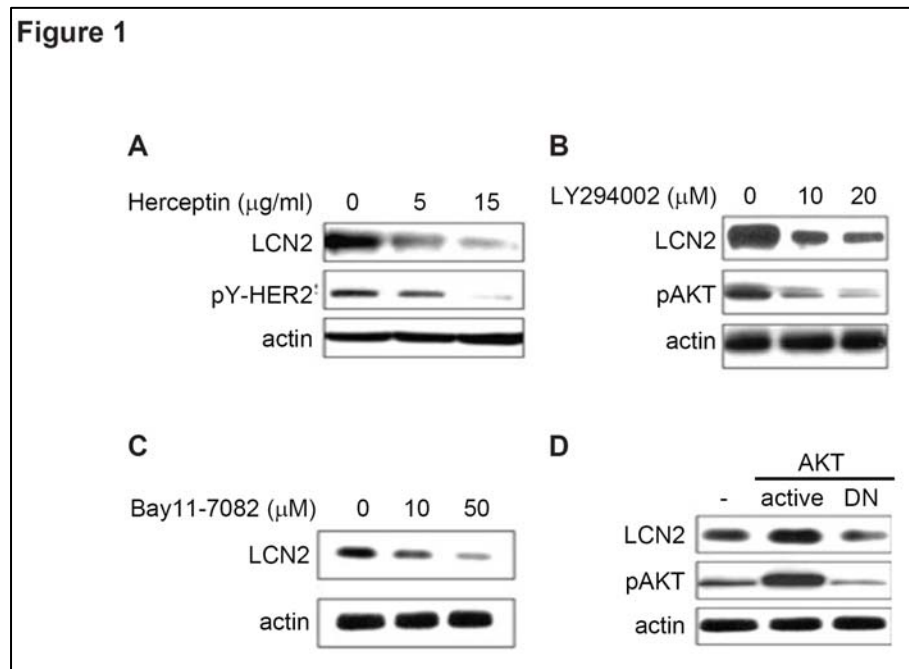


Figure 1. LCN2 (NGAL) is a down-stream target of HER2/PI-3K/AKT/NF- κ B pathway. *A*, Western blotting of LCN2 levels in HER2⁺ SKBr3 cells treated with Herceptin. *B*, Western blotting of LCN2 levels in SKBr3 cells incubated with the PI-3K inhibitor LY294002. *C*, Western blotting of LCN2 levels in SKBr3 cells incubated with the NF- κ B inhibitor Bay 11-7082. *D*, Western blotting of LCN2 levels in SKBr3 cells transiently expressing either constitutive active or dominant negative AKT.

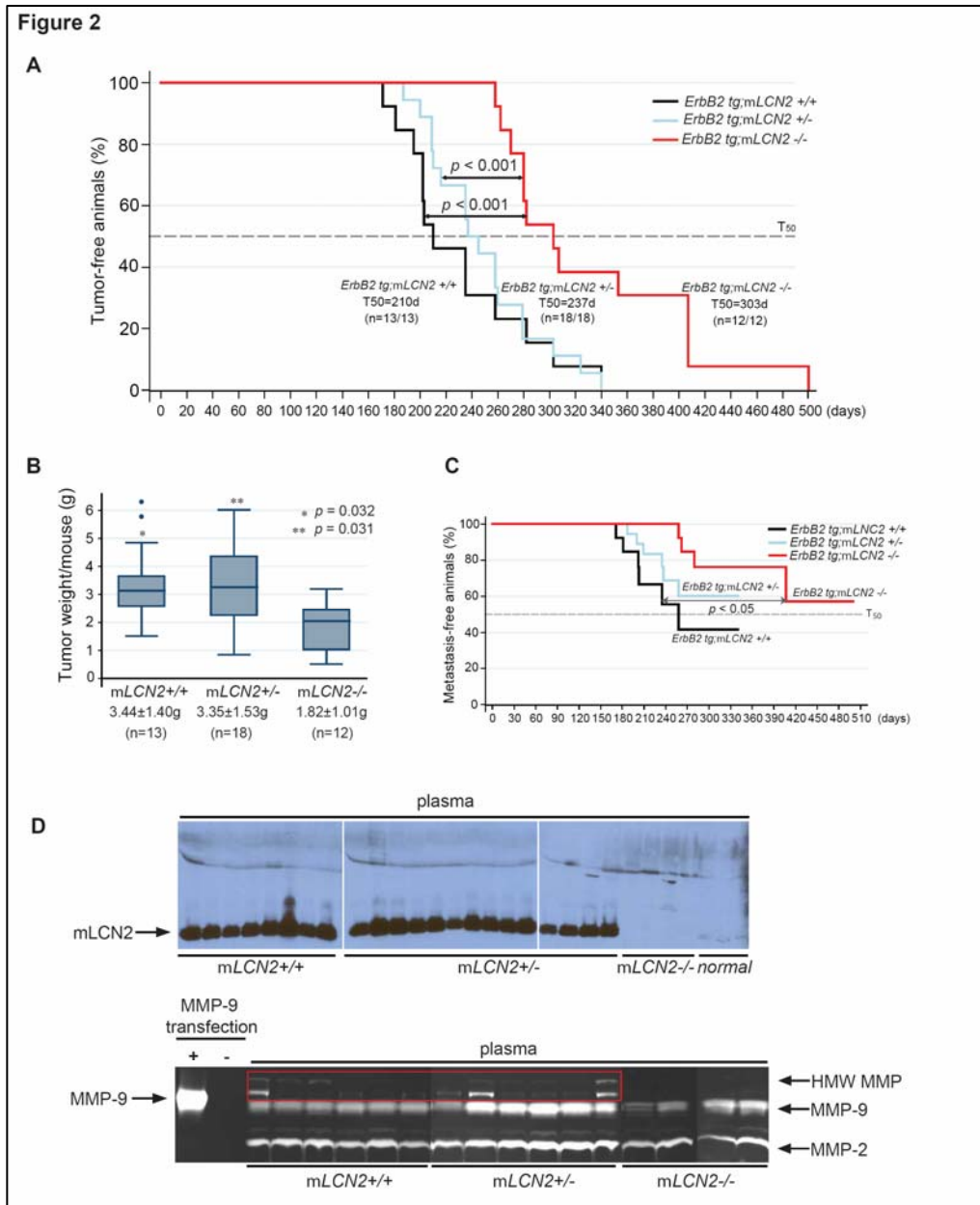


Figure 2. Effects of mLCN2 on the mammary tumor formation and metastasis in the MMTV-*ErbB2*(V664E) transgenic mouse model. **A**, time course of primary mammary tumor development in MMTV-*ErbB2*(V664E) transgenic mice in the three genetic backgrounds: *mLCN2*^{+/+}, *mLCN2*^{+/-}, and *mLCN2*^{-/-}, depicted by the Kaplan-Meier analysis. T_{50} is a calculated statistical value incorporating both time and incidence of tumor formation when 50% of the mice in the same group developed mammary tumors. **B**, summary of total breast tumor volumes per mouse in the three mice groups. **C**, Kaplan-Meier analysis of lung metastasis of the three mice groups. **D**, levels of mLCN2 (upper panel) in the plasma of the three mice groups at euthanization. mLCN2 level in the plasma from normal healthy mice was shown in the last three lanes. MMP activity in the plasma of the three mice groups were analyzed using 10% zymogram gel (lower panel). Conditioned medium from MMP-9-transfected 293T cells were used to mark MMP-9 position. Red box outlined the high molecular weight of MMP activity (HMW MMP).

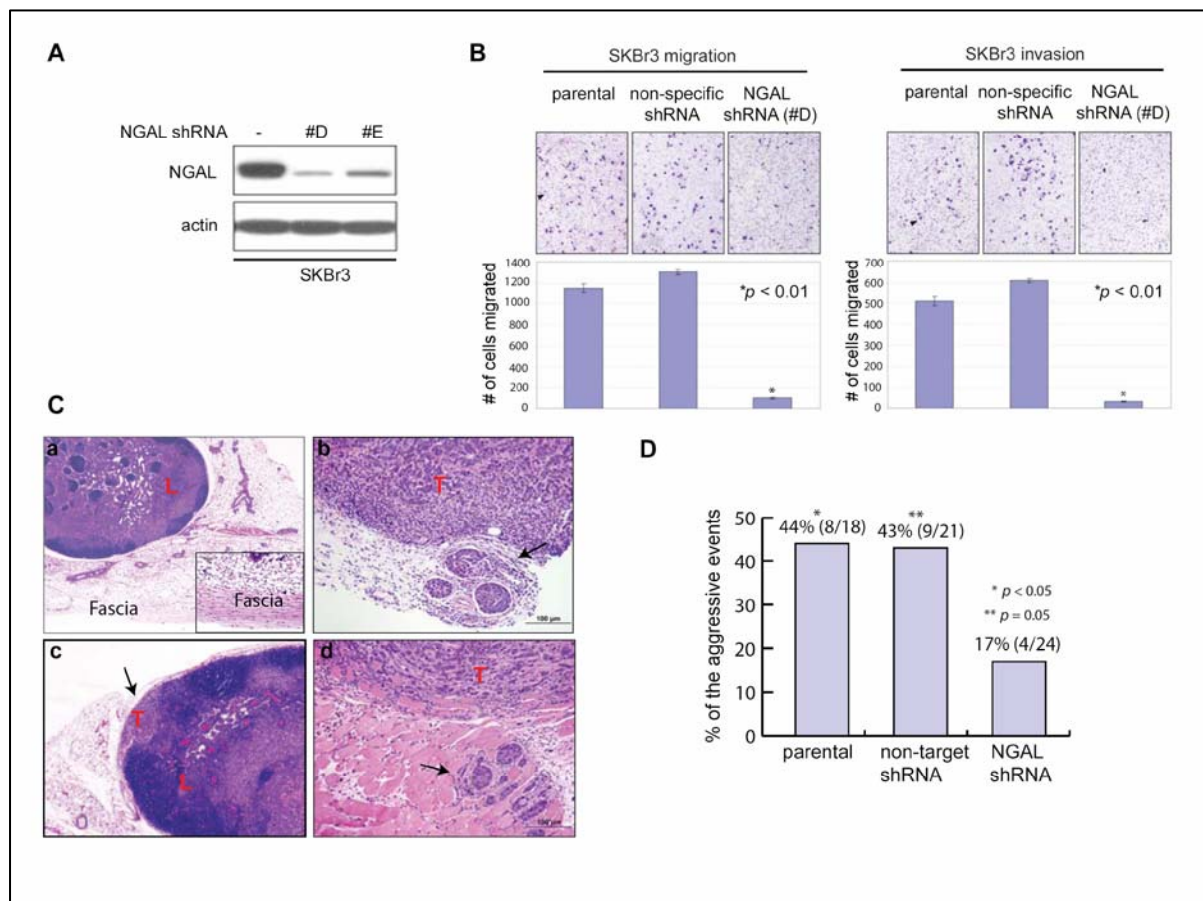


Figure 3. LCN2 (NGAL) expression stimulates cell invasion and metastasis of human breast cancer cells. **A**, Western blotting of NGAL levels in SKBr3 with shRNA targeting NGAL. **B**, cell migration (left panel) and invasion (right panel) assays of SKBr3 cells, SKBr3 cells expressing non-specific shRNA, or SKBr3 cells expressing NGAL shRNA. **C**, H&E staining of the 4th mammary gland from normal mouse (a) showing normal intramammary lymph node structure and fascia as a barrier to block tumor cells from invading into the chest/abdominal wall. Pathological features (black arrow) including lymphovascular invasion (b), intramammary lymph node metastasis (c) and chest/abdominal wall invasion (d) were used to measure the metastatic and locally aggressive events in the whole mount mammary fat pad in the experiment performed in (D). “L” represents lymph node; “T” represents tumor. **D**, summary of the lymph node metastasis and locally aggressive events observed in the mammary glands injected with MDA-MB-468 cells, or MDA-MB-468 cells expressing either non-targeted shRNA or NGAL shRNA. Statistical analysis was performed with Pearson Chi Square test.

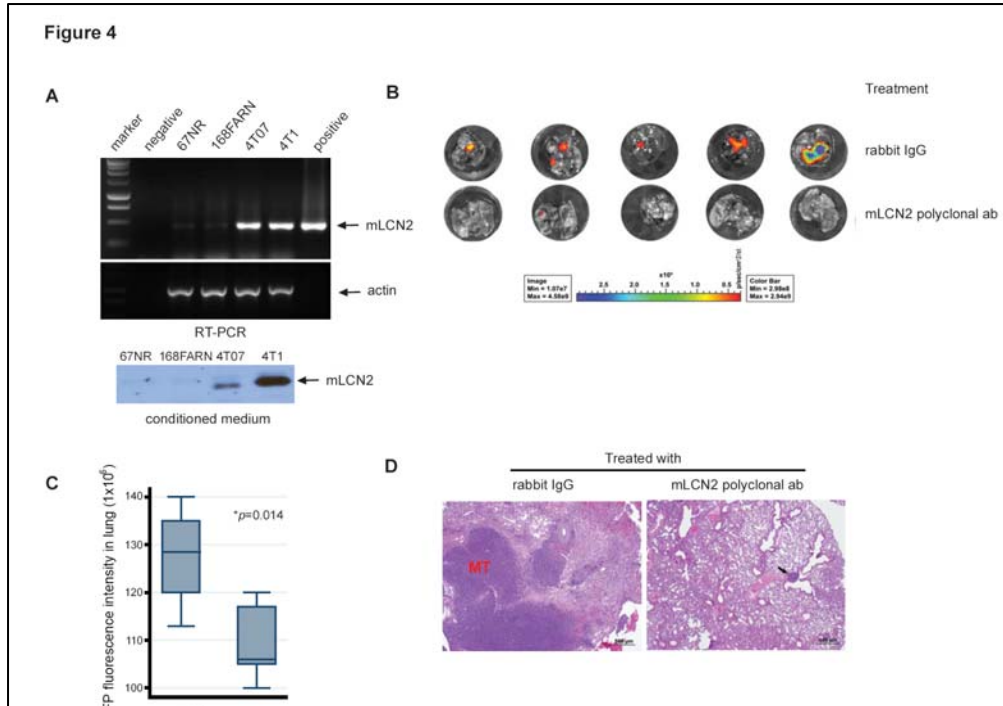


Figure 4. Reduction of lung metastasis by mLCN2 antibody injection in the 4T1 breast cancer model. **A**, expression of mLCN2 is associated with aggressive mouse mammary tumor cell lines. Upper panel: RT-PCR of mLCN2 transcripts in 67NR, 168FARN, 4T07 and 4T1 cells; actin as internal control and a plasmid containing mLCN2 as positive control. Lower panel: Western blotting of mLCN2 levels in the conditioned medium of the four cell lines. **B**, *in vivo* metastasis assay of 4T1 cells of mice injected with purified rabbit IgG (upper panel) or purified rabbit anti-mLCN2 polyclonal antibody (lower panel). Lung metastasis burden was monitored using Xenogen IVIS 200 Imaging System. The color scale depicts the GFP signal emitted from the metastatic 4T1 cells. **C**, comparison of total GFP signal intensity in either the control antibody-treated group or the anti-mLCN2 antibody-treated group. **D**, representative H&E staining of lung metastasis of 4T1 tumor bearing mice treated with purified rabbit IgG (left panel) or purified anti-mLCN2 antibody (right panel); “MT” represents metastatic tumor. Black arrow points to a small metastatic tumor lesion.

Acknowledgements

We are grateful to Dr. Alan Aderem for the *LCN2* KO mice, to Dr. Roland Strong for the recombinant LCN2 proteins and to Dr. Zhou Songyang for the AKT expression constructs. We thank Drs. Yibing Deng and Sendurai Mani for discussions and suggestions of the data in the manuscript. This research was supported in part by grants from the Susan G. Komen Foundation (KG081319 to Y.W. and R.B. A.), the Department of Defense (BC076078 to R.B.A.), and start up fund from UT MDACC to Y.W. R.B.A. is a holder of the Stringer Chair of Cancer Research.

Conclusions

Published studies and our unpublished clinical data indicate the association of elevated LCN2 expression with HER2-positive breast cancer patients, but its role in breast cancer is not known. Here, we used a mouse mammary tumor model to elucidate the role of LCN2 in breast tumorigenesis. We found that LCN2 knockout mice have a significant delay in mammary tumor formation and metastasis in MMTV-ErbB2(V664E)-transgenic background. Importantly, an affinity purified polyclonal antibody against mouse LCN2 largely blocks lung metastasis in mice with established 4T1 mammary tumors, suggesting that inhibitory molecules against human LCN2 will have medical utility for breast cancer treatment.

References

1. Lin H, Monaco G, Sun T, et al. Bcr-Abl-mediated suppression of normal hematopoiesis in leukemia. *Oncogene* 2005; 24: 3246-56.
2. Villalva C, Sorel N, Bonnet ML, et al. Neutrophil gelatinase-associated lipocalin expression in chronic myeloid leukemia. *Leuk Lymphoma* 2008; 49: 984-8.
3. Leng X, Lin H, Ding T, et al. Lipocalin 2 is required for BCR-ABL-induced tumorigenesis. *Oncogene* 2008; 27: 6110-9.
4. Devarajan P. Neutrophil gelatinase-associated lipocalin: new paths for an old shuttle. *Cancer Ther* 2007; 5: 463-70.
5. Flower DR, North AC, Sansom CE. The lipocalin protein family: structural and sequence overview. *Biochim Biophys Acta* 2000; 1482: 9-24.
6. Goetz DH, Holmes MA, Borregaard N, Bluhm ME, Raymond KN, Strong RK. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. *Mol Cell* 2002; 10: 1033-43.
7. Berger T, Togawa A, Duncan GS, et al. Lipocalin 2-deficient mice exhibit increased sensitivity to *Escherichia coli* infection but not to ischemia-reperfusion injury. *Proc Natl Acad Sci U S A* 2006; 103: 1834-9.
8. Flo TH, Smith KD, Sato S, et al. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestering iron. *Nature* 2004; 432: 917-21.
9. Devireddy LR, Teodoro JG, Richard FA, Green MR. Induction of apoptosis by a secreted lipocalin that is transcriptionally regulated by IL-3 deprivation. *Science* 2001; 293: 829-34.
10. Devireddy LR, Gazin C, Zhu X, Green MR. A cell-surface receptor for lipocalin 24p3 selectively mediates apoptosis and iron uptake. *Cell* 2005; 123: 1293-305.
11. Fernandez CA, Yan L, Louis G, Yang J, Kutok JL, Moses MA. The matrix metalloproteinase-9/neutrophil gelatinase-associated lipocalin complex plays a role in breast tumor growth and is present in the urine of breast cancer patients. *Clin Cancer Res* 2005; 11: 5390-5.
12. Bauer M, Eickhoff JC, Gould MN, Mundhenke C, Maass N, Friedl A. Neutrophil gelatinase-associated lipocalin (NGAL) is a predictor of poor prognosis in human primary breast cancer. *Breast Cancer Res Treat* 2008; 108: 389-97.
13. Hu L, Hittelman W, Lu T, et al. NGAL decreases E-cadherin-mediated cell-cell adhesion and increases cell motility and invasion through Rac1 in colon carcinoma cells. *Lab Invest* 2009.
14. Yang J, Bielenberg DR, Rodig SJ, et al. Lipocalin 2 promotes breast cancer progression. *Proc Natl Acad Sci U S A* 2009; 106: 3913-8.

15. Kubben FJ, Sier CF, Hawinkels LJ, et al. Clinical evidence for a protective role of lipocalin-2 against MMP-9 autodegradation and the impact for gastric cancer. *Eur J Cancer* 2007; 43: 1869-76.
16. Yan L, Borregaard N, Kjeldsen L, Moses MA. The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL). Modulation of MMP-9 activity by NGAL. *J Biol Chem* 2001; 276: 37258-65.
17. Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell* 1988; 54: 105-15.
18. Iannetti A, Pacifico F, Acquaviva R, et al. The neutrophil gelatinase-associated lipocalin (NGAL), a NF-kappaB-regulated gene, is a survival factor for thyroid neoplastic cells. *Proc Natl Acad Sci U S A* 2008; 105: 14058-63.
19. Aslakson CJ, Miller FR. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. *Cancer Res* 1992; 52: 1399-405.
20. Roy R, Louis G, Loughlin KR, et al. Tumor-specific urinary matrix metalloproteinase fingerprinting: identification of high molecular weight urinary matrix metalloproteinase species. *Clin Cancer Res* 2008; 14: 6610-7.

Bibliography

Publications

Leng XH, Ding T, Lin H, Wang Y, Hu L, Hu J, Feig B, Zhang W, Pusztai L, Symmans WF, Wu Y, Arlinghaus RB. **Inhibition of Lipocalin 2 Impairs Breast Tumorigenesis and Metastasis.** *Cancer Research*, in press.

Leng X, Ding T, Wu Y, Arlinghaus R. **Requirement of lipocalin 2 in breast cancer.** *Adv Enzyme*, 2010 (invited article).

Meetings

Poster

100 AACR annual meeting, April 18-22, 2009, Denver, CO

Inhibition of lipocalin 2 impairs breast tumorigenesis and metastasis

Xiaohong Leng^{1,3}, Tian Ding^{1,3}, Hui Lin¹, Yan Wang¹, and Yun Wu^{2,4}, and Ralph B. Arlinghaus^{1,4}

¹ Department of Molecular Pathology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030

² Department of Pathology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030

³ These authors contributed equally to this work

⁴ Co-corresponding author

Keywords: lipocalin 2, NGAL, breast cancer, metastasis

Abstract

Despite of the detection of higher molecular weight complex containing MMP9 and human lipocalin 2 (LCN2, also NGAL) in the urine samples of breast cancer patients, the clinical significance and the biological mechanism underlying this observation are not yet known. In this study, we analyzed LCN2 expression in more than 300 samples of breast cancer cases using molecular genetic profiling and found that elevated LCN2 expression is associated with aggressive and advanced types of breast cancers such as ER negative, HER2 positive, advanced disease stages, high nuclear grade and positive for node metastasis. More importantly, we have validated the role of LCN2 in breast tumorigenesis using a mouse genetic model and other experimental approaches. By crossing LCN2(-/-) mice with MMTV-HER2(V664E)^{tg} mice, we found that HER2(V664E)^{tg} mice without LCN2 allele showed a significant delay in primary breast tumor formation and a much reduced tumor volume compared to HER2(V664E)^{tg} mice with LCN2 allele(s). In addition, MMTV-HER2(V664E)^{tg};LCN2(-/-) mice displayed a great reduction in lung metastasis compared to HER2(V664E)^{tg} mice with LCN2 allele(s). We found that HER2-induced LCN2 expression is through PI3K/AKT/NF κ B pathway. Modulation of LCN2 expression in established human breast cancer cell lines has profound effects on cell motility and invasive ability. Ectopic expression of LCN2 in non-tumorigenic human breast

cancer cell ZR75.1 induced tumor formation in a xenograft model, whereas reducing LCN2 expression in tumorigenic human breast cancer cell MDA-MB468 greatly blocked its invasion activity in vitro and in vivo. Using a murine breast cancer cell line (4T1), we found that i.v. injection of antibody against LCN2 largely blocked lung metastasis. Our results indicated that expression and secretion of LCN2 is an essential player in breast cancer tumor formation and metastasis. Inhibition of its function using such as an antibody against LCN2 could be a feasible approach for designing new cancer therapy.

Presentation

50th Advances in Enzyme Regulation Symposium, Bologna September 27-29.

Abstract

Mechanistic knowledge of molecular markers (e.g. HER2) has increased our understanding of breast tumorigenesis, and has lead to effective treatments and diagnoses. To determine its role in breast cancer, we analyzed human LCN2 (NGAL) expression using data derived from 318 primary breast cancer samples and found increased NGAL expression is associated with certain aggressive forms of breast cancer. We also analyzed a series of human breast cancer cell lines, and identified high LCN2 expression in HER2 and epidermal growth factor receptor positive cell lines (such as SKBr3 and MDA-MB-468) and low expression in estrogen receptor (ER) positive cell lines (such as ZR75.1 and MCF-7). Using both the human and mouse mammary tumor cell lines, we found that elevated LCN2 expression increased cell migration and invasion both in cell culture and in mouse tumor studies. The elevated LCN2 expression is up regulated by HER2/PI-3 kinase/AKT/NF-kB pathways. To further characterize the role of LCN2 in breast cancer formation and progression, we established a mouse genetic model. We demonstrated that mice lacking LCN2 were significantly impaired for induction of ErbB2-induced breast tumor formation. Importantly, injection of affinity-purified antibody against 24p3 in mice with established 4T1-induced breast tumors significantly reduced lung metastasis. Taken together, our finds illustrated the molecular mechanism involved in breast cancer progression promoted by LCN2, and indicate that inhibition of LCN2 is a potential new therapeutic approach for treating human breast cancer, in particular, by blocking metastasis of aggressive types of breast cancers.

List of Personnel (not salaries) receiving pay from the research effort

Leng, Xiaohong
Arlinghaus, Ralph B.
Wang, Yang

Appendices

Email about the paper acceptance in Cancer Research

From: kyle.overturf@aacr.org [mailto:kyle.overturf@aacr.org]
Sent: Tuesday, September 22, 2009 8:39 AM
To: Arlinghaus,Ralph B
Subject: CANCER RESEARCH CAN-09-1934R -- Decision Rendered

Dr. Ralph B Arlinghaus
The University of Texas M.D. Anderson Cancer Center
Dept of Molecular Pathology
7435 Fannin St
Unit 951
Houston, TX 77054

Re: CAN-09-1934R
Inhibition of Lipocalin 2 Impairs Breast Tumorigenesis and Metastasis

Dear Dr. Arlinghaus:

I am pleased to inform you that your CANCER RESEARCH manuscript CAN-09-1934R, Inhibition of Lipocalin 2 Impairs Breast Tumorigenesis and Metastasis, has been accepted for publication in CANCER RESEARCH.

The scheduled print publication date of your article will be provided on your proofs, which you will receive within 2 to 3 weeks. If you expect your address to change within that time, please inform us immediately by contacting the Editorial Office either via e-mail to cancerres@aacr.org, or via fax to (215)440-9354. If your manuscript submission information included an e-mail address, you will receive electronic page proofs. If you do not have access to e-mail, you will receive hard copy page proofs. Please note that all AACR journals now offer online first publication of accepted articles. The version of the manuscript used for online first publication will be the final edited version incorporating any corrections or changes made at the proof stage. Therefore, it is imperative that you return your corrected proofs within two business days. Instructions on how to return proofs are provided below.

Please note that the assignment to which section of the journal your article will appear is made at the discretion of the editors.

We would like to call your particular attention to the following:

1) When page proofs are received, please read, correct, and return them to the Editorial Office via e-mail to CanResProofs@aacr.org or via fax to (267)765-1056 within two business days. Our schedule takes weekends and holidays into account. IMPORTANT: PLEASE NOTE THAT IF PROOFS ARE NOT RETURNED WITHIN THIS TIMEFRAME, PUBLICATION OF THE ARTICLE WILL BE POSTPONED.

2) Changes in proofs entail considerable expense and publication delay and thus are discouraged unless absolutely necessary.

3) When reading proofs, please answer carefully all of the copy editor's queries and pay particular attention to the figures and legends. Please read the entire manuscript carefully to verify that no changes in meaning have been introduced through copy editing.

4) Retain a copy of your corrected proof in the event that there are any problems with the original.

5) The Editors may select your article to be featured in "CANCER RESEARCH Highlights: Selected Articles from This Issue." Please provide, in WORD format only, a few short statements on why the study was important, what was done, what was found, and the study's implications in the context of cancer science. This summary should be no more than 75-100 words. You may view samples of previous Highlights at <http://www.aacr.org/home/scientists/publications-of-the-aacr/highlights-cancer-research.aspx>. You may also include for consideration a figure of at least 300-dpi TIFF or EPS file in CMYK color format, but note that the images will appear only if space permits. Name the files containing the summary and the figure HL_CAN-09-1934R and send them to kristin.murray@aacr.org as soon as possible (do not await the arrival of proof or notification of issue assignment). Please be advised that the final selection of articles to be included in the Highlights section is made at the discretion of the Editor-in-Chief from among the summary statements submitted. You will be informed only if your article is selected for inclusion.

To generate media coverage for your paper, please notify your institute's PR department and ask them to contact Jeremy Moore, Senior Manager for Science Communications at the American Association for Cancer Research (jeremy.moore@aacr.org). Although we cannot guarantee media coverage, the AACR will work with your local office to maximize opportunities.

***** IMPORTANT NOTICE REGARDING FUNDING BODIES' ARCHIVING MANDATES (INCLUDING THE NIH PUBLIC ACCESS POLICY) *****

AACR journals will help you to fulfill many funders' mandates to archive your accepted manuscript by archiving your manuscript for you in PubMed Central (PMC) or UK PubMed Central (UKPMC). If your research is funded by the National Institutes of Health, Howard Hughes Medical Institute, Wellcome Trust, Cancer Research UK, and/or the Medical Research Council, AND IF YOU SELECTED THE ARCHIVING OPTION WHEN YOU SUBMITTED YOUR PAPER, you need do nothing else. The accepted version of the manuscript will be automatically uploaded to the repository with a release date of twelve months (for National Institutes of Health) or six months (for Howard Hughes Medical Institute, Wellcome Trust, Cancer Research UK, and the Medical Research Council) post-publication. The repository may contact you or the Principal Investigator specified for approval of your submission.

If you did not select the archiving option and wish to make use of the AACR journals' archiving

service for authors, please contact the Editorial Office for assistance.

More information about AACR archiving, copyright, and permission policies is available at <http://www.aacr.org/home/scientists/publications-of-the-aacr/copyright-and-permissions-policy.aspx>.

Thank you in advance for your cooperation.

Sincerely,
Rakesh Kumar, Ph.D.
Senior Editor