AD_____

Award Number: W81XWH-10-1-0152

TITLE: Endoplasmic Reticulum-Associated Degradation Factor ERLIN2: Oncogenic Roles and Molecular Targeting of Breast Cancer

PRINCIPAL INVESTIGATOR: Dr. Zeng-Quan Yang

CONTRACTING ORGANIZATION: Wayne State University
Detroit, MI 48202

REPORT DATE: June 2011

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-06-2011	Annual	15 MAY 2010 - 14 MAY 2011

4. TITLE AND SUBTITLE

Endoplasmic Reticulum-Associated Degradation Factor ERLIN2: Oncogenic Roles and Molecular Targeting of Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER

W81XWH-10-1-0152

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dr. Zeng-Quan Yang

E-Mail: Yangz@karmanos.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Wayne State University
Detroit, MI 48202

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

Amplification of the chromosome 8p11-12 region has been found in approximately 15% of human breast cancer and is associated with poor prognosis. Previous genomic analysis has led us to identify an endoplasmic reticulum (ER) lipid raft-associated 2 (ERLIN2) gene as one of candidate oncogenes within the 8p11-12 amplicon in human breast cancer. ERLIN2 is an ER membrane protein that was recently identified as a novel mediator of ER-associated degradation. In the present study, Gain- and loss-of-function approaches demonstrated that ERLIN2 is a novel oncogenic factor that is associated with the ER stress response pathway. We found that ERLIN2 likely facilitates the adaptation of breast epithelial cells to ER stress by supporting cell growth and protecting the cancer cells from ER stress-induced cell death. Thus, ERLIN2 may confer human breast cancer cells a selective growth advantage by promoting a cytoprotective response to various cellular stresses associated with oncogenesis.

15. SUBJECT TERMS

Gene amplification, Endoplasmic reticulum, ERLIN2

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	9	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Page

Introduction

Breast cancer cells contain a large number of genetic alterations that act in combination to create the malignant phenotype. Oncogenes, such as *Her2,* play important roles in uncontrolled proliferation and survival of breast cancer cells. However, cancer cells must acquire the ability to tolerate a series of oncogenesis-associated cellular stresses such as DNA damage, proteotoxic, mitotic, metabolic and oxidative stresses (1, 2). To date, very little is known about the genomic basis and molecular mechanisms that allow breast cancer cells to tolerate and adapt to these stresses. Amplification of chromosome 8p11-12 occurs in approximately 15% of human breast cancer (HBC). This region of amplification is significantly associated with disease-specific survival and distant recurrence in breast cancer patients (3-6). Previous work in our laboratory, together with others, have identified an *endoplasmic reticulum (ER) lipid raft-associated 2* (*ERLIN2*, also known as *SPFH2*, *C8ORF2*) gene as one of several candidate oncogenes within the 8p11-12 amplicon based on statistical analysis of copy number increase and over expression (3, 4, 7). Yet, the transforming roles of ERLIN2 and molecular mechanisms by which ERLIN2 coordinates ER pathways in breast cancer have not been elucidated. In this research project, we hypothesized that *ERLIN2* plays an important role in the maintenance of malignancy and therapy-resistance through modulation of ERAD signaling in aggressive forms of human breast cancer. Accordingly, we propose that ERLIN2 represents a novel class of oncogenic factors and that targeting ERLIN2 may reduce the therapy resistance of aggressive breast cancers and thus improve the effectiveness of conventional anti-cancer drugs.

Body

1. Specific Aims

This project consists of 3 specific aims:
Aim 1: To investigate the role of ERLIN2 in the maintenance of stress- and apoptosis-resistant phenotypes of aggressive breast cancer cells.
Aim 2: To elucidate the molecular mechanism by which ERLIN2 increases ER protein folding capacity and suppresses ER stress-induced apoptosis in breast cancer cells.
Aim 3: To determine whether inhibition of ERLIN2 activity can enhance the effectiveness of the conventional anti-cancer drugs in aggressive breast cancers.

2. Studies and Results

Task 1 (Dr. Zeng-Quan Yang). **To investigate the role of endogenous ERLIN2 up-regulation in the maintenance of stress- and apoptosis-resistant phenotypes of aggressive breast cancer cells.** Month 1-16

To investigate the pathophysiological function of endogenous ERLIN2 in breast cancer cells in which the *ERLIN2* gene is amplified, we stably silenced the *ERLIN2* gene in ERLIN2-amplified SUM-44 and SUM-225 cells using the lentiviral-based shRNA system. To perform RNAi knockdown experiments, we utilized pGIPZ-ERLIN2 shRNA expression constructs in which TurboGFP and shRNA are part of a bicistronic transcript allowing for the visual marking of the shRNA-expressing stable cells. Semi-quantitative RT-PCR and Western blot analysis indicated that expression levels of ERLIN2 mRNA and protein were markedly reduced in the stable ERLIN2-shRNA-transduced SUM-44 and SUM-225 cell lines, compared to the control cell lines that were infected with a non-silencing shRNA lentiviral control. Among the two targeted vectors used, the knockdown effect was more striking for ERLIN2-shRNA vector #1, which resulted in a nearly complete loss of ERLIN2 protein expression in SUM-225 cells (Figure 1A). No change of ERLIN1 mRNA and protein levels was detected in ERLIN2-shRNA knockdown cells, thus ruling out the possible off-target effects of ERLIN2-shRNAs. Cell growth and proliferation analyses showed

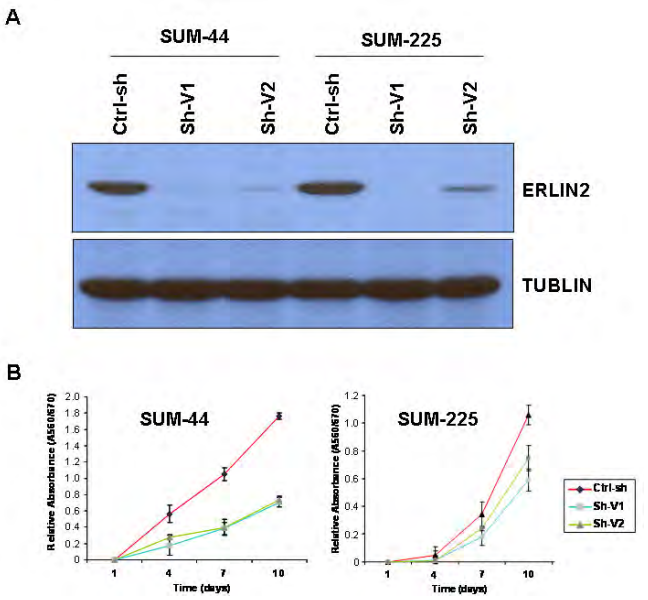

Figure 1. shRNA-mediated knockdown of ERLIN2 inhibits growth of breast cancer cells SUM-44 and SUM-225 with ERLIN2 amplification. (A) ERLIN2 expression in SUM-44 and SUM-225 cells was analyzed by western blot after infection with non-silencing control shRNA (Ctrl-sh) or ERLIN2 specific shRNAs (sh-V1 and V2). (B) MTT assay to measure cell viability in SUM-44 and SUM-225 cells after knocked down ERLIN2.

that knockdown of ERLIN2 slowed the proliferation rate of SUM-44 and SUM-225 cells (Figure 1B), but not MCF-10A cells. Knockdown of ERLIN2 in SUM-225 cells also suppressed anchorage-independent growth in soft agar (Data not shown). Taken together, results from knockdown experiments suggested that *ERLIN2* may play an oncogenic role in a subset of human breast cancer. In the next year, we will evaluate the impact of ERLIN2 knockdown or over-expression in ER stress response and apoptotic events in human breast cancer cells.

Task2 (Dr. Kezhong Zhang). **To elucidate the molecular mechanism by which ERLIN2 regulates ER calcium levels, increases ER capacity, and suppresses ER stress-induced apoptosis in breast cancers.** Months 6-20

To determine the mechanism by which ERLIN2 increase ER protein folding capacity and suppresses stress-induced apoptosis, we first tested the involvement of ERLIN2 in sustaining high levels of ER calcium through mediating degradation of activated IP3 receptor. By determining the ER calcium and cytosolic calcium concentrations in the ERLIN2-knockdown and over-expression cells, we found that calcium signal alteration/ER calcium release is not likely the cause of resistance to apoptosis by ERLIN2 over-expression. Furthermore, we examined the ER expansion in ERLIN2-over-expression cells. The results suggested that ERLIN2 over-expression leads to expansion of ER compartment, a possible mechanism that account for stress- and apoptosis-resistance in ERLIN2-over-expressed cells. For this approach, we utilized confocal fluorescence imaging to visualize the expression and sub-cellular localization of ERLIN2 in ERLIN2-amplified human breast cancer cell lines SUM44 and SUM225. The confocal analysis showed that over-expressed ERLIN2 protein localizes to the ER compartment that is significantly expanded in these breast cancer cells (Figure 2). To further investigate the capacity of ERLIN2 to modulate the structure and abundance of the ER, we exogenously and stably expressed ERLIN2 in MCF10A cells using a lentiviral expression system. Over expression of ERLIN2 mRNA and protein in two ERLIN2-transduced MCF10A pool clones was confirmed with semiquantitative RT-PCR and western blot (Data not shown). Immunofluorescence microscopy demonstrated that enforced expression of ERLIN2 in MCF10A cells triggered ER expansion (Figure 2). These results suggest that increased expression of ERLIN2 would be necessary to equip an enlarged ER compartment for more efficient ER-associated function in a subset of cancer cells. This ER expansion mechanism driven by ERLIN2 over-expression may provide a basis for cancer cell growth and resistance to apoptosis during oncogenesis.

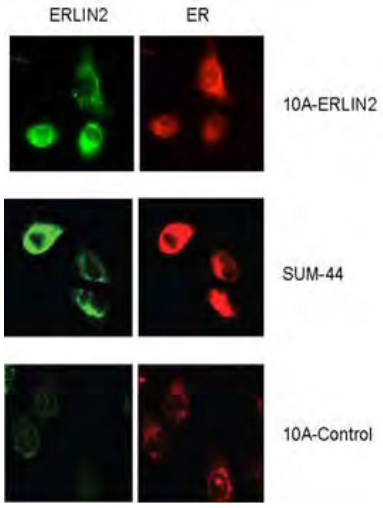

Figure 2. Confocal analysis of ERLIN2 expression and subcellular localization in MCF10A-ERLIN2, SUM-44 and MCF10A control cells.

Task 3 (Dr. Zeng-Quan Yang). **To determine whether inhibition of ERLIN2 activity can enhance the effectiveness of conventional anti-cancer drugs in aggressive breast cancers *in***

***vitro* and *in vivo* and to evaluate the potential of ERLIN2 as a therapeutic target in aggressive breast cancer.** Months 8-24

In order to detect whether ERLIN2 knockdown can enhance the effectiveness of targeted- and chemo-therapeutic drugs *in vitro*, we first examined sensitivities of human breast cancer cell lines with or without the *ERLIN2* gene amplification and over-expression to ER stress-inducing reagents. Figure 3A shows the IC50 values for the ER stress-inducing reagent Tunicamycin (Tm), in ten breast cancer cell lines as well as nontransformed human mammary epithelial cell line MCF10A. We revealed that SUM-44 and SUM-225 cells with *ERLIN2* amplification exhibited a significant higher in TM IC50 values ($P<0.05$), compared to the cell lines without *ERLIN2* amplification. We obtained the similar results with Thapsigargin (Tg) treatment in SUM-44 and SUM-225 cells. Next, we treated the ERLIN2 knocked down SUM-225 cells with Tm and Tg for 72 hours and evaluated their viability using the MTT assay. Knockdown of ERLIN2 resulted in increased sensitivity to Tg or Tm-induced cell death (Figure 3b). Together, these results suggest that over expression of ERLIN2 may facilitate the adaptation of breast epithelial cells to ER stress by supporting cell growth. In the next year, we will investigate whether inhibition of ERLIN2 activity can enhance the effectiveness of anti-cancer drugs by utilizing our established *in vitro* models and in xenograft mouse models.

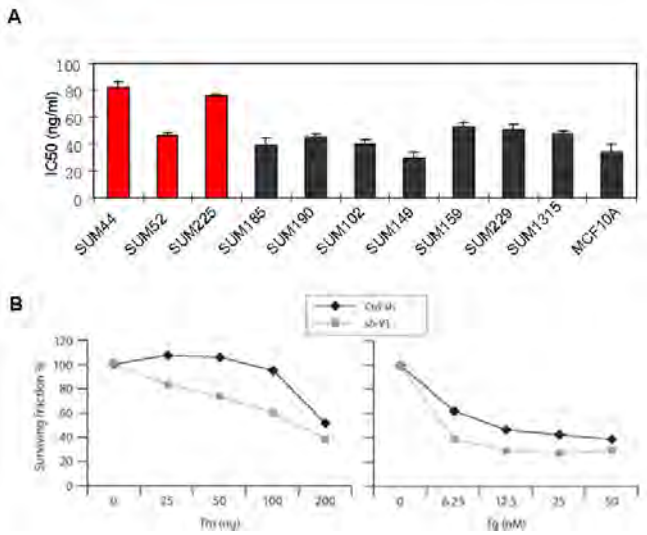

Figure 3. (A) IC50 values for the Tunicamycin (Tm), in ten breast cancer cell lines as well as nontransformed human mammary epithelial cell line MCF10A (B) Cell viability of ERLIN2 knockdown and control SUM-225 cells was measured with MTT assays after exposure to different concentrations of the Tm or Tg for 72 hours.

Key Research Accomplishments

In the present study, we investigated the transforming properties of newly identified 8p11-12 candidate oncogene ERLIN2 *in vitro*. We found knockdown of ERLIN2 in 8p11-12 amplified human breast cancer cells resulted in profound loss of growth and survival of these cells. Furthermore, ERLIN2 has the ability to protect breast cancer cells from ER stress-induced cell death. We revealed that ER expansion mechanism driven by ERLIN2 amplification and over-expression may provide a basis for cancer cell growth and resistance to apoptosis during oncogenesis.

Reportable Outcomes

Manuscript:
"**ERLIN2 promotes breast cancer cell survival by modulating endoplasmic reticulum stress pathways**" In preparation

Abstracts:
"Endoplasmic reticulum factor ERLIN2 plays an oncogenic role by modulating ER stress response in breast cancer" DOD BCRP Era of Hope Meeting 2011

"Endoplasmic Reticulum Factor ERLIN2 Preserves Oncogenesis by Regulating De novo Lipogenesis" DOD BCRP Era of Hope Meeting 2011

Conclusion

We have made significant progress in the past year in characterizing the endoplasmic reticulum factor ERLIN2 in human breast cancer. We found that ERLIN2 is amplified and over expressed in a subset of human breast cancer cells. Gain- and loss-of-function approaches demonstrated that ERLIN2 is a novel oncogenic factor that is associated with the ER stress response pathway. We show that over expression of ERLIN2 facilitated the adaptation of breast epithelial cells to ER stress by supporting cell growth and protecting the cells from ER stress-induced cell death. Thus, ERLIN2 may confer breast cancer cells a selective growth advantage for by promoting a cytoprotective response to various cellular stresses associated with oncogenesis.

References

1. Luo J, Solimini NL, Elledge SJ. Principles of cancer therapy: oncogene and non-oncogene addiction. Cell 2009; 136: 823-37.
2. Solimini NL, Luo J, Elledge SJ. Non-oncogene addiction and the stress phenotype of cancer cells. Cell 2007; 130: 986-8.
3. Yang ZQ, Streicher KL, Ray ME, Abrams J, Ethier SP. Multiple interacting oncogenes on the 8p11-p12 amplicon in human breast cancer. Cancer Research 2006; 66: 11632-43.
4. Gelsi-Boyer V, Orsetti B, Cervera N, et al. Comprehensive profiling of 8p11-12 amplification in breast cancer. Mol Cancer Res 2005; 3: 655-67.
5. Garcia MJ, Pole JC, Chin SF, et al. A 1 Mb minimal amplicon at 8p11-12 in breast cancer identifies new candidate oncogenes. Oncogene 2005; 24: 5235-45.
6. Yang ZQ, Albertson D, Ethier SP. Genomic organization of the 8p11-p12 amplicon in three breast cancer cell lines. Cancer Genet Cytogenet 2004; 155: 57-62.
7. Kwek SS, Roy R, Zhou H, et al. Co-amplified genes at 8p12 and 11q13 in breast tumors cooperate with two major pathways in oncogenesis. Oncogene 2009.