AD_____
(Leave blank)

Award Number: W81XWH-10-1-0153

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

PRINCIPAL INVESTIGATOR: Kezhong Zhang

CONTRACTING ORGANIZATION: WAYNE STATE UNIVERSITY, Detroit, MI 48201

REPORT DATE: June 2013

TYPE OF REPORT: Final

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 *(DD-MM-YYYY)*
June 2013

2. REPORT TYPE
Final

3. DATES COVERED *(From - To)*
15 May 2010 - 14 May 2013

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

5a. CONTRACT NUMBER
W81XWH-10-1-0153

5b. GRANT NUMBER
W81XWH-10-1-0153

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Kezhong Zhang and Zeng-Quan Yang
kzhang@med.wayne.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Wayne State University
Detroit, Michigan 48201

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
Previous genomic analysis has led us to identify the endoplasmic reticulum (ER) lipid raft-associated 2 (ERLIN2) gene as one of the candidate oncogenes within the 8p11-12 amplicon in a subset of aggressive breast cancer. We proposed that ERLIN2, an ER membrane protein, plays an unconventional oncogenic role through the endoplasmic reticulum (ER) stress pathway. In this study, we found: (1) ERLIN2 is required for cell proliferation and maintenance of transforming phenotypes in aggressive, ERLIN2-amplified breast cancer; (2) the UPR pathway, through the IRE1α/XBP1 axis, modulated the high-level expression of the ERLIN2 protein; (3) ERLIN2 also plays a key role in maintaining lipogenic phenotype of breast cancer cells by regulating activation of Sterol Regulatory Element-Binding Protein 1c (SREBP1c), the key lipogenic trans-activator; (4) ERLIN2 regulates activation of SREBP1c by interacting with Insulin-induced Gene 1 (INSIG1); (5) ERLIN2 had the ability to protect breast cancer cells from ER stress-induced cell death. The information provided here sheds new light on the mechanism of the novel ER factor ERLIN2 in promoting breast cancer progression.

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**	UU	95	USAMRMC
U	U	U			**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Page

Introduction…….……..….. 1

Body……..2

Key Research Accomplishments…….…….. 5

Reportable Outcomes…… 6

Conclusion…… 6

References……7

Appendices…… 8

Introduction

Breast cancer is the most common type of cancer for women in the United States and ranks second as a cause of cancer-related mortality. Increased *de novo* lipogenesis is one of the most important metabolic hallmarks of breast cancer [1-5]. Breast cancer cells contain a large number of genetic alterations that act in concert to create the malignant phenotype. For example, the up-regulation of oncogenes, such as *Her2, c-MYC and CCND1,* directly contributes to the uncontrolled proliferation of breast cancer cells. For cancer cells to survive, they must acquire the ability to tolerate a series of oncogenesis-associated cellular stress, such as proteotoxic-, mitotic-, metabolic-, and oxidative-stress [1, 2]. However, 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 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 the *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 biological roles of ERLIN2 and molecular mechanisms by which ERLIN2 contributes to breast carcinogenesis remain unclear. In this research project, we hypothesized that *ERLIN2* plays an important role in the maintenance of malignancy and therapy-resistance through modulation of ER-associated signaling pathways 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 and Dr. Kezhong Zhang's labs). **To investigate the role of endogenous ERLIN2 up-regulation in the maintenance of stress- and apoptosis-resistant phenotypes of aggressive breast cancer cells. *(Completed).***

In our previous annual reports, we have stated that we successfully knocked down ERLIN2 in *ERLIN2*-amplified SUM-44 and SUM-225 cells using the lentiviral-based shRNA system. Cell growth and proliferation analyses showed that knockdown of ERLIN2 slowed the proliferation rate of SUM-44 and SUM-225 cells, but not MCF10A control cells. We also revealed that knockdown of ERLIN2 in SUM-44 and SUM-225 cells also suppressed anchorage-independent growth in soft agar, one of the hallmark characteristics of aggressive cancer cells. Additionally, we found that amplification and over-expression of *ERLIN2* enhances the resistance to a variety of stressors, such as the ER stress-inducing reagents Tunicamycin or Thapsigargin, to promote breast cancer cell survival. Taken together, these results suggested ERLIN2 plays a role in cell proliferation and maintenance of transforming phenotypes in breast cancer cells with the *ERLIN2* amplification.

Previously, we evaluated the expression of ERLIN2 in normal and breast cancer tissues using immunohistochemistry (IHC) in tissue arrays. We found that the ERLIN2 protein is significantly upregulated in a subset of primary breast cancer cells compared with normal breast cells. Very recently, we searched the Cancer Genome Atlas database that contains 744 breast invasive carcinomas with survival data. We found a significantly worse overall survival for patients with ERLIN2 alteration, where the major samples are gene amplified and/or over-expressed ($p<0.05$) (Figure 1). This new data further supports the findings that ERLIN2 plays an important role in promoting breast cancer progression.

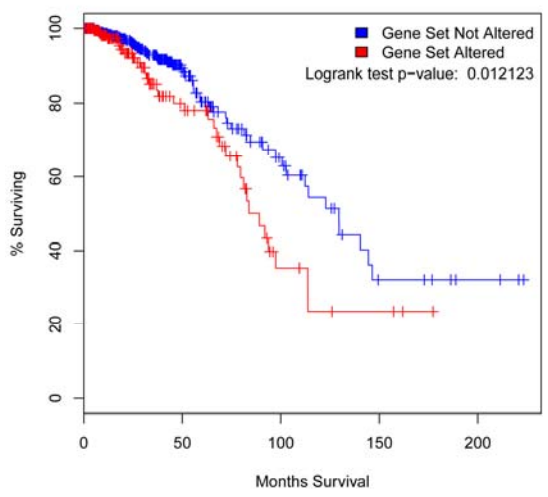

Fig 1. The overall survival of breast cancer patients with or without ERLIN2 gene alterations. The red curve in the Kaplan-Meier plot includes all tumors with an ERLIN2 copy number or expression alteration, and the blue curve includes all samples without the alteration.

Task 2 (Dr. Kezhong Zhang and Dr. Zeng-Quan Yang's labs). **To elucidate the molecular mechanism by which ERLIN2 regulates ER calcium levels, increases ER capacity, and suppresses ER stress-induced apoptosis in breast cancers. *(Completed).***

In the past progress reports, we have reported the following findings related to task 2: 1) the UPR signaling regulates ERLIN2 protein expression through IRE1α- XBP1 in human breast epithelial cells; 2) IRE1α-mediated regulation of ERLIN2 is through the IRE1α RNase activity, but not its kinase activity; 3) Over-expression of ERLIN2 leads to expansion of the ER compartment, a possible mechanism for stress- and apoptosis-resistance of human breast cancer cells; 4) Over-expression of ERLIN2 in human breast cancer cells did not affect ER calcium homeostasis, and thus, calcium signal alteration/ER calcium release is not likely the cause of resistance to apoptosis by ERLIN2 over-expression; 5) human breast cancer cells gain stress-adaption and apoptosis-resistance by up-regulating IRE1α/XBP1 UPR pathway but repressing ER stress-induced apoptotic pathway through CHOP in response to ER stress reagents or anti-cancer chemotherapeutic drugs; and 6) over-expression of ERLIN2 in human breast cancer cells promotes lipid droplet accumulation, a new mechanism to help cancer cells gain a growth advantage and protect from stress-induced apoptosis.

In the past year, we have accomplished all the remained tasks originally proposed for the Zhang lab. We not only accomplished all the experiments originally proposed in task 2, but also extended our study on delineating an unexpected molecular mechanism through which human breast cancer cells gain stress-resistance and apoptosis-evading capability. Specifically, we have made the following new processes:

Our studies suggested ERLIN2 does not function as a mediator of ER-associated protein degradation (ERAD) in human breast cancer cells. The levels of activated or polyubiquitinated IP3 receptor proteins, the key regulators of ER calcium homeostasis, were not changed in ERLIN2 over-expressing or knockdown breast cancer cells. As mentioned in the last report, ERLIN2 unlikely regulates ER calcium levels to prevent ER stress-induced apoptosis in breast cancers. This is opposite to our original hypothesis in potential roles of ERLIN2 in ER calcium homeostasis. However, we discovered that ERLIN2 over-expression promotes *de novo* lipogenesis and accumulation of cytosolic lipid droplets in breast cancer cells (reported in the last progress report), a new mechanism that help cancer cells survive from oncogenic stress and gain therapy resistance.

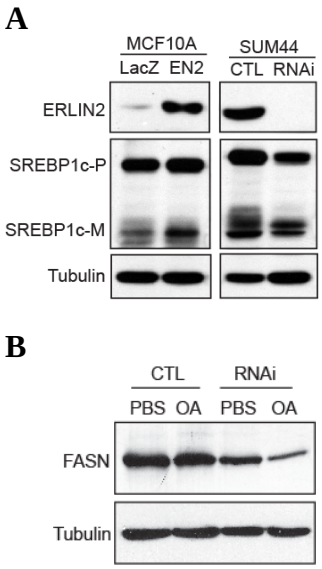

Fig 2. (**A**) Western blot analysis of ERLIN2 and SREBP1c levels in MCF10A cells expressing ERLIN2 or LacZ control and in ERLIN2-knockdown and control SUM44 cells. SREBP1c-P, SREBP1c precursor; SREBP1c-M, mature SREBP1c. (**B**) Western blot analysis of levels of fatty acid synthase (FASN) and tubulin (as a control) in ERLIN2 control (CTL) and knockdown (RNAi) Huh- 7cells. The cells were treated with vehicle PBS or oleic acid (OA) (0.5 mM) for 12 hr.

We investigate the molecular mechanism by which ERLIN2 regulates *de novo* lipogenesis and lipid droplet accumulation in human breast cancer cells. We examined activation of SREBP1c, an ER-transmembrane protein that plays the central roles in de novo lipogenesis[8], in MCF10A that over-expresses exogenous ERLIN2 or LacZ control. Levels of the

activated form of SREBP1c were significantly higher in the MCF10A cells over-expressing ERLIN2, compared to that of the control cells (Figure 2A). Supporting a role of ERLIN2 in regulating SREBP1c activation in ERLIN2-amplified breast cancer cells, levels of cleaved SREBP1c protein were lower in the ERLIN2-knockdown SUM44 breast cancer cells than that in the control cells (Figure 2A). Supporting a regulatory role of ERLIN2 in SREBP1c activation, expression of fatty acid synthase (FASN), a key enzyme in de novo lipo genesis and a target of SREBP1c, was decreased in ERLIN2-knockdown hepatoma cell line Huh7 in the absence or presence of oleic acid (OA), a monounsaturated fatty acid that can stimulate cytosolic lipid accumulation (Figure 2B). Taken together, our gain- and loss-of-function analyses indicate that ERLIN2 regulates activation of SREBP1c, the key de novo lipogenic activator, in human breast cancer cells. This finding explains why the *ERLIN2*-amplified breast cancer cell lines, such as SUM225, possess abundant cytosolic lipid droplets, as we mentioned in the last progress report.

Next, we analyzed ERLIN2-binding partners in human breast cancer cells in order to understand the molecular mechanism underlying regulation of SREBP1c activation by ERLIN2. It is known that activation of SREBP is controlled by interactions involving ER-resident proteins that are regulated by metabolic signals [8, 9]]. In particular, the SREBP precursor proteins interact with ER membrane-SREBP cleavage-activating protein (SCAP), and SCAP binds to another ER membrane protein called insulin-induced gene 1 (INSIG1) to maintain SREBPs in an inactive state. In response to metabolic stimuli, INSIG1 dissociates with SCAP and is subsequently degraded through ERAD, thus allowing SREBP activation. We performed immunoprecipitation (IP)-western blot analysis to detect the interaction between ERLIN2 and INSIG1 in human breast cancer cells. In the absence of stress challenges, we detected only a nominal interaction between ERLIN2 and INSIG1 (Figure 3A). However, significant amounts of INSIG1 proteins associated with ERLIN2 were detected in the cells challenged by insulin or under the culture of lipoprotein-deficient serum (LPDS)-containing medium, a stress culture condition that stimulates de novo lipogenesis (Figure 3A) [8] . Additionally, we found that only a small portion of SCAP proteins associated with ERLIN2 in the presence of insulin or LPDS challenge. Moreover, we found that ERLIN2 does not function as a mediator of ERAD in facilitating INSIG1 degradation, as the levels of INSIG1 proteins were not significantly changed in ERLIN2-overexpressing cells compared to that in the control cells over-expressing LacZ (Figure 3B).

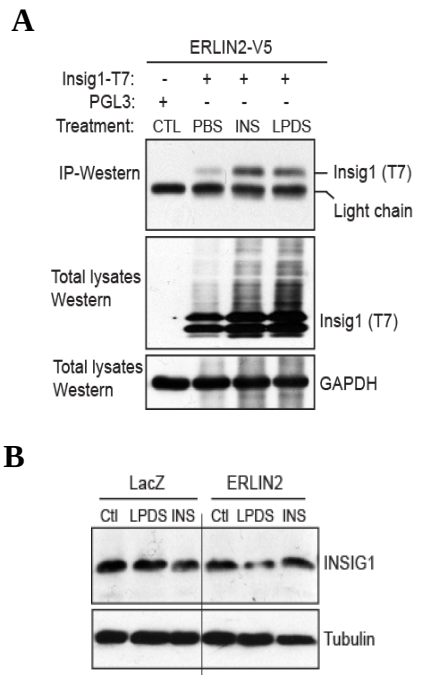

Fig 3. (**A**) IP-western blot analysis of the interaction between ERLIN2 and INSIG1. CHO cells stably expressing V5-tagged ERLIN2 were transfected with a plasmid vector expressing T7-tagged INSIG1. The transfected CHO cells were challenged with PBS, insulin (INS, 100 nM) for 6 hrs, or LPDS medium for 12 hrs. As a control, CHO cells were transfected with a plasmid vector PGL3. ER protein fractions were precipitated with an anti-V5 antibody and subjected to immunoblotting to detect the interaction between ERLIN2 and INSIG1. (**B**) Western blot analysis of INSIG1 protein levels in the Huh-7 cells overexpressing LacZ or ERLIN2. Cell lysates were prepared from the Huh-7 cells cultured in normal medium (Ctl), LPDS medium, or challenged with insulin (INS) for 6 hrs.

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.**

In our previous annual reports, we have detailed that SUM-225 breast cancer cells with or without ERLIN2 knock-down were treated with conventional chemotherapeutic drugs, proteasome inhibitor Bortezonib or EGFR family inhibitors (lapatinib or erlotinib). We found that SUM-225 cells with ERLIN2 knockdown exhibited significantly lower Bortezonib IC50 values as compared with control SUM-225 cells without ERLIN2 knockdown. However, we did not detect significant changes of IC50 values for EGFR family inhibitors in SUM-225 cells with or without ERLIN2 knockdown. It is claimed that Bortezomib induces cell death by disrupting the ER stress responses in a wide variety of cancer cell lines. Our data suggests that the synergistic cooperation between knockdown of ERLIN2 and a proteasome inhibitor might lead to a significant decrease in proliferation in a subset of breast cancer cells *in vitro*.

In the past year, we performed *in vivo* studies with the two most effective ERLIN2 shRNAs determined in our previous *in vitro* experiments. Two breast cancer cell lines, SUM-225 and SUM-52, with the ERLIN2 gene amplification were used. The cells were orthotopically transplanted into the mammary fat pads of nude mice. Mice were examined for tumor growth by palpation two times every week. In order to avoid tumor necrosis and in compliance with regulations for use of vertebrate animals in research, the animals were euthanized when the largest tumors reached approximately 1.5 cm in diameter. We found that knockdown of ERLIN2 likely inhibits tumor growth of the SUM225 breast cancer line. Unfortunately, SUM-52 breast cancer cells didn't grow in mammary fat pads of nude mice. In our future experiments, we will mainly use the SUM-225 breast cancer line.

Key Research Accomplishments

The highlights of our accomplishments from the past years are: (1) we discovered that ERLIN2 is amplified and over-expressed in both aggressive luminal B and HER2 subtypes of breast cancer; (2) we stably knocked down or over-expressed ERLIN2 in various breast model cells utilizing lentiviral-based shRNA or over-expression systems, and demonstrated that ERLIN2 is required for cell proliferation and maintenance of transforming phenotypes in aggressive, ERLIN2-amplified breast cancer; (3) we determined that expression of ERLIN2 in human breast cancer cells is regulated by the unfolded protein response (UPR) pathway through the ER stress sensor IRE1α and its downstream trans-activator XBP1; (4) we revealed that ERLIN2 facilitates breast cancer cell adaptation to ER stress and resistance to ER stress-induced apoptosis; (5) we found that ERLIN2-amplified breast cancer cell lines, such as SUM225 and SUM44, possess abundant cytosolic lipid droplets; (6) we demonstrated that the levels of cytosolic lipid droplet and cellular triglyceride contents were significantly reduced in the ERLIN2-knockdown breast cancer cells; (7) we revealed that ERLIN2 modulates activation of ER-resident lipogenic regulators, including sterol regulatory element-binding protein 1c (SREBP1c) and fatty acid synthase (FASN), in breast cancer cells; (8) we discovered that ERLIN2 regulates activation of SREBP1c by interacting with the ER-resident, SREBP/SCAP retention protein INSIG1; (9)we found

that knockdown of ERLIN2 enhances efficacy of chemo-therapeutic drugs, including proteasome inhibitor Bortezonib, in breast cancer *in vitro*.

Reportable Outcomes

Manuscript:

"ERLIN2 promotes breast cancer cell survival by modulating endoplasmic reticulum stress pathways" *BMC Cance*r 2012, 12: 225.

"Endoplasmic Reticulum Factor ERLIN2 Regulates Cytosolic Lipid Contents in Cancer Cells" *Biochem J.* 2012, 446(3):415-425.

"Pharmacological ER stress promotes hepatic lipogenesis and lipid droplet formation" *Am J Transl Res.* 2012, 4:102-113.

"Measurement of ER stress response and inflammation in the mouse model of nonalcoholic fatty liver disease" *Methods Enzymol.* 2011, 489:329-348.

"Role of unfolded protein response in lipogenesis" *World J Hepatol.* 2010, 2: 203–207

"Integration of ER stress, oxidative stress and the inflammatory response in health and disease" *Int J Clin Exp Med.* 2010, 3: 33–40.

"Endoplasmic reticulum stress response in cancer: molecular mechanism and therapeutic potential" *Am J Transl Res.* 2010, 2: 65–74.

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 completed all the tasks related to the partnering PI Dr. Kezhong Zhang (tasks 1 and 2). During the funding period, we made important discoveries in understanding the function and mechanism of the endoplasmic reticulum factor ERLIN2 in human breast cancer. We found that ERLIN2 confers a selective growth advantage on breast cancer cells by facilitating a cytoprotective response to various

cellular stresses associated with oncogenesis. We demonstrated that ERLIN2 also plays a key role in maintaining lipogenic phenotype of breast cancer cells by regulating activation of SREBP, the key lipogenic trans-activator. Under oncogensis-associated metabolic stress, ERLIN2 interacts with INSIG1, thus leading to dissociation of SCAP from INSIG1 and subsequent activation of SREBP1c in human breast cancer cells. The activation of SREBP1c triggered by the interaction between ERLIN2 and INSIG1 represents an important mechanism by which breast cancer cells increase *de novo* lipogenesis to gain growth advantage and stress-resistance capability. The information provided here sheds new light on the mechanism of breast cancer malignancy.

References

[1] J. Luo, N.L. Solimini, S.J. Elledge, Principles of cancer therapy: oncogene and non-oncogene addiction, Cell, 136 (2009) 823-837.

[2] N.L. Solimini, J. Luo, S.J. Elledge, Non-oncogene addiction and the stress phenotype of cancer cells, Cell, 130 (2007) 986-988.

[3] Z.Q. Yang, K.L. Streicher, M.E. Ray, J. Abrams, S.P. Ethier, Multiple interacting oncogenes on the 8p11-p12 amplicon in human breast cancer, Cancer Research, 66 (2006) 11632-11643.

[4] V. Gelsi-Boyer, B. Orsetti, N. Cervera, P. Finetti, F. Sircoulomb, C. Rouge, L. Lasorsa, A. Letessier, C. Ginestier, F. Monville, S. Esteyries, J. Adelaide, B. Esterni, C. Henry, S.P. Ethier, F. Bibeau, M.J. Mozziconacci, E. Charafe-Jauffret, J. Jacquemier, F. Bertucci, D. Birnbaum, C. Theillet, M. Chaffanet, Comprehensive profiling of 8p11-12 amplification in breast cancer, Molecular cancer research : MCR, 3 (2005) 655-667.

[5] M.J. Garcia, J.C. Pole, S.F. Chin, A. Teschendorff, A. Naderi, H. Ozdag, M. Vias, T. Kranjac, T. Subkhankulova, C. Paish, I. Ellis, J.D. Brenton, P.A. Edwards, C. Caldas, A 1 Mb minimal amplicon at 8p11-12 in breast cancer identifies new candidate oncogenes, Oncogene, 24 (2005) 5235-5245.

[6] Z.Q. Yang, D. Albertson, S.P. Ethier, Genomic organization of the 8p11-p12 amplicon in three breast cancer cell lines, Cancer Genet Cytogenet, 155 (2004) 57-62.

[7] S.S. Kwek, R. Roy, H. Zhou, J. Climent, J.A. Martinez-Climent, J. Fridlyand, D.G. Albertson, Co-amplified genes at 8p12 and 11q13 in breast tumors cooperate with two major pathways in oncogenesis, Oncogene, (2009).

[8] J.D. Horton, J.L. Goldstein, M.S. Brown, SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver, J Clin Invest, 109 (2002) 1125-1131.

[9] J.N. Lee, J. Ye, Proteolytic activation of sterol regulatory element-binding protein induced by cellular stress through depletion of Insig-1, J Biol Chem, 279 (2004) 45257-45265.

Wang et al. BMC Cancer 2012, 12:225
http://www.biomedcentral.com/1471-2407/12/225

BMC Cancer

RESEARCH ARTICLE Open Access

ERLIN2 promotes breast cancer cell survival by modulating endoplasmic reticulum stress pathways

Guohui Wang[1,2,8†], Gang Liu[1†], Xiaogang Wang[1†], Seema Sethi[3,6], Rouba Ali-Fehmi[4], Judith Abrams[3,7], Ze Zheng[2], Kezhong Zhang[1,2,5*], Stephen Ethier[1,9] and Zeng-Quan Yang[1,3*]

Abstract

Background: Amplification of the 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 the *endoplasmic reticulum (ER) lipid raft-associated 2* (*ERLIN2*) gene as one of the candidate oncogenes within the 8p11-12 amplicon in human breast cancer, particularly in the luminal subtype. ERLIN2, an ER membrane protein, has recently been identified as a novel mediator of ER-associated degradation. Yet, the biological roles of ERLIN2 and molecular mechanisms by which ERLIN2 coordinates ER pathways in breast carcinogenesis remain unclear.

Methods: We established the MCF10A-ERLIN2 cell line, which stably over expresses ERLIN2 in human nontransformed mammary epithelial cells (MCF10A) using the pLenti6/V5-ERLIN2 construct. ERLIN2 over expressing cells and their respective parental cell lines were assayed for *in vitro* transforming phenotypes. Next, we knocked down the ERLIN2 as well as the ER stress sensor IRE1α activity in the breast cancer cell lines to characterize the biological roles and molecular basis of the ERLIN2 in carcinogenesis. Finally, immunohistochemical staining was performed to detect ERLIN2 expression in normal and cancerous human breast tissues

Results: We found that amplification of the *ERLIN2* gene and over expression of the ERLIN2 protein occurs in both luminal and Her2 subtypes of breast cancer. Gain- and loss-of-function approaches demonstrated that ERLIN2 is a novel oncogenic factor associated with the ER stress response pathway. The IRE1α/XBP1 axis in the ER stress pathway modulated expression of ERLIN2 protein levels in breast cancer cells. We also showed 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.

Conclusions: ERLIN2 may confer a selective growth advantage for breast cancer cells by facilitating a cytoprotective response to various cellular stresses associated with oncogenesis. The information provided here sheds new light on the mechanism of breast cancer malignancy

Keywords: Gene amplification, Breast cancer, Endoplasmic reticulum, ERLIN2

Background

Breast cancer cells contain a large number of genetic alterations that act in concert to create the malignant phenotype. For example, the up-regulation of oncogenes, such as *Her2, c-MYC and CCND1,* directly contributes to the uncontrolled proliferation of breast cancer cells. For cancer cells to survive, they must acquire the ability to tolerate a series of oncogenesis-associated cellular stressors, which include DNA damage, proteotoxic-, mitotic-, metabolic-, and oxidative-stress [1,2]. However, very little is currently known about the genomic basis and molecular mechanisms that allow breast cancer cells to tolerate and adapt to these stresses. Amplification of 8p11-12 occurs in approximately 15% of human breast cancer (HBC). This region of amplification is significantly associated with

* Correspondence: kzhang@med.wayne.edu; yangz@karmanos.org
†Equal contributors
[1]Karmanos Cancer Institute, Wayne State University, Detroit, MI 48201, USA
[2]Center for Molecular Medicine and Genetics, Wayne State University, Detroit, MI 48201, USA
Full list of author information is available at the end of the article

BioMed Central

© 2012 Wang et al.; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

disease-specific survival and distant recurrence in breast cancer patients [3-6]. Previous work in our laboratory, together with others, have identified the *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 biological roles of ERLIN2 and molecular mechanisms by which ERLIN2 coordinates ER pathways in breast carcinogenesis remain unclear.

The ER is a cellular organelle primarily responsible for protein folding, lipid and sterol biosynthesis, and calcium storage. Physiological processes that increase protein folding demand or stimuli that disrupt the ER protein folding process can create an imbalance between ER protein folding load and capacity. This imbalance leads to the accumulation of unfolded or misfolded proteins in the ER: a condition referred to as "ER stress" [8,9]. The ER has evolved highly specific signaling pathways, collectively termed the "unfolded protein response" (UPR), to ensure protein folding fidelity and to protect the cell from ER stress. Upon activation of UPR, inositol-requiring protein 1 (IRE1α), the conserved ER stress sensor from yeasts to mammals, mediates splicing of the mRNA encoding X-box binding protein 1 (XBP1). XBP1 serves as a potent UPR *trans*-activator that helps protein refolding, transportation, and degradation in order to bolster ER capacity and facilitate cell adaptation to stress [8]. However, if UPR fails to restore ER homeostasis, ER stress-associated apoptosis will occur [10]. As part of the UPR program, ER-associated degradation (ERAD) targets aberrantly folded proteins in the ER. In addition to this "quality control" function, ERAD also accounts for the degradation of several metabolically-regulated ER proteins [11].

Recent studies provide evidence that UPR and ERAD components are highly expressed in various tumors, including human breast cancer [12-21]. During tumor development and progression, increased amounts of misfolded proteins caused by gene mutations, hypoxia, nutrient starvation, and high-levels of reactive oxygen species lead to ER stress [22,23]. The activation of UPR and ERAD induces an adaptive response in which the tumor cell attempts to overcome ER stress to facilitate cytoprotection. In this study, we demonstrated that amplification and the resultant over expression of ERLIN2 occurred in both luminal and Her2 subtypes of breast cancer. We also found that the UPR pathway, through the IRE1α/XBP1 axis, modulated the high-level expression of ERLIN2 protein. Furthermore, ERLIN2 had the ability to protect breast cancer cells from ER stress-induced cell death. Thus, ERLIN2 is a novel mediator of ER stress response and thus amplification and over expression of ERLIN2 may facilitate the adaptation of breast cancer cells to the various cellular stresses associated with oncogenesis.

Materials and methods

Cell lines and cell culture conditions

The culture conditions of of SUM breast cancer cells and the immortalized non-tumorigenic MCF10A cells are described in the Additional file 1: Materials and Methods.

Genomic array CGH

Genomic array CGH experiments were performed using the Agilent 44 K human genome CGH microarray chip (Agilent Technologies, Palo Alto, CA). Agilent's CGH Analytics software was used to calculate various measurement parameters, including log2 ratio of total integrated Cy-5 and Cy-3 intensities for each probe.

Semiquantitative RT-PCR reactions

Total RNA was prepared from human breast cancer cell lines and the MCF10A cell line by standard methods [3,24]. For RT-PCR reactions, RNA was converted into cDNA *via* a reverse transcription reaction using random hexamer primers. Primers were ordered from Invitrogen (Carlsbad, CA). A GAPDH primer set was used as a control. Semiquantitative RT-PCR was done using the iQSYBR Green Supermix (Bio-Rad, Hercules, CA).

Lentivirus construction and transduction of cells

The lentiviral expression construct containing the *ERLIN2* gene (pLenti-ERLIN2), was established as previously described [3]. The lentivirus for pLenti-ERLIN2 was generated and used to infect the immortalized, non-transformed mammary epithelial MCF10A cells. Control infections with pLenti-LacZ virus were performed in parallel with the pLenti-ERLIN2 infections. Selection began 48 h after infection in growth medium with 10 μg/mL blasticidin in the absence of either insulin or epidermal growth factor (EGF). Upon confluence, selected cells were passaged and serially cultured.

Three-dimensional morphogenesis assays in matrigel

For three-dimensional morphogenesis assays in Matrigel, cells grown in monolayer culture were detached by trypsin/EDTA treatment and seeded in Matrigel (BD Biosciences, San Jose, CA) precoated 8-well chamber slides. The appropriate volume of medium was added and cells were maintained in culture for 10–18 days. Phase-contrast images and immunostaining images were taken using bright-field and confocal microscopy.

Lentivirus-mediated shRNA knockdown of gene expression

We knocked down the expression of the human *ERLIN2* gene in breast cancer cell lines and in the MCF10A cell line using the Expression Arrest GIPZ lentiviral shRNA-mir system (OpenBiosystems, Huntsville, AL). Lentivirus was produced by transfecting 293FT cells with the combination of the lentiviral expression plasmid DNA and Trans-Lentiviral packaging mix (OpenBiosystems. Huntsville, AL). For cell infection, viral supernatants were supplemented with 6 μg/mL polybrene and incubated with cells for 24 hours. Cells expressing shRNA were selected with puromycin for 2–3 weeks for functional studies (cell proliferation and colony formation assays) and for 4 to 10 days after infection for RNA extraction.

Recombinant adenoviral or retrovirus infection

Adenoviruse vectors for expressing flag-tagged IRE1α isoforms, including wild type IRE1α (Ad-IRE1α WT), IRE1α kinase mutant (Ad-IRE1α K599A), and IRE1α RNase mutant (Ad-IRE1α K907A), were kindly provided by Dr. Yong Liu (Institute for Nutritional Sciences, Shanghai, China) and amplified using the AdEasy System (Stratagene) [25,26]. Retrovirus expressing spliced XBP1 was kindly provided by Dr. Lauri Glimcher (Harvard University) [27]. For infection of cells with adenovirus and retrovirus, cells were seeded in six-well plates. After 24 h, cells were infected with adenovirus expressing wild type IRE1α (Ad-IRE1α WT), IRE1α kinase mutant (Ad-IRE1α K599A), IRE1α RNase mutant (Ad-IRE1α K907A), and retrovirus expressing spliced XBP1 as described previously [28,29].

Tissue array and immunohistochemistry (IHC) staining

Human breast cancer tissue array was obtained from Nuclea Biotechnologies (Pittsfield, MA). Immunohistochemistry was performed on tumor tissue sections using the standard laboratory protocols [30]. Briefly, after deparaffinizing and hydrating with phosphate-buffered saline (PBS) buffer (pH 7.4), the sections were pretreated with hydrogen peroxide (3%) for 10 minutes to remove endogenous peroxidase, followed by antigen retrieval *via* steam bath for 20 minutes in EDTA. A primary antibody was applied, followed by washing and incubation with the biotinylated secondary antibody for 30 minutes at room temperature. Detection was performed with diaminobenzidine (DAB) and counterstaining with Mayer hematoxylin followed by dehydration and mounting. Immunostained slides were blindly evaluated under a transmission light microscope. Areas of highest staining density were identified for evaluating the expression in tumors.

Results

ERLIN2 is amplified and over expressed in human breast cancer cells

Recently, we used quantitative genomic PCR and array comparative genomic hybridization (CGH) to profile copy number alterations in 10 human breast cancer cell lines and 90 primary human breast cancers [3,6,31]. Analysis of our array CGH data showed that ERLIN2 gene was commonly amplified in 30% of the cell lines tested, as well as in 7.8% of breast cancer specimens tested (Figure 1a). Previously, we and several other laboratories have demonstrated that the 8p11-12 amplicon occurs mainly in the luminal subtype of breast cancer cells, such as the SUM-44 and SUM-52 cell lines. However, SUM-225 is a *Her2*-amplified HBC cell line [31,32]. We also found two primary tumors, 10173 and 9895, which have *Her2* gene amplifications in addition to the amplification of the ERLIN2 gene (Figure 1a). To obtain further support for a potential involvement of the ERLIN2 region in breast cancer, we searched the published database of the Affymetrix 250 K array CGH [33]. We found that 42 of the 243 HBC lines and primary samples in the array exhibited amplification of the ERLIN2 region. Interestingly, eight of the *ERLIN2*-amplified samples showed co-amplification of the *Her2* gene (Additional file 1: Figure S1). Next, we measured ERLIN2 protein levels in ten breast cancer cell lines by Western blot analysis. In correlation with *ERLIN2* gene amplification, ERLIN2 protein levels in SUM-44, SUM-52, and SUM-225 cells were dramatically greater than the levels in breast cancer cell lines without *ERLIN2* gene amplification (Figure 1b). The presence of the ERLIN2 amplification in both luminal and Her2 subtypes of breast cancer prompted us to further investigate the role of the *ERLIN2* gene in breast cancer progression.

ERLIN2 plays a functional role in breast cancer cells

Next, we addressed whether *ERLIN2* possess transforming properties. We transduced the immortalized, non-transformed mammary epithelial cell line, MCF10A, with lentivirus expressing ERLIN2 or control LacZ. Semi-quantitative RT-PCR (qRT-PCR), Western blot and immunofluorescence staining confirmed the over expression of ERLIN2 protein in MCF10A-ERLIN2 cells (Figure 2a and Additional file 1: Figure S2). The infected MCF10A cells were then subjected to analyses for growth rates, growth factor-independent proliferation, anchorage-independent growth, and three-dimensional morphogenesis assays. Growth curves and colony formation assays in MCF10A cells showed that forced expression of ERLIN2 resulted in growth factor-independent proliferation in insulin-like growth factor-deficient media. To further examine the effects of ERLIN2 in a context that more closely resembles *in vivo* mammary architecture, we assessed the consequences of ERLIN2

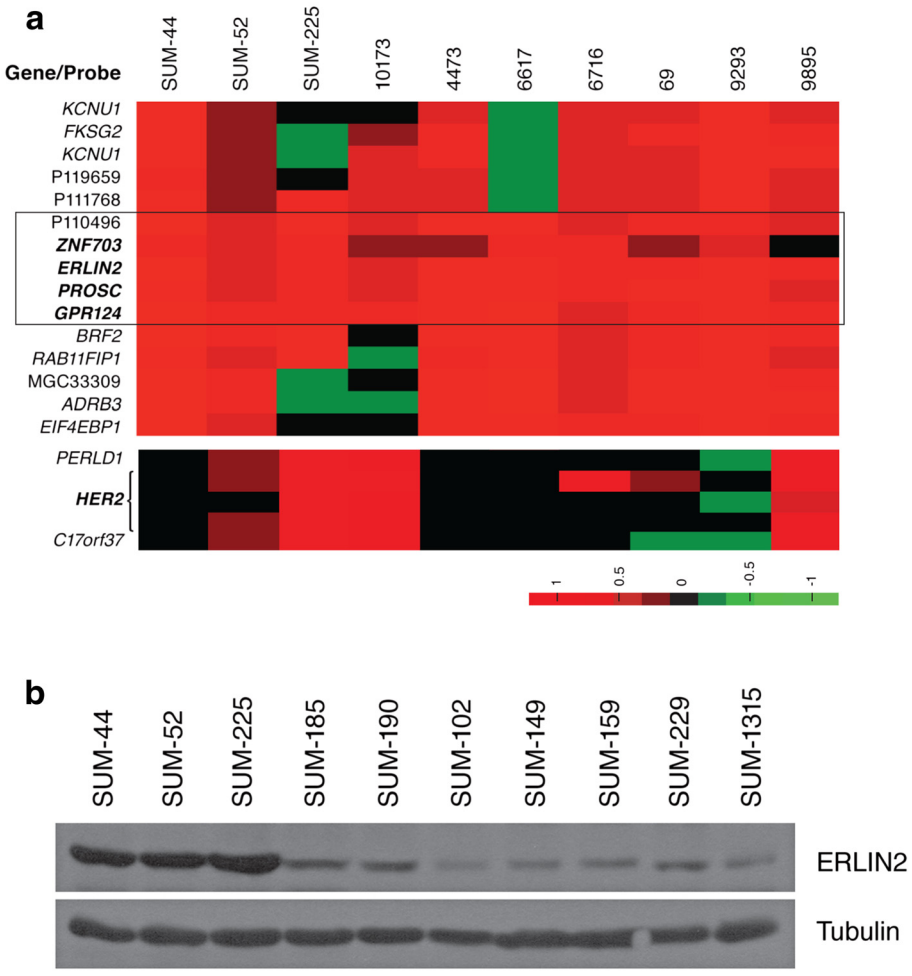

Figure 1 (a) Genomic copy number profiles of the ERLIN2 region analyzed on the Agilent oligonucleotide array CGH in 3 SUM breast cancer cell lines and 7 primary breast cancer specimens. Tumors are displayed vertically and array probes are displayed horizontally by genome position. Log2 ratio in a single sample is relative to normal female DNA and is depicted according to the color scale (bottom). Red indicates relative copy number gain, whereas green indicates relative copy number loss. (**b**) ERLIN2 protein levels were analyzed by Western blot in ten breast cancer cell lines with or without *ERLIN2* amplification.

over expression on three-dimensional morphogenesis in Matrigel. Although MCF10A cells formed polarized, growth-arrested acinar structures with hollow lumens similar to the glandular architecture *in vivo*, MCF10A-ERLIN2 cells formed abnormal acini at a high frequency that was grossly disorganized, and contained filled lumens (Figure 2b).

To further explore the pathophysiological function of ERLIN2 over expression, we stably silenced the *ERLIN2* gene in SUM-44 and SUM-225 breast cancer cells using the lentiviral-based shRNA system. To perform RNAi knockdown experiments, we utilized pGIPZ-ERLIN2 shRNA expression constructs in which TurboGFP and shRNA were part of a bicistronic transcript allowing for the visual marking of the shRNA-expressing stable cells. qRT-PCR and Western blot analysis indicated a marked reduction in expression levels of ERLIN2 mRNA and protein in the stable ERLIN2-shRNA-transduced SUM-44 and SUM-225 cell lines as compared with the control cell lines infected with a non-silencing shRNA lentiviral control (Figure 3a). Among the two targeted vectors used, ERLIN2-shRNA vector #1 produced a more striking knockdown effect: infected SUM-225 cells had a nearly complete loss of ERLIN2 protein expression (Figure 3a). We did not detect any change in ERLIN1 mRNA and protein levels in ERLIN2-shRNA knockdown cells, thus ruling out the possiblity of off-target effects by ERLIN2-shRNAs (Data not shown). Cell growth and proliferation analyses showed that knockdown of ERLIN2 slowed the proliferation rate of SUM-44 and SUM-225 cells, but had only a minor effect on SUM-102 and MCF10A cells, which lack ERLIN2 amplification (Figure 3b). Importantly, knockdown of ERLIN2 in SUM-44 and SUM-225 cells also suppressed anchorage-independent growth in soft agar, one of

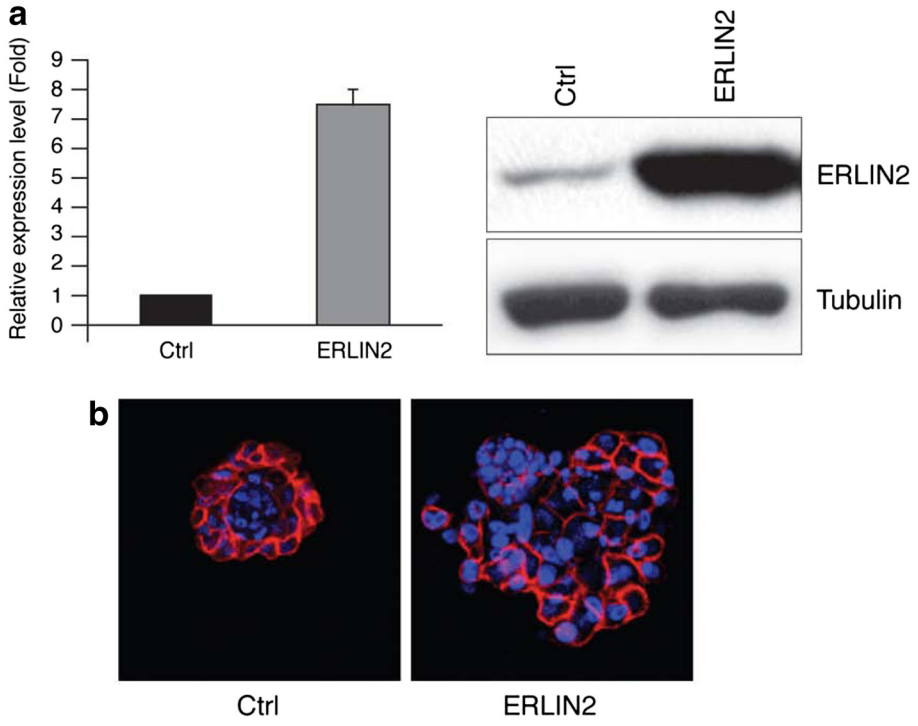

Figure 2 (a) Stable overexpressing ERLIN2 in MCF10A cells (MCF10A-ERLIN2) with the pLenti6/V5-ERLIN2 construct. Over expression of ERLIN2 mRNA and protein in this cell line was confirmed with semi-quantitative RT-PCR (right panel) and western blot assays (left panel). (**b**) Effects of ERLIN2 on mammary acinar morphogenesis. MCF10A-ERLIN2 and control cells were cultured on a bed of Matrigel. Representative images show the structures with staining for actin with phalloidin conjugated to Alexa Fluor-568 (red), and DAPI as a marker of nuclei (blue).

the hallmark characteristics of aggressive cancer cells. (Figure 3c). Taken together, results from over expression and knockdown experiments suggested *ERLIN2* plays a role in cell proliferation and maintenance of transforming phenotypes in breast cancer cells with the 8p11-12 amplification.

Expression of ERLIN2 is regulated by the ER pathway through IRE1α/XBP1

Recent studies have identified ERLIN2 both as a novel component of lipid raft domains in the ER membrane and as a substrate recognition factor during ERAD of activated inositol triphosphate receptors (IP3R) as well as other substrates [34-36]. IRE1α is the primary ER stress sensor implicated in the regulation of the ERAD pathway [37]. Under ER stress, IRE1α undergoes autophosphorylation to activate its RNase activity, which triggers one of the UPR cascades through splicing *Xbp1* mRNA [8]. Previous work has demonstrated that breast cancer cells over express XBP1 [38,39], while we observed that SUM-44, SUM-52 and SUM-225 cell lines over expressed total and activated XBP1 (Additional file 1: Figure S3 Additional file 2: Table S1). To evaluate the possibility of an association between ERLIN2 expression and the IRE1α-mediated UPR pathway in HBC, we inhibited IRE1α RNase or kinase activity in breast cancer cells. To accomplish this, we used adenoviral-based expression system to introduce the previously characterized IRE1 kinase dominant-negative mutant (IRE1 K599A) or the IRE1 RNase dominant-negative mutant (IRE1 K907A) into breast cancer cells [26,40,41]. We chose SUM-44 cells for this experiment because the SUM-44 cells are very amenable to adenovirus-mediated expression. The inhibition of the IRE1α RNase activity significantly reduced protein levels of ERLIN2 in SUM-44 cells (Figure 4a). In addition, forcible expression of wild-type IRE1α or spliced XBP1 in MCF10A cells resulted in increased expression levels of endogenous ERLIN2 protein (Figure 4b and c). However, quantitative real-time RT-PCR analysis showed that over expression of IRE1α or spliced XBP1 did not increase expression of the *ERLIN2* mRNA (Data not shown). Next, we asked whether ERLIN2 expression was induced by stress inducers in normal mammary epithelial cells. Our group routinely cultures MCF10A cells in serum-free, growth factor-supplemented media. Oncogenesis-associated conditions, such as nutrient or growth factor depletion, can cause pathophysiologic ER stress [22,23]. When MCF10A cells were cultured in media lacking insulin or EGF, expression levels of endogenous ERLIN2 protein in MCF10A were increased as compared with levels in cells cultured in normal media (Figure 4d). Our observations

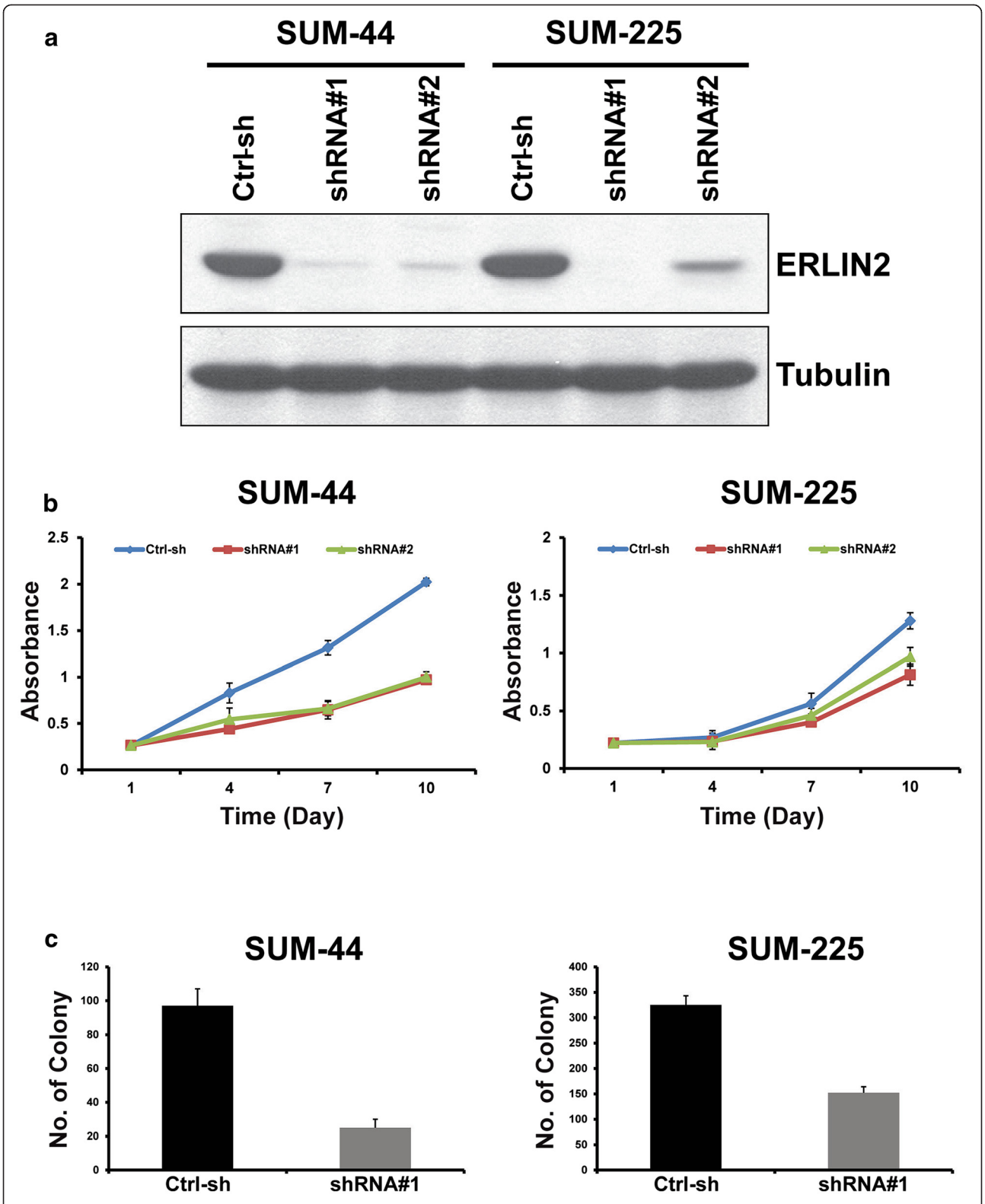

Figure 3 shRNA-mediated knockdown of ERLIN2 inhibits monolayer and anchorage-independent cell growth in breast cancer cells with ERLIN2 amplification. (**a**) Knockdown of ERLIN2 expression in SUM-44 and SUM-225 cells with two different shRNAs was confirmed by western blot. (**b**) *In vitro* growth rate of SUM-44 and SUM-225 cells with ERLIN2 shRNA treatment compared to cells with control shRNA treatment. (**c**) Knockdown of ERLIN2 reduces colony formation in soft agar. SUM-44 and SUM-225 cells were tranfected with ERLIN2 shRNA#1 or control shRNA. The colony numbers were counted 3 weeks later ($P < 0.05$).

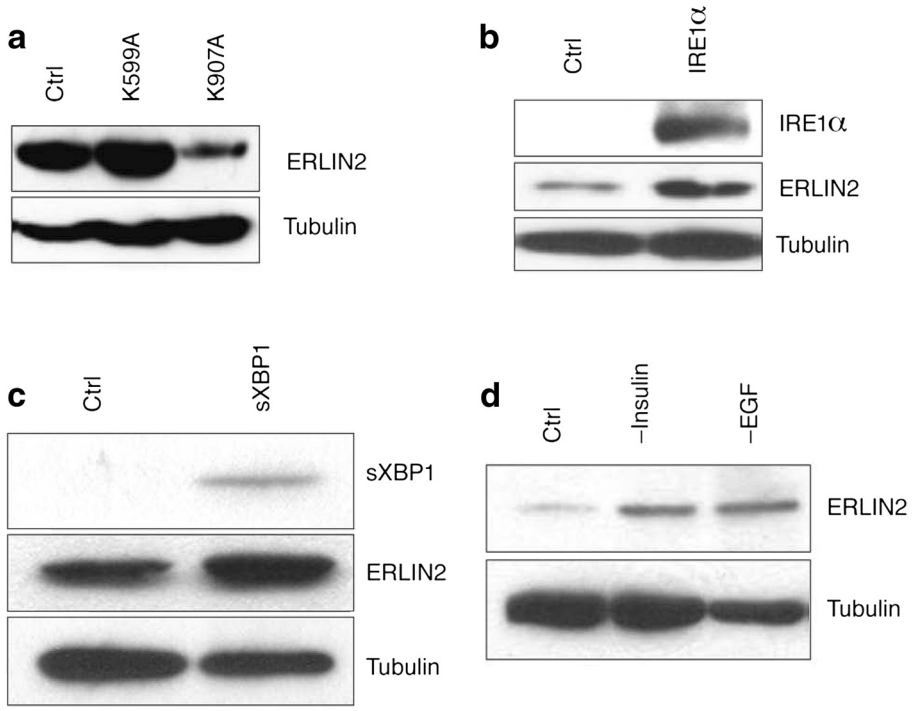

Figure 4 (a) The knockdown of the IRE1α RNase activity (K907A) reduced levels of ERLIN2 protein in SUM-44 cells. Forced expression of wild-type IRE1α (**b**) and its substrate, spliced XBP1 (**c**), leads to increased expression of ERLIN2 at protein level in MCF10A cells. (**d**) ERLIN2 expression in MCF10A cells was analyzed by western blot after culture 48 hours in insulin- or EGF-depleted media, compared to that in normal culture media.

suggest that the ER stress pathway likely regulates ERLIN2 protein expression through IRE1α-actived XBP1 in human breast epithelial cells.

ERLIN2 promotes breast cancer cell survival

Next, we tested if amplification and over expression of ERLIN2 enhances the resistance to a variety of stressors to promote cancer cell survival. Figure 5a shows the IC_{50} values for the ER stress-inducing reagent Tunicamycin (Tm), in ten breast cancer cell lines as well as in the nontransformed human mammary epithelial cell line MCF10A. SUM-44 and SUM-225 cells, which have ERLIN2 amplification, had significantly higher TM IC_{50} values than cell lines without ERLIN2 amplification ($P < 0.05$). We obtained similar results with Thapsigargin (Tg) treatment of SUM-225 cells (data not shown). Expression of the CCAAT/enhancer-binding protein (C/EBP) homology protein (CHOP) is characteristic of the ER stress–mediated apoptotic pathway. In response to treatment with Tm or Tg, expression of CHOP dramatically increased in control MCF10A cells (Figure 5b). However, induction of CHOP by Tm and Tg treatment was weaker or barely detectable in SUM-44 and SUM-225 cells (Figure 5b). Next, to determine whether suppressing ERLIN2 in breast cancer cells re-sensitize them to ER-stress, we challenged stable ERLIN2-knockdown SUM-44 and 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 Tm or Tg -induced cell death (Figure 5c). Our data suggested that over expression of ERLIN2 may facilitate the adaptation of breast epithelial cells to ER stress by supporting cell growth. Future investigations are required to more precisely address the mechanism by which ERLIN2 promotes breast cancer cell survival.

Expression of ERLIN2 in breast tissues: Carcinomas and normal

We evaluated the expression of ERLIN2 in normal and cancerous human breast tissues using immunohisyochemistry (IHC) in breast cancer tissue arrays. We first confirmed the specificity and sensitivity of the ERLIN2 antibody for visualizing ERLIN2 expression in formalin-fixed, paraffin-embedded breast cancer cell lines. Consistent with the immunoblotting data, SUM-225 cells displayed significantly higher levels of positive staining as compared with the MCF10A control cells (Additional file 1: Figure S4). The tissue array included 34 breast

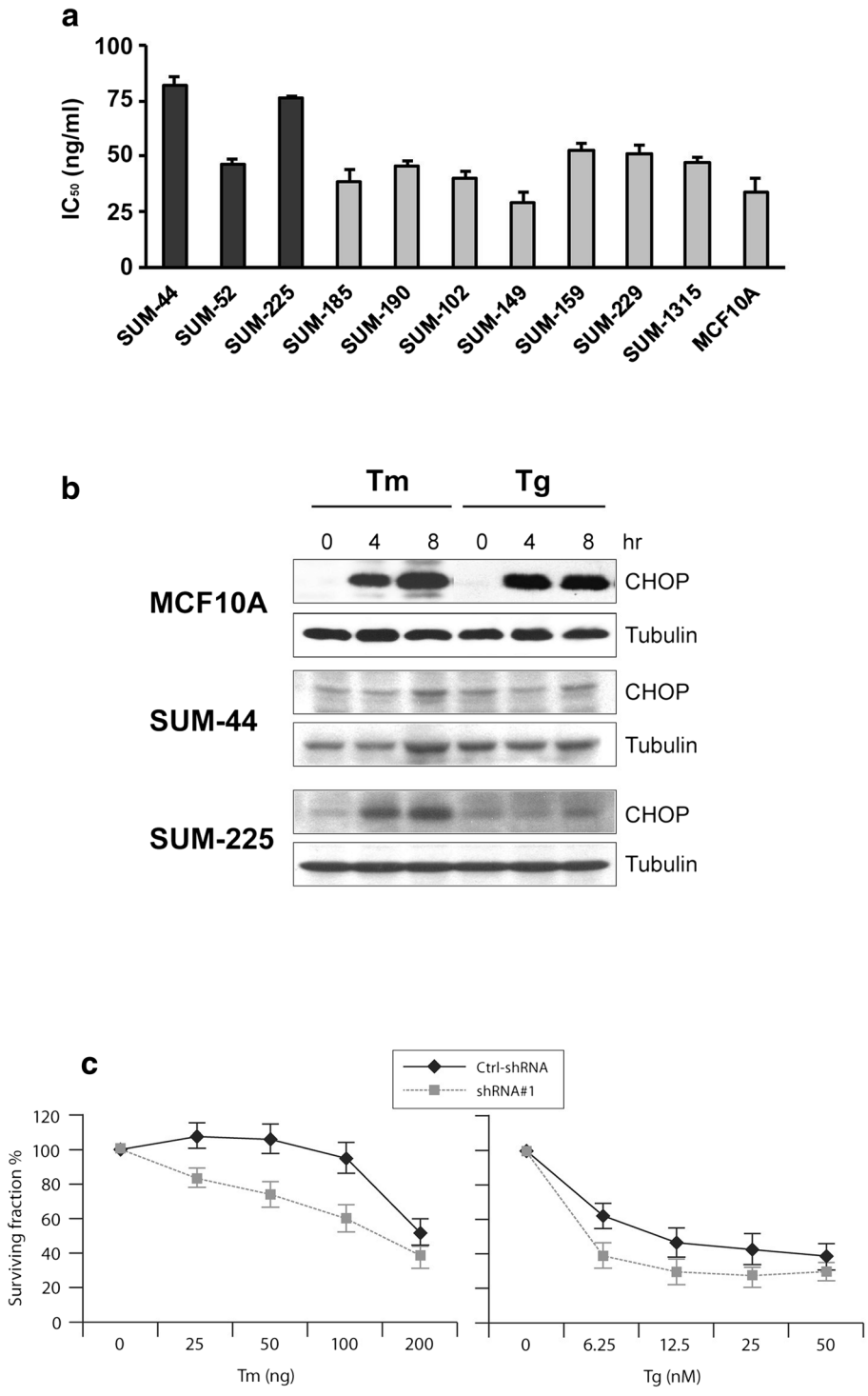

Figure 5 (a) IC_{50} **values for the ER stress-inducing reagent Tm, in ten breast cancer cell lines as well as in the MCF10A cells (b) The expression level of CHOP in SUM-225, SUM-44 breast cancer cells and MCF10A control cells was analyzed with Western blot after Tm (500 ng) or Tg (400 nM) treatment.** (**c**) 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.

carcinomas and 17 normal breast tissue, which included 14 cases of adjacent normal counterparts. ERLIN2 expression was scored based on the staining intensity: 0 (negative), 1 + (weak), 2 + (low); 3+ (moderate) or 4 + (strong). In breast carcinomas samples, 11 (32.4%) stained ERLIN2 strongly and 13 (38.2%) moderately

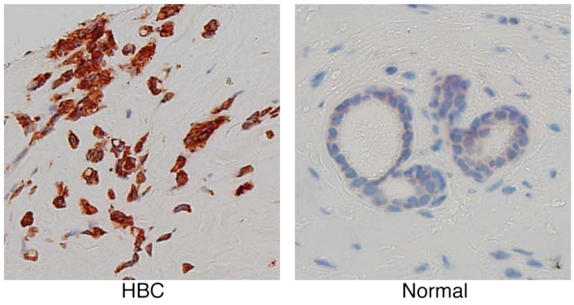

Figure 6 Immunohistotochemical staining of ERLIN2 protein on a representative HBC sample and a normal control.

(Figure 5, Additional file 1: Figure S5 Additional file 2: Table S2). In contrast, no strong or moderate staining was observed in the 17 normal breast tissues. The staining intensities of ERLIN2 were significantly higher in tumor cells than in normal tissue cells (P = 0.001).

Discussion

The 8p11-12 amplicon in HBC has been the subject of a number of studies using high-resolution genomic analysis of copy number and gene expression [3-6,42,43]. We previously found that the 8p11-12 amplicon has a highly complex genomic structure and that the size of the amplicon in three HBC lines, SUM-44, SUM-52 and SUM-225, is highly variable [6,31]. Moreover, the sub-amplicon of 8p11-12 that carries the *ERLIN2* gene amplification was more frequently identified in HBCs [4,7]. Previous studies have demonstrated that the 8p11-12 amplicon occurs mainly in the luminal subset of breast cancer cells, such as SUM-44 cells, a subset that also expresses the estrogen receptor [3,4,7,44-46]. Here we report that the co-amplification of the *ERLIN2* region occurred in a subset of *HER2*-amplified breast cancer cells, including SUM-225 cells. Our recent studies with Her2 model cells demonstrated that over expression of Her2 alone is not sufficient to induce full transformation *in vitro* and is not tumorigenic *in vivo* [47]. In contrast, *Her2*-amplified SUM225 breast cancer cells are fully transformed *in vitro* and tumorgic *in vivo* [48]. In this study, *in vitro* transforming and shRNA assays provided evidence that ERLIN2 is the most likely non- classical oncogene within this 8p11-12 minimal common amplified region. Our results suggest that the *ERLIN2* plays a role in cell proliferation and maintenance of transforming phenotypes in breast cancer cells with the 8p11-12 amplification.

ERLIN2 belongs to a larger family of proteins that share an evolutionarily conserved stomatin/prohibitin/flotillin/HflK/C (SPFH) domain. SPFH-containing proteins localize to different membranes, but have common characteristics. For example, N-terminal sequences are required for subcellular localization and membrane attachment, while the coiled-coil motifs located at the C-terminal side of SPFH domain mediate the assembly of high-molecular-weight complexes [49]. ERLIN2 and its homologue ERLIN1 were originally identified as components of lipid rafts that localize to the ER [36]. More recently, ERLIN2 has been recognized as a novel mediator of ERAD [34-36,50]. ERLIN2 binds to activated IP3Rs and other ERAD substrates, leading to polyubiquitination and subsequent degradation of these substrates [34,35].

Of particular interest in this study, we found that the UPR pathway modulated ERLIN2 protein expression in breast cancer cells through the IRE1α/XBP1 axis. Forced expression of IRE1α, or spliced XBP1, the target of IRE1α under ER stress, up-regulated expression of the ERLIN2 protein, while knockdown of IRE1α RNase activity decreased ERLIN2 expression in the *ERLIN2*-amplified breast cancer cells. These gain- and loss-of-function studies provided support that the IRE1α/XBP1-mediated UPR pathway in HBC regulated production of ERLIN2. Importantly, our study also showed that the depletion of nutrient and growth signals, a condition that is associated with oncogenesis and ER stress, can increase ERLIN2 production in breast epithelial cells. However, over expression of IRE1α or spliced XBP1 did not increase expression of the *ERLIN2* mRNA level, suggesting regulation at the post-transcription level. In the present study, we also showed that expression of primary breast cancer cells significantly up regulated ERLIN2 protein expression as compared with normal breast cells. As we had described earlier, amplification of the *ERLIN2* gene, as part of the 8p11-12 amplicon, occurs in approximately 15% of human breast cancer. It is reported that XBP1 is over expressed in aggressive breast cancer and associated with cancer cell survival and therapy resistance [51]. In the ten SUM breast cancer cell lines we investigated, three lines have both ERLIN2 gene amplification and up-regulation of activated XBP1, resulting in dramatically high-level expression of ERLIN2 protein. In contrast, two lines with up-regulation of the XBP1, but no ERLIN2 gene amplification, had moderately high-expression of the ERLIN2 protein. Taken together, our results raise an intriguing notion that the breast cancer cells may utilize gene amplification and the UPR pathway to regulate ERLIN2 protein over-production under oncogenic stress conditions.

In response to ER stress, cells activate UPR to reprogram gene transcription and translation. Depending on the type and/or degree of the stress, cells can differentially activate the UPR pathways in order to make survival or death decisions [52]. The literature indicates that the UPR branch, through IRE1α/XBP1, plays a critical role in cell adaptation to ER stress by increasing protein refolding and degradation of misfolded proteins, and by bolstering the protein-folding capacity and

secretion potential of the ER [20,52,53]. Cancer cells may adapt to the cellular stress and evade stress-induced apoptotic pathways by differentially activating the UPR branches. Indeed, tumor microenvironment has been characterized by a 'baseline' level of ER stress response that promotes tumor development and metastasis [20].

Conclusions

In the present study, we show that over expression of ERLIN2 may facilitate the adaptation of breast epithelial cells to ER stress by supporting cell growth and protecting the cells from ER stress-induced apoptosis. These results suggest that ERLIN2 confers a selective growth advantage for breast cancer cells by facilitating a cytoprotective response to various cellular stresses associated with oncogenesis. The information provided here sheds new light the mechanism of breast cancer malignancy.

Additional files

Additional file 1: Materials and Methods [54-58].

Additional file 2: Table S1. Expression Levels of XBP1, ERLIN1 and ERLIN2 in Ten SUM BreastCancer Cell Lines Using Our Affymetrix Array Database. Table S2: Expression of ERLIN2 in breast tissues:carcinomas and normal. Figure S1. Figure S2. Figure S3. Figure S4. Figure S5.

Abbreviations

Her2: v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 neuro/glioblastoma derived oncogene homolog (avian); c-MYC: v-myc myelocytomatosis viral oncogene homolog (avian); CCND1: cyclin D1; HBC: Human breast cancer; ER: Endoplasmic reticulum; ERLIN2: Endoplasmic reticulum lipid raft-associated 2; UPR: Unfolded protein response; IRE1: Inositol-requiring protein 1; XBP1: X-box binding protein 1; ERAD: ER-associated degradation; CGH: Comparative genomic hybridization; shRNA: Short hairpin RNA; IP3R: Inositol triphosphate receptors; Tm: Tunicamycin; Tg: Thapsigargin; CHOP: The CCAAT/enhancer-binding protein (C/EBP) homology protein; IHC: Immunohisyochemistry.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GHW, GL and XGW performed most of the experiments, participated in designing the study, analyzing the data. SS, RAF and ZZ were involved in IHC staining experiments. SE participated in design of the study. KZZ and ZQY conceived, coordinated, designed and procured funding for the study and wrote the manuscript. All authors gave final approval for the manuscript to be published.

Acknowledgements

This work was supported by the Department of Defense Breast Cancer Program grants BC083945 and BC09517 to Zeng-Quan Yang and BC095179P1 to Kezhong Zhang, the National Institutes of Health (NIH) grant 1R21ES017829-01A1 to Kezhong Zhang, NIH grant RO1 CA100724 to Stephen P. Ethier, and a Karmanos Cancer Institute pilot grant to Zeng-Quan Yang and Kezhong Zhang. The array CGH work was facilitated by the Microarray and Bioinformatics Core Facility of the Wayne State University Environmental Health Sciences Center, NIEHS P30 ES06639. The Biorepository core and the Biostatistics Core of the Karmanos Cancer Institute are supported by the National Institutes of Health grant P30-CA022453-29. We thank Michele L. Dziubinski and Katie L. Streicher for technical assistance on the cell culture and three-dimensional morphogenesis assays. We thank Dr. Aliccia Bollig-Fischer and Kimberly Lyons for discussions and careful reading of a draft manuscript.

Author details

[1]Karmanos Cancer Institute, Wayne State University, Detroit, MI 48201, USA. [2]Center for Molecular Medicine and Genetics, Wayne State University, Detroit, MI 48201, USA. [3]Department of Oncology, Wayne State University, Detroit, MI 48201, USA. [4]Department of Pathology, Wayne State University, Detroit, MI 48201, USA. [5]Department of Immunology and Microbiology, Wayne State University, Detroit, MI 48201, USA. [6]Biorepository Core, Wayne State University, Detroit, MI 48201, USA. [7]Biostatistics Core of the Karmanos Cancer Institute, Wayne State University, Detroit, MI 48201, USA. [8]Department of Medical Imaging and Interventional Radiology, State Key Laboratory of Oncology in South China, Cancer Center, Sun Yat-Sen University, No. 651, Dongfeng Road East, Guangzhou, 510060, China. [9]Department of Pathology and Laboratory Medicine, Hollings Cancer Center, Medical University of South Carolina, BEB 412, 68 President St, Charleston, SC 29425, USA.

Received: 28 November 2011 Accepted: 14 May 2012
Published: 8 June 2012

References

1. Luo J, Solimini NL, Elledge SJ: **Principles of cancer therapy: oncogene and non-oncogene addiction.** *Cell* 2009, **136**(5):823–837.
2. Solimini NL, Luo J, Elledge SJ: **Non-oncogene addiction and the stress phenotype of cancer cells.** *Cell* 2007, **130**(6):986–988.
3. Yang ZQ, Streicher KL, Ray ME, Abrams J, Ethier SP: **Multiple interacting oncogenes on the 8p11-p12 amplicon in human breast cancer.** *Cancer Res* 2006, **66**(24):11632–11643.
4. Gelsi-Boyer V, Orsetti B, Cervera N, Finetti P, Sircoulomb F, Rouge C, Lasorsa L, Letessier A, Ginestier C, Monville F, *et al*: **Comprehensive profiling of 8p11-12 amplification in breast cancer.** *Molecular cancer research: MCR* 2005, **3**(12):655–667.
5. Garcia MJ, Pole JC, Chin SF, Teschendorff A, Naderi A, Ozdag H, Vias M, Kranjac T, Subkhankulova T, Paish C, *et al*: **A 1 Mb minimal amplicon at 8p11-12 in breast cancer identifies new candidate oncogenes.** *Oncogene* 2005, **24**(33):5235–5245.
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**(1):57–62.
7. Kwek SS, Roy R, Zhou H, Climent J, Martinez-Climent JA, Fridlyand J, Albertson DG: **Co-amplified genes at 8p12 and 11q13 in breast tumors cooperate with two major pathways in oncogenesis.** *Oncogene* 2009 .
8. Ron D, Walter P: **Signal integration in the endoplasmic reticulum unfolded protein response.** *Nat Rev Mol Cell Biol* 2007, **8**(7):519–529.
9. Zhang K, Kaufman RJ: **From endoplasmic-reticulum stress to the inflammatory response.** *Nature* 2008, **454**(7203):455–462.
10. Zhang K, Kaufman RJ: **Identification and characterization of endoplasmic reticulum stress-induced apoptosis in vivo.** *Methods Enzymol* 2008, **442**:395–419.
11. Schroder M, Kaufman RJ: **ER stress and the unfolded protein response.** *Mutat Res* 2005, **569**(1–2):29–63.
12. Dong D, Ni M, Li J, Xiong S, Ye W, Virrey JJ, Mao C, Ye R, Wang M, Pen L, *et al*: **Critical role of the stress chaperone GRP78/BiP in tumor proliferation, survival, and tumor angiogenesis in transgene-induced mammary tumor development.** *Cancer Res* 2008, **68**(2):498–505.
13. Pyrko P, Schonthal AH, Hofman FM, Chen TC, Lee AS: **The unfolded protein response regulator GRP78/BiP as a novel target for increasing chemosensitivity in malignant gliomas.** *Cancer Res* 2007, **67**(20):9809–9816.
14. Daneshmand S, Quek ML, Lin E, Lee C, Cote RJ, Hawes D, Cai J, Groshen S, Lieskovsky G, Skinner DG, *et al*: **Glucose-regulated protein GRP78 is up-regulated in prostate cancer and correlates with recurrence and survival.** *Hum Pathol* 2007, **38**(10):1547–1552.
15. Fu Y, Li J, Lee AS: **GRP78/BiP inhibits endoplasmic reticulum BIK and protects human breast cancer cells against estrogen starvation-induced apoptosis.** *Cancer Res* 2007, **67**(8):3734–3740.
16. Hetz C: **The UPR as a survival factor of cancer cells: More than folding proteins?** *Leuk Res* 2009.
17. Ran Y, Hu H, Hu D, Zhou Z, Sun Y, Yu L, Sun L, Pan J, Liu J, Liu T, *et al*: **Derlin-1 is overexpressed on the tumor cell surface and enables antibody-mediated tumor targeting therapy.** *Clin Cancer Res* 2008, **14**(20):6538–6545.
18. Virrey JJ, Dong D, Stiles C, Patterson JB, Pen L, Ni M, Schonthal AH, Chen TC, Hofman FM, Lee AS: **Stress chaperone GRP78/BiP confers chemoresistance**

to tumor-associated endothelial cells. *Molecular cancer research: MCR* 2008, **6**(8):1268–1275.
19. Moenner M, Pluquet O, Bouchecareilh M, Chevet E: **Integrated endoplasmic reticulum stress responses in cancer.** *Cancer Res* 2007, **67**(22):10631–10634.
20. Wang G, Yang ZQ, Zhang K: **Endoplasmic reticulum stress response in cancer: molecular mechanism and therapeutic potential.** *Am J Transl Res* 2010, **2**(1):65–74.
21. Tsai YC, Weissman AM: **The Unfolded Protein Response, Degradation from Endoplasmic Reticulum and Cancer.** *Genes Cancer* 2010, **1**(7):764–778.
22. Healy SJ, Gorman AM, Mousavi-Shafaei P, Gupta S, Samali A: **Targeting the endoplasmic reticulum-stress response as an anticancer strategy.** *Eur J Pharmacol* 2009, **625**(1–3):234–246.
23. Rutkowski DT, Hegde RS: **Regulation of basal cellular physiology by the homeostatic unfolded protein response.** *J Cell Biol* 2010, **189**(5):783–794.
24. Yang ZQ, Imoto I, Fukuda Y, Pimkhaokham A, Shimada Y, Imamura M, Sugano S, Nakamura Y, Inazawa J: **Identification of a novel gene, GASC1, within an amplicon at 9p23-24 frequently detected in esophageal cancer cell lines.** *Cancer Res* 2000, **60**(17):4735–4739.
25. Qiu Y, Mao T, Zhang Y, Shao M, You J, Ding Q, Chen Y, Wu D, Xie D, Lin X, *et al*: **A crucial role for RACK1 in the regulation of glucose-stimulated IRE1alpha activation in pancreatic beta cells.** *Sci Signal*, **3**(106):ra7.
26. Tirasophon W, Lee K, Callaghan B, Welihinda A, Kaufman RJ: **The endoribonuclease activity of mammalian IRE1 autoregulates its mRNA and is required for the unfolded protein response.** *Genes Dev* 2000, **14**(21):2725–2736.
27. Iwakoshi NN, Lee AH, Vallabhajosyula P, Otipoby KL, Rajewsky K, Glimcher LH: **Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1.** *Nat Immunol* 2003, **4**(4):321–329.
28. Zhang K, Wang S, Malhotra J, Hassler JR, Back SH, Wang G, Chang L, Xu W, Miao H, Leonardi R, *et al*: **The unfolded protein response transducer IRE1alpha prevents ER stress-induced hepatic steatosis.** *EMBO J* 2011, **30**(7):1357–1375.
29. Yang ZQ, Moffa AB, Haddad R, Streicher KL, Ethier SP: **Transforming properties of TC-1 in human breast cancer: interaction with FGFR2 and beta-catenin signaling pathways.** *Int J Cancer* 2007, **121**(6):1265–1273.
30. Ali-Fehmi R, Che M, Khalifeh I, Malone JM, Morris R, Lawrence WD, Munkarah AR: **The effect of cyclooxygenase-2 expression on tumor vascularity in advanced stage ovarian serous carcinoma.** *Cancer* 2003, **98**(7):1423–1429.
31. Ray ME, Yang ZQ, Albertson D, Kleer CG, Washburn JG, Macoska JA, Ethier SP: **Genomic and expression analysis of the 8p11-12 amplicon in human breast cancer cell lines.** *Cancer Res* 2004, **64**(1):40–47.
32. Forozan F, Veldman R, Ammerman CA, Parsa NZ, Kallioniemi A, Kallioniemi OP, Ethier SP: **Molecular cytogenetic analysis of 11 new breast cancer cell lines.** *Br J Cancer* 1999, **81**(8):1328–1334.
33. Beroukhim R, Mermel CH, Porter D, Wei G, Raychaudhuri S, Donovan J, Barretina J, Boehm JS, Dobson J, Urashima M, *et al*: **The landscape of somatic copy-number alteration across human cancers.** *Nature* 2010, **463**(7283):899–905.
34. Pearce MM, Wang Y, Kelley GG, Wojcikiewicz RJ: **SPFH2 mediates the endoplasmic reticulum-associated degradation of inositol 1,4,5-trisphosphate receptors and other substrates in mammalian cells.** *J Biol Chem* 2007, **282**(28):20104–20115.
35. Pearce MM, Wormer DB, Wilkens S, Wojcikiewicz RJ: **An ER membrane complex composed of SPFH1 and SPFH2 mediates the ER-associated degradation of IP3 receptors.** *J Biol Chem* 2009.
36. Browman DT, Resek ME, Zajchowski LD, Robbins SM: **Erlin-1 and erlin-2 are novel members of the prohibitin family of proteins that define lipid-raft-like domains of the ER.** *J Cell Sci* 2006, **119**(Pt 15):3149–3160.
37. Yoshida H, Matsui T, Hosokawa N, Kaufman RJ, Nagata K, Mori K: **A time-dependent phase shift in the mammalian unfolded protein response.** *Dev Cell* 2003, **4**(2):265–271.
38. Fujimoto T, Onda M, Nagai H, Nagahata T, Ogawa K, Emi M: **Upregulation and overexpression of human X-box binding protein 1 (hXBP-1) gene in primary breast cancers.** *Breast Cancer* 2003, **10**(4):301–306.
39. Davies MP, Barraclough DL, Stewart C, Joyce KA, Eccles RM, Barraclough R, Rudland PS, Sibson DR: **Expression and splicing of the unfolded protein response gene XBP-1 are significantly associated with clinical outcome of endocrine-treated breast cancer.** *Int J Cancer* 2008, **123**(1):85–88.
40. Zhang K, Wong HN, Song B, Miller CN, Scheuner D, Kaufman RJ: **The unfolded protein response sensor IRE1alpha is required at 2 distinct steps in B cell lymphopoiesis.** *J Clin Invest* 2005, **115**(2):268–281.
41. Qiu Y, Mao T, Zhang Y, Shao M, You J, Ding Q, Chen Y, Wu D, Xie D, Lin X, *et al*: **A crucial role for RACK1 in the regulation of glucose-stimulated IRE1alpha activation in pancreatic beta cells.** *Sci Signal* 2010, **3**(106):ra7.
42. Pole JC, Courtay-Cahen C, Garcia MJ, Blood KA, Cooke SL, Alsop AE, Tse DM, Caldas C, Edwards PA: **High-resolution analysis of chromosome rearrangements on 8p in breast, colon and pancreatic cancer reveals a complex pattern of loss, gain and translocation.** *Oncogene* 2006, **25**(41):5693–5706.
43. Haverty PM, Fridlyand J, Li L, Getz G, Beroukhim R, Lohr S, Wu TD, Cavet G, Zhang Z, Chant J: **High-resolution genomic and expression analyses of copy number alterations in breast tumors.** *Genes Chromosomes Cancer* 2008, **47**(6):530–542.
44. Holland DG, Burleigh A, Git A, Goldgraben MA, Perez-Mancera PA, Chin SF, Hurtado A, Bruna A, Ali HR, Greenwood W, *et al*: **ZNF703 is a common Luminal B breast cancer oncogene that differentially regulates luminal and basal progenitors in human mammary epithelium.** *EMBO Mol Med* 2011, **3**(3):167–180.
45. Sircoulomb F, Nicolas N, Ferrari A, Finetti P, Bekhouche I, Rousselet E, Lonigro A, Adelaide J, Baudelet E, Esteyries S, *et al*: **ZNF703 gene amplification at 8p12 specifies luminal B breast cancer.** *EMBO Mol Med* 2011, **3**(3):153–166.
46. Chin K, DeVries S, Fridlyand J, Spellman PT, Roydasgupta R, Kuo WL, Lapuk A, Neve RM, Qian Z, Ryder T, *et al*: **Genomic and transcriptional aberrations linked to breast cancer pathophysiologies.** *Cancer Cell* 2006, **10**(6):529–541.
47. Woods Ignatoski KM, Dziubinski ML, Ammerman C, Ethier SP: **Cooperative interactions of HER-2 and HPV-16 oncoproteins in the malignant transformation of human mammary epithelial cells.** *Neoplasia* 2005, **7**(8):788–798.
48. Behbod F, Kittrell FS, LaMarca H, Edwards D, Kerbawy S, Heestand JC, Young E, Mukhopadhyay P, Yeh HW, Allred DC, *et al*: **An intraductal human-in-mouse transplantation model mimics the subtypes of ductal carcinoma in situ.** *Breast Cancer Res* 2009, **11**(5):R66.
49. Browman DT, Hoegg MB, Robbins SM: **The SPFH domain-containing proteins: more than lipid raft markers.** *Trends Cell Biol* 2007, **17**(8):394–402.
50. Jo Y, Sguigna PV, DeBose-Boyd RA: **Membrane-associated ubiquitin ligase complex containing gp78 mediates sterol-accelerated degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase.** *J Biol Chem* 2011, **286**(17):15022–15031.
51. Shajahan AN, Riggins RB, Clarke R: **The role of X-box binding protein-1 in tumorigenicity.** *Drug News Perspect* 2009, **22**(5):241–246.
52. Shen X, Zhang K, Kaufman RJ: **The unfolded protein response–a stress signaling pathway of the endoplasmic reticulum.** *J Chem Neuroanat* 2004, **28**(1–2):79–92.
53. Hetz C, Martinon F, Rodriguez D, Glimcher LH: **The Unfolded Protein Response: integrating Stress Signals Through the Stress Sensor IRE1 {alpha}.** *Physiol Rev* 2011, **91**(4):1219–1243.
54. Soule HD, Maloney TM, Wolman SR, *et al*: **Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10.** *Cancer Res* 1990, **50**:6075–86.
55. Ethier SP, Mahacek ML, Gullick WJ, Frank TS, Weber BL: **Differential isolation of normal luminal mammary epithelial cells and breast cancer cells from primary and metastatic sites using selective media.** *Cancer Res* 1993, **53**:627–35.
56. Ethier SP, Kokeny KE, Ridings JW, Dilts CA: **erbB family receptor expression and growth regulation in a newly isolated human breast cancer cell line.** *Cancer Res* 1996, **56**:899–907.
57. Forozan F, Veldman R, Ammerman CA, *et al*: **Molecular cytogenetic analysis of 11 new breast cancer cell lines.** *Br J Cancer* 1999, **81**:1328–34.
58. Ray ME, Yang ZQ, Albertson D, *et al*: **Genomic and expression analysis of the 8p11-12 amplicon in human breast cancer cell lines.** *Cancer Res* 2004, **64**:40–7.

doi:10.1186/1471-2407-12-225
Cite this article as: Wang *et al.*: ERLIN2 promotes breast cancer cell survival by modulating endoplasmic reticulum stress pathways. *BMC Cancer* 2012 **12**:225.

BJ Cell

View this PDF in Utopia Documents for more interactive functionality
Download FREE software now

Biochem. J. (2012) **446**, 415–425 (Printed in Great Britain) doi:10.1042/BJ20112050

online data

Endoplasmic reticulum factor ERLIN2 regulates cytosolic lipid content in cancer cells

Guohui WANG*[1], Xuebao ZHANG*[1], Jin-Sook LEE*, Xiaogang WANG†, Zeng-Quan YANG†‡[2] and Kezhong ZHANG*†§[2]

*Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, MI 48201, U.S.A., †Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI 48201, U.S.A., ‡Department of Oncology, Wayne State University School of Medicine, Detroit, MI 48201, U.S.A., and §Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, MI 48201, U.S.A.

Increased *de novo* lipogenesis is a hallmark of aggressive cancers. Lipid droplets, the major form of cytosolic lipid storage, have been implicated in cancer cell proliferation and tumorigenesis. Recently, we identified the *ERLIN2* [ER (endoplasmic reticulum) lipid raft-associated 2) gene that is amplified and overexpressed in aggressive human breast cancer. Previous studies demonstrated that ERLIN2 plays a supporting oncogenic role by facilitating the transformation of human breast cancer cells. In the present study, we found that ERLIN2 supports cancer cell growth by regulating cytosolic lipid droplet production. ERLIN2 is preferably expressed in human breast cancer cells or hepatoma cells and is inducible by insulin signalling or when cells are cultured in lipoprotein-deficient medium. Increased expression of ERLIN2 promotes the accumulation of cytosolic lipid droplets in breast cancer cells or hepatoma cells in response to insulin or overload of unsaturated fatty acids. ERLIN2 regulates activation of SREBP (sterol regulatory element-binding protein) 1c, the key regulator of *de novo* lipogenesis, in cancer cells. ERLIN2 was found to bind to INSIG1 (insulin-induced gene 1), a key ER membrane protein that blocks SREBP activation. Consistent with the role of ERLIN2 in regulating cytosolic lipid content, down-regulation of ERLIN2 in breast cancer or hepatoma cells led to lower cell proliferation rates. The present study revealed a novel role for ERLIN2 in supporting cancer cell growth by promoting the activation of the key lipogenic regulator SREBP1c and the production of cytosolic lipid droplets. The identification of ERLIN2 as a regulator of cytosolic lipid content in cancer cells has important implications for understanding the molecular basis of tumorigenesis and the treatment of cancer.

Key words: cancer, endoplasmic reticulum (ER), ER membrane lipid raft-associated 2 (ERLIN2), lipogenesis, oncogenesis.

INTRODUCTION

Increased lipid and energy metabolism is a prominent feature of cancer [1]. It has been demonstrated that the lipogenic phenotype and activation of lipogenic enzymes correlate with a poorer prognosis and shorter disease-free survival for patients with different tumour types [2]. Production of lipid droplets, the main cytosolic lipid storage organelle in eukaryotic cells, is closely associated with *de novo* lipogenesis [3]. Indeed, elevated lipid droplet content is implicated in cancer cell proliferation and tumorigenesis and has been increasingly recognized as a hallmark of aggressive cancers [2,4].

ERLIN2 {ER (endoplasmic reticulum) membrane lipid raft-associated 2; also known as SPFH2 [SPFH (stomatin/prohibitin/flotillin/HflK/C) domain family, member 2] and C8ORF2 [chromosome 8 open reading frame 2]} and its homologue ERLIN1 belong to a larger family of proteins that share an evolutionarily conserved SPFH domain [5]. Recent high-resolution genomic analyses of copy number in human breast cancer specimens demonstrated that high-level amplification of the *ERLIN2* region occurs in 28 % of cases [6]. On the basis of statistical analysis of copy number increase and overexpression, we and others have identified the *ERLIN2* gene as one of several candidate oncogenes within the 8p11-12 amplicon [7–11]. Together with other identified oncogenes in the 8p11-12 amplicon, *ERLIN2* promotes transformation of human breast cancer cells, although it does not behave as a classical transforming oncogene, such as receptor tyrosine kinases and the small GTPase Ras [7,10–12]. Previous studies suggested that ERLIN2 might mediate ER-associated protein degradation [5,13,14]. ERLIN1 and ERLIN2 interact with each other to form a functional complex. ERLIN2 can bind to the activated inositol trisphosphate receptors and other ERAD (ER-associated degradation) substrates, leading to polyubiquitination and subsequent degradation of these substrates [13,14]. ERLIN2 can also interact with ER-resident proteins GP78 [AMFR (autocrine motility factor receptor)] and TMUB1 (transmembrane and ubiquitin-like domain containing 1) to mediate degradation of HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase [15]. However, previous studies were focused on the biochemical characterization of ERLIN2 as a mediator of the ERAD pathway. The precise role and mechanism of ERLIN2 in aggressive cancer cells, where the *ERLIN2* gene is amplified and overexpressed, remain poorly understood.

In the present study, we found that ERLIN2 plays an important role in regulating cytosolic lipid content and activation of SREBP (sterol regulatory element-binding protein) 1c, a key lipogenic

Abbreviations used: AHF, atherogenic high-fat; BODIPY, boron dipyrromethene; CHO, Chinese hamster ovary; DAPI, 4′,6-diamidino-2-phenylindole; DGAT, diacylglycerol *O*-acyltransferase; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERLIN2, ER membrane lipid raft-associated 2; FAS, fatty acid synthase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; INSIG1, insulin-induced gene 1; IP, immunoprecipitation; LPDS, lipoprotein-deficient serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; NP-40, Nonidet P40; PGC, peroxisome-proliferator-activated receptor γ coactivator; RT, reverse transcription; shRNA, small hairpin RNA; SPFH, stomatin/prohibitin/flotillin/HflK/C; SREBP, sterol regulatory element-binding protein; SCAP, SREBP cleavage-activating protein; TG, triglyceride.

[1] These authors contributed equally to this work.

[2] Correspondence may be addressed to either of these authors (email kzhang@med.wayne.edu or yangz@karmanos.org).

© The Authors Journal compilation © 2012 Biochemical Society

Biochemical Journal www.biochemj.org

regulator in human breast cancer cells and hepatoma cells. Furthermore, knockdown of endogenous ERLIN2 led to reduced cancer cell proliferation rates. Our finding that ERLIN2 regulates lipogenesis in cancer cells contributes to our understanding of the molecular basis governing lipid metabolism in tumorigenesis and could have important applications in cancer therapy.

MATERIALS AND METHODS

Materials

Chemicals were purchased from Sigma unless indicated otherwise. Synthetic oligonucleotides were purchased from Integrated DNA Technologies. Human insulin was purchased from Eli Lilly. Antibodies against FLAG, ERLIN1 and α-tubulin were purchased from Sigma. An antibody against ERLIN2 was purchased from Cell Signaling Technology. An antibody against SREBP1c was purchased from Thermo Scientific. ERLIN1 and ERLIN2 ON-TARGETplus siRNA SMARTpool® reagents were purchased from Dharmacon. Antibodies against T7, V5, INSIG1 (insulin-induced gene 1), SREBP1a and FAS (fatty acid synthase) were purchased from Santa Cruz Biotechnology. An antibody against SCAP (SREBP cleavage-activating protein) was purchased from Abcam. The monoclonal antibody against C-terminal SREBP2 was purchased from BD Pharmingen, and the polyclonal antibody against N-terminal SREBP2 was from Cayman Chemicals. The photo-reactive amino acids kit was from Thermo Scientific Pierce. The kit for measuring TGs (triglycerides) was from BioAssay System. The plasmid expressing T7-tagged human INSIG1 used in the present study was provided by Dr Jin Ye (University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.) [16].

Cancer cell culture, lentivirus construction and transduction of cells

The human breast cancer cell lines SUM44 and SUM225, and the human mammary epithelial cell line MCF10A were cultured as described previously [12,17,18]. The human breast cancer cell line ZR-75-1 was purchased from the A.T.C.C. The human hepatocellular carcinoma cell line Huh-7 was cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10 % FBS (fetal bovine serum), L-glutamine and antibiotics at 37 °C in a 5 % CO_2 environment [19]. The details regarding the origins and culture conditions of these cancer cell lines are described in the Supplementary Online data (at http://www.BiochemJ.org/bj/446/bj4460415add.htm). The lentiviral expression construct containing human *ERLIN2* (pLenti-ERLIN2) was established as described previously [7]. The lentivirus for pLenti-ERLIN2 was used to infect MCF10A or Huh-7 cells. Control infections with pLenti-LacZ virus were performed in parallel with the pLenti-ERLIN2 infections. Selection began 48 h after infection in growth medium with 10 μg/ml blasticidin. Upon confluence, selected cells were passaged and serially cultured.

Lentivirus-mediated shRNA (small hairpin RNA) knockdown of gene expression

We knocked down the expression of the human *ERLIN2* gene in human breast cancer cell lines SUM225 and SUM44 or human hepatoma cell line Huh-7 by using the Expression Arrest GIPZ lentiviral shRNAmir system (OpenBiosystems). Lentivirus was produced by transfecting HEK (human embryonic kidney)-293FT cells with a combination of the lentiviral expression plasmid DNA and the Trans-Lentiviral packaging mix (OpenBiosystems). For cell infection, viral supernatants were supplemented with 6 μg/ml polybrene and incubated with the cells for 24 h. The cells expressing shRNA were selected with puromycin for 2–3 weeks for functional studies (cell proliferation assays) and for 4–10 days after infection for RNA extraction.

BODIPY (boron dipyrromethene) staining of lipid droplets

The cells were washed with PBS, fixed with 3 % formaldehyde for 15 min and stained with BODIPY 493/503 (stock 1 mg/ml, working solution 1:1000 dilution; Invitrogen) for 15 min at room temperature (25°C). Cells were then mounted with Prolong gold antifade reagent containing DAPI (4′,6-diamidino-2-phenylindole; Invitrogen).

Oil Red O staining of lipid droplets

Frozen liver tissue sections were stained with Oil Red O according to the standard protocol to visualize lipid droplet content. Briefly, frozen liver tissue sections of 8 μm were air-dried and then fixed in formalin. The fixed sections were rinsed with 60 % propan-2-ol before they were stained with freshly prepared Oil Red O solution for 15 min. After Oil Red O staining, the liver sections were rinsed again with 60 % propan-2-ol followed by washing with water.

Incorporation of photo-reactive amino acids and UV cross-linking to analyse the protein binding complex

Cells at 60–70 % confluence were washed twice with PBS and cultivated with DMEM limiting medium (without L-leucine and L-methionine) containing 2 mM photo-reactive leucine and 4 mM photo-reactive methionine analogues supplemented with 10 % dialysed FBS for 24 h. After washing twice with PBS, cells were UV-irradiated using a Stratalinker 1800 (UVA output at 1 cm = 3000 μW/cm^2) for 12 min. The cell lysates were then collected in NP-40 (Nonidet P40) lysis buffer for IP (immunoprecipitation) Western blot analysis. The photo-reactive amino acids kit, DMEM limiting medium and dialysed FBS were purchased from Thermo Scientific Pierce.

Cell proliferation assay

Cell proliferation rates were determined using CellTiter 96 non-radioactive cell proliferation assay {MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide]} kits from Promega. Briefly, approximately 5000 cells per well were seeded in triplicate in 96-well culture plates and allowed to attach for 24 h. After cell culture for 3, 6, 9 and 12 days, 20 μl of MTT (5 mg/ml) solution was added to 200 μl of medium in each well. Cells were cultured for an additional 4 h to allow MTT to be well metabolized. After that, the medium was aspirated, and 200 μl of DMSO was added into the well to dissolve the purple formazan crystals. The absorbance of the plate was measured at 570 nm using a plate reader.

Western blot and IP Western blot analyses

To determine expression levels of ERLIN1, ERLIN2, SREBPs, INSIG1, α-tubulin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), total cell lysates were prepared from cultured cells or liver tissue by using NP-40 lysis as described previously [20]. Denatured proteins were separated by SDS/PAGE (10 %

© The Authors Journal compilation © 2012 Biochemical Society

Tris-Glycine polyacrylamide gels) and transferred on to a 0.45-mm PVDF membrane (GE Healthcare). Membrane-bound antibodies were detected by an enhanced chemiluminescence detection reagent (GE Healthcare). For IP Western blot analysis, total protein lysates from *in vitro* cultured cells were immunoprecipitated with the anti-ERLIN1, anti-ERLIN2 or anti-V5 antibody, followed by Western blot analysis with the anti-ERLIN1, anti-SCAP or anti-T7 antibody.

Quantitative real-time RT (reverse transcription)–PCR analysis

For real-time RT–PCR analysis, total cellular RNA was prepared using TRIzol reagent (Invitrogen) and reverse-transcribed to cDNA using a random primer. The real-time PCR reaction mixture containing cDNA template, primers and SYBR Green PCR Master Mix (Invitrogen) was run in a 7500 Fast Real-time PCR System (Applied Biosystems). The sequences of the PCR primers used in the present study are provided in the Supplementary Table S1 (at http://www.BiochemJ.org/bj/446/bj4460415add.htm). Fold changes of mRNA levels were determined after normalization to internal control β-actin RNA levels.

Statistics

Experimental results (quantitative real-time RT–PCR analysis, MTT assay and quantitative Western blot analysis) were calculated as means $\pm$ S.E.M. (for variation between animals or experiments). The differences among means between multiple (more than two) groups were analysed by one-way ANOVA followed by post-hoc comparisons of group means with the Tukey–Kramer test. The mean values for biochemical data from two experimental groups were compared by a paired or unpaired two-tailed Student's *t* test. Differences of $P < 0.05$ were considered statistically significant.

RESULTS

ERLIN2 is overexpressed in human cancer cells and inducible by metabolic stress signals

Previously we and others demonstrated that the *ERLIN2* gene was highly amplified in human breast cancer cell lines and breast tumours of aggressive forms [7–9]. Western blot analysis indicated that ERLIN2 was overexpressed in the aggressive human breast cancer cell lines SUM225, ZR-75-1, SUM44 and SUM52, and it was modestly expressed in the non-transformed human mammary epithelial cell line MCF10A (Figure 1A, [7]). Moreover, ERLIN2 was expressed in human hepatoma cell lines, including HepG2 and Huh-7, but only slightly expressed in murine primary hepatocytes (Figure 1A). Evidence suggests that elevated lipogenesis is essential for tumour cell survival and malignancy maintenance [2,4]. To elucidate the involvement of ERLIN2 in the lipogenic phenotype of human cancer cells, we exposed murine primary hepatocytes to metabolic stress conditions that are associated with lipogenesis and/or cancer cell growth. First, we found that expression of endogenous ERLIN2 in murine primary hepatocytes was inducible by insulin, a metabolic signal that induces activation of SREBP1c and *de novo* lipogenesis in the liver (Figure 1B) [21]. Secondly, expression of endogenous ERLIN2 was higher in primary hepatocytes cultured in medium containing LPDS (lipoprotein-deficient serum), a metabolic stress condition that triggers SREBP activation and *de novo* lipogenesis, in a time-dependent manner (Figure 1C). To further elucidate the effect of the insulin signal or LPDS on the induction of ERLIN2, we examined the induction of *ERLIN2* mRNA in murine primary hepatocytes in response to insulin or LPDS challenge. Quantitative real-time RT–PCR analysis indicated that expression of *ERLIN2* mRNA in primary hepatocytes is inducible by insulin or LPDS (Supplementary Figure S1 at http://www.BiochemJ.org/bj/446/bj4460415add.htm). However, there is a discrepancy in expression patterns between the ERLIN2 protein and mRNA levels. The discrepancy between protein and mRNA levels, which may be due to post-transcriptional regulation, has been observed with many genes involved in lipid metabolism [21–24].

We also evaluated the expression of ERLIN2 in the liver of mice receiving normal chow or an AHF (atherogenic high-fat) diet known to induce atherosclerosis and fatty liver disease in murine models [25,26]. Expression levels of the ERLIN2 protein in the steatoic livers of the mice fed the AHF diet were much higher than those in mice fed the normal diet (Figure 1D). Quantitative real-time RT–PCR analysis confirmed that expression of *ERLIN2* mRNA was significantly higher in the livers of the mice fed the AHF diet compared with the mice fed normal chow (Figure 1E). Together, the results suggest that expression of ERLIN2 is up-regulated in cancer cells and inducible by insulin or LPDS challenge. These findings motivated us to investigate the involvement of ERLIN2 in lipid metabolism associated with tumorigenesis.

ERLIN2 is required for cytosolic lipid droplet accumulation in cancer cells after insulin stimulation or overload of oleic acid

To assess the potential involvement of ERLIN2 in lipid metabolism in cancer cells, we analysed cytosolic lipid content in human mammary epithelial cells, breast cancer cells or hepatoma cells in which exogenous ERLIN2 was overexpressed or endogenous ERLIN2 was knocked down. Using a lentiviral expression system, we established a human mammary epithelial cell line (MCF10A) that stably expresses ERLIN2 or control LacZ (Figure 2A). Cytosolic lipid droplets, as indicated by BODIPY staining, accumulated in the MCF10A cells overexpressing exogenous ERLIN2, but not the LacZ control (Figure 2B). The human breast cancer cell line SUM225, in which the *ERLIN2* gene was amplified and overexpressed, possesses abundant cytosolic lipid droplet contents (Figures 2C and 2D). However, when the endogenous *ERLIN2* gene was knocked down, the levels of lipid droplet contents in the ERLIN2-knockdown SUM225 cells were significantly reduced. The reduction of lipid droplet contents in the absence of ERLIN2 was consistent with cellular TG levels in the ERLIN2-knockdown breast cancer cells (Supplementary Figure S2 at http://www.BiochemJ.org/bj/446/bj4460415add.htm). Together, these results suggest a potential role for ERLIN2 in promoting lipid droplet production in human breast cancer cells.

To verify the role of ERLIN2 in cytosolic lipid accumulation, we used the human hepatocellular carcinoma cell line Huh-7 [19], which has been used to study tumorigenesis or hepatic lipid metabolism. The *ERLIN2* gene was stably silenced in Huh-7 cells by using a lentivirus-based shRNA system. The result of Western blot analysis indicated that expression levels of ERLIN2 protein were markedly reduced in the ERLIN2-knockdown Huh-7 cells, compared with the control cells transduced by a non-silencing shRNA control (Figure 3A). The ERLIN2-knockdown Huh-7 cells had much lower levels of lipid droplet content, indicated by Oil Red O staining, than the control cells (Figure 3B). Overexpression of ERLIN2 significantly increased lipid droplet

© The Authors Journal compilation © 2012 Biochemical Society

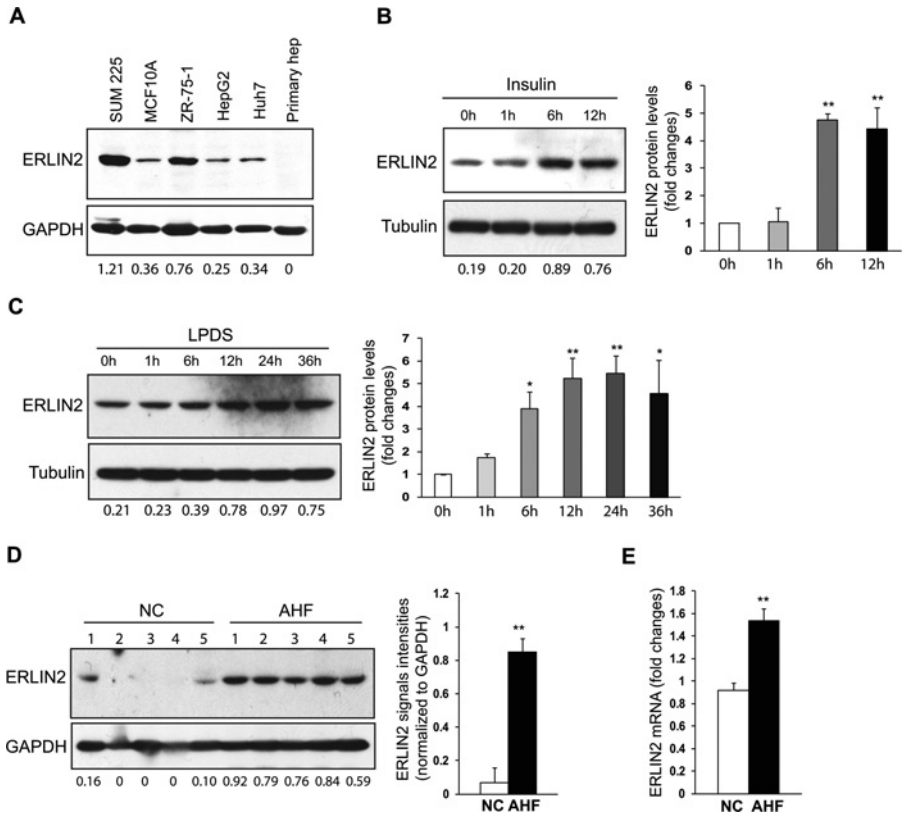

Figure 1 ERLIN2 is preferentially expressed in cancer cells and is inducible by metabolic signals

(**A**) Western blot analysis of ERLIN2 protein levels in the human breast cancer cell lines SUM225 and ZR-75-1, non-tumorigenic mammary epithelial cell line MCF10A, human hepatoma cell lines HepG2 and Huh-7, and murine primary hepatocytes (hep). Levels of GAPDH were included as loading controls. The values below the gels indicate ERLIN2 protein signal intensities [quantified using ImageJ (http://rsbweb.nih.gov/ij/)] after normalization to GAPDH signal intensities. (**B**) Western blot analysis of ERLIN2 protein levels in murine primary hepatocytes challenged with insulin (100 nM) for 1, 6 or 12 h. Murine primary hepatocytes were cultured in normal medium with vehicle buffer PBS added as a control (0 h under insulin). Tubulin was included as a loading control. The values below the gels indicate the ERLIN2 protein signal intensities after normalization to tubulin signal intensities. The graph beside the images shows fold changes of normalized ERLIN2 protein signal intensities (compared with 0 h control). Results are means ± S.E.M. ($n = 3$ experimental repeats); $^{**}P < 0.01$. (**C**) Western blot analysis of ERLIN2 protein levels in murine primary hepatocytes cultured in medium containing LPDS for 1, 6, 12, 24 or 36 h. As a control, murine primary hepatocytes were cultured in normal medium containing 10 % FBS (0 h under LPDS). Tubulin was included as a loading control. The values below the gels indicate the ERLIN2 protein signal intensities after normalization to tubulin signal intensities. The graph beside the images shows fold changes of normalized ERLIN2 protein signal intensities (compared with 0 h control). Results are means ± S.E.M. ($n = 3$ experimental repeats); $^{*}P < 0.05$; $^{**}P < 0.01$. (**D**) Western blot analysis of ERLIN2 protein levels in liver tissues of mice given normal chow (NC) or an AHF diet for 6 months. GAPDH was included as a loading control. The values below the gels indicate the ERLIN2 protein signal intensities after normalization to GAPDH signal. The graph beside the images shows ERLIN2 protein signal intensities in the livers of mice fed with normal chow or AHF diet after normalization to GAPDH. Results are means ± S.E.M. ($n = 5$); $^{**}P < 0.01$. For (**A**–**D**), the experiments were repeated three times and representative data are shown. (**E**) Quantitative real-time RT–PCR analysis of expression of *ERLIN2* mRNA in the liver of age-matched male mice given normal chow (NC) or the AHF diet for 6 months. Expression values were normalized to β-actin mRNA levels. The baseline of the *ERLIN2* mRNA level in one of the NC-fed mice was set to 1. Fold changes of the *ERLIN2* mRNA levels in other mice were calculated by comparing with the baseline mRNA level. Results are means ± S.E.M. ($n = 6$ mice per group). $^{**}P < 0.01$.

accumulation in Huh-7 cells, consistent with the observation that ERLIN2 overexpression increased lipid droplet production in MCF10A cells (Figure 2B). It is known that insulin can trigger *de novo* lipogenesis in hepatocytes, whereas overload of oleic acid, a monounsaturated fatty acid, can lead to cytosolic lipid droplet accumulation and steatosis in cultured hepatoma cells [27,28]. To further delineate the effect of ERLIN2 on cytosolic lipid droplet production, we challenged non-silencing control, ERLIN2-knockdown and ERLIN2-overexpressing Huh-7 cells with insulin or oleic acid. The ERLIN2-knockdown Huh-7 cells exhibited significantly less lipid droplet accumulation, whereas the ERLIN2-overexpressing Huh-7 cells displayed markedly greater hepatic steatosis compared with control Huh-7 cells, in response to insulin stimulation or oleic acid feeding (Figure 3). The lipid droplet staining results were consistent with the biochemical quantification of cellular TG levels in the related Huh-7 cells (Supplementary Figure S2). Together, these findings support the role of ERLIN2 in *de novo* lipogenesis and lipid droplet production.

ERLIN2 is associated with the activation of SREBPs in cancer cells

The ER is the organelle responsible for lipid and sterol biosynthesis. SREBP1 and SREBP2 are ER transmembrane proteins that play central roles in controlling expression of genes encoding key regulators and enzymes in *de novo* lipogenesis [21]. Among others, SREBP1c is inducible in liver and adipose tissue by insulin change or fasting/refeeding conditions, and it plays a critical role in nutritional regulation of lipogenic gene expression [21]. Induction of SREBP1c, but not SREBP1a or SREBP2, is evidenced in numerous primary human breast tumours and breast cancer cell lines [29]. Previous studies indicated that fatty acid synthesis and expression of lipogenic genes in breast

© The Authors Journal compilation © 2012 Biochemical Society

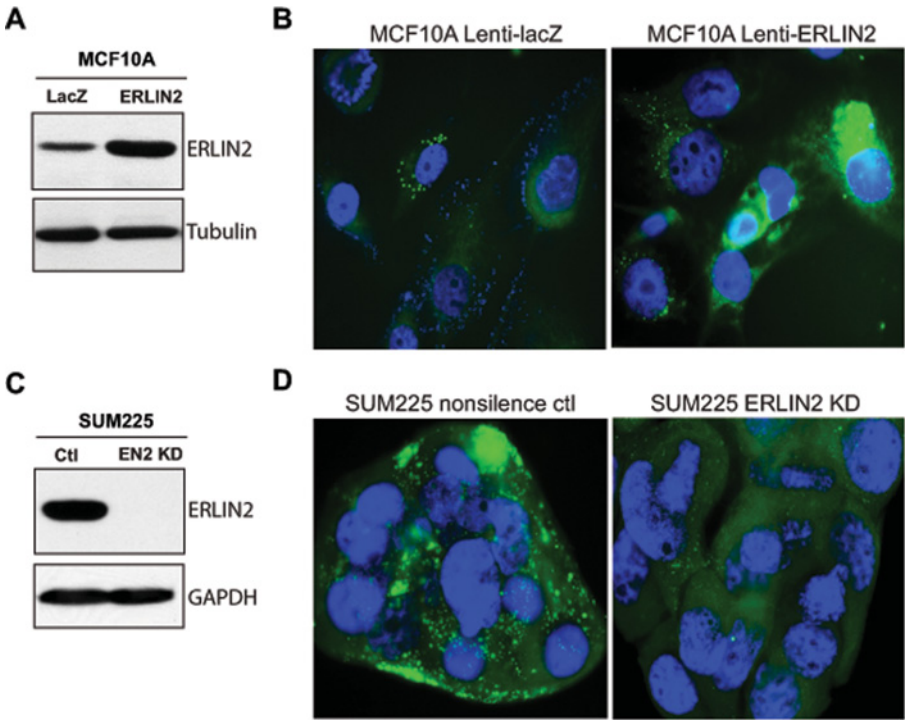

Figure 2 ERLIN2 regulates levels of cytosolic lipid content in human breast cancer cells

(**A**) Western blot analysis of ERLIN2 levels in MCF10A cells stably overexpressing LacZ or ERLIN2 through a lentivirus-based expression system. Tubulin was used as a loading control. (**B**) BODIPY staining of lipid droplets in MCF10A cells stably overexpressing LacZ or ERLIN2. The cells were counterstained with DAPI. Magnification is ×630. (**C**) Western blot analysis of ERLIN2 levels in human breast cancer cell line SUM225, in which ERLIN2 is stably knocked down (EN2 KD) and its control cell line (Ctl). GAPDH was used as a loading control. (**D**) BODIPY staining of lipid droplets in the ERLIN2 knockdown (KD) and control (ctl) SUM225 cells. Magnification is ×630. For (**A**–**D**) the experiments were repeated three times and representative data are shown.

cancer is achieved through modulation of SREBP1c, similar to its regulation in liver and adipose tissue, although the upstream regulation of lipogenesis differs in these tissues [21,29].

It has been shown that activation of SREBP is controlled by interactions involving ER-resident proteins that are regulated by metabolic signals [21]. In particular, the SREBP precursor proteins interact with ER membrane SCAP, and SCAP binds to another ER membrane protein called INSIG1 to maintain SREBPs in an inactive state [30]. In response to low sterol or insulin stimuli, INSIG1 dissociates from SCAP and is subsequently degraded through ERAD, thus allowing SREBP activation [30–32]. Because ERLIN2 is an ER lipid raft protein and has been characterized as a mediator of ERAD [13,14], we suspected that ERLIN2 might regulate lipogenesis by modulating the activation of SREBPs and/or ER-associated degradation of INSIG1. To explore this possibility, we first examined activation of SREBP1c in a mammary epithelial cell line (MCF10A) that overexpresses exogenous ERLIN2 or LacZ control. Levels of the cleaved/activated form of SREBP1c were significantly higher in the MCF10A cells overexpressing ERLIN2, compared with that of the cells overexpressing LacZ (Figure 4A). We further evaluated activation of SREBP1c in SUM44, an aggressive human breast cancer cell line in which the endogenous *ERLIN2* gene is amplified and overexpressed [7] (Figure 2C). We generated SUM44 stable cell lines in which ERLIN2 was knocked down through a lentivirus-based shRNA expression system. Supporting a role for ERLIN2 in regulating SREBP activation, the levels of total cleaved SREBP1c protein were lower in the ERLIN2 knockdown SUM44 cells, compared with that in the control cells (Figure 4B). Interestingly, SREBP cleavage products in SUM44 cells appeared as multiple isoforms that might represent phosphorylated or SUMOylated forms of mature SREBP under different metabolic conditions [33,34]. Moreover, the levels of the SREBP1c precursor were also modestly lower in the ERLIN2-knockdown SUM44 cells (Figure 4B). Additionally, we examined levels of SREBP1a in the ERLIN2-knockdown or control breast cancer cells. Consistent with the previous observation that SREBP1c, but not SREBP1a or SREBP2, is induced in human breast tumours and breast cancer cell lines [29], the ERLIN2 knockdown or overexpressing breast cancer cell lines express only trace levels of SREBP1a (Supplementary Figure S3 at http://www.BiochemJ.org/bj/446/bj4460415add.htm).

Next, we confirmed the involvement of ERLIN2 in SREBP activation by using the hepatoma cell line Huh-7. In liver hepatocytes, activation of SREBP1c is tightly controlled by feedback regulation [21]. To circumvent the potential adaptation of SREBP activation in stable ERLIN2 knockdown Huh-7 cells, we transiently knocked down ERLIN2 and/or its functional binding partner, ERLIN1, in Huh-7 cells by using ON-TARGETplus siRNA SMARTpool® reagents [35]. Transient knockdown of ERLIN2 and/or ERLIN1 significantly reduced the levels of mature SREBP1c proteins in the Huh-7 cells in the absence or presence of insulin (Figure 4C). Moreover, levels of cleaved SREBP2 proteins were also reduced in the ERLIN2 and/or ERLIN1 knockdown Huh-7 cells, compared with those in control Huh-7 cells (Figure 4C and Supplementary Figure S4 at http://www.BiochemJ.org/bj/446/bj4460415add.htm). These results suggest that ERLIN2 plays a role in regulating SREBP activation. Note that the results obtained with ERLIN1-knockdown cells suggested that ERLIN1 may also be involved in regulation of SREBP activation. Because ERLIN2 is known to

© The Authors Journal compilation © 2012 Biochemical Society

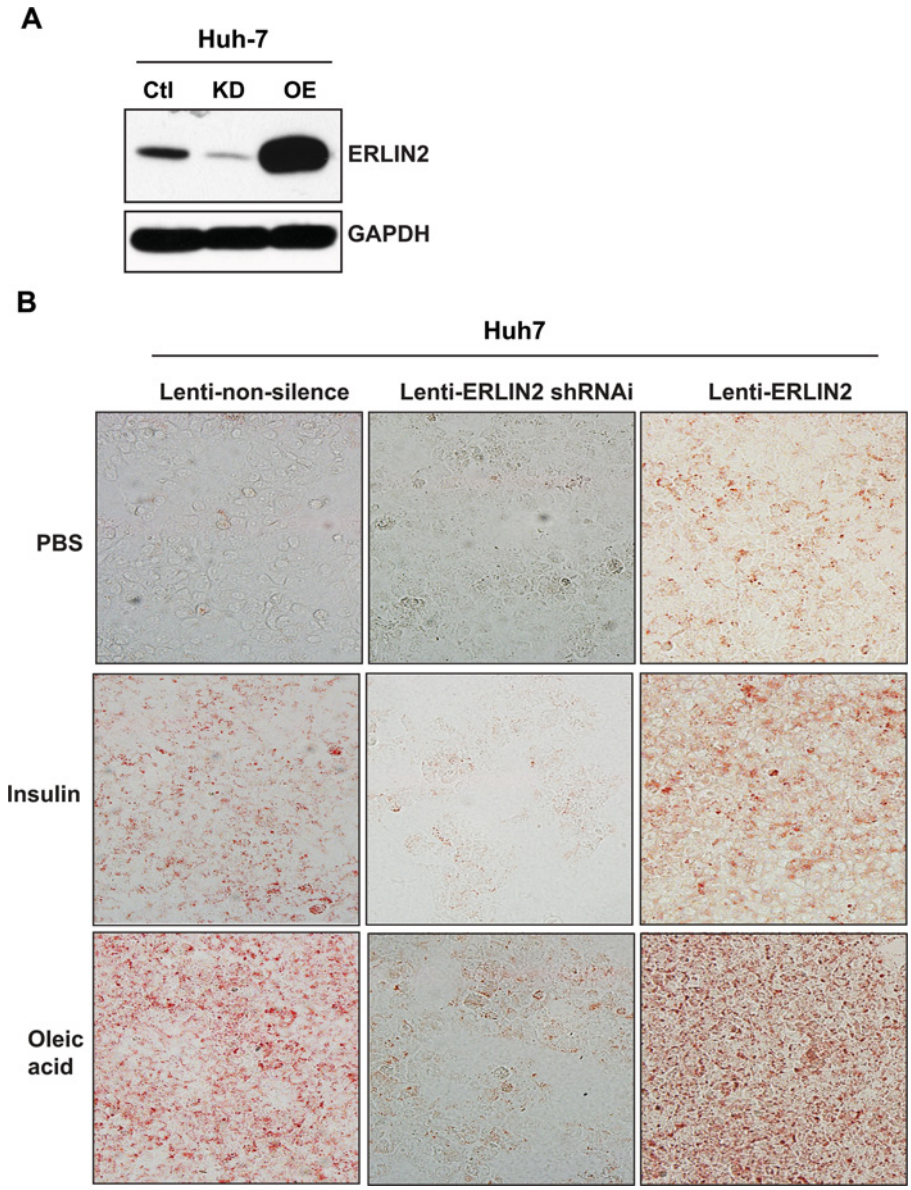

Figure 3 ERLIN2 regulates levels of cytosolic lipid content in human hepatoma cells in the absence or presence of metabolic signals

(**A**) Western blot analysis of ERLIN2 levels in the Huh-7 stable cell line, which was transduced with non-silencing control shRNA (Ctl), knocked down by ERLIN2 shRNAi (KD) or overexpressed ERLIN2 (OE) via lentivirus. GAPDH was included as a loading control. (**B**) Oil Red O staining of lipid droplets in the Huh-7 stable cell line, which was transduced with non-silencing control (lenti-non-silence), ERLIN2 shRNAi (lenti-ERLIN2 shRNAi) or ERLIN2 overexpression (lenti-ERLIN2) lentivirus and treated with vehicle PBS, insulin (100 nM) or oleic acid (0.5 mM) for 12 h. Magnification is ×200. The experiments were repeated three times and representative data are shown.

dimerize with ERLIN1 to form a functional complex [5,13,14], it is possible that knockdown of ERLIN1 may destabilize ERLIN2 and thus reduce SREBP activation in cancer cells. The involvement of ERLIN1 in regulating SREBP activation needs to be further elucidated in the future. Since ERLIN2 has been proposed as a mediator of ERAD [13,14], we wondered whether ERLIN2 regulates SREBP activation by facilitating INSIG1 degradation through the ERAD mechanism. The levels of INSIG1 proteins were not significantly changed in the ERLIN1- and/or ERLIN2-knockdown cells compared with the control cells (Figure 4C). Together, our data suggest that, although expression of ERLIN2 has a marginal effect on INSIG1 degradation, ERLIN2 regulates SREBP activation in cancer cells.

To further elucidate the role of ERLIN2 in *de novo* lipogenesis, we used ERLIN2 knockdown and control SUM44 or Huh-7 cells to examine the expression of genes that encode key lipogenic enzymes or regulators. Quantitative real-time RT–PCR analysis indicated that expression levels of SREBP1-regulated lipogenic genes, including *ACC1* (acetyl-CoA carboxylase 1) and *SCD1* (stearoyl-CoA desaturase 1) and other key lipogenic genes, including *DGAT* (diacylglycerol *O*-acyltransferase) 1, *DGAT*2, *ADRP* (adipose differentiation-related protein), *FIT1* (fat-inducing transcript 1), *FATP2* (fatty acid transport protein 2) and *FSP27* (fat-specific protein 27), were lower in ERLIN2 knockdown SUM44 and Huh-7 cells (Supplementary Figures S5A–S5C at http://www.BiochemJ.org/bj/446/bj4460415add.htm). We also

© The Authors Journal compilation © 2012 Biochemical Society

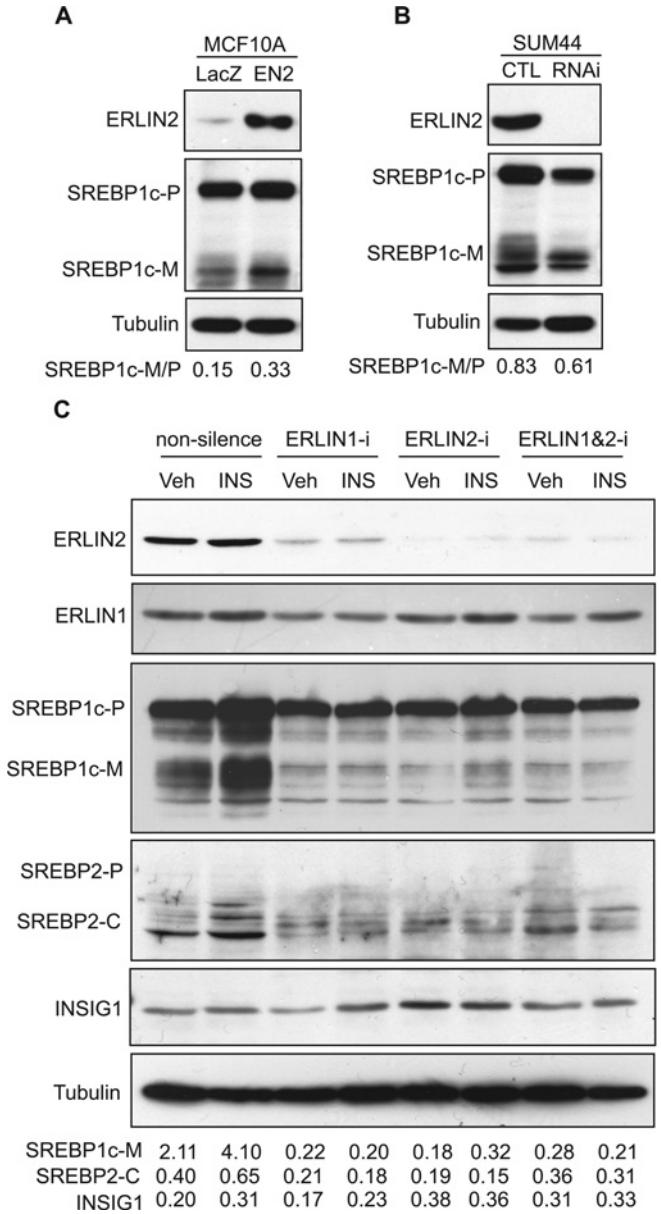

Figure 4 ERLIN2 regulates activation of SREBP1c

(**A**) Western blot analysis of ERLIN2 and SREBP1c levels in MCF10A cells that stably express exogenous ERLIN2 or LacZ control. Tubulin was included as a loading control. The values below the gels represent the ratios of signal intensities of mature SREBP1c to SREBP1c precursor. (**B**) Western blot analysis of ERLIN2, SREBP1c and tubulin levels in stable ERLIN2 knockdown SUM44 cells and control (CTL) SUM44 cells that were transduced by non-silencing shRNA. The values below the gels represent the ratios of signal intensities of mature SREBP1c to SREBP1c precursor. (**C**) Western blot analysis of ERLIN1, ERLIN2, SREBP1c, SREBP2 and INSIG1 protein levels in Huh-7 cells in which ERLIN1 and/or ERLIN2 were transiently knocked down. The *ERLIN1* and/or *ERLIN2* genes were transiently knocked down in Huh-7 cells by using ON-TARGETplus siRNA SMARTpool® reagents (Dharmacon). The Huh-7 cells transduced with non-silencing siRNA were included as the control. After 36 h, the control and knockdown cell lines were treated with PBS vehicle or insulin (100 nM) for 6 h followed by a collection of total cell lysates for Western blot analysis. The SREBP2 signal was detected by using a monoclonal antibody against a C-terminal SREBP protein fragment (BD Pharmingen). The values below the gels represent the ratios of mature SREBP1c to SREBP1c precursor, cleaved SREBP2 to tubulin, and INSIG1 to tubulin signal intensities. INS, insulin; RNAi, RNA interference; SREBP2-C, cleaved SREBP2 (C-terminal); SREBP1c-M, mature SREBP1c; SREBP1c-P, SREBP1c precursor; Veh, vehicle.

examined expression of lipogenic *trans*-activators in ERLIN2 knockdown and control Huh-7 cells. Expression levels of the genes encoding the lipogenic *trans*-activators *PGC* [PPARγ (peroxisome-proliferator-activated receptor γ) coactivator]-1α and *PGC-1*β were significantly lower in ERLIN2 knockdown Huh-7 cells (Supplementary Figure 5D). Additionally, we examined expression of FAS, a key SREBP1-regulated enzyme in *de novo* lipogenesis. Expression levels of *FAS* mRNA were only marginally altered by the absence of ERLIN2 (results not shown), whereas FAS protein levels were reduced in ERLIN2 knockdown Huh-7 cells (Supplementary Figure S6 at http://www.BiochemJ.org/bj/446/bj4460415add.htm). The discrepancy in the expression patterns between the *FAS* mRNA and protein levels might be due to feedback regulation of the genes involved in lipid homoeostasis [21,23,24].

ERLIN2 interacts with SCAP and INSIG1

To gain further insight into the molecular basis by which ERLIN2 regulates SREBP activation, we tested whether ERLIN2 can interact with ER-resident protein factors that control SREBP activation. Through IP Western blot analysis, we first confirmed the strong interaction between ERLIN1 and ERLIN2 in Huh-7 cells (Figure 5A). We then examined potential interactions between ERLIN2 and the protein factors that regulate SREBP activation in the ER, particularly SCAP and INSIG1. Because ERLIN2, SCAP and INSIG1 are ER-resident proteins, we performed IP Western blot analyses with ER protein fractions to detect the interaction between ERLIN2, SCAP and INSIG1. IP Western blot analysis indicated that only a small portion of endogenous SCAP proteins associated with ERLIN2 in CHO (Chinese hamster ovary) cells exogenously expressing ERLIN2 and INSIG1 in the presence of insulin or LPDS challenge (Figure 5B). Moreover, we failed to detect ERLIN2 protein associated with SCAP in protein lysates pulled down by an anti-SCAP antibody (results not shown). Therefore the present study excludes the possibility of any strong or direct interaction between ERLIN2 and SCAP. Next, we evaluated the interaction between ERLIN2 and INSIG1. Because of a limitation of the anti-INSIG1 antibody in IP analysis, we expressed T7-tagged INSIG1 and V5-tagged ERLIN2 in CHO cells for IP Western blot analysis. In the absence of challenges, we detected only a nominal interaction between ERLIN2 and INSIG1 (Figure 5C). However, significant amounts of INSIG1 proteins associated with ERLIN2 were detected in the cells challenged by insulin or LPDS (Figure 5C). These results suggest a strong interaction between ERLIN2 and INSIG1 after insulin or LPDS challenge, the metabolic condition that triggers SREBP activation and *de novo* lipogenesis [21]. To further delineate the interaction between ERLIN2 and INSIG1, we endogenously incorporated photo-reactive amino acid analogues into the primary sequence of proteins during synthesis and then UV activated them to covalently cross-link proteins within protein–protein interaction domains in their native environment [36–38]. This powerful method enabled us to detect the intact protein interaction complex within live cells without the use of completely foreign chemicals or molecular modifiers that might adversely affect the interaction being studied [37]. Utilizing a photo-reactive amino acids kit, we incorporated photo-reactive leucine and methionine analogues into the CHO cells expressing both ERLIN2 and INSIG1. IP Western blot analysis with photo-reactive amino acid-incorporated UV cross-linked protein lysates revealed a significant amount of ERLIN2–INSIG1 binding complex formed in the CHO cells after insulin or LPDS challenge (Figure 5D).

© The Authors Journal compilation © 2012 Biochemical Society

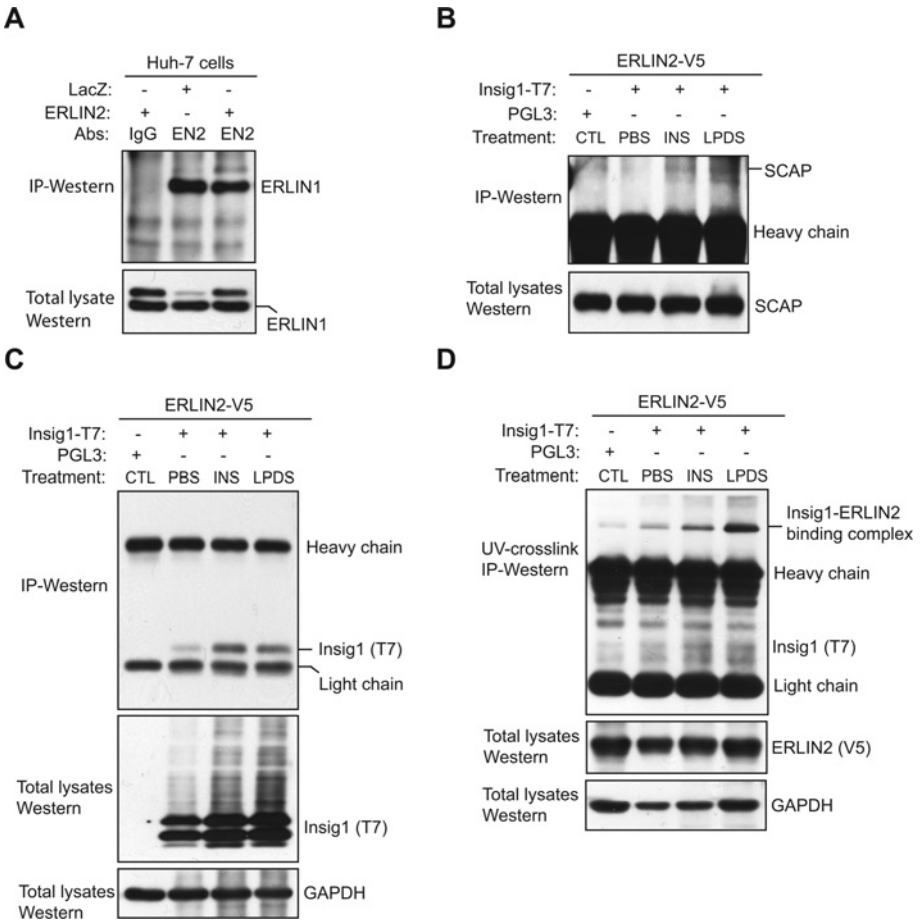

Figure 5 ERLIN2 interacts with SCAP and INSIG1

(**A**) IP Western blot analysis of the interaction between ERLIN1 and ERLIN2 in Huh-7 cells. T cell lysates from Huh-7 cells expressing exogenous ERLIN2 or LacZ were immunoprecipitated with an anti-ERLIN2 antibody (EN2) or rabbit IgG (negative control). The pulled-down proteins were subjected to immunoblotting analysis using an anti-ERLIN1 antibody. The levels of ERLIN1 in total cell lysates were included as input controls. (**B**) IP Western blot analysis of the interaction between ERLIN2 and SCAP. CHO cells stably expressing exogenous ERLIN2 protein with a V5 tag were transfected with a plasmid vector expressing INSIG1 protein with a T7 tag. The transfected CHO cells were treated with PBS vehicle, insulin (INS; 100 nM) for 6 h or were cultured in LPDS medium for 12 h. As a control (CTL), CHO cells stably expressing exogenous ERLIN2 protein were transfected with a plasmid vector control PGL3 and cultured in normal medium without insulin or LPDS challenge. ER protein fractions isolated from the CHO cells were immunoprecipitated with an anti-V5 antibody and then subjected to immunoblotting analysis using an anti-SCAP antibody to detect the interaction between ERLIN2 and SCAP. The levels of SCAP in total cell lysates were determined as input controls. (**C**) IP Western blot analysis of the interaction between ERLIN2 and INSIG1. The CHO cells, the plasmid transfection procedure, and the treatments are the same as described for (**B**). The ER protein fractions isolated from the CHO cells were immunoprecipitated with an anti-V5 antibody and then subjected to immunoblotting analysis using an anti-T7 antibody to detect the interaction between ERLIN2 and INSIG1. The levels of T7-tagged INSIG1 in total cell lysates were determined as input controls. (**D**) IP Western blot analysis of ERLIN2–INSIG1 binding complex by using a photo-reactive amino acid incorporation approach. The CHO cells, the plasmid transfection procedure, and the treatments are the same as described in (**B**), except that the cells were cultivated with DMEM limiting medium containing photo-reactive leucine and methionine analogues for 24 h before UV cross-linking and cell lysate collection. The photo-reactive amino acids incorporated UV cross-linked protein lysates were immunoprecipitated with an anti-V5 antibody and then subjected to immunoblotting analysis using an anti-T7 antibody to detect the ERLIN2–INSIG1 binding complex. The levels of ERLIN2 in total cell lysates were determined as input controls.

To verify whether ERLIN2 is involved in ER-associated degradation of INSIG1, we determined the levels of INSIG1 in Huh-7 cells overexpressing ERLIN1 or ERLIN2 after insulin or LPDS challenge. Consistent with our previous observation using transient ERLIN2-knockdown cells (Figure 4C), the levels of INSIG1 were marginally lower in the Huh-7 cells overexpressing ERLIN2, compared with those in cells overexpressing LacZ or ERLIN1 (Supplementary Figure S7 at http://www.BiochemJ.org/bj/446/bj4460415add.htm). Therefore ERLIN2 is not likely to play a significant role in mediating INSIG1 degradation. The interaction between ERLIN2 and INSIG1, and loosely with SCAP, might be required for efficient SREBP activation by facilitating the dissociation of the SREBP–SCAP complex from INSIG1 in cancer cells (Supplementary Figure S8 at http://www.BiochemJ.org/bj/446/bj4460415add.htm). This hypothesis needs to be further investigated in future studies.

Knockdown of ERLIN2 leads to reduced proliferation rates in cancer cells

Cancer cells, especially aggressive forms, have a high demand for lipid supplies for unlimited cell proliferation. The SREBP activities and *de novo* lipogenesis are functionally relevant to the cell proliferation rate [39]. Having established the role of ERLIN2 in regulating SREBP activation and lipid droplet production, we determined whether down-regulation of endogenous ERLIN2 in cancer cells affects cancer cell growth. To address this question, we examined cell proliferation rates in the human hepatoma cell line Huh-7 or the human breast cancer cell line SUM225 in which ERLIN2 had been knocked down. We observed that in the absence of ERLIN2 the Huh-7 cells or SUM225 cells displayed a significant reduction in both size and number of cell aggregates (Figures 6A and 6B).

© The Authors Journal compilation © 2012 Biochemical Society

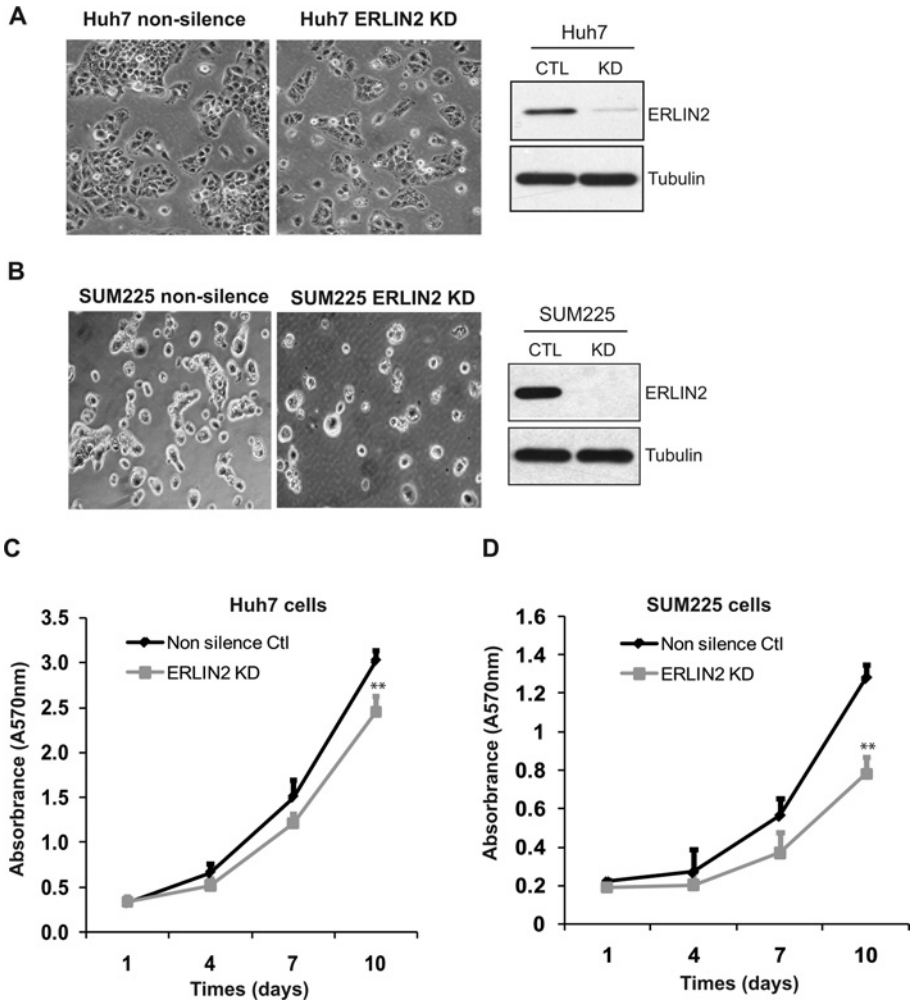

Figure 6 Down-regulation of ERLIN2 reduces cancer cell proliferation rates

(**A**) Left-hand panel, morphology of ERLIN2 knockdown and control Huh-7 cells. Magnification is ×400. Right-hand panel, levels of ERLIN2 protein in knockdown (KD) and control (CTL) Huh-7 cells were determined by Western blot analysis. (**B**) Morphology of ERLIN2 knockdown and control SUM225 cells. Magnification is ×400. Levels of ERLIN2 protein in knockdown and control SUM225 cells were determined by Western blot analysis. (**C** and **D**) Cell proliferation assays with the stable ERLIN2 knockdown Huh-7 (**C**) or SUM225 (**D**) cells and control cells. On day 1, the same number of ERLIN2 knockdown or control cells was seeded (5000 cells per well). The cell proliferation rate at each time point was represented by a 570-nm absorbance reading determined by MTT assay. Results are means ± S.E.M. ($n = 3$ biological samples). $^{**}P < 0.01$.

Cell growth and proliferation analyses indicated that knockdown of ERLIN2 reduced proliferation rates of Huh-7 cells and SUM225 cells (Figures 6C and 6D). After 10 days in cell culture, the proliferation rates of ERLIN2 knockdown Huh-7 cells or SUM225 cells were significantly lower than that of control cells. The reduced cancer cell proliferation rates, caused by down-regulation of ERLIN2, are consistent with the role of ERLIN2 in regulating *de novo* lipogenesis. This result implies that targeting ERLIN2 could be an effective therapeutic approach for aggressive cancers by down-regulating *de novo* lipogenesis in cancer cells.

DISCUSSION

The present study provides important new information about the role and mechanism of the ER lipid raft protein factor ERLIN2 in lipid metabolism associated with tumour cell growth and malignancy maintenance. The *ERLIN2* gene is amplified and overexpressed in the luminal subtype of human breast cancer that is associated with reduced metastasis-free survival rate [7–9,12]. Our work demonstrated that ERLIN2 is preferably expressed in aggressive breast cancer cell lines and in mouse fatty liver tissue, and it is inducible by insulin or LPDS-containing culture medium (Figure 1). We found that ERLIN2 modulates the activation of SREBP1c, the key regulator of lipid and cholesterol metabolism, in cancer cells (Figure 4). Consistently, cytosolic lipid droplet production, a reflection of *de novo* lipid/cholesterol metabolism, could be modulated by up- or down-regulation of ERLIN2 in human breast cancer cells or hepatoma cells (Figures 2 and 3). As a result of decreased lipogenesis, and other possible effects, cancer cell proliferation rates were reduced when ERLIN2 was down-regulated (Figure 6). These findings not only contribute to our understanding of the regulatory mechanism of activation of SREBPs in cancer cells, but could also inform novel therapy and pharmaceutical interventions to control cancers, especially aggressive forms.

© The Authors Journal compilation © 2012 Biochemical Society

Previously, ERLIN2 was characterized as a mediator of ERAD of activated inositol trisphosphate receptors, the key component of the ER Ca^{2+}-release channel, and of the cholesterol biosynthetic enzyme HMG-CoA reductase [13–15]. The ERLIN1–ERLIN2 complex interacts with the membrane-bound ubiquitin ligase GP78 and the substrate inositol trisphosphate receptors or HMG-CoA reductase, leading to polyubiquitination and subsequent degradation of these substrates. Activation of SREBPs, the key regulators of lipid and sterol biosynthesis, is regulated by the binding activities of ER-resident proteins, including INSIG1 and SCAP [21]. Because SCAP escorts SREBP from the ER to the Golgi for proteolytic processing into an active transcription factor, the binding of SCAP by INSIG1 effectively prevents SREBP activation [30]. Degradation of the INSIG1 protein through ERAD is an important process that is associated with the activation of SREBPs [31,32]. The present study showed that ERLIN2 interacts with INSIG1 and is weakly associated with SCAP after insulin signalling or LPDS culture (Figure 5). Because INSIG1 interacts with SCAP, it is possible that ERLIN2 indirectly associates with SCAP by interacting with INSIG1. We showed that ERLIN2 regulates cleavage of SREBP1c in human breast cancer cells or hepatoma cells (Figure 4). However, ERLIN2 does not likely play a significant role in degrading INSIG1, although it interacts directly with INSIG1 in response to metabolic signals (Figure 4C and Supplementary Figure S7). On the basis of these results, we propose that ERLIN2 might interact with the INSIG1–SCAP binding complex by directly binding to INSIG1 after insulin or LPDS challenge. Consequently, ERLIN2 and INSIG1 interaction facilitates the dissociation of SCAP from INSIG1, thus promoting SREBP activation and *de novo* lipogenesis in cancer cells (Supplementary Figure S8). The ERLIN2-mediated regulation of SREBP and thus of *de novo* lipogenesis might represent an important enhancing mechanism in lipid and energy metabolism that helps cancer cells gain their growth advantage.

During tumorigenesis, uncontrolled proliferation of cancer cells requires elevated *de novo* lipogenesis to meet the high demand for lipids and energy [2]. Shifting lipid acquisition toward *de novo* lipogenesis dramatically changes membrane properties and protects cells from both endogenous and exogenous insults. Our work suggests that ERLIN2, which is highly expressed in aggressive human breast cancer cells, supports malignancy by promoting *de novo* lipogenesis. Down-regulation of ERLIN2 can reduce cytosolic lipid droplet content and slow the proliferation rate of cancer cells. Therefore targeting ERLIN2 might reduce resistance of aggressive cancers to therapy and thus improve the effectiveness of conventional anti-cancer drugs. The present study has also raised many immediate and important questions. For example, what is the precise mechanism by which ERLIN2 regulates activation of SREBPs? Does ERLIN2 interact with other ER-resident lipogenic regulators, such as the hepatocyte-specific CREBH (cAMP responsive element binding protein) [40,41], to regulate lipid metabolism in cancer cells? Our data showed that ERLIN2 is involved in lipid droplet accumulation in the cells incubated with oleic acid (Figure 3B). Given that the oleic acid-induced response is not dependent upon *de novo* lipogenesis, ERLIN2 may also act on other pathways to facilitate cytosolic lipid accumulation. In the future, it will be interesting to investigate additional roles of ERLIN2 in promoting lipid accumulation. Additionally, the present study only demonstrated the regulation of SREBP activation and lipogenesis by ERLIN2 in a panel of cancer cells. Interestingly, expression of ERLIN2 was elevated in fatty liver tissues (Figure 1). Therefore, it is plausible to speculate that ERLIN2 may also regulate lipid metabolism in fatty liver disease. All of these questions merit future research in ERLIN2.

AUTHOR CONTRIBUTION

Kezhong Zhang, Zeng-Quan Yang and Guohui Wang designed the study; Guohui Wang, Xuebao Zhang, Jin-Sook Lee, Xiaogang Wang and Kezhong Zhang performed the experiments; Guohui Wang, Xuebao Zhang, Kezhong Zhang and Zeng-Quan Yang analysed the data; and Kezhong Zhang and Zeng-Quan Yang wrote the paper.

ACKNOWLEDGEMENTS

We thank Dr Steven Ethier (Medical University of South Carolina, Charleston, SC, U.S.A.) for providing human SUM breast cancer cell lines, Dr Jin Ye (University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.) for providing the plasmid expressing human INSIG1, and Mr Steven Horne and Ms Batoul Abdallah for editorial assistance prior to submission.

FUNDING

This work was supported by the National Institutes of Health (NIH) [grant numbers DK090313 and ES017829 (to K.Z.)], the Department of Defense Breast Cancer Program [grant numbers BC095179P1 (to K.Z.), BC083945 (to Z.Y.) and BC095179 (to Z.Y.)] and a Karmanos Cancer Institute pilot grant (to Z.Y. and K.Z.).

REFERENCES

1 Luo, J., Solimini, N. L. and Elledge, S. J. (2009) Principles of cancer therapy: oncogene and non-oncogene addiction. Cell **136**, 823–837
2 Hilvo, M., Denkert, C., Lehtinen, L., Muller, B., Brockmoller, S., Seppanen-Laakso, T., Budczies, J., Bucher, E., Yetukuri, L., Castillo, S. et al. (2011) Novel theranostic opportunities offered by characterization of altered membrane lipid metabolism in breast cancer progression. Cancer Res. **71**, 3236–3245
3 Brown, D. A. (2001) Lipid droplets: proteins floating on a pool of fat. Curr. Biol. **11**, R446–R449
4 Menendez, J. A. and Lupu, R. (2007) Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nat. Rev. Cancer **7**, 763–777
5 Browman, D. T., Resek, M. E., Zajchowski, L. D. and Robbins, S. M. (2006) Erlin-1 and erlin-2 are novel members of the prohibitin family of proteins that define lipid-raft-like domains of the ER. J. Cell Sci. **119**, 3149–3160
6 Staaf, J., Jonsson, G., Ringner, M., Vallon-Christersson, J., Grabau, D., Arason, A., Gunnarsson, H., Agnarsson, B. A., Malmstrom, P. O., Johannsson, O. T. et al. (2010) High-resolution genomic and expression analyses of copy number alterations in HER2-amplified breast cancer. Breast Cancer Res. **12**, R25
7 Yang, Z. Q., Streicher, K. L., Ray, M. E., Abrams, J. and Ethier, S. P. (2006) Multiple interacting oncogenes on the 8p11-p12 amplicon in human breast cancer. Cancer Res. **66**, 11632–11643
8 Gelsi-Boyer, V., Orsetti, B., Cervera, N., Finetti, P., Sircoulomb, F., Rouge, C., Lasorsa, L., Letessier, A., Ginestier, C., Monville, F. et al. (2005) Comprehensive profiling of 8p11-12 amplification in breast cancer. Mol. Cancer Res. **3**, 655–667
9 Garcia, M. J., Pole, J. C., Chin, S. F., Teschendorff, A., Naderi, A., Ozdag, H., Vias, M., Kranjac, T., Subkhankulova, T., Paish, C. et al. (2005) A 1 Mb minimal amplicon at 8p11-12 in breast cancer identifies new candidate oncogenes. Oncogene **24**, 5235–5245
10 Holland, D. G., Burleigh, A., Git, A., Goldgraben, M. A., Perez-Mancera, P. A., Chin, S. F., Hurtado, A., Bruna, A., Ali, H. R., Greenwood, W. et al. (2011) ZNF703 is a common Luminal B breast cancer oncogene that differentially regulates luminal and basal progenitors in human mammary epithelium. EMBO Mol. Med. **3**, 167–180
11 Sircoulomb, F., Nicolas, N., Ferrari, A., Finetti, P., Bekhouche, I., Rousselet, E., Lonigro, A., Adelaide, J., Baudelet, E., Esteyries, S. et al. (2011) ZNF703 gene amplification at 8p12 specifies luminal B breast cancer. EMBO Mol. Med. **3**, 153–166
12 Yang, Z. Q., Liu, G., Bollig-Fischer, A., Giroux, C. N. and Ethier, S. P. (2010) Transforming properties of 8p11-12 amplified genes in human breast cancer. Cancer Res. **70**, 8487–8497
13 Pearce, M. M., Wang, Y., Kelley, G. G. and Wojcikiewicz, R. J. (2007) SPFH2 mediates the endoplasmic reticulum-associated degradation of inositol 1,4,5-trisphosphate receptors and other substrates in mammalian cells. J. Biol. Chem. **282**, 20104–20115
14 Pearce, M. M., Wormer, D. B., Wilkens, S. and Wojcikiewicz, R. J. (2009) An endoplasmic reticulum (ER) membrane complex composed of SPFH1 and SPFH2 mediates the ER-associated degradation of inositol 1,4,5-trisphosphate receptors. J. Biol Chem. **284**, 10433–10445
15 Jo, Y., Sguigna, P. V. and DeBose-Boyd, R. A. (2011) Membrane-associated ubiquitin ligase complex containing gp78 mediates sterol-accelerated degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. J. Biol. Chem. **286**, 15022–15031
16 Lee, J. N., Gong, Y., Zhang, X. and Ye, J. (2006) Proteasomal degradation of ubiquitinated Insig proteins is determined by serine residues flanking ubiquitinated lysines. Proc. Natl. Acad. Sci. U.S.A. **103**, 4958–4963

© The Authors Journal compilation © 2012 Biochemical Society

17 Ethier, S. P., Mahacek, M. L., Gullick, W. J., Frank, T. S. and Weber, B. L. (1993) Differential isolation of normal luminal mammary epithelial cells and breast cancer cells from primary and metastatic sites using selective media. Cancer Res. **53**, 627–635
18 Forozan, F., Veldman, R., Ammerman, C. A., Parsa, N. Z., Kallioniemi, A., Kallioniemi, O. P. and Ethier, S. P. (1999) Molecular cytogenetic analysis of 11 new breast cancer cell lines. Br. J. Cancer **81**, 1328–1334
19 Nakabayashi, H., Taketa, K., Miyano, K., Yamane, T. and Sato, J. (1982) Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. Cancer Res. **42**, 3858–3863
20 Laing, S., Wang, G., Briazova, T., Zhang, C., Wang, A., Zheng, Z., Gow, A., Chen, A. F., Rajagopalan, S., Chen, L. C. et al. (2010) Airborne particulate matter selectively activates endoplasmic reticulum stress response in the lung and liver tissues. Am. J. Physiol. Cell Physiol. **299**, C736–C749
21 Horton, J. D., Goldstein, J. L. and Brown, M. S. (2002) SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J. Clin. Invest. **109**, 1125–1131
22 Tian, Q., Stepaniants, S. B., Mao, M., Weng, L., Feetham, M. C., Doyle, M. J., Yi, E. C., Dai, H., Thorsson, V., Eng, J. et al. (2004) Integrated genomic and proteomic analyses of gene expression in mammalian cells. Mol. Cell. Proteomics **3**, 960–969
23 Lee, J. S., Mendez, R., Heng, H. H., Yang, Z. Q. and Zhang, K. (2012) Pharmacological ER stress promotes hepatic lipogenesis and lipid droplet formation. Am. J. Transl. Res. **4**, 102–113
24 Lee, J. S., Zheng, Z., Mendez, R., Ha, S. W., Xie, Y. and Zhang, K. (2012) Pharmacologic ER stress induces non-alcoholic steatohepatitis in an animal model. Toxicol. Lett. **211**, 29–38
25 Paigen, B., Morrow, A., Holmes, P. A., Mitchell, D. and Williams, R. A. (1987) Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis **68**, 231–240
26 Matsuzawa, N., Takamura, T., Kurita, S., Misu, H., Ota, T., Ando, H., Yokoyama, M., Honda, M., Zen, Y., Nakanuma, Y. et al. (2007) Lipid-induced oxidative stress causes steatohepatitis in mice fed an atherogenic diet. Hepatology **46**, 1392–1403
27 Wong, R. H. and Sul, H. S. (2010) Insulin signaling in fatty acid and fat synthesis: a transcriptional perspective. Curr. Opin. Pharmacol. **10**, 684–691
28 Ricchi, M., Odoardi, M. R., Carulli, L., Anzivino, C., Ballestri, S., Pinetti, A., Fantoni, L. I., Marra, F., Bertolotti, M., Banni, S. et al. (2009) Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. J. Gastroenterol. Hepatol. **24**, 830–840
29 Yang, Y. A., Morin, P. J., Han, W. F., Chen, T., Bornman, D. M., Gabrielson, E. W. and Pizer, E. S. (2003) Regulation of fatty acid synthase expression in breast cancer by sterol regulatory element binding protein-1c. Exp. Cell Res. **282**, 132–137

Received 20 November 2011/11 June 2012; accepted 13 June 2012
Published as BJ Immediate Publication 13 June 2012, doi:10.1042/BJ20112050

30 Yang, T., Espenshade, P. J., Wright, M. E., Yabe, D., Gong, Y., Aebersold, R., Goldstein, J. L. and Brown, M. S. (2002) Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. Cell **110**, 489–500
31 Lee, J. N. and Ye, J. (2004) Proteolytic activation of sterol regulatory element-binding protein induced by cellular stress through depletion of Insig-1. J. Biol. Chem. **279**, 45257–45265
32 Gong, Y., Lee, J. N., Lee, P. C., Goldstein, J. L., Brown, M. S. and Ye, J. (2006) Sterol-regulated ubiquitination and degradation of Insig-1 creates a convergent mechanism for feedback control of cholesterol synthesis and uptake. Cell Metab. **3**, 15–24
33 Kotzka, J., Lehr, S., Roth, G., Avci, H., Knebel, B. and Muller-Wieland, D. (2004) Insulin-activated Erk-mitogen-activated protein kinases phosphorylate sterol regulatory element-binding Protein-2 at serine residues 432 and 455 *in vivo*. J. Biol. Chem. **279**, 22404–22411
34 Hirano, Y., Murata, S., Tanaka, K., Shimizu, M. and Sato, R. (2003) Sterol regulatory element-binding proteins are negatively regulated through SUMO-1 modification independent of the ubiquitin/26 S proteasome pathway. J. Biol. Chem. **278**, 16809–16819
35 Birmingham, A., Anderson, E. M., Reynolds, A., Ilsley-Tyree, D., Leake, D., Fedorov, Y., Baskerville, S., Maksimova, E., Robinson, K., Karpilow, J. et al. (2006) 3′ UTR seed matches, but not overall identity, are associated with RNAi off-targets. Nat. Methods **3**, 199–204
36 Jumper, C. C. and Schriemer, D. C. (2011) Mass spectrometry of laser-initiated carbene reactions for protein topographic analysis. Anal. Chem. **83**, 2913–2920
37 Suchanek, M., Radzikowska, A. and Thiele, C. (2005) Photo-leucine and photo-methionine allow identification of protein–protein interactions in living cells. Nat. Methods **2**, 261–267
38 Vila-Perello, M., Pratt, M. R., Tulin, F. and Muir, T. W. (2007) Covalent capture of phospho-dependent protein oligomerization by site-specific incorporation of a diazirine photo-cross-linker. J. Am. Chem. Soc. **129**, 8068–8069
39 Sherr, C. J. (2000) The Pezcoller lecture: cancer cell cycles revisited. Cancer Res. **60**, 3689–3695
40 Zhang, K., Shen, X., Wu, J., Sakaki, K., Saunders, T., Rutkowski, D. T., Back, S. H. and Kaufman, R. J. (2006) Endoplasmic reticulum stress activates cleavage of CREBH to induce a systemic inflammatory response. Cell **124**, 587–599
41 Zhang, C., Wang, G., Zheng, Z., Maddipati, K. R., Zhang, X., Dyson, G., Williams, P., Duncan, S. A., Kaufman, R. J. and Zhang, K. (2012) Endoplasmic reticulum-tethered transcription factor cAMP responsive element-binding protein, hepatocyte specific, regulates hepatic lipogenesis, fatty acid oxidation, and lipolysis upon metabolic stress in mice. Hepatology **55**, 1070–1082

© The Authors Journal compilation © 2012 Biochemical Society

Biochem. J. (2012) **446**, 415–425 (Printed in Great Britain) doi:10.1042/BJ20112050

SUPPLEMENTARY ONLINE DATA

Endoplasmic reticulum factor ERLIN2 regulates cytosolic lipid content in cancer cells

Guohui WANG*[1], Xuebao ZHANG*[1], Jin-Sook LEE*, Xiaogang WANG†, Zeng-Quan YANG†‡[2] and Kezhong ZHANG*†§[2]

*Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, MI 48201, U.S.A., †Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI 48201, U.S.A., ‡Department of Oncology, Wayne State University School of Medicine, Detroit, MI 48201, U.S.A., and §Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, MI 48201, U.S.A.

Origins and culture conditions for the cancer cell lines used in the present study

The SUM44 cell line was established from pleural effusion-derived breast cancer cells [1]. SUM44 cells were cultured in Ham's F12 medium supplemented with 0.1 % BSA, fungizone (0.5 μg/ml), gentamicin (5 μg/ml), ethanolamine (5 mmol/l), Hepes (10 mmol/l), transferrin (5 μg/ml), T3 (3,3,′5-triiodo-L-thyronine; 10 μmol/l), selenium (50 μmol/l), hydrocortisone (1 μg/ml) and insulin. The SUM225 cell line was established from a chest wall recurrence of ductal carcinoma *in situ* of breast [2]. SUM225 cells were cultured with 5 % FBS, fungizone (0.5 μg/ml), gentamicin (5 μg/ml), hydrocortisone (1 μg/ml) and insulin (5 μg/ml). Each of these cell lines, from a single human patient, represents a different subtype of breast cancer [1, 2]. All of the currently known oncogenes with altered expression patterns in breast cancer are well represented and have been characterized in the SUM lines. These cell lines have been described in over 50 peer-reviewed publications in cancer research.

MCF10A is a spontaneously immortalized, but non-transformed, human mammary epithelial cell line derived from the breast tissue of a 36-year-old patient with fibrocystic changes [3]. MCF10A cells were cultured in Ham's F12 medium supplemented with 0.1 % BSA, fungizone (0.5 μg/ml), gentamicin (5 μg/ml), ethanolamine (5 mmol/l), Hepes (10 mmol/l), transferrin (5 μg/ml), T3 (10 μmol/l), selenium (50 μmol/l), hydrocortisone (1 μg/ml), insulin (5 μg/ml) and 10 ng/ml epidermal growth factor.

Huh-7 is a hepatocellular carcinoma cell line that was originally derived from a liver tumour in a Japanese male [4]. This cell line was cultured in DMEM containing 10 % FBS, L-glutamine and antibiotics at 37 °C in a 5 % CO_2 environment.

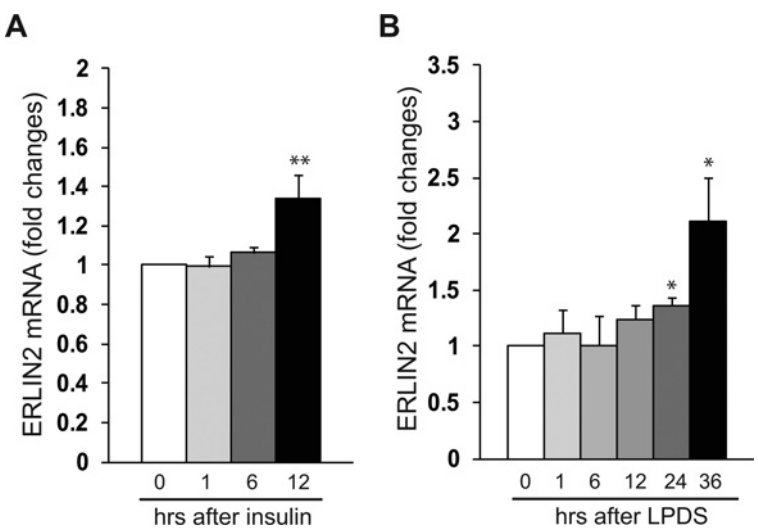

Figure S1 Quantitative real-time RT–PCR analysis of *ERLIN2* mRNA expression in murine primary hepatocytes in response to insulin or LPDS challenge

(**A**) Murine primary hepatocytes were challenged with insulin (100 nM) for 1, 6 or 12 h. Murine primary hepatocytes were cultured in normal medium with vehicle buffer PBS added as a control (0 h under insulin). (**B**) Murine primary hepatocytes were cultured in medium containing LPDS for 1, 6, 12, 24 or 36 h. As a control, murine primary hepatocytes were cultured in normal medium containing 10 % FBS (0 h under LPDS). For (**A** and **B**), total RNAs were isolated from the primary hepatocytes after the treatment, and quantitative real-time RT–PCR was performed to determine *ERLIN2* mRNA expression levels in the primary hepatocytes after insulin or LPDS challenge. The mRNA expression values were determined after normalization to internal control *GAPDH* mRNA levels. The baseline of the *ERLIN2* mRNA level in the hepatocytes challenged with insulin or LPDS at 0 h was set to 1. After the treatments, fold changes of *ERLIN2* mRNA levels in the hepatocytes were calculated by comparison to the baseline mRNA level. Results are means ± S.E.M. ($n = 3$ experiments). *$P < 0.05$; **$P < 0.01$.

[1] These authors contributed equally to this work.
[2] Correspondence may be addressed to either of these authors (email kzhang@med.wayne.edu and yangz@karmanos.org).

© The Authors Journal compilation © 2012 Biochemical Society

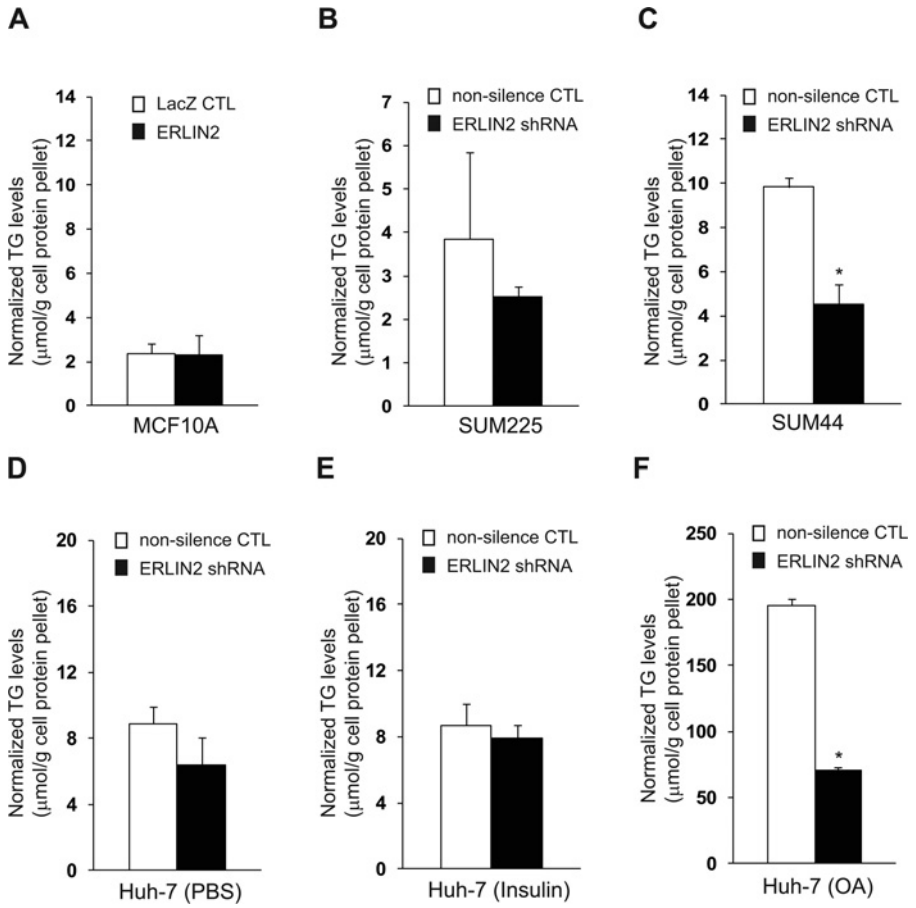

Figure S2 Biochemical quantification of cellular TG levels in MCF10A, SUM225, SUM44 and Huh-7 cells

Levels of total cellular TG were determined using a TG measurement kit from BioAssay System. TG levels were presented after normalization to net weights of cellular pellets. For Huh-7 cells, ERLIN2 knockdown or control (CTL) stable cell lines were incubated with vehicle PBS, insulin (100 nM), or oleic acid (OA) (0.5 mM) for 12 h. (**A**) Cellular TG levels in MCF10A cells expressing exogenous LacZ or ERLIN2. (**B**) Cellular TG levels in ERLIN2 knockdown or control SUM225 cells. (**C**) Cellular TG levels in ERLIN2 knockdown or control SUM44 cells. (**D**) Cellular TG levels in ERLIN2 knockdown or control Huh-7 cells treated with PBS vehicle. (**E**) Cellular TG levels in ERLIN2 knockdown or control Huh 7 cells treated with insulin. (**F**) Cellular TG levels in ERLIN2-knockdown or control Huh-7 cells incubated with oleic acid. Results are means $\pm$ S.E.M. ($n = 3$). $^*P < 0.05$.

© The Authors Journal compilation © 2012 Biochemical Society

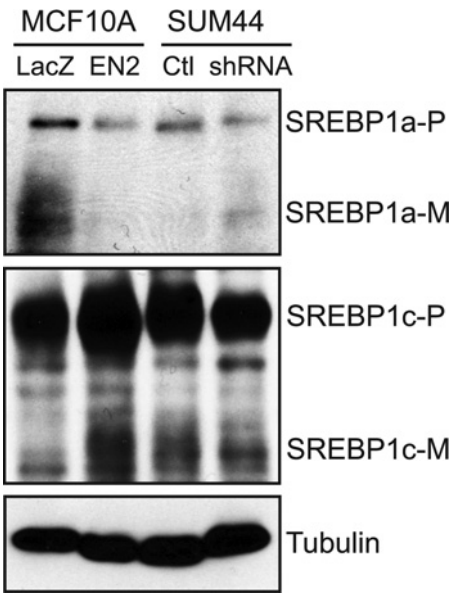

Figure S3 Western blot analysis of SREBP1c and SREBP1a protein levels in MCF10A expressing LacZ control or ERLIN2 and in ERLIN2 knockdown or control SUM44 cells

Tubulin was included as a loading control. LacZ, LacZ overexpression; EN2, ERLIN2 overexpression; Ctl, non-silence control; shRNA, ERLIN2 shRNA knockdown; SREBP1a-P, SREBP1a precursor; SREBP1a-M, mature SREBP1a; SREBP1c-P, SREBP1c precursor; and SREBP1c-M, mature SREBP1c. Note that our data showed that SUM44 and MCF10A cells only express trace levels of SREBP1a, which is consistent with the published conclusion that SREBP1c, but not SREBP1a or SREBP2, is induced in human breast cancer cell lines [5]. Interestingly, our data shows that expression levels of SREBP1a were decreased in MCF10A cells overexpressing ERLIN2, whereas SREBP1a levels were increased in ERLIN2 knockdown SUM44 cells. The correlation of SREBP1a levels with ERLIN2 induction and malignancy states is an interesting question to be elucidated in the future.

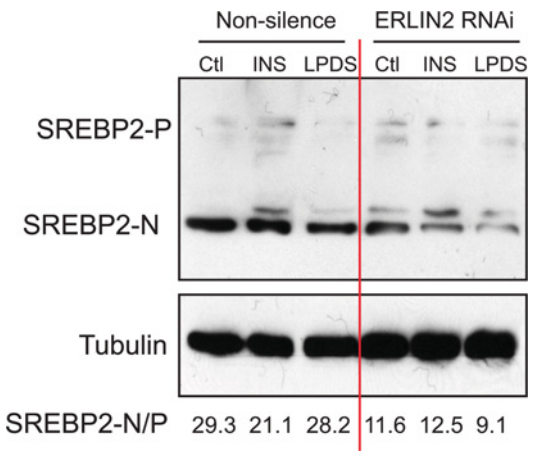

Figure S4 Western blot analysis of cleaved SREBP2 protein levels in Huh-7 cells that were transduced by non-silencing or ERLIN2 shRNAi lentivirus

The Huh-7 cells were treated with vehicle PBS or insulin (100 nM) for 6 h or cultured in LPDS-containing medium for 12 h. Tubulin was included as a loading control. SREBP2 protein signals were detected by using an antibody against the N-terminal SREBP protein fragment (Cayman Chemicals). The values below the gels represent the ratios of mature cleaved SREBP2 to SREBP2 precursor signal intensities. Ctl, control cells treated with vehicle PBS; INS, insulin; SREBP2-N, cleaved SREBP2 (N-terminal); SREBP2-P, SREBP2 precursor.

© The Authors Journal compilation © 2012 Biochemical Society

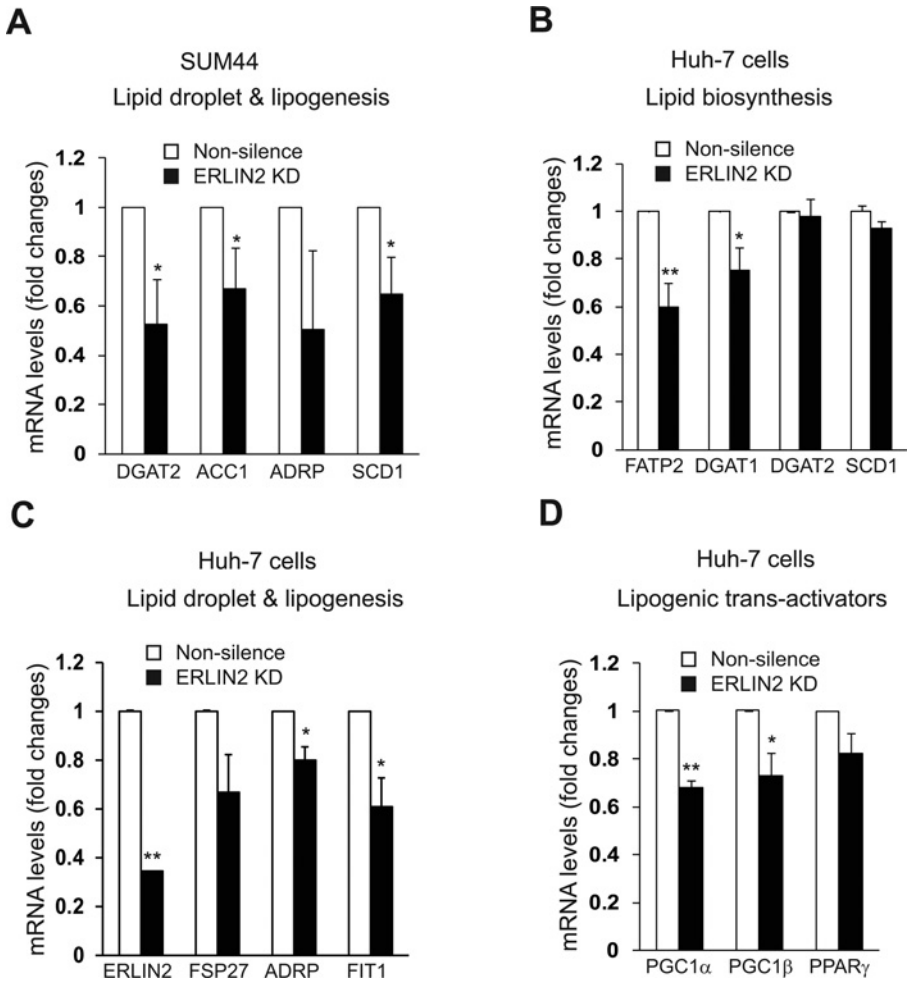

Figure S5 Quantitative real-time RT–PCR analysis of expression of lipogenic genes in ERLIN2 knockdown and non-silenced control Huh-7 or SUM44 cells

(**A**) Expression of the genes involved in lipid droplet formation and lipogenesis, including *DAGT2*, *ACC1*, *ADRP*, and *SCD1*, in ERLIN2-knockdown and control SUM44 cells. (**B**) Expression of the genes involved in lipid biosynthesis, including *FATP2*, *DGAT1*, *DAGT2* and *SCD1*, in ERLIN2 knockdown and control Huh-7 cells. (**C**) Expression of the *ERLIN2* gene and the genes involved in lipid droplet formation and lipogenesis, including *FSP27*, *ADRP* and *FIT1*, in ERLIN2 knockdown and control Huh-7 cells. (**D**) Expression of the genes encoding the lipogenic *trans*-activators PGC1α, PGC1β and PPARγ2 in ERLIN2 knockdown and control Huh-7 cells. For (**A**–**D**), total RNAs were isolated from the cells, and quantitative real-time RT–PCR was performed to determine mRNA expression levels. The mRNA expression values were determined after normalization to internal control *GAPDH* mRNA levels. To determine the expression profile for a particular gene, the baseline mRNA level in control cells was set to 1. Fold changes in the mRNA levels of the ERLIN2-knockdown cells were calculated by comparison with the baseline mRNA level. Results are means $\pm$ S.E.M. ($n = 3$ experimental repeats). $^{*}P < 0.05$; $^{**}P < 0.01$. ACC1, acetyl-CoA carboxylase 1; ADRP, adipose differentiation-related protein; FATP2, fatty acid transport protein 2; FIT1, fat-inducing transcript 1; FSP27, fat-specific protein 27; PPAR, peroxisome-proliferator-activated receptor.

© The Authors Journal compilation © 2012 Biochemical Society

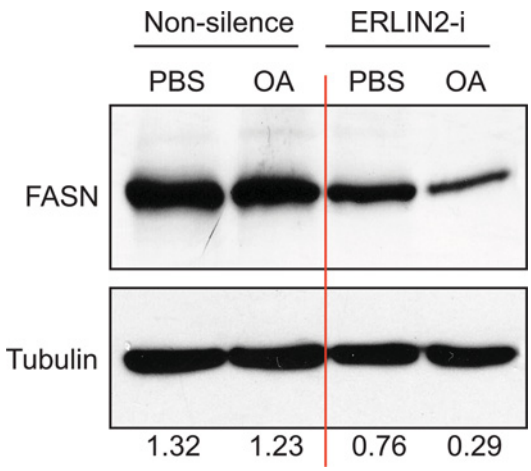

Figure S6 Western blot analysis of FAS protein levels in Huh-7 cells that were transduced by non-silencing or ERLIN2 shRNAi lentivirus in the presence or absence of oleic acid treatment

The non-silencing control or ERLIN2 knockdown Huh–7 cells were treated with vehicle PBS or oleic acid (OA) (0.5 mM) for 12 h. Tubulin was included as a loading control. The values below the gels represent the ratio of FAS to tubulin signal intensities.

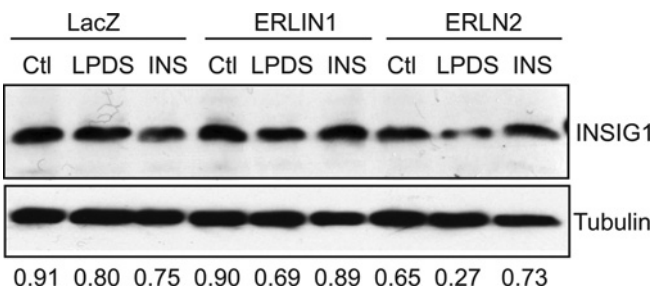

Figure S7 Western blot analysis of INSIG1 protein levels in the Huh-7 cell line that was transduced by lentivirus overexpressing LacZ, ERLIN1 or ERLIN2

Cell lysates were prepared from the Huh-7 cell lines cultured in normal medium (Ctl), LPDS medium for 12 h, or challenged with insulin (INS, 100 nM) for 6 h. Tubulin was included as a loading control. The values below the gels represent INSIG1 signal intensities after normalization to tubulin signal intensities.

© The Authors Journal compilation © 2012 Biochemical Society

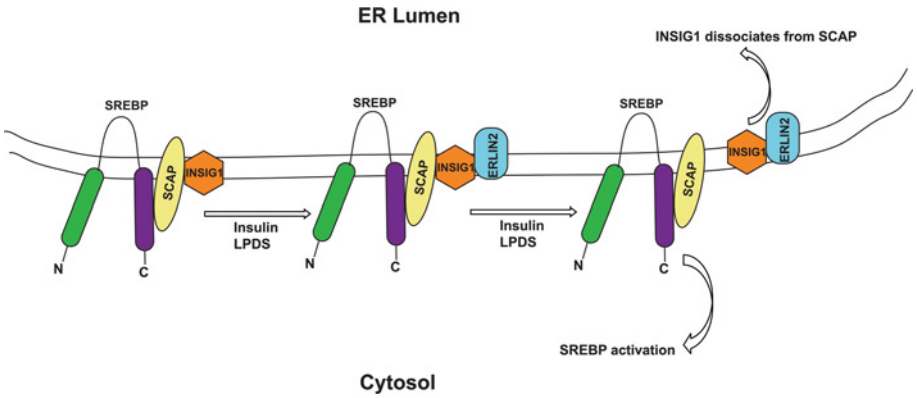

Figure S8 A working model for regulation of SREBP activation by ERLIN2

In response to insulin or LPDS challenge, ERLIN2 interacts with the INSIG1–SCAP binding complex by directly binding to INSIG1. The interaction between ERLIN2 and INSIG1 facilitates the dissociation of SCAP from INSIG1, thus promoting SREBP–SCAP complex release from the ER to Golgi for SREBP processing.

Table S1 Sequence information for the real-time PCR analysis

ACC1, acetyl-CoA carboxylase 1; ACTB, β-actin; ADRP, adipose differentiation-related protein; FATP2, fatty acid transport protein 2; FIT1, fat-inducing transcript 1; FSP27, fat-specific protein 27.

Gene symbol	Forward primer (5′→3′)	Reverse primer (5′→3′)
PGC1α	TATGGAGTGACATAGAGTGTGCT	CCACTTCAATCCACCCAGAAAG
PGC1β	AGAAGCTCCTCCTGGCCACATCCT	GCCTTCTTGTCTTGGGTGCTGTCC
ERLIN2	GAACCAGTGGTGGTGTGATG	TATGAACGCTGCAGAACTGG
FSP27	GAGTCCAACGCAGTCCAGCTGAC	GCAGCTGCTGGGTCACCACAG
ACC1	AGGGCTAGGTCTTTCTGGAAGTGGA	TCAGCTCCAGAGGTTGGGCCA
DGAT1	CCGTGAGCTACCCGGACAAT	AGGATCCGTCGCAGCAGAA
DGAT2	TTTCGAGACTACTTTCCCATCCA	TGGCCTCTGTGCTGAAGTTG
ADRP	GATGGCAGAGAACGGTGTGAA	TCAATCCTGTCTAGCCCCTTACAG
SCD1	CTGCCCCTACGGCTCTTTCT	ACGTCGGGAATTATGAGGATCA
PPARγ2	CCTATTGACCCAGAAAGCGATT	CATTACGGAGAGATCCACGGA
FIT1	TTCGCCAGCCACGGCAACTT	GCGCCGTGTAGCCAGGAACA
FATP2	CCACAGGTCTTCCAAAAGCAGCCA	GTGCAGCACTGTGGTAAAAGGGCA
ACTB	AGCCTCGCCTTTGCCGATCCG	ACATGCCGGAGCCGTTGTCGA

Received 20 November 2011/11 June 2012; accepted 13 June 2012
Published as BJ Immediate Publication 13 June 2012, doi:10.1042/BJ20112050

REFERENCES

1 Ethier, S. P., Mahacek, M. L., Gullick, W. J., Frank, T. S. and Weber, B. L. (1993) Differential isolation of normal luminal mammary epithelial cells and breast cancer cells from primary and metastatic sites using selective media. Cancer Res. **53**, 627–635
2 Forozan, F., Veldman, R., Ammerman, C. A., Parsa, N. Z., Kallioniemi, A., Kallioniemi, O. P. and Ethier, S. P. (1999) Molecular cytogenetic analysis of 11 new breast cancer cell lines. Br. J. Cancer **81**, 1328–1334
3 Soule, H. D., Maloney, T. M., Wolman, S. R., Peterson, Jr, W. D., Brenz, R., McGrath, C. M., Russo, J., Pauley, R. J., Jones, R. F. and Brooks, S. C. (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res. **50**, 6075–6086
4 Nakabayashi, H., Taketa, K., Miyano, K., Yamane, T. and Sato, J. (1982) Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. Cancer Res. **42**, 3858–3863
5 Yang, Y. A., Morin, P. J., Han, W. F., Chen, T., Bornman, D. M., Gabrielson, E. W. and Pizer, E. S. (2003) Regulation of fatty acid synthase expression in breast cancer by sterol regulatory element binding protein-1c. Exp. Cell Res. **282**, 132–137

© The Authors Journal compilation © 2012 Biochemical Society

Am J Transl Res 2012;4(1):102-113
www.ajtr.org /ISSN:1943-8141/AJTR1112005

Original Article

Pharmacological ER stress promotes hepatic lipogenesis and lipid droplet formation

Jin-Sook Lee[1], Roberto Mendez[1], Henry H Heng[1,3], Zeng-quan Yang[3,4], Kezhong Zhang[1,2,3]

[1]Center for Molecular Medicine and Genetics, [2]Department of Immunology and Microbiology, [3]Karmanos Cancer Institute, [4]Department of Oncology, Wayne State University School of Medicine, Detroit, MI 48201, USA.

Received December 30, 2011; accepted January 5, 2012; Epub January 6, 2012; Published January 15, 2012

Abstract: Endoplasmic Reticulum (ER) stress refers to a condition of accumulation of unfolded or misfolded proteins in the ER lumen. A variety of biochemical stimuli or pathophysiologic conditions can directly or indirectly induce ER stress, leading to activation of an ER-originated adaptive signaling response called Unfolded Protein Response (UPR). Recent studies demonstrated that ER stress and UPR signaling are critically involved in the initiation and progression of many diseases, such as metabolic disease, cardiovascular disease, neurodegenerative disease, and cancer. In this study, we show that ER stress induced by pharmacologic reagents, including tunicamycin (TM) and thapsigargin (Tg), promotes hepatic lipogenesis and lipid droplet formation. Using quantitative gene expression analysis, we identified 3 groups of key lipogenic regulators or enzymes that are inducible by pharmacological ER stress in a human hepatoma cell line Huh-7. These ER stress-inducible lipogenic factors include: 1) lipogenic trans-activators including CCAAT/enhancer binding protein alpha (C/EBPα), peroxisome proliferator-activated receptor gamma (PPARγ), PPARγ coactivator 1-alpha (PGC1α), and Liver X receptor alpha (LXRα); 2) components of lipid droplets including fat-specific protein 27 (FSP27), adipose differentiation related protein (ADRP), fat-inducing transcript 2 (FIT2), and adipocyte lipid-binding protein (AP2); 3) key enzymes involved in *de novo* lipogenesis including acetyl-CoA carboxylase 1 (ACC1) and stearoyl-CoA desaturase-1 (SCD1). Supporting the role of pharmacologic ER stress in up-regulating *de novo* lipogenesis, TM or Tg treatment significantly increased accumulation of cytosolic lipid droplet formation in the hepatocytes. Moreover, we showed that forced expression of an activated form of X-box binding protein 1 (XBP1), a potent UPR *trans*-activator, can dramatically increase expression of PPARγ and C/EBPα in Huh-7 cells. The identification of ER stress-inducible lipogenic regulators provides important insights into the molecular basis by which acute ER stress promotes *de novo* lipogenesis. In summary, the findings from this study have important implication in understanding the link between ER stress and metabolic disease.

Keywords: Endoplasmic reticulum (ER) stress, hepatic lipogenesis, lipid droplet formation

Introduction

In eukaryotic cells, the ER is the site of folding of membrane and secreted proteins, synthesis of lipids and sterols, and storage of free calcium [1, 2]. As a protein-folding compartment, the ER is exquisitely sensitive to alterations in homeostasis, and provides stringent quality control systems to ensure that only correctly folded proteins transit to the Golgi and unfolded or misfolded proteins are retained in the ER and ultimately degraded. A number of biochemical stimuli and physiological and pathological processes, such as perturbation in calcium homeostasis, elevated secretory protein synthesis, and expression of misfolded proteins, can disrupt ER homeostasis, impose stress to the ER, and subsequently lead to accumulation of unfolded or misfolded proteins in the ER lumen. To cope with accumulation of unfolded or misfolded proteins in the ER, the cell has evolved highly specific signaling pathways called the unfolded protein response (UPR) to reduce the amount of new proteins translocated into the ER lumen, increase retrotranslocation and degradation of ER-localized proteins, and bolster the protein-folding capacity and secretion potential of the ER [2]. The UPR is orchestrated by transcriptional activation of multiple genes mediated by the protein kinase/endoribonuclease IRE1

(inositol-requiring 1) and the b-ZiP transcription factor ATF6 (activating transcription factor 6), and a general decrease in translation initiation and the selective translation of specific mRNAs mediated by the protein kinase PERK (double-strand RNA-activated kinase-like ER kinase) [1-4].

Liver is a major organ responsible for lipid and glucose metabolism. Dysregulation of hepatic lipid metabolism is closely associated with the initiation and progression of metabolic syndrome. Recent studies suggest that ER stress response plays important roles in maintaining lipid homeostasis [5-9]. The UPR branches through IRE1α and/or ATF6 is required to prevent hepatic steatosis upon acute ER stress [6, 7, 10]. It has also been shown that the IRE1α/XBP1 UPR branch is activated by the dietary high-carbohydrate and controls the expression of lipogenic enzymes, such as ACC2, DGAT2 and SCD1, that are essential for fatty acid and cholesterol biosynthesis [11]. Moreover, the UPR pathway through PERK/eIF2α was documented to be required for the expression of lipogenic genes and the development of hepatic steatosis [9]. Together, these observations suggest that ER stress and the UPR signaling are critically involved in regulating hepatic lipid metabolism.

In this study, we utilized two structurally-unrelated ER stress-inducing reagents, tunicamycin (TM) and Thapsigargin (Tg), to induce pharmacologic ER stress in Huh-7, a human hepatoma cell line that maintains key features of hepatic lipid metabolism [12]. Through this approach, we confirmed the effect of pharmacologic ER stress in up-regulating *de novo* lipogenesis and lipid droplet formation. Importantly, we have identified a subset of genes encoding key lipogenic trans-activators and enzymes, which are inducible by acute ER stress. The results from this study provide important insights into ER stress-induced hepatic lipogenesis

Materials and methods

Materials

Chemicals were purchased from Sigma unless indicated otherwise. Synthetic oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Antibodies against XBP1, C/EBPα, and PPARγ were from Santa Cruz Biotechnologies, Inc (Santa Cruz, CA). Antibodies against GAPDH and β-actin were purchased from Sigma (St. Louis, MO). Tunicamycin was from Sigma. BODIPY staining kit was purchased from Invitrogen. Human hepatoma cell line Huh-7 was kindly provided by Drs. Christopher M. Schonhoff (Tufts University Cummings School of Veterinary Medicine).

Huh-7 cell culture and TM and Tg treatment

HuH-7 cells were cultured at 37 °C and 5% CO_2 in DMEM containing high glucose (25 mM) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Huh-7 cells at 70% confluency were treated with tunicamycin (TM; 5, 10, and 20 μg/ml) or thapsigargin (Tg; 0.5, 1, and 1.5 μM) or vehicle PBS for 6, 12, and 24 hrs.

BODIPY staining of lipid droplets

Cells were washed with PBS, fixed with 3% formaldehyde for 15 min, and stained with BODIPY 493/503 (Invitrogen, stock concentration 1mg/ml, working solution 1:1000 dilution) for 15min at room temperature. Cells were then mounted with Prolong gold anti-fade reagent (Invitrogen) followed by washing in PBS for 3 times.

Western Blot and IP-Western blot Analyses

To determine expression levels of XBP1, PPARγ, C/EBPα, and GAPDH, total cell lysates were prepared from cultured Huh-7 cells using NP-40 lysis as previously described [13]. Denatured proteins were separated by SDS-PAGE on 10% Tris-glycine polyacrylamide gels and transferred to a 0.45-mm PVDF membrane (GE Healthcare). Membrane-bound antibodies were detected by an enhanced chemiluminescence detection reagent (GE Healthcare).

Recombinant adenoviral infection

Huh-7 cells at 60% confluency were infected by recombinant adenovirus expressing GFP or an activated form of XBP1 protein at an MOI of 100 for 48 hours before cell lysates were collected for Western blot analysis. Adenovirus expressing spliced XBP1 was kindly provided by Dr. Umut Ozcan (Harvard University) [14]. Recombinant adenovirus expressing GFP was kindly provided by Dr. Jiande Lin (University of Michigan).

Quantitative real-time RT-PCR analysis

For real-time PCR analysis, the reaction mixture containing cDNA template, primers, and SYBR Green PCR Master Mix (Invitrogen) was run in a 7500 Fast Real-time PCR System (Applied Biosystems, Carlsbad, CA). The real-time PCR primer sequences used in this study are described in supplemental information. Fold changes of mRNA levels were determined after normalization to internal control β-actin RNA levels.

Statistics analysis

Experimental results are shown as mean ± STDEV (for variation between experiments). The mean values for biochemical data from the experimental groups were compared by a paired or unpaired, 2-tailed Student's *t* test. Statistical tests with $P < 0.05$ were considered significant.

Results

Pharmacologic ER stress induced by TM or Tg promotes lipid droplet formation

To study the effect of pharmacologic ER stress on hepatic lipid metabolism, we challenged a human hepatoma cell line, Huh-7, with two structurally-unrelated ER stress-inducing drugs, tunicamycin (TM) and Thapsigargin (Tg). Huh-7 is a human hepatocellular carcinoma cell line that has been used for studying hepatic lipid metabolism [12]. TM is a bacterial nucleoside antibiotic that can block N-linked glycoproteins and cause accumulation of unfolded or misfolded proteins in the ER [15]. Tg is a specific inhibitor of intracellular SERCA-type Ca^{2+} pumps present in the sarcoplasmic/ER [16, 17]. Tg treatment can disrupt ER calcium homeostasis, leading to accumulation of unfolded or misfolded proteins in the ER lumen. Both TM and Tg have been routinely used as experimental tools to induce pharmacologic ER stress [18]. To delineate gene expression profiles in hepatic lipid metabolism upon pharmacologic ER stress challenge, Huh-7 cells were treated with TM at doses ranging from 5 to 20 μg/ml or Tg at doses ranging from 0.5 to 1.5 μM. The time intervals for each treatment were 6, 12, and 24 hours. Quantitative real-time RT-PCR analysis indicated that expression of the UPR target mRNA or genes, including spliced *Xbp1* mRNA, *Bip*, and *Chop*, was increased upon TM or Tg treatment in a dose-and time-dependent manner (**Figure 1**). This result suggests that TM and Tg can efficiently induce ER stress and activation of the UPR signaling in Huh-7 cells. Note that the levels of the spliced *XBP1*, *CHOP*, and *BiP* mRNAs in Huh-7 cells under different doses of TM treatment were comparable at 24 hours post TM treatment (**Figure 1A-C**), suggesting that Huh-7 cells can adapt to ER stress at the late stage of TM treatment.

Next, we evaluated the impact of pharmacologic ER stress in *de novo* hepatic lipogenesis, a key lipid synthesis progress that is tightly regulated by multiple layers of metabolic and stress signals [19, 20]. We examined the production of cytosolic lipid droplets, a major indicator of *de novo* lipogenesis, in the Huh-7 cells upon TM or Tg challenge. Production of cytosolic lipid droplets, as indicated by Bodipy staining, was significantly increased in the Huh-7 cells after 6 hours of TM or Tg treatment, compared to that after vehicle treatment (**Figure 2**). This result suggests that pharmacologic ER stress induced by TM or Tg can promote hepatic lipid droplet formation.

Challenge of TM or TG up-regulates expression of the genes encoding key lipogenic trans-activators

To understand the mechanism by which pharmacologic ER stress promotes lipid droplet formation, we first tested whether TM or Tg can up-regulate *trans*-activators in *de novo* lipogenesis. Huh-7 cells were treated with different doses of TM or Tg for a time course from 6, 12, to 24 hours. Quantitative real-time RT-PCR analysis was performed with the TM or Tg-treated Huh-7 cells to determine ER stress-inducible target genes in *trans*-activation of *de novo* lipogenesis. Among known lipogenic *trans*-activators we examined, expression of the genes encoding lipogenic *trans*-activators including CCAAT/enhancer binding protein alpha (C/EBPα), peroxisome proliferator-activated receptor gamma 2 (PPARγ2), PPARγ coactivator 1-alpha (PGC1α), and Liver X receptor alpha (LXRα) was significantly increased in the Huh-7 cells under the treatment of TM or Tg (**Figure 3A-D**). Although all these genes are inducible by TM or Tg, the expression dynamics of these genes were different upon TM or Tg challenge. Expression of the *C/EBPα*, *LXRα*, and *PGC1α* genes was inducible by TM or Tg in the time windows from 6 to 24

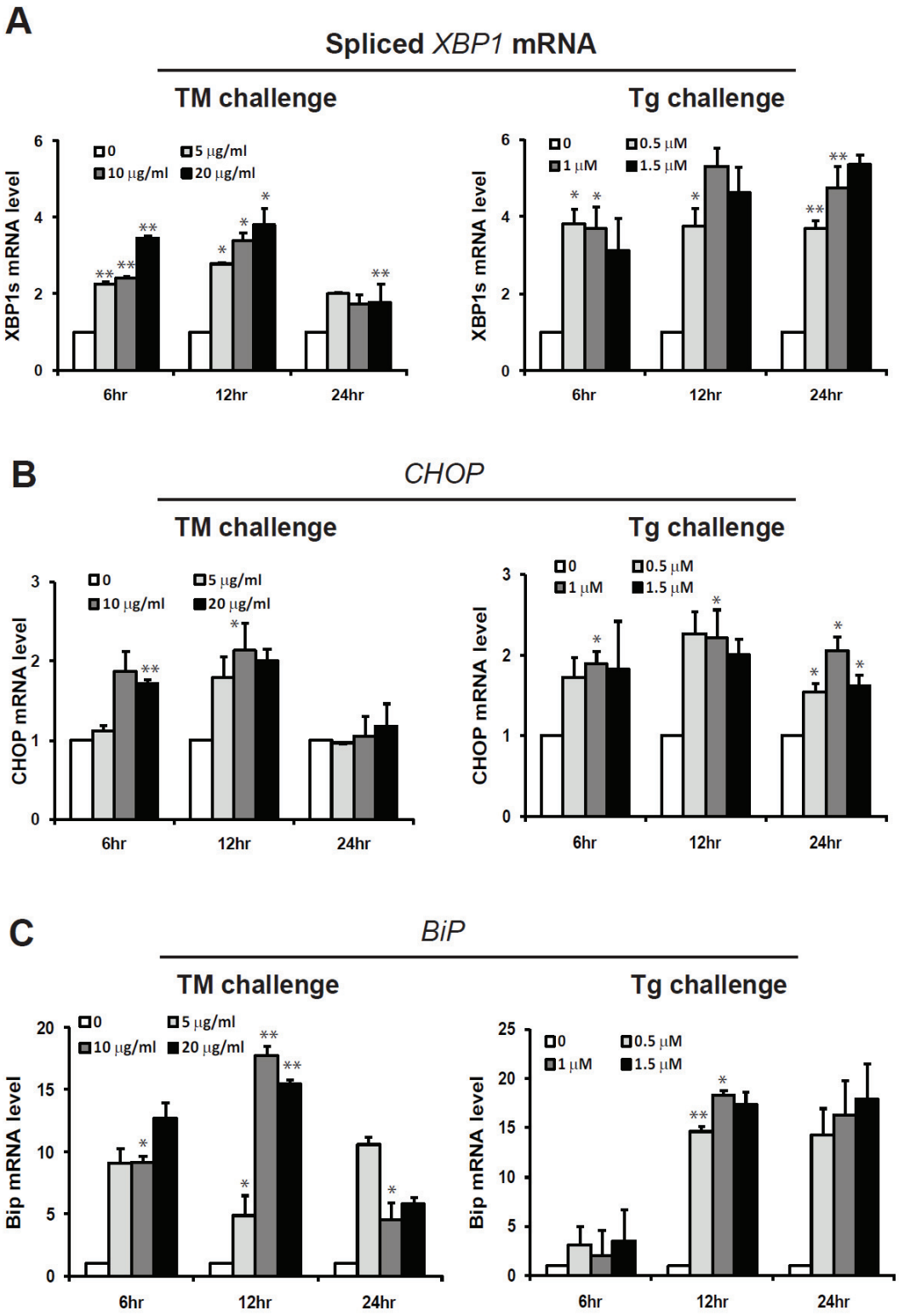

Figure 1. Quantitative real-time RT-PCR analysis of the mRNAs encoding spliced XBP1 (A), CHOP (B), and BiP (C) in Huh-7 cells. Total RNAs were isolated from Huh-7 cells treated with TM (5, 10, and 20 μg/ml) or Tg (0.5, 1, and 1.5 μM) for 6, 12 and 24 hrs. Fold changes of mRNA are shown by comparing to the vehicle-treated control. Each bar denotes mean ± SEM (n= 3). * $p<0.05$; ** $p<0.01$.

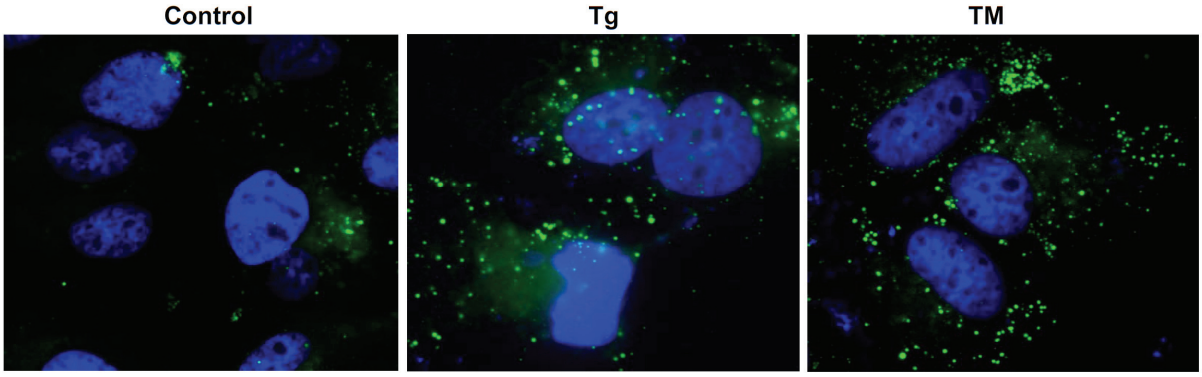

Figure 2. Bodipy staining of lipid droplets in Huh-7 cells. Huh-7 cells were treated with TM (10 μg/ml) or Tg (1 μM) for 6 hrs and then stained with Bodipy for lipid droplets. Magnification: 630 ×.

hours post treatment (**Figure 3A**, **C** and **D**). However, expression of the gene encoding PPARγ2 was only inducible at 12 hours after TM or Tg treatment (**Figure 3B**). Both TM and Tg challenge failed to increase PPARγ2 expression at either early 6 hours or late 24 hours post treatment. It has been documented that PPARγ2, which is usually expressed in adipose tissue, is inducible in steatotic livers, and contributes to increased *de novo* lipogenesis [19, 21-23]. Our data suggest that pharmacologic ER stress can induce expression of PPARγ2 in hepatocytes that may contribute to ER stress-induced lipogenesis and lipid droplet formation.

TM or TG treatment promotes expression of genes encoding key enzymes in lipid droplet formation and triglyceride synthesis

We extended our effort in understanding pharmacologic ER stress-induced hepatic lipogenesis by identifying ER stress-inducible target genes in lipid droplet formation and triglyceride synthesis [19]. Through quantitative real-time RT-PCR analysis, we found that expression of the genes encoding key factors in lipid droplet formation, including fat-specific protein 27 (FSP27), adipose differentiation related protein (ADRP), fat-inducing transcript 2 (FIT2), and adipocyte lipid-binding protein (AP2), was increased in Huh-7 cells challenged with TM or Tg (**Figure 4**). All these genes were inducible by TM or Tg at 6 hours post treatment. However, expression of ADRP and FSP27 was reduced in Huh-7 cells at 24 hours post TM treatment (**Figure 4A-B**). The expression patterns of *ADRP* and *FSP27* were similar to those of the classic ER stress targets including *XBP1*, *CHOP*, and *BiP* (**Figure 1A-C**), implying that an ER stress-associated negative feedback regulation may exist for expression of the *ADRP* and *FSP27* genes (**Figure 4**). In contrast, expression of AP2, a protein factor involved in lipid transport and storage in lipogenesis and lipolysis [24], was increased in response to TM or Tg treatment from 6 to 24 hours (**Figure 4D**), suggesting a prominent regulation of AP2 gene expression by ER stress. Moreover, we identified two key enzymes required for triglyceride synthesis, acetyl-CoA carboxylase 1 (ACC1), and stearoyl-CoA desaturase-1 (SCD1), were inducible by TM or Tg in Huh-7 cells in a dose- and time-dependent manner (**Figure 5**). Because triglyceride is the core component of lipid droplet, increased expression of key enzymes or protein regulators in triglyceride synthesis and lipid droplet formation may account for ER stress-induced lipid droplet formation.

Activated XBP1 increases expression of PPARγ and C/EBPα in hepatoma cells

To verify the role of the UPR signaling in regulating expression of lipogenic genes, we forcibly expressed an activated form of human XBP1, an ER stress-inducible transcription factor, in Huh-7 cells by utilizing an adenoviral-based overexpression system. As a control, Huh-7 cells were infected by an adenovirus over-expressing GFP. Under ER stress, the UPR transducer IREα is activated to function as an RNase that splices the mRNA encoding X-box binding protein 1 (XBP1) [25-27]. The spliced *XBP1* mRNA, but not the unspliced *XBP1* mRNA, encodes an activated transcription factor that potently activates the UPR target genes. Through Western blot

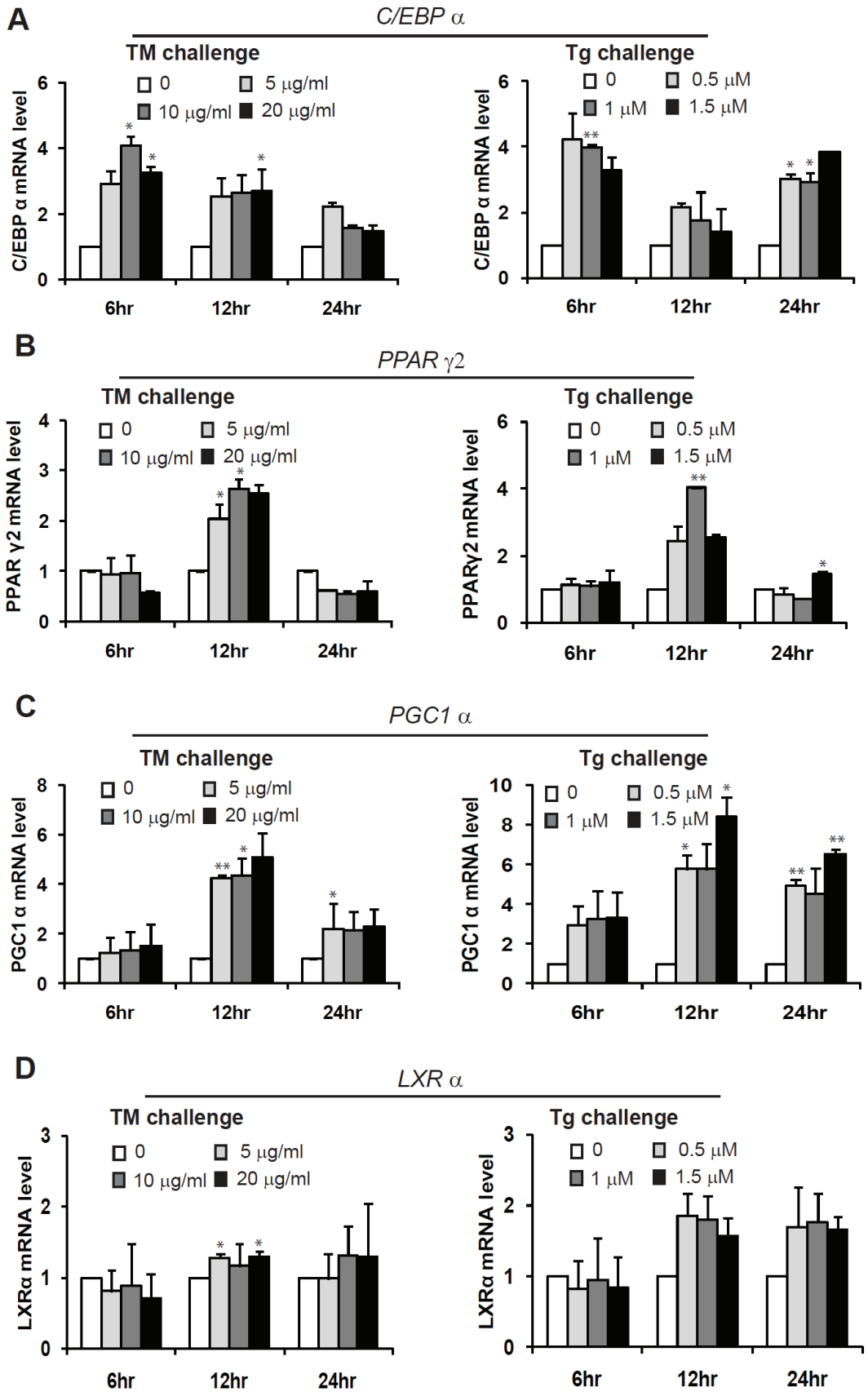
A
C/EBP α
TM challenge
Tg challenge
0
5 μg/ml
10 μg/ml
20 μg/ml
0.5 μM
1 μM
1.5 μM
C/EBP α mRNA level
6hr
12hr
24hr
B
PPAR γ2
PPAR γ2 mRNA level
PPARγ2 mRNA level
C
PGC1 α
PGC1 α mRNA level
D
LXR α
LXRα mRNA level

Figure 3. Quantitative real-time RT-PCR analysis of the mRNAs encoding key lipogenic trans-activators, including C/EBPα (A), PPARγ2 (B), PGC1α (C), and LXRα (D), in Huh-7 cells. Total RNAs were isolated from Huh-7 cells treated with TM (5, 10, and 20 μg/ml) or Tg (0.5, 1, and 1.5 μM) for 6, 12 and 24 hrs. Fold changes of mRNA are shown by comparing to the vehicle-treated control. Each bar denotes mean ± SEM (n= 3). * $p<0.05$; ** $p<0.01$.

analysis, we confirmed that expression levels of the activated form of XBP1 protein (encoded by the spliced *XBP1* mRNA), but not the inactivated XBP1 protein, were significantly increased in Huh-7 cells infected by the adenovirus expressing the activated form of human XBP1 (**Figure 6A-B**). Consistent with the gene expression analysis, Huh-7 cells expressing the activated XBP1 produced much higher levels of PPARγ and C/EBPα, two key lipogenic *trans*-activators, compared to those expressing GFP (**Figure 6A-B**). These results confirm the role of the UPR in activating hepatic lipogenesis. It should be noted that over-expression of the activated XBP1 has mild or no effects on activating expression of other lipogenic regulators or enzymes that are inducible by ER stress challenge (data not shown), suggesting that ER stress may regulate the ER stress-inducible lipogenic factors through the other UPR branches. Nevertheless, our study indentified two ER stress-inducible lipogenic *trans*-activators, C/EBPα and PPARγ, that are under the regulation of the IRE1α/XBP1-mediated UPR pathway.

Discussion

In this study, we demonstrated that pharmacologic ER stress, induced by two structurally-unrelated ER stress-inducing reagents TM and Tg, can promote *de novo* lipogenesis in the hepatoma cell line Huh-7. Both lipid droplet phenotype and gene expression profile further validated the effect of pharmacologic ER stress in promoting lipogenesis and lipid droplet formation (**Figures 2-5**). Importantly, we identified three groups of ER stress-inducible regulators and enzymes in *de novo* lipogenesis (**Figures 3-5**). In particular, we demonstrated that the ER stress-inducible lipogenic *trans*-activators, C/EBPα and PPARγ are regulated by the UPR *trans*-activator XBP1 (**Figure 6**). These results have important implications in the understanding of the upstream signals that facilitate *de novo* lipogenesis.

The UPR signaling is an adaptive response that protects cells from ER stress [28]. The UPR signaling mediated through IRE1α/XBP1, ATF6, and PERK/eIF2α reprograms transcription and translation of stressed cells, leading to alterations in cell physiology that helps the stressed cells adapt to ER stress. However, when ER stress gets more severe or prolonged, the same UPR signaling can activate cell death programs to remove the stressed cells. Lipid droplet is a dynamic organelle composed of a monolayer phospholipid embedded with numerous proteins without trans-membrane spanning domains, and a hydrophobic core that contains triglycerides and sterol esters [29]. Under normal physiological conditions, hepatic lipid droplets are important to maintain lipid and energy homeostasis at the cellular and organismal levels. As a defense response to acute liver injuries, accumulation of lipid droplets is increased in the liver of animal models [6, 7, 30]. Our study suggests that the UPR-regulated *de novo* lipogenesis and accumulation of cytosolic lipid droplets may be parts of the protective response of liver hepatocytes to pharmacologic ER stress. On the other hand, excessive accumulation of lipid droplets is closely associated with the development of metabolic disease [31]. If ER stress-induced lipid droplet accumulation cannot be resolved, prolonged hepatic lipid droplet accumulation may result in metabolic deterioration. This is consistent with the dual roles of the UPR in mediating survival and death signals in the context of cell pathophysiology.

Our work demonstrated that pharmacologic ER stress represents a strong stimulus that triggers *de novo* lipogenesis and lipid storage. In addition to TM or Tg, many pharmaceutical drugs, for example, clinically-used anti-cancer drug Bortezomib, are strong inducers of pharmacologic ER stress [7, 32, 33]. Although the mechanisms involved in its anticancer activity are still being elucidated, Bortezomib has been shown to cause the accumulation of misfolded proteins in the ER by inhibiting the 26S proteasome activity and subsequent ER-associated protein degradation machinery [34-36]. Previously we demonstrated that Bortezomib induces pharmacologic ER stress, causes hepatic steatosis, and increases hepatotoxicity in an animal model [7]. Our work here confirmed that phar-

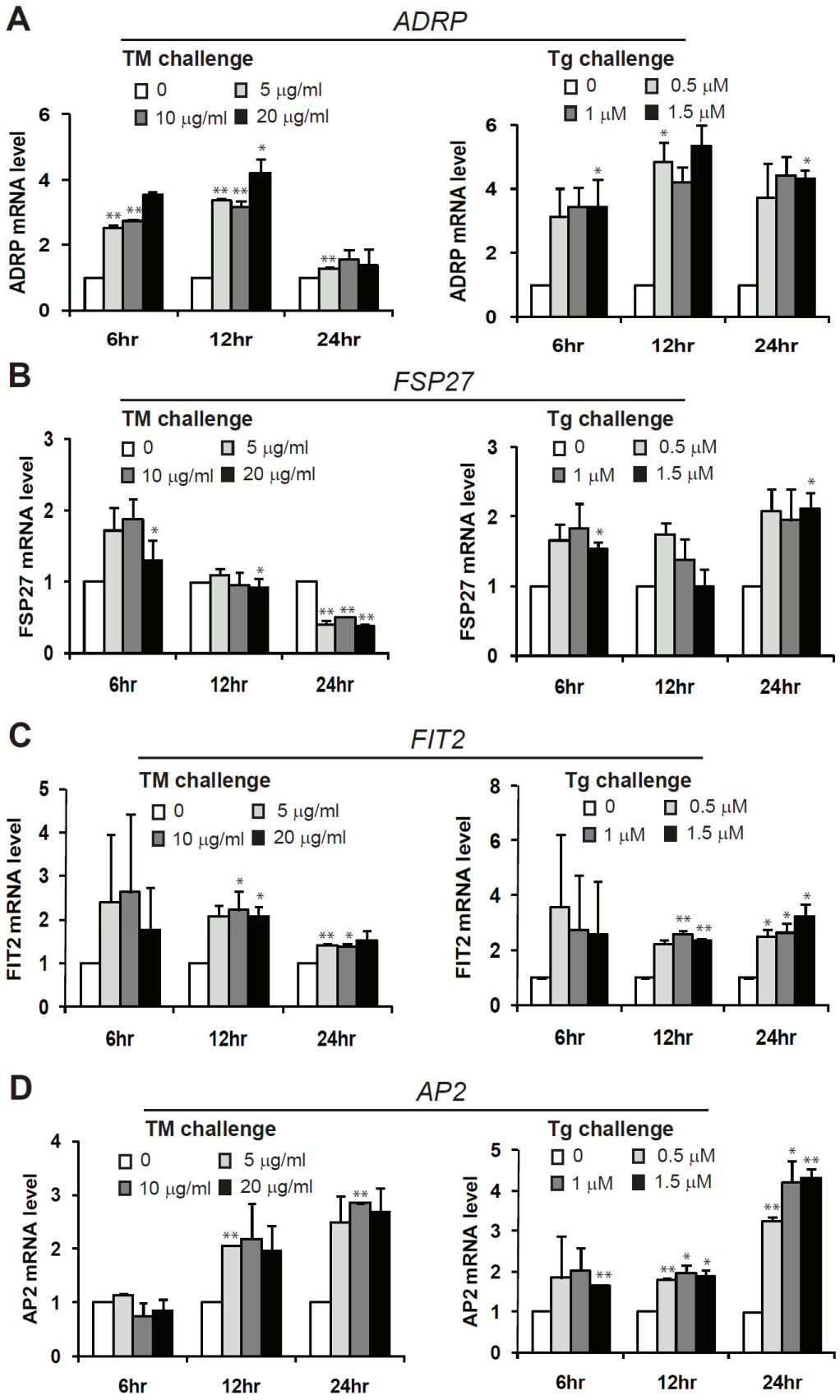
A
ADRP
TM challenge
0
5 μg/ml
10 μg/ml
20 μg/ml
ADRP mRNA level
6hr
12hr
24hr
Tg challenge
0
0.5 μM
1 μM
1.5 μM
B
FSP27
FSP27 mRNA level
C
FIT2
FIT2 mRNA level
D
AP2
AP2 mRNA level

Figure 4. Quantitative real-time RT-PCR analysis of the mRNAs encoding protein factors in lipid droplet formation, including ADRP (A), FSP27 (B), FIT2 (C), and AP2 (D), in Huh-7 cells. Total RNAs were isolated from Huh-7 cells treated with TM (5, 10, and 20 μg/ml) or Tg (0.5, 1, and 1.5 μM) for 6, 12 and 24 hrs. Fold changes of mRNA are shown by comparing to the vehicle-treated control. Each bar denotes mean ± SEM (n= 3). * $p<0.05$; ** $p<0.01$.

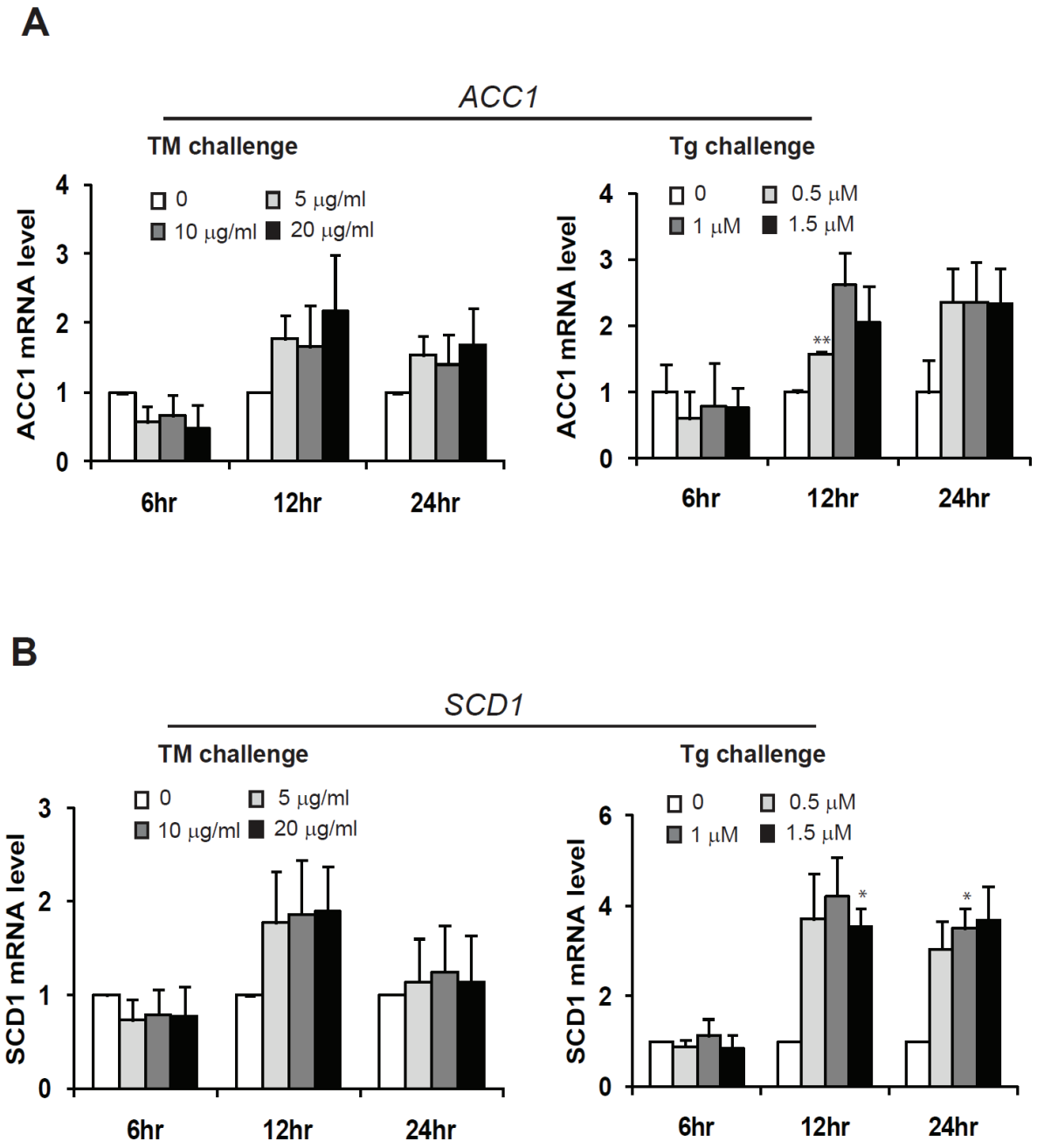

Figure 5. Quantitative real-time RT-PCR analysis of the mRNAs encoding key enzymes in triglyceride synthesis, including ACC1 (A) and SCD1 (B), in Huh-7 cells. Total RNAs were isolated from Huh-7 cells treated with TM (5, 10, and 20 μg/ml) or Tg (0.5, 1, and 1.5 μM) for 6, 12 and 24 hrs. Fold changes of mRNA are shown by comparing to the vehicle-treated control. Each bar denotes mean ± SEM (n= 3). * $p<0.05$; ** $p<0.01$.

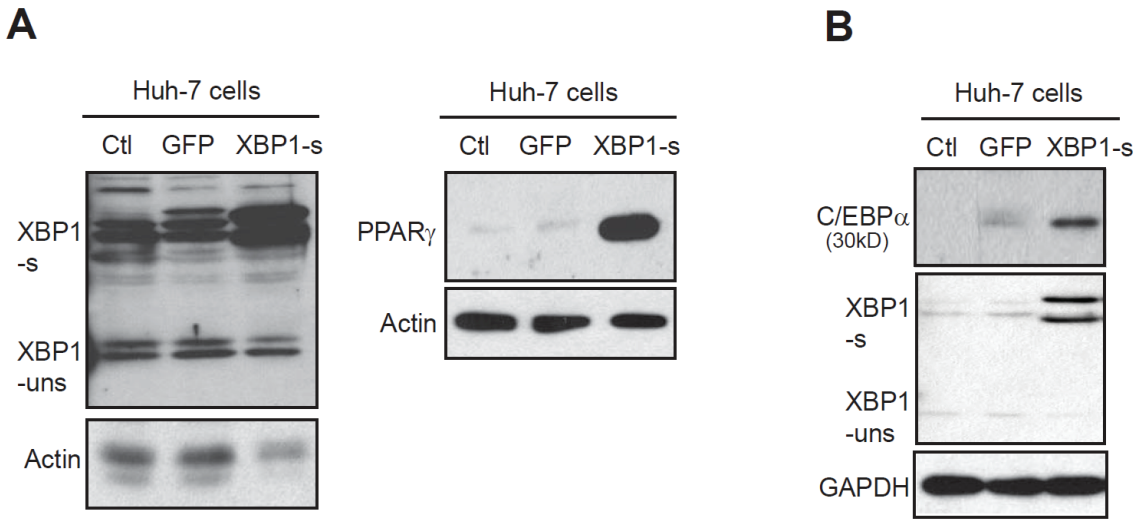

Figure 6. Western blot analysis of expression levels of PPARγ2 (A) and C/EBPα (B) proteins in Huh-7 cells. Huh-7 cells were infected with an adenovirus expressing an activated form of human XBP1 protein or GFP control. Levels of β-actin or GAPDH were determined as a loading control. XBP1-s, the activated form of XBP1 protein encoded by the spliced *XBP1* mRNA; XBP1-uns, the inactivated XBP1 protein encoded by the un-spliced *XBP1* mRNA.

maceuticals that directly or indirectly induce ER stress *in vivo* may have side-effects on induction of hepatic steatosis by promoting lipogenesis and lipid deposition.

In summary, our study provides mechanistic evidence that pharmacologic ER stress and its associated UPR signaling can directly regulate hepatic lipid metabolism by stimulating lipogenesis and lipid droplet accumulation. The identification of the ER stress-inducible lipogenic regulators and enzymes provides important insights into the molecular link between ER stress and lipid metabolism. Additional investigations need to be done in the future in order to delineate the regulation of these individual ER stress-inducible targets by the UPR branches. Nevertheless, the findings from this study significantly contribute to our understanding of pathophysiological roles of ER stress and the UPR as well as potential side effects of ER stress-inducing clinically-used drugs.

Acknowledgement

Portions of this work were supported by American Heart Association Grants 0635423Z and 09GRNT2280479 (KZ), National Institutes of Health (NIH) grants DK090313 and ES017829 (KZ), and the Department of Defense Breast Cancer Program grants BC095179P1 (to KZ and ZY).

Conflict of Interest

None.

Abbreviations: ER, endoplasmic reticulum; UPR, unfolded protein response; TM, tunicamycin; Tg, thapsigargin. PPARγ, peroxisome proliferator-activated receptor gamma; C/EBPα, CCAAT/enhancer binding protein alpha; XBP1, X-box binding protein 1.

Please address correspondence to: Kezhong Zhang, PhD, Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, 540 E. Canfield Avenue, Detroit, MI 48201, USA. Tel: 313-577-2669; Email: kzhang@med.wayne.edu

References

[1] D Ron and P Walter. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol 2007; 8: 519-529.

[2] RJ Kaufman. Orchestrating the unfolded protein response in health and disease. J Clin Invest 2002; 110: 1389-1398.

[3] Y Kozutsumi, M Segal, K Normington, MJ Gething and J Sambrook. The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. Nature 1988; 332: 462-464.

[4] AJ Dorner, LC Wasley and RJ Kaufman. Increased synthesis of secreted proteins in-

duces expression of glucose regulated proteins in butyrate treated CHO cells. Journal of Biological Chemistry 1989; 264: 20602-20607.
[5] C Zhang, G Wang, Z Zheng, KR Maddipati, X Zhang, G Dyson, P Williams, SA Duncan, RJ Kaufman and K Zhang. ER-tethered transcription factor crebh regulates hepatic lipogenesis, fatty acid oxidation, and lipolysis upon metabolic stress. Hepatology 2011; doi: 10.1002/hep.24783
[6] DT Rutkowski, J Wu, SH Back, MU Callaghan, SP Ferris, J Iqbal, R Clark, H Miao, JR Hassler, J Fornek, MG Katze, MM Hussain, B Song, J Swathirajan, J Wang, GD Yau and RJ Kaufman. UPR pathways combine to prevent hepatic steatosis caused by ER stress-mediated suppression of transcriptional master regulators. Dev Cell 2008; 15: 829-840.
[7] K Zhang, S Wang, J Malhotra, JR Hassler, SH Back, G Wang, L Chang, W Xu, H Miao, R Leonardi, YE Chen, S Jackowski and RJ Kaufman. The unfolded protein response transducer IRE1alpha prevents ER stress-induced hepatic steatosis. EMBO J 2011; 30: 1357-1375.
[8] MW Lee, D Chanda, J Yang, H Oh, SS Kim, YS Yoon, S Hong, KG Park, IK Lee, CS Choi, RW Hanson, HS Choi and SH Koo. Regulation of hepatic gluconeogenesis by an ER-bound transcription factor, CREBH. Cell Metab 2010; 11: 331-339.
[9] S Oyadomari, HP Harding, Y Zhang, M Oyadomari and D Ron. Dephosphorylation of translation initiation factor 2alpha enhances glucose tolerance and attenuates hepatosteatosis in mice. Cell Metab 2008; 7: 520-532.
[10] K Yamamoto, K Takahara, S Oyadomari, T Okada, T Sato, A Harada and K Mori. Induction of liver steatosis and lipid droplet formation in ATF6alpha-knockout mice burdened with pharmacological endoplasmic reticulum stress. Mol Biol Cell 2010; 21: 2975-2986.
[11] AH Lee, EF Scapa, DE Cohen and LH Glimcher. Regulation of hepatic lipogenesis by the transcription factor XBP1. Science 2008; 320: 1492-1496.
[12] H Nakabayashi, K Taketa, K Miyano, T Yamane and J Sato. Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. Cancer Res 1982; 42: 3858-3863.
[13] S Laing, G Wang, T Briazova, C Zhang, A Wang, Z Zheng, A Gow, AF Chen, S Rajagopalan, LC Chen, Q Sun and K Zhang. Airborne particulate matter selectively activates endoplasmic reticulum stress response in the lung and liver tissues. Am J Physiol Cell Physiol 2010; 299: C736-749.
[14] SW Park, Y Zhou, J Lee, A Lu, C Sun, J Chung, K Ueki and U Ozcan. The regulatory subunits of PI3K, p85alpha and p85beta, interact with XBP-1 and increase its nuclear translocation. Nat Med 16: 429-437.
[15] IA King and A Tabiowo. Effect of tunicamycin on epidermal glycoprotein and glycosaminoglycan synthesis in vitro. Biochem J 1981; 198: 331-338.
[16] TR Jackson, SI Patterson, O Thastrup and MR Hanley. A novel tumour promoter, thapsigargin, transiently increases cytoplasmic free Ca2+ without generation of inositol phosphates in NG115-401L neuronal cells. Biochem J 1988; 253: 81-86.
[17] Y Sagara and G Inesi. Inhibition of the sarcoplasmic reticulum Ca2+ transport ATPase by thapsigargin at subnanomolar concentrations. J Biol Chem 1991; 266: 13503-13506.
[18] RJ Kaufman. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes.Dev. 1999; 13: 1211-1233.
[19] G Musso, R Gambino and M Cassader. Recent insights into hepatic lipid metabolism in nonalcoholic fatty liver disease (NAFLD). Prog Lipid Res 2009; 48: 1-26.
[20] C Postic and J Girard. Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. J Clin Invest 2008; 118: 829-838.
[21] L Dubuquoy, S Dharancy, S Nutten, S Pettersson, J Auwerx and P Desreumaux. Role of peroxisome proliferator-activated receptor gamma and retinoid X receptor heterodimer in hepatogastroenterological diseases. Lancet 2002; 360: 1410-1418.
[22] SE Schadinger, NL Bucher, BM Schreiber and SR Farmer. PPARgamma2 regulates lipogenesis and lipid accumulation in steatotic hepatocytes. Am J Physiol Endocrinol Metab 2005; 288: E1195-1205.
[23] T Yamazaki, S Shiraishi, K Kishimoto, S Miura and O Ezaki. An increase in liver PPARgamma2 is an initial event to induce fatty liver in response to a diet high in butter: PPARgamma2 knockdown improves fatty liver induced by high-saturated fat. J Nutr Biochem 2010;
[24] L Banaszak, N Winter, Z Xu, DA Bernlohr, S Cowan and TA Jones. Lipid-binding proteins: a family of fatty acid and retinoid transport proteins. Adv Protein Chem 1994; 45: 89-151.
[25] H Yoshida, T Matsui, A Yamamoto, T Okada and K Mori. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 2001; 107: 881-891.
[26] X Shen, RE Ellis, K Lee, CY Liu, K Yang, A Solomon, H Yoshida, R Morimoto, DM Kurnit, K Mori and RJ Kaufman. Complementary signaling pathways regulate the unfolded protein response and are required for C. elegans development. Cell 2001; 107: 893-903.
[27] M Calfon, H Zeng, F Urano, JH Till, SR Hubbard, HP Harding, SG Clark and D Ron. IRE1

couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature 2002; 415: 92-96.
[28] K Zhang and RJ Kaufman. From endoplasmic-reticulum stress to the inflammatory response. Nature 2008; 454: 455-462.
[29] DA Brown. Lipid droplets: proteins floating on a pool of fat. Curr Biol 2001; 11: R446-449.
[30] L Yang, R Jhaveri, J Huang, Y Qi and AM Diehl. Endoplasmic reticulum stress, hepatocyte CD1d and NKT cell abnormalities in murine fatty livers. Lab Invest 2007; 87: 927-937.
[31] SE Thomas, LE Dalton, ML Daly, E Malzer and SJ Marciniak. Diabetes as a disease of endoplasmic reticulum stress. Diabetes Metab Res Rev 2010; 26: 611-621.
[32] PG Richardson, C Mitsiades, T Hideshima and KC Anderson. Bortezomib: proteasome inhibition as an effective anticancer therapy. Annu Rev Med 2006; 57: 33-47.
[33] PG Richardson, B Barlogie, J Berenson, S Singhal, S Jagannath, D Irwin, SV Rajkumar, G Srkalovic, M Alsina, R Alexanian, D Siegel, RZ Orlowski, D Kuter, SA Limentani, S Lee, T Hideshima, DL Esseltine, M Kauffman, J Adams, DP Schenkein and KC Anderson. A phase 2 study of bortezomib in relapsed, refractory myeloma. N Engl J Med 2003; 348: 2609-2617.
[34] AH Lee, NN Iwakoshi, KC Anderson and LH Glimcher. Proteasome inhibitors disrupt the unfolded protein response in myeloma cells. Proc Natl Acad Sci U S A 2003; 100: 9946-9951.
[35] A Fribley, Q Zeng and CY Wang. Proteasome inhibitor PS-341 induces apoptosis through induction of endoplasmic reticulum stress-reactive oxygen species in head and neck squamous cell carcinoma cells. Mol Cell Biol 2004; 24: 9695-9704.
[36] ST Nawrocki, JS Carew, K Dunner, Jr., LH Boise, PJ Chiao, P Huang, JL Abbruzzese and DJ McConkey. Bortezomib inhibits PKR-like endoplasmic reticulum (ER) kinase and induces apoptosis via ER stress in human pancreatic cancer cells. Cancer Res 2005; 65: 11510-11519.

CHAPTER NINETEEN

Measurement of ER Stress Response and Inflammation in the Mouse Model of Nonalcoholic Fatty Liver Disease

Ze Zheng,[*,†] Chunbin Zhang,[*] *and* Kezhong Zhang[*,‡,1]

Contents

1. Introduction 330
2. Methods to Measure ER Stress Response and Hepatic Inflammation in the Mouse Model of NAFLD 332
   2.1. Establishing the mouse model of NAFLD 332
   2.2. Detecting activation of the UPR pathways in mouse fatty liver tissue 333
   2.3. Detecting hepatic inflammation in the mouse model of NAFLD 339
3. Visualizing ER Stress Response Associated with Liver Pathology in the Mouse Model of NAFLD 341
   3.1. Staining ER stress markers in mouse fatty liver tissue 341
   3.2. Staining hepatic lipids 343
   3.3. Staining liver fibrosis 343
Acknowledgments 345
References 345

Abstract

In eukaryotic cells, the endoplasmic reticulum (ER) is the organelle that is responsible for protein folding and assembly, lipid and sterol biosynthesis, and intracellular calcium storage. Biochemical or pathophysiological stimuli that disrupt protein-folding reaction or increase protein-folding load can cause accumulation of unfolded or misfolded proteins in the ER lumen, a condition called "ER stress". As an adaptive intracellular stress response initiated from the ER, unfolded protein response (UPR) alleviates the accumu-

* The Center for Molecular Medicine and Genetics, The Wayne State University School of Medicine, Detroit, Michigan, USA
† Currently a graduate student in the program of Biological Science at the Wayne State University
‡ The Department of Immunology and Microbiology, The Wayne State University School of Medicine, Detroit, Michigan, USA
1 Corresponding author.

Methods in Enzymology, Volume 489
ISSN 0076-6879, DOI: 10.1016/B978-0-12-385116-1.00019-4
© 2011 Elsevier Inc.
All rights reserved.

lation of unfolded or misfolded proteins in the ER. It has been demonstrated that the UPR is a fundamental intracellular signal transduction response that is critical for health and disease. ER stress and other cellular stress responses, such as inflammation and oxidative stress, are integrated in many pathophysiological processes. Particularly, recent research demonstrated that ER stress and the UPR signaling are critically involved in the initiation and progression of nonalcoholic fatty liver disease (NAFLD). Under metabolic stress conditions, the UPR regulates transcriptional and translational programs that are associated with hepatic steatosis and inflammation, the major characteristics of NAFLD. In this chapter, we summarize reliable methods to quantitatively analyze the UPR and hepatic inflammation in the mouse model of NAFLD.

1. Introduction

In eukaryote, the endoplasmic reticulum (ER) is the organelle for protein folding and assembly, lipid and sterol biosynthesis, and free calcium storage inside the cell (Gething and Sambrook, 1992). As a protein-folding compartment and calcium storage, the ER maintains a very sensitive homeostasis that can be perturbed by numerous stress signals, such as toxic chemicals, oxidative stress, nutrient depletion, hypoxia, expression of secretory proteins, DNA damage, and bacterial and/or viral infection (Kaufman, 1999; Ron and Walter, 2007). As a consequence, protein-folding process is interrupted, leading to the accumulation of unfolded or misfolded proteins in the ER lumen. To deal with this stressful condition, the ER has evolved a highly specific signaling pathway called unfolded protein response (UPR) to remodel the transcriptional and/or translational programs of stressed cells. There are three basic UPR signal transduction molecules that localize to the ER membrane: inositol-requiring 1α (IRE1α), double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6). These three UPR transducers are coordinately activated to reduce the amount of newly synthesized proteins translocated into the ER, to increase protein-folding capacity, and to promote the degradation of misfolded proteins in the ER (Kaufman, 1999; Ron and Walter, 2007).

IRE1α is a protein kinase/endoribonuclease. Upon ER stress, IRE1α is activated to splice the mRNA encoding X-box binding protein 1 (XBP1), a basic leucine zipper (bZIP) transcription factor that can activate expression of UPR target genes encoding functions involved in ER expansion, protein folding and secretion, and degradation of misfolded proteins (Lee *et al.*, 2003b; Yamamoto *et al.*, 2007; Yoshida *et al.*, 2001). The activated IRE1α also functions as a scaffold protein to recruit tumor-necrosis factor (TNF)-receptor-associated factor 2 (TRAF2), leading to activation of the Jun amino-terminal kinase (JNK)-mediated signaling pathway under ER stress (Urano *et al.*, 2000). PERK is a PKR-like ER kinase that can phosphorylate

the α-subunit of eukaryotic translation–initiation factor 2 (eIF2α) (Harding *et al.*, 1999, 2000b; Scheuner *et al.*, 2001). To reduce the amount of newly synthesized proteins entering into the ER, PERK mediates phosphorylation of eIF2α, leading to a general translational attenuation (Harding *et al.*, 2000b). However, phosphorylated eIF2a can selectively induce translation of a specific subset of mRNAs that harbor multiple upstream open reading frames in 5′-untranslated regions (Harding *et al.*, 2000a; Scheuner *et al.*, 2001; Yaman *et al.*, 2003). For examples, phosphorylated eIF2α can induce translation of the mRNA encoding activating transcription factor 4 (ATF4) in mammalian cells under prolonged ER stress. ATF4 subsequently activates transcription of PERK-dependent UPR target genes, such as CHOP and NF-E2-related factor-2 (Nrf2), to mediate antioxidative response and/or ER stress-induced apoptosis (Cullinan *et al.*, 2003; Harding *et al.*, 2000a; Marciniak *et al.*, 2004). ATF6 is an ER-transmembrane bZIP transcription factor of ATF/CREB family (Haze *et al.*, 1999). Upon activation of UPR, ATF6 is released and translocated to the Golgi compartment where it is cleaved by site-1 protease (S1P) and site-2 protease (S2P) to release an active transcription factor (Ye *et al.*, 2000). Activated ATF6 induces expression of the UPR target genes encoding ER-resident molecular chaperones, folding catalysts, and ERAD machinery (Okada *et al.*, 2002; Yamamoto *et al.*, 2007).

Nonalcoholic fatty liver disease (NAFLD), the hepatic manifestation of metabolic syndrome, is characterized by excessive hepatic lipid accumulation, inflammation, and fibrosis in the absence of significant ethanol consumption, viral infection, or other specific etiologies (Angulo, 2002; Brunt, 2001). Recently, increasing evidence suggested that the UPR is critical for the development of NAFLD. Chronic excess of metabolic factors, such as lipids, glucose, and inflammatory cytokines, can interrupt the protein-folding process in the ER and subsequently activate physiological UPR in specialized cell types, such as macrophages and hepatocytes (Feng *et al.*, 2003; Kharroubi *et al.*, 2004; Wei *et al.*, 2006; Zhang and Kaufman, 2008a; Zhang *et al.*, 2006). Increased saturated fatty acids delivered to or accumulated in the liver have been shown to induce ER stress and activation of the UPR that leads to dysregulated lipid metabolism and hepatic injuries. Therefore, ER stress represents an intrinsic hit that triggers NAFLD, and delineation of ER stress signaling in fatty livers is particularly informative to novel therapeutic designs toward a cure of NAFLD (Wang *et al.*, 2006; Wei *et al.*, 2006). Moreover, increasing evidence suggest that the UPR pathways mediated through IRE1α, PERK, and ATF6 are differentially involved in regulating hepatic lipid metabolism upon metabolic diets or acute liver injuries (Lee *et al.*, 2008; Oyadomari *et al.*, 2008; Rutkowski *et al.*, 2008). Therefore, delineating ER stress and the UPR pathways in the liver of mouse model of NAFLD provides important avenues to address ER stress-associated mechanism and possible novel therapeutic targets in prevention and treatment of NAFLD.

2. Methods to Measure ER Stress Response and Hepatic Inflammation in the Mouse Model of NAFLD

In this section, we describe optimal conditions and procedures to detect activation of three major UPR pathways and hepatic inflammation in mouse fatty liver tissue. To establish an animal model of NAFLD, we feed wild-type mice of C57BL/6J strain background with either a normal chow diet or an atherogenic high-fat (AHF) diet that has been demonstrated to induce hepatic steatosis and steatohepatitis in the mice (Larter and Yeh, 2008; Matsuzawa *et al.*, 2007). Liver tissues and blood samples will be isolated from the animals after the AHF or normal chow diet for Western blot, quantitative real-time PCR, enzyme-linked immunosorbent assay (ELISA), and immunohistochemical staining analyses.

2.1. Establishing the mouse model of NAFLD

Required materials:

- Wild-type mice of C57BL/6J strain background, male, 3 months old (Cat # 000664, The Jackson Laboratory).
- AHF diet, also called Paigen Diet (Paigen *et al.*, 1987): 15% fat, 1.25% cholesterol, and 0.5% cholic acid (Cat # TD.88051, Harlan Laboratories, Inc.).
- 10× phosphate buffered saline (PBS): 8% sodium chloride (NaCl), 0.2% potassium chloride (KCl), 1.44% sodium phosphate, dibasic (Na_2HPO_4), and 0.24% potassium phosphate, monobasic (KH_2PO_4), pH 7.4.
- 10% formalin (Cat # HT501128, Sigma)
- Tissue-Tek O.C.T. compound (Cat # 1SLB4583EA, Sakura Finetek USA, California)

Procedures: Wild-type male C57BL/6J mice of 3 months old are fed with either the AHF diet or normal chow diet for 6 months (Larter and Yeh, 2008; Matsuzawa *et al.*, 2007). After 6 months of AHF diet feeding, the mice are euthanized and liver tissues and blood samples are collected: (1) The mouse liver tissues are snap frozen in liquid nitrogen and stored at −80 °C for future Western blot and quantitative real-time reverse-transcription (RT)-PCR analyses. (2) Fresh liver tissue pieces are washed briefly with PBS buffer and then fixed in 10% PBS-buffered formalin. Fixed liver samples are paraffin-embedded, and 4 μm sections are prepared and mounted on glass slides for immunohistochemical staining. (3) Fresh liver tissue pieces are embedded in specimen molds with Tissue-Tek O.C.T. compound and frozen using dry ice. The O.C.T.-embedded frozen liver tissues are cut

into 8 μm sections and stored at −80 °C for future staining of hepatic lipid droplets. (4) Blood plasma samples will be collected and stored at −80 °C for future detection of acute-phase proteins using ELISA.

2.2. Detecting activation of the UPR pathways in mouse fatty liver tissue

Western blot analysis or immunoprecipitation (IP)–Western blot analysis is utilized to determine activation of the major UPR pathways mediated by ER stress sensors IRE1α, PERK, and ATF6, respectively. Quantitative real-time RT-PCR analysis is used to quantify spliced *Xbp1* mRNA and expression of other UPR target genes. As lipid contents are enriched in the liver tissues of mice after the AHF diet, lipid extraction is required in preparing liver cellular lysates for Western blot analysis.

Required materials:

- NP-40 lysis buffer: 1% NP-40, 50 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 0.05% SDS, 0.5 m*M* Na vanadate, 100 m*M* NaF, 50 m*M* β-glycerophosphate, and 1 m*M* phenylmethylsulfonyl fluoride (PMSF) supplemented with protease inhibitors (EDTA-free Complete Mini, Roche).
- 2× SDS-PAGE sample buffer: 100 m*M* Tris, pH 6.8, 20% glycerol, 4% SDS, 0.2% bromophenol blue, 200 m*M* dithiothreitol.
- Trizol reagent (Cat # 15596026, Invitrogen).
- DEPC water: add 0.1 ml DEPC in 100 ml distilled water (dH_2O) and incubate the solution for 12 h at 37 °C before subjecting to autoclave to remove any traces of DEPC.
- IP washing buffer: 50 m*M* Tris–HCl, pH 7.5, 500 m*M* sodium chloride, 0.1% Nonidet 40, 0.05% sodium deoxycholate.

Liver cellular protein lysates preparation: (1) approximately 100 mg fresh liver tissue from the mice after the AHF or normal chow diet is briefly washed with PBS and then homogenized in 800 μl of ice-cold NP-40 lysis buffer; (2) the homogenized liver tissue lysate is incubated on ice for 40 min; (3) liver tissue lysate is centrifuged at 4 °C at 15,000 rpm for 15 min; (4) collect the lysate sample in the middle phase between lipid contents (upper phase) and tissue debris (bottom); (5) add 0.5% SDS solution into the protein lysate, and centrifuge the lysate sample at 4 °C at 15,000 rpm for 10 min to extract lipid contents; (6) collect the lysate sample in the middle phase, and repeat the lipid extraction step using 0.5% SDS; (7) the lysate sample in the middle phase is collected for Western blot analysis, IP–Western blot analysis, or stored at −80 °C for future use.

Preparation of total RNA and cDNA from mouse liver tissue: (1) Total RNA is isolated from liver tissue with the Trizol reagent; (2) synthesis of

cDNA from murine total RNA is performed using a SuperScript III reverse transcriptase kit (Cat # 11752, Invitrogen). The reaction mixture (20 μl) contains 500 ng total RNA, 10 μl 2× RT reaction mix, and 2 μl reverse transcriptase enzyme mix; (3) the reverse transcription reaction is incubated at 25 °C for 10 min, followed by incubation at 50 °C for 30 min, and finally reverse transcriptase is inactivated at 85 °C for 5 min; (4) the reaction mix is diluted 10-fold by the addition of 180 μl nuclease-free water. The diluted cDNA mix from the reverse transcription reaction is subjected to quantitative real-time PCR analysis.

2.2.1. Measuring IRE1-mediated UPR signaling

2.2.1.1. Phosphorylation of IRE1α IRE1α is the most conserved UPR transducer (Kaufman, 1999). Activation of IRE1α requires its *homo*-dimerization and *auto*-phosphorylation. Phosphorylation of IRE1α is the key indicator of activation of IRE1α-mediated UPR pathway. Expression and phosphorylation of IRE1α can be analyzed by an upward mobility shift upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

The conditions and procedures to detect levels of total and phosphorylated IRE1α proteins are as follows: (1) liver protein lysates are prepared from approximately 100 mg of fresh or frozen liver tissue as described in Section 2.1; (2) protein concentrations of the lysate are determined by the Bradford assay using the commercial kit (Cat # 500-0205, Bio-Rad Laboratories); (3) appropriate amounts of 2× SDS-PAGE sample buffer are added into the protein lysate solution followed by heating at 95° for 5 min; (4) approximately 80 μg of denatured proteins are separated by SDS-PAGE on 10% Tris–glycine polyacrylamide gels and transferred to a 0.45-μm PVDF membrane (RPN1416F, GE Healthcare); (5) to detect levels of total IRE1α protein, the blots are incubated with a rabbit anti-human IRE1α antibody (Cat # 3294, Cell Signaling Technology) at a 1:1000 dilution in 0.1% Tween 20–PBS buffer containing 5% skim milk overnight at 4 °C. To detect levels of phosphorylated IRE1α protein, the blots are incubated with a rabbit anti-human phosphorylated IRE1α antibody (Cat # ab48187, Abcam) at a 1:1000 dilution in 0.1% Tween 20–PBS buffer containing 5% skim milk overnight at 4 °C; (6) after the primary antibody incubation, the blots are washed and then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Cat # W401B, Promega) at a 1:3000 dilution for 2 h at room temperature; (7) membrane-bound antibodies are detected by an enhanced chemiluminescence (ECL) detection reagent (Cat # 32106, Thermo Scientific). The size of detected mouse IRE1α protein is approximately 120 kDa. The size of phosphorylated IRE1α protein is increased slightly.

2.2.1.2. Xbp1 mRNA splicing and expression of spliced XBP1 protein Under ER stress, IRE1α is activated to render its endoribonuclease/RNase activity (Ron and Walter, 2007). IRE1α RNase cleaves *XBP1* mRNA to remove a 26-nucleotide intron, generating a translational frameshift. Spliced Xbp1 mRNA encodes a functional XBP1 protein of approximately 55 kDa. Unspliced *Xbp1* mRNA can also encode a protein of approximately 30 kDa, which is rapidly degraded by proteasomes (Lee *et al.*, 2003a; Yoshida *et al.*, 2006). It has been suggested that the XBP1 protein encoded by unspliced *Xbp1* mRNA can act as a dominant-negative inhibitor of the spliced form during the recovery phase of ER stress or when the protein is stabilized in the presence of proteasome inhibitor. Quantitative analyses of the *Xbp1* mRNA splicing and the XBP1 protein expression are critical in determining the activation of IRE1α-mediated UPR pathway.

For quantitative real-time RT-PCR analysis of mouse Xbp1 mRNA splicing, a pair of real-time PCR primers is designed to specifically amplify the spliced Xbp1 mRNA (Zhang and Kaufman, 2008b; Zhang *et al.*, 2005): 5′-GAGTCCGCAGCAGGTG-3′ and 5′-GTGTCAGAGTCCATGG-GA-3′. Additionally, a pair of real-time PCR primers is used to amplify β-*actin* mRNA as an internal control: 5′-GATCTGGCAC-CACACCTTCT-3′ and 5′-GGGGTGTTGAAGGTCTCAAA-3′. The procedures to quantitatively analyze spliced *Xbp1* mRNA by quantitative real-time RT-PCR: (1) SYBR GreenER q-PCR Supermix (Cat # 11762-500, Invitrogen) is used for quantitative real-time RT-PCR reactions. 12.5 ng cDNA templates prepared from mouse liver total RNA as described in Section 2.1, 500 n*M* forward and reverse primers, 10 μl SYBR GreenER q-PCR Supermix, and DEPC-treated water are mixed for 20 μl reaction volume; (2) the reaction is performed in Stratagene MX3000P Q-PCR system. The thermal cycle parameters are step 1, 95 °C for 10 min; step 2, 95 °C for 30 s, 59 °C for 1 min, and then 72 °C for 30 s; and step 3, 59 °C for 1 min. Step 2 is repeated for 40 cycles. The final step is incubation at 4 °C to terminate the reaction. (3) Fold changes of spliced mRNA levels were determined after normalization to internal control *β-actin* mRNA levels using the 2(-Delta Delta C(T)) Method (Livak and Schmittgen, 2001).

The procedure to determine levels of XBP1 proteins: (1) Liver protein lysates are prepared from approximately 100 mg of fresh or frozen liver tissue as described in Section 2.1; (2) protein concentrations of the lysates are determined by the Bradford assay, and then denatured in SDS-PAGE sample buffer by heating the samples at 95° for 5 min; (4) approximately 80 μg of denatured proteins are separated by SDS-PAGE on 10% Tris–glycine polyacrylamide gels and transferred to a 0.45-μm PVDF membrane; (5) the blots are incubated with a rabbit anti-mouse XBP1 antibody (Cat # sc-7160, Santa Cruz Biotechnologies) at a 1:200 dilution in 0.1% Tween

20–PBS buffer containing 5% skim milk overnight at 4 °C; (6) after the primary antibody incubation, the blots are washed and then incubated with a 1:3000 dilution of HRP-conjugated anti-mouse antibody (Cat # W402B, Promega) for 2 h at room temperature; (7) membrane-bound antibodies are detected by an ECL detection reagent. The size of murine XBP1 protein encoded by spliced Xbp1 mRNA is approximately 55 kDa; and the size of XBP1 protein encoded by unspliced Xbp1 mRNA is approximately 28 kDa.

2.2.1.3. IRE1α interaction with TRAF2 In addition to its endoribonuclease activity, activated IRE1α can serve as a scaffold protein that recruits TRAF2, leading to activation of JNK and/or nuclear factor-κB (NF-κB) under ER stress (Hu *et al.*, 2006; Urano *et al.*, 2000). This might provide stressed cells with survival or death signals by activating JNK- and/or NF-κB-mediated signaling pathways that are associated with the progression of liver diseases. To evaluate the activity of IRE1α in mediating JNK and/or NF-κB activation in mouse fatty liver, a key experiment is to determine interaction between IRE1α and TRAF2 using IP–Western blot analysis.

The procedures to detect interaction between IRE1α and TRAF2 in mouse fatty liver tissue: (1) a rabbit anti-human TRAF2 antibody (10 μl; Cat # sc-876, Santa Cruz Biotechnologies) is incubated with 50 μl protein A beads (Cat # 11134515, Roche) in NP-40 lysis buffer for 1 h on an end-over-end rotator at room temperature; (2) the antibody-bound beads are washed for three times by NP-40 lysis buffer in 1.5 ml Eppendorf tubes, rotating at 4 °C for 10 min; (3) the liver protein lysates of approximately 300 μg (~1 ml) are added into the tubes with washed antibody-bound beads and then incubated overnight at 4 °C on a rotator; (4) after incubation, the beads are washed for three times with 1 ml lysis buffer and one time with 1 ml IP washing buffer; (5) the solution from the tube containing the beads is removed and then 40 μl of 2× SDS-PAGE sample buffer is added into the tube followed by heating at 95° for 5 min; (6) Denatured proteins are separated by 10% SDS-PAGE gels and transferred to a PVDF membrane; (7) the blots are incubated with a rabbit anti-human IRE1α antibody (Cat # 3294, Cell Signaling Technology) at a 1:1000 dilution overnight at 4 °C; (8) after the incubation with the primary antibody, the blots are incubated with a 1:1000 dilution of HRP-conjugated TrueBlot anti-rabbit antibody (Cat # 18-8816, eBioscience) for 2 h at room temperature. The TrueBlot anti-rabbit antibody preferentially detects the nonreduced form of rabbit IgG with minimal reactivity to the reduced, SDS-denatured form of IgG; (9) membrane-bound antibodies are detected by the ECL detection reagent described above. The size of detected mouse IRE1α protein that interacts with TRAF2 is approximately 120 kDa.

2.2.2. Measuring PERK-mediated UPR pathway

2.2.2.1. Phosphorylation of PERK Similar to IRE1α, phosphorylation of PERK is a hallmark of activation of PERK-mediated UPR pathway. However, due to its low expression level, detection of endogenous levels of PERK in liver tissues by direct Western blot analysis is very difficult. To overcome this difficulty, liver protein lysates prepared from the liver tissues of mice after the high-fat or normal chow diet are immunoprecipitated with an anti-PERK antibody followed by Western blot analysis using the same antibody.

The procedures to detect endogenous PERK expression in mouse liver tissue: (1) rabbit anti-human PERK polyclonal antibody (5 μl; Cat # sc-13073, Santa Cruz Biotechnologies) is incubated with 50 μl protein A beads (Cat # 11134515, Roche) in NP-40 lysis buffer for 1 h on an end-over-end rotator at room temperature; (2) the antibody-bound beads are washed three times by NP-40 lysis buffer in 1.5 ml Eppendorf tubes, rotating at 4 °C for 10 min; (3) the liver protein lysates of approximately 300 μg (~1 ml) are added into the tubes with washed antibody-bound beads and then incubated overnight at 4 °C on a rotator; (4) after incubation, the beads are washed three times with 1 ml lysis buffer and one time with 1 ml IP washing buffer; (5) the solution from the tube containing the beads is removed and then 40 μl of 2× SDS-PAGE sample buffer is added to the tube followed by heating at 95° for 5 min; (6) denatured proteins are separated by 10% SDS-PAGE gels and transferred to a PVDF membrane; (7) the blots are incubated with the same rabbit anti-human PERK antibody at a 1:200 dilution overnight at 4 °C; (8) after incubation with the primary antibody, the blots are incubated with a 1:1000 dilution of HRP-conjugated TrueBlot anti-rabbit antibody (Cat # 18-8816, eBioscience) for 2 h at room temperature; (9) membrane-bound antibodies are detected by the ECL detection reagent described above. The size of detected mouse PERK protein is approximately 170 kDa. The size of PERK protein is increased slightly due to phosphorylation.

2.2.2.2. Phosphorylation of eIF2α In response to ER stress, PERK phosphorylates translational initiation factor eIF2α, leading to general translation attenuation as well as selective translation of specific mRNAs (Ron and Walter, 2007). Quantitative analysis of phosphorylated versus total eIF2α protein is important for evaluating activation of the PERK-mediated UPR pathway.

Procedures to determine levels of phosphorylated and total eIF2α proteins in mouse liver tissue are as follows: (1) The liver protein lysates of approximately 80 μg are denatured, separated by 10% SDS-PAGE gels, and transferred to a PVDF membrane as described above. (2) To detect the phosphorylated eIF2α protein, the blots are incubated with a rabbit

anti-Ser51-phosphorylated eIF2α antibody (Cat # 9721, Cell Signaling Technology) at a 1:1000 dilution overnight at 4 °C. To detect the total eIF2α protein, the blots are incubated with a rabbit total eIF2α antibody (Cat # 9722, Cell Signaling Technology) at a 1:1000 dilution overnight at 4 °C. (3) After the antibody incubations, the blots are incubated with the ECL detection reagent to detect chemiluminescent signals as described above. The size of detected mouse eIF2α protein is approximately 38 kDa. The size of phosphorylated eIF2α protein is slightly increased.

2.2.2.3. Expression of ATF3, ATF4, CHOP, and GADD34 Upon activation of PERK-mediated UPR pathway, phosphorylated eIF2α can selectively induce expression of ATF4, a bZIP transcription activator, in mammalian cells (Ron and Walter, 2007). Subsequently, ATF4 activates expression of additional bZIP regulators, CHOP/GADD153, and ATF3, which function to regulate cell metabolism, redox status of the cell, and apoptosis. Furthermore, CHOP directly activates *GADD34*, a growth arrest and DNA damage-inducible protein that can form a complex with the catalytic subunit of protein phosphatase 1 (Novoa *et al.*, 2001). Under ER stress, GADD34 promotes ER client protein biosynthesis by dephosphorylating phospho-Ser51 of eIF2α in stressed cells. GADD34-mediated dephosphorylation of eIF2α provides a negative feedback loop that promotes recovery from translational inhibition in the UPR by decreasing ER client protein load and changing redox conditions within the ER (Marciniak *et al.*, 2004). Liver plays a major role in metabolism and detoxification. Expression of ATF3, ATF4, CHOP, and GADD34 are induced in the liver, especially under physiological or pathological stress. Expression levels of these proteins in liver tissues are important indicators of ER stress response to the metabolic stress that causes NAFLD.

Procedures to determine levels of ATF4, ATF3, CHOP, and GADD34 in the liver tissue of NAFLD mice are as follows: (1) The liver protein lysates of approximately 80 μg from the mice after the AHF or normal chow diet are denatured, separated by 10% SDS-PAGE gels, and transferred to a PVDF membrane as described above. (2) To detect the ATF4, ATF3, CHOP, or GADD34 protein, the blots are incubated with a rabbit anti-ATF4 antibody (Cat # sc-200, Santa Cruz Biotechnologies), a rabbit anti-ATF3 antibody (Cat # sc-188, Santa Cruz Biotechnologies), a rabbit anti-CHOP antibody (Cat # sc-793, Santa Cruz Biotechnologies), or a rabbit anti-GADD34 antibody (Cat # sc-8327, Santa Cruz Biotechnologies) at a 1:200 dilution for 2 h at room temperature or overnight at 4 °C followed by incubation with a 1:3000 dilution of HRP-conjugated anti-rabbit antibody (Cat # W401B, Promega) for 2 h at room temperature. (3) The blots are incubated with the ECL detection reagent to detect chemiluminescent signals as described above. The sizes of detected mouse ATF4, ATF3, CHOP, and GADD34 proteins are approximately 38, 37, 26, and 75 kDa, respectively.

2.3. Detecting hepatic inflammation in the mouse model of NAFLD

Hepatic inflammation is one of key features of NAFLD. The UPR signaling and inflammatory responses can interact and be integrated in specialized cell types in metabolic disease models (Zhang and Kaufman, 2008a). In addition to ER stress response, measurement of hepatic inflammation is necessary for evaluating stress response associated with the pathogenesis of NAFLD. Acute-phase response, represented by production of acute-phase proteins by hepatocytes, such as serum amyloid component-P (SAP), serum amyloid A (SAA), and C-reactive protein (CRP), is one of the indicators of hepatic inflammation. Recent research suggests that ER stress mechanism is critically involved in hepatic acute-phase response (Macintyre *et al.*, 1994; Zhang *et al.*, 2006). Moreover, liver lobular inflammation and hepatocyte ballooning are two primary pathologic criteria for hepatic inflammation in NAFLD (Bondini *et al.*, 2007; Haukeland *et al.*, 2005; Papandreou *et al.*, 2007). In this section, we describe the methods to measure hepatic acute-phase response (production of SAP, SAA, and CRP) as well as the procedures to characterize lobular inflammation and hepatocyte ballooning in the mouse model of NAFLD induced by the metabolic diet.

We use highly sensitive two-site ELISA kits from Immunology Consultants Laboratory, Inc. (Newberg, USA) for measuring levels of plasma SAP, SAA, and CRP in the mice after the high-fat or normal chow diet. To determine levels of plasma SAP using the ELISA kit (Cat # E-90SAP), mouse blood plasma samples are diluted 1500 times; to determine levels of plasma CRP using the ELISA kit (Cat # E-90CRP), mouse blood plasma samples are diluted five times; to determine levels of plasma SAA using the ELISA kit (Cat # E-90SAA), mouse blood plasma samples are diluted 1000 times. The experimental procedures are detailed in the manufacture instructions.

Liver histological staining can provide direct and reliable evidence for hepatic inflammation. We use hematoxylin and eosin (HE) staining of formalin-fixed, paraffin-embedded liver tissue section to identify lobular inflammation and hepatocyte ballooning in the liver tissue of NAFLD mice. The procedures of staining and analyzing lobular inflammation and hepatocyte ballooning with the liver tissue sections from the mice after the AHF or normal chow diet are described below.

Required materials:

Graded alcohol (from 50%, 70%, 80%, 95% to 100%)
Xylenes (Cat # A5597, Sigma)
Harris hematoxylin solution (Cat # HHS32, Sigma)
Eosin Y solution alcoholic (Cat # HT110132, Sigma)
Scott's tap water substrate concentrate (Cat # S5134-6X, Sigma)
0.25% acid alcohol; Permount (Cat # SP15-100, Fisher)

Procedures for characterizing hepatic inflammation by HE staining are as follows: (1) Deparaffinize and hydrate mouse liver tissue sections of 4 μm through fresh xylenes and graded alcohol. (2) Rinse the tissue section slides in distilled water for 5 min. (3) Stain the sections in hematoxylin for 3 min. (4) Dip the section slides quickly in bluing solution (1× Scott's tap water) for five times. (5) Rinse the sections in running tap water for 10 min. (6) Dip the sections quickly in acid alcohol for three times to decolorize everything except nucleus. (7) Rinse the section slides in tap water for 5 min. (8) Counterstain the sections in eosin for 2 min. (9) Dehydrate the sections through the regressive graded alcohol and fresh xylenes. (10) Mount the section slides with Permount.

The histological features of hepatic inflammation revealed by HE-stained liver tissue sections include hepatocyte ballooning and lobular inflammation (Fig. 19.1). Hepatocyte ballooning is a phenomenon of hepatocyte degeneration in form of parenchymal cell death. The cellular shape in this condition can be distinguished by its two to three times of body size compared to the adjacent hepatocytes. Additionally, ballooning hepatocytes are different from adipocytes. Ballooning hepatocytes have their nucleus in the center of the cell, while the nucleus of adipocytes locates in the periphery of the cell (Goldstein *et al.*, 1991). Lobular inflammation, another hallmark of liver inflammation, indicates accelerated liver cells renewal. It appears as binuclear hepatocytes, swelling hepatocytes, and shrinking apoptotic cells (Buligescu and Antonescu, 1992). Moreover, Mallory's hyaline is a form of inclusion in the cytoplasm of hepatocytes. It is made of intermediate keratin filament proteins which have been

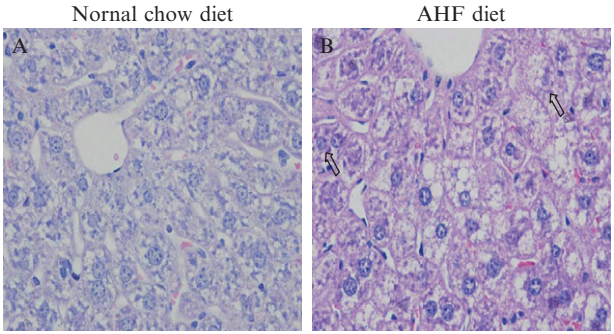

Figure 19.1 Hematoxylin and eosin staining of paraffin-embedded liver tissue sections from C57BL/6 mice fed with either normal chow diet (A) or the AHF diet (B) for 6 months. Ballooning hepatocytes is characterized by its swelling cellular body with nucleus in the center (pointed by the right arrow). Lobular inflammation is characterized by binuclear hepatocytes (pointed by the left arrow) in the mice fed with the AHF diet. Magnification: 600×.

ubiquinated, or bound by heat shock proteins and p62 proteins (Zatloukal *et al.*, 2002). The process of hepatocyte ballooning is accompanied by reducing density or losing cytoplasmic intermediate filaments network. Along this process, misfolded and aggregated keratin accumulation is the reason for Mallory hyaline. The histopathological features of NASH include (1) hepatocellular steatosis and ballooning, (2) mixed acute and chronic lobular inflammation, (3) perisinusoidal fibrosis, and (4) others such as Mallory's hyaline (Brunt, 2000; Kleiner *et al.*, 2005). The methods to measure liver fibrosis and hepatocellular steatosis will be described in the following section.

Note: The hematoxylin solution needs to be filtered before each use to remove oxidized particles. It is not necessary to wash the sections in water after counterstain them in eosin.

3. Visualizing ER Stress Response Associated with Liver Pathology in the Mouse Model of NAFLD

It is important to elucidate the correlation of ER stress response with the liver pathology of NAFLD. The major characteristics of liver pathology in NAFLD include hepatic steatosis, hepatic inflammation and ballooning, fibrosis, as well as apoptosis. It is informative to visualize the intensity and localization of ER stress response in correlation to the liver pathology. We have developed immunohistochemistry staining methods to visualize ER stress response in the liver tissue of NAFLD mice. In addition to the methods to evaluate hepatic inflammation and ballooning, here we describe the methods to detect hepatic steatosis and fibrosis in the liver.

3.1. Staining ER stress markers in mouse fatty liver tissue

Induction of the XBP1 protein encoded by spliced *Xbp1* mRNA is a hallmark of activation of IRE1α-mediated UPR pathway. The UPR pathway through ATF6 or IRE1α/XBP1 can upregulate expression of ER chaperone BiP/GRP78 (Yamamoto *et al.*, 2007). Increased expression of ER chaperone BiP/GRP78 indicates ER stress and activation of the UPR pathway through ATF6 and/or IRE1α/XBP1. Together with expression of spliced XBP1 and BiP, induction of CHOP is an indicator of the activation of PERK-mediated UPR pathway and ER stress-induced apoptosis. We developed immunohistochemistry staining methods for visualizing induction of XBP1, CHOP, and BiP in the liver tissues of NAFLD mice.

Required material:

- 10× Tris buffered saline: 24.2 g Trizma base ($C_4H_{11}NO_3$) and 80 g NaCl in 1 L dH_2O, pH 7.6.
- Washing buffer: 100 ml 10× TBS and 1 ml Tween 20 in dH_2O with a total volume of 1 L.
- 10× PBS: 80 g NaCl, 2 g potassium chloride (KCl), 14.4 g sodium phosphate, dibasic (Na_2HPO_4), and 2.4 g potassium phosphate, monobasic (KH_2PO_4) in 1 L dH_2O, pH 7.4.
- Blocking solution: 5% normal goat serum in washing buffer.
- VECTASTAIN Elite ABC kit (Cat # PK-1600, Vector Laboratories)
- DAB peroxidase substrate solution (Vector Laboratories SK-4100)

Optimized procedures to stain XBP1, CHOP, and BiP in mouse liver tissue sections are as follows: (1) Prepare formalin-fixed, paraffin-embedded liver tissue sections (4 μ*M*) from the mice after the AHF or normal chow diet as described in Section 2.1. The section slides are subjected to routine deparaffinization/hydration process. (2) Rinse the section slides with dH_2O for 3 min. (3) Quench the sections for 2 h in 0.3% H_2O_2 in methanol to block the endogenous peroxidase activity in the mouse liver tissue. (4) Wash the section slides with PBS buffer followed by incubating sections with the blocking buffer for 30 min. (5) Incubate the tissue sections with a rabbit anti-human XBP1 antibody (Cat # ab37152, Abcam) at 1:100 dilution, a rabbit anti-mouse BiP antibody (Cat # ab21685, Abcam) at 1:300 dilution, or a rabbit anti-mouse CHOP antibody (Cat # sc-575, Santa Cruz Biotechnologies) at 1:50 dilution to stain XBP1, BiP, or CHOP in the mouse liver tissue. The section slides are then incubated with the antibody overnight at 4 °C in a humidified chamber. Negative controls include omission of the primary antibody. (6) Wash the sections with PBS and incubate them with biotinylated anti-rabbit secondary antibody (Cat # BA-1000, Vector Laboratories) for 30 min. (7) Wash the tissue sections with PBS and prepare VECTASTAIN Elite ABC reagent freshly. Add exactly one drop of REAGENT A to 2.5 ml of 1× PBS followed by adding one drop of REAGENT B. Allow ABC reagent to stand for about 30 min before use. (8) Incubate the tissue sections with freshly made VECTASTAIN Elite ABC Reagent for 30 min. Wash the tissue sections and incubate them with DAB peroxidase substrate solution (Cat # SK-4100, Vector Laboratories) for 2–3 min until desired stain intensity develops. (9) Rinse sections with dH_2O and then counterstain with Harris Hematoxylin for 10 s followed by dipping section slides in Bluing solution 10 times. (10) Rinse the slides in tap water, dehydrate in graded alcohol, and soak in xylenes. (18) Mount the section slides with Permount.

Note: VECTASTAIN Elite ABC Reagent should be prepared freshly according to manufacturer's instruction. Allow the ABC reagent to stand for about 30 min before use. For the quenching process, increased volume

and incubation time of H_2O_2 may be required, because endogenous peroxidase activity is high in the liver tissues.

3.2. Staining hepatic lipids

Hepatic lipid accumulation is a prominent feature of NAFLD. Hepatic steatosis is characterized by accumulation of lipids, mainly triglycerides, in the cytoplasm of hepatocytes. In hepatocytes, triglycerides are synthesized in the ER and stored in the cytoplasm surrounded by a monolayer of phospholipid in distinct lipid droplet structures (Martin and Parton, 2006). Under normal physiological conditions, lipid droplets maintain energy balance at the cellular and organismal levels. Under conditions of overnutrition or acute liver injury, lipid deposition is significantly increased in hepatocytes (Rutkowski *et al.*, 2008; Yang *et al.*, 2007). Oil Red O is a type of Sudan lysochromes that stain lipid and triglyceride contents in frozen liver tissue sections. When staining for hepatic lipids, the Oil Red O staining method can give a much deeper red color compared with similar dyes such as Sudan III, Sudan IV, and Sudan Black B.

Required materials:

- Oil Red O stock solution: Add Oil Red O (Cat # CI 26125, Polysciences, Inc.) 0.5 g to 100 ml isopropanol. Dissolve the dye by using a 37 °C water bath for 30 min.
- Oil Red O working solution: Prepare freshly. Dilute 24 ml of the stock solution with 12 ml of distilled water. Allow it to stand for 10 min before filtering it into a Coplin jar.

Staining procedures are as follows: (1) Prepared O.C.T.-embedded frozen liver tissue sections of 8 μ*M* from the mice after the AHF or normal chow diet as described in Section 2.1. Air-dry the sections at the room temperature for 10 min. (2) Fix the tissue sections in 10% formalin for 15 min at room temperature. (3) Wash the section slides in running tap water for 5 min. (4) Rinse the tissue sections in 60% isopropanol for 5 min. (5) Stain the tissue sections in Oil Red O working solution for 15 min. (6) Rinse the tissue sections in 60% isopropanol for 5 min followed by counterstaining of nuclei with Harris Hematoxylin. (7) Rinse the tissue sections in running dH_2O for 10 min. (8) Mount in aqueous mounting medium and dry overnight in the room temperature.

3.3. Staining liver fibrosis

Liver fibrosis is the excessive accumulation of extracellular matrix proteins, mainly collagen, which is associated with hepatic steatosis, inflammation, and cell stress in advanced NAFLD (Bataller and Brenner, 2005).

Determination of liver fibrosis along with ER stress markers, hepatic inflammation, and steatosis is important to evaluate the progression of NAFLD. Sirius-red staining is a widely used method to stain collagen as red with a pale yellow background under bright-field microscopy (Junqueira *et al.*, 1979). The following Sirius-red staining protocol works stably and reliably for staining liver fibrosis in the mouse model of NAFLD.

Required materials:

- Saturated picric acid: Add 3.75 g wet picric acid to 250 ml distilled water and stir overnight. The saturation of picric acid is important.
- Picrosirius red solution: 0.25 g Sirius-red (Cat # F3B C.I. 35782, Direct Red 80, Sigma) in 250 ml saturated picric acid solution.
- Acidified water: 5 ml glacial acetic acid in 1 L dH_2O.

The staining procedure: (1) Prepare formalin-fixed, paraffin-embedded liver tissue sections (4 μ*M*) from the mice after the AHF or normal chow diet as described in Section 2.1. The section slides are then subjected to standard deparaffinization/hydration process. (2) Stain the tissue sections with picrosirius red solution for 1 h. (3) Wash the section slides with acidified water and subsequent dH_2O. (4) Dehydrate the slide sections through the regressive graded alcohol followed by soaking the sections in fresh xylenes. (7) Mount with Permount.

The stage of liver fibrosis depends on the extent of fibrosis and tissue architectural remodeling (Brunt *et al.*, 1999). Four stages of fibrosis have been defined: stage 1, perisinusoidal fibrosis in liver tissue zone 3, the center of acinus that has the poorest oxygenation; stage 2, zone 3 perisinusoidal fibrosis plus portal fibrosis; stage 3, perisinusoidal fibrosis, portal fibrosis, plus bridging fibrosis; and stage 4, cirrhosis. Collagen type I represents 1% of the

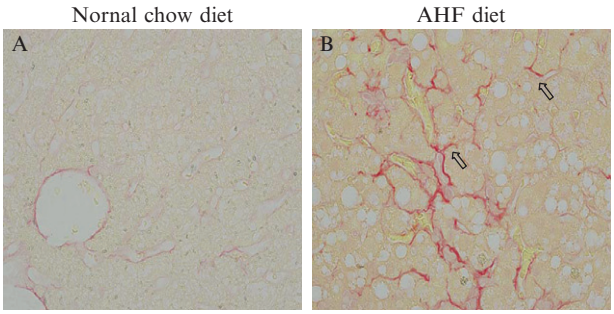

Figure 19.2 Sirius-red staining of paraffin-embedded liver tissue sections from C57BL/6 mice fed with either normal chow diet (A) or the AHF diet (B) for 6 months. The staining shows the progression to stage 2 fibrosis, which is represented by increased deposition of collagen in hepatic zone 3 perisinusoidal and portal area (pointed by arrows), in the liver tissue of the mice after the AHF diet (B). Magnification: 200×.

total liver proteins in normal liver, but it represents 50% of the total liver proteins in cirrhotic liver (Bataller and Brenner, 2005; Stefanovic *et al.*, 1997). In the livers of mice after the AHF diet for 6 months, liver fibrosis of stage 3 can be observed, suggesting severe steatohepatitis developed under the metabolic diet (Fig. 19.2).

Note: Picrosirius red solution is stable at room temperature for up to 3 years and could be used many times. The staining time for picrosirius red solution should be exactly 1 h. Shorter time would not stabilize the color. Washing the tissue sections in the acidified water is important for maintaining the dye.

ACKNOWLEDGMENTS

The research in the Zhang laboratory is partially supported by the American Heart Association (AHA) grant 09GRNT2280479, the Department of Defense (DOD) Breast Cancer Research Program grant BC095179P1, and the National Institutes of Health (NIH) grant 1R21ES017829-01A1 to KZ. We thank Ms. Ginny Bao for her assistance in editing this chapter.

REFERENCES

Angulo, P. (2002). Nonalcoholic fatty liver disease. *N Engl. J. Med.* **346,** 1221–1231.

Bataller, R., and Brenner, D. A. (2005). Liver fibrosis. *J. Clin. Invest.* **115,** 209–218.

Bondini, S., Kleiner, D. E., Goodman, Z. D., Gramlich, T., and Younossi, Z. M. (2007). Pathologic assessment of non-alcoholic fatty liver disease. *Clin. Liver Dis.* **11,** 17–23, vii.

Brunt, E. M. (2000). Grading and staging the histopathological lesions of chronic hepatitis: The Knodell histology activity index and beyond. *Hepatology* **31,** 241–246.

Brunt, E. M. (2001). Nonalcoholic steatohepatitis: Definition and pathology. *Semin. Liver Dis.* **21,** 3–16.

Brunt, E. M., Janney, C. G., Di Bisceglie, A. M., Neuschwander-Tetri, B. A., and Bacon, B. R. (1999). Nonalcoholic steatohepatitis: A proposal for grading and staging the histological lesions. *Am. J. Gastroenterol.* **94,** 2467–2474.

Buligescu, L., and Antonescu, O. (1992). The significance of lobular inflammation in chronic active hepatitis. *Med. Interna* **44,** 20–27.

Cullinan, S. B., Zhang, D., Hannink, M., Arvisais, E., Kaufman, R. J., and Diehl, J. A. (2003). Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Mol. Cell. Biol.* **23,** 7198–7209.

Feng, B., Yao, P. M., Li, Y., Devlin, C. M., Zhang, D., Harding, H. P., Sweeney, M., Rong, J. X., Kuriakose, G., Fisher, E. A., Marks, A. R., Ron, D., *et al.* (2003). The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages. *Nat. Cell Biol.* **5,** 781–792.

Gething, M. J., and Sambrook, J. (1992). Protein folding in the cell. *Nature* **355,** 33–45.

Goldstein, N. S., Hart, J., and Lewin, K. J. (1991). Diffuse hepatocyte ballooning in liver biopsies from orthotopic liver transplant patients. *Histopathology* **18,** 331–338.

Harding, H. P., Zhang, Y., and Ron, D. (1999). Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase [In Process Citation]. *Nature* **397,** 271–274.

Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000a). Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol. Cell* **6,** 1099–1108.

Harding, H. P., Zhang, Y., Bertolotti, A., Zeng, H., and Ron, D. (2000b). Perk is essential for translational regulation and cell survival during the unfolded protein response. *Mol. Cell* **5,** 897–904.

Haukeland, J. W., Konopski, Z., Linnestad, P., Azimy, S., Marit Loberg, E., Haaland, T., Birkeland, K., and Bjoro, K. (2005). Abnormal glucose tolerance is a predictor of steatohepatitis and fibrosis in patients with non-alcoholic fatty liver disease. *Scand. J. Gastroenterol.* **40,** 1469–1477.

Haze, K., Yoshida, H., Yanagi, H., Yura, T., and Mori, K. (1999). Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Mol. Biol. Cell* **10,** 3787–3799.

Hu, P., Han, Z., Couvillon, A. D., Kaufman, R. J., and Exton, J. H. (2006). Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1alpha-mediated NF-kappaB activation and down-regulation of TRAF2 expression. *Mol. Cell. Biol.* **26,** 3071–3084.

Junqueira, L. C., Bignolas, G., and Brentani, R. R. (1979). Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem. J.* **11,** 447–455.

Kaufman, R. J. (1999). Stress signaling from the lumen of the endoplasmic reticulum: Coordination of gene transcriptional and translational controls. *Genes Dev.* **13,** 1211–1233.

Kharroubi, I., Ladriere, L., Cardozo, A. K., Dogusan, Z., Cnop, M., and Eizirik, D. L. (2004). Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: Role of nuclear factor-kappaB and endoplasmic reticulum stress. *Endocrinology* **145,** 5087–5096.

Kleiner, D. E., Brunt, E. M., Van Natta, M., Behling, C., Contos, M. J., Cummings, O. W., Ferrell, L. D., Liu, Y. C., Torbenson, M. S., Unalp-Arida, A., Yeh, M., McCullough, A. J., *et al.* (2005). Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* **41,** 1313–1321.

Larter, C. Z., and Yeh, M. M. (2008). Animal models of NASH: Getting both pathology and metabolic context right. *J. Gastroenterol. Hepatol.* **23,** 1635–1648.

Lee, A. H., Iwakoshi, N. N., Anderson, K. C., and Glimcher, L. H. (2003a). Proteasome inhibitors disrupt the unfolded protein response in myeloma cells. *Proc. Natl. Acad. Sci. USA* **100,** 9946–9951.

Lee, A. H., Iwakoshi, N. N., and Glimcher, L. H. (2003b). XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol. Cell. Biol.* **23,** 7448–7459.

Lee, A. H., Scapa, E. F., Cohen, D. E., and Glimcher, L. H. (2008). Regulation of hepatic lipogenesis by the transcription factor XBP1. *Science* **320,** 1492–1496.

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25,** 402–408.

Macintyre, S., Samols, D., and Dailey, P. (1994). Two carboxylesterases bind C-reactive protein within the endoplasmic reticulum and regulate its secretion during the acute phase response. *J. Biol. Chem.* **269,** 24496–24503.

Marciniak, S. J., Yun, C. Y., Oyadomari, S., Novoa, I., Zhang, Y., Jungreis, R., Nagata, K., Harding, H. P., and Ron, D. (2004). CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes Dev.* **18,** 3066–3077.

Martin, S., and Parton, R. G. (2006). Lipid droplets: A unified view of a dynamic organelle. *Nat. Rev. Mol. Cell Biol.* **7,** 373–378.

Matsuzawa, N., Takamura, T., Kurita, S., Misu, H., Ota, T., Ando, H., Yokoyama, M., Honda, M., Zen, Y., Nakanuma, Y., Miyamoto, K., and Kaneko, S. (2007). Lipid-induced oxidative stress causes steatohepatitis in mice fed an atherogenic diet. *Hepatology* **46,** 1392–1403.

Novoa, I., Zeng, H., Harding, H. P., and Ron, D. (2001). Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. *J. Cell Biol.* **153,** 1011–1022.

Okada, T., Yoshida, H., Akazawa, R., Negishi, M., and Mori, K. (2002). Distinct roles of ATF6 and PERK in transcription during the mammalian unfolded protein response. *Biochem. J.* **366,** 585–594.

Oyadomari, S., Harding, H. P., Zhang, Y., Oyadomari, M., and Ron, D. (2008). Dephosphorylation of translation initiation factor 2alpha enhances glucose tolerance and attenuates hepatosteatosis in mice. *Cell Metab.* **7,** 520–532.

Paigen, B., Morrow, A., Holmes, P. A., Mitchell, D., and Williams, R. A. (1987). Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis* **68,** 231–240.

Papandreou, D., Rousso, I., and Mavromichalis, I. (2007). Update on non-alcoholic fatty liver disease in children. *Clin. Nutr.* **26,** 409–415.

Ron, D., and Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev. Mol. Cell Biol.* **8,** 519–529.

Rutkowski, D. T., Wu, J., Back, S. H., Callaghan, M. U., Ferris, S. P., Iqbal, J., Clark, R., Miao, H., Hassler, J. R., Fornek, J., Katze, M. G., Hussain, M. M., *et al.* (2008). UPR pathways combine to prevent hepatic steatosis caused by ER stress-mediated suppression of transcriptional master regulators. *Dev. Cell* **15,** 829–840.

Scheuner, D., Song, B., McEwen, E., Liu, C., Laybutt, R., Gillespie, P., Saunders, T., Bonner-Weir, S., and Kaufman, R. J. (2001). Translational control is required for the unfolded protein response and in vivo glucose homeostasis. *Mol. Cell* **7,** 1165–1176.

Stefanovic, B., Hellerbrand, C., Holcik, M., Briendl, M., Aliebhaber, S., and Brenner, D. A. (1997). Posttranscriptional regulation of collagen alpha1(I) mRNA in hepatic stellate cells. *Mol. Cell. Biol.* **17,** 5201–5209.

Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H. P., and Ron, D. (2000). Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* **287,** 664–666.

Wang, D., Wei, Y., and Pagliassotti, M. J. (2006). Saturated fatty acids promote endoplasmic reticulum stress and liver injury in rats with hepatic steatosis. *Endocrinology* **147,** 943–951.

Wei, Y., Wang, D., Topczewski, F., and Pagliassotti, M. J. (2006). Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells. *Am. J. Physiol. Endocrinol. Metab.* **291,** E275–E281.

Yamamoto, K., Sato, T., Matsui, T., Sato, M., Okada, T., Yoshida, H., Harada, A., and Mori, K. (2007). Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6alpha and XBP1. *Dev. Cell* **13,** 365–376.

Yaman, I., Fernandez, J., Liu, H., Caprara, M., Komar, A. A., Koromilas, A. E., Zhou, L., Snider, M. D., Scheuner, D., Kaufman, R. J., and Hatzoglou, M. (2003). The zipper model of translational control: A small upstream ORF is the switch that controls structural remodeling of an mRNA leader. *Cell* **113,** 519–531.

Yang, L., Jhaveri, R., Huang, J., Qi, Y., and Diehl, A. M. (2007). Endoplasmic reticulum stress, hepatocyte CD1d and NKT cell abnormalities in murine fatty livers. *Lab Invest.* **87,** 927–937.

Ye, J., Rawson, R. B., Komuro, R., Chen, X., Dave, U. P., Prywes, R., Brown, M. S., and Goldstein, J. L. (2000). ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. *Mol. Cell* **6,** 1355–1364.

Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001). XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* **107,** 881–891.

Yoshida, H., Oku, M., Suzuki, M., and Mori, K. (2006). pXBP1(U) encoded in XBP1 pre-mRNA negatively regulates unfolded protein response activator pXBP1(S) in mammalian ER stress response. *J. Cell Biol.* **172,** 565–575.

Zatloukal, K., Stumptner, C., Fuchsbichler, A., Heid, H., Schnoelzer, M., Kenner, L., Kleinert, R., Prinz, M., Aguzzi, A., and Denk, H. (2002). p62 Is a common component of cytoplasmic inclusions in protein aggregation diseases. *Am. J. Pathol.* **160,** 255–263.

Zhang, K., and Kaufman, R. J. (2008a). From endoplasmic-reticulum stress to the inflammatory response. *Nature* **454,** 455–462.

Zhang, K., and Kaufman, R. J. (2008b). Identification and characterization of endoplasmic reticulum stress-induced apoptosis in vivo. *Methods Enzymol.* **442,** 395–419.

Zhang, K., Wong, H. N., Song, B., Miller, C. N., Scheuner, D., and Kaufman, R. J. (2005). The unfolded protein response sensor IRE1alpha is required at 2 distinct steps in B cell lymphopoiesis. *J. Clin. Invest.* **115,** 268–281.

Zhang, K., Shen, X., Wu, J., Sakaki, K., Saunders, T., Rutkowski, D. T., Back, S. H., and Kaufman, R. J. (2006). Endoplasmic reticulum stress activates cleavage of CREBH to induce a systemic inflammatory response. *Cell* **124,** 587–599.

World Journal of Hepatology

Online Submissions: http://www.wjgnet.com/1948-5182office
wjh@wjgnet.com
doi:10.4254/wjh.v2.i6.203

World J Hepatol 2010 June 27; 2(6): 203-207
ISSN 1948-5182 (online)
© 2010 Baishideng. All rights reserved.

EDITORIAL

Role of unfolded protein response in lipogenesis

Ze Zheng, Chunbin Zhang, Kezhong Zhang

Ze Zheng, Chunbin Zhang, Kezhong Zhang, Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, MI 48201, United States
Kezhong Zhang, Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, MI 48201, United States
Author contributions: Zheng Z and Zhang CB were involved in material collection, discussion and the writing of the manuscript; Zhang KZ organized the materials, discussed with Zheng Z and Zhang CB and wrote the manuscript.
Supported partially by the American Heart Association (AHA) Scientist Development Award (0635423Z), the AHA Grant-in-Aid (09GRNT2280479), the Department of Defense Breast Cancer Research Program (BC095179P1) and the Karmanos Cancer Institute Pilot Grant
Correspondence to: Kezhong Zhang, PhD, Center for Molecular Medicine and Genetics, 540 E, Canfield Street, Wayne State University School of Medicine, Detroit, MI 48201, United States. kzhang@med.wayne.edu
Telephone: +1-313-5772669 Fax: +1-313-5775218
Received: February 23, 2010 Revised: June 8, 2010
Accepted: June 15, 2010
Published online: June 27, 2010

Abstract

The signal transduction network in regulating lipid metabolism is a hot topic of biomedical research. Recent research endeavors reveal that intracellular stress signaling from a cellular organelle called endoplasmic reticulum (ER) is critically involved in lipid homeostasis and the development of metabolic disease. The ER is a site where newly-synthesized proteins are folded and assembled into their three-dimensional structures, modified and transported to their precise cellular destinations. A wide range of biochemical, physiological and pathological stimuli can interrupt the protein folding process in the ER and cause accumulation of unfolded or misfolded proteins in the ER lumen, a condition referred to as ER stress. To cope with this stress condition, the ER has evolved highly-specific signaling pathways collectively termed Unfolded Protein Response (UPR) or ER stress response. The UPR regulates transcriptional and translational programs, affecting broad aspects of cellular metabolism and cell fate. Lipogenesis, the metabolic process of *de novo* lipid biosynthesis, occurs primarily in the liver where metabolic signals regulate expression of key enzymes in glycolytic and lipogenic pathways. Recent studies suggest that the UPR plays crucial roles in modulating lipogenesis under metabolic conditions. Here we address some of recent representative evidence regarding the role of the UPR in lipogenesis.

© 2010 Baishideng. All rights reserved.

Key words: Endoplasmic reticulum stress; Unfolded protein response; Lipogenesis; Hepatic lipid metabolism; Metabolic disease

Peer reviewer: Nattiya Hirankarn, MD, Associated Professor, Immunology Unit, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Rama 4 road, Bangkok 10330, Thailand

Zheng Z, Zhang CB, Zhang KZ. Role of unfolded protein response in lipogenesis. *World J Hepatol* 2010; 2(6): 203-207 Available from: URL: http://www.wjgnet.com/1948-5182/full/v2/i6/203.htm DOI: http://dx.doi.org/10.4254/wjh.v2.i6.203

INTRODUCTION

In the liver, the lipid content is regulated by dietary fatty acids or carbohydrates uptake, hepatic fatty acids biosynthesis, esterification, oxidation and export. Metabolic signals such as an excess of fatty acids, glucose or insulin can regulate the activity or abundance of key transcription factors to modulate hepatic lipid metabolism[1]. Many hepatic transcription factors have been identified as prospective targets for *de novo* lipogenesis and fatty acids oxidation including sterol regulatory element binding protein-1c (SREBP-1c), liver X receptor (LXRα), peroxisome proliferator-activated receptors (PPARα, δ, γ1, and γ2) and carbohydrate-responsive element-binding protein

(chREBP)[2-5]. These factors integrate signals from various pathways and coordinate the activity of the metabolic machinery necessary for hepatic lipid metabolism with the supply of energy and fatty acids.

Recently, accumulating evidence suggests that endoplasmic reticulum (ER) stress response is critically involved in hepatic lipid metabolism. The presence of ER stress is evidenced in the liver of high fat diet-induced obese mice[6,7]. The specific type of fat deposited in the liver may directly induce ER stress response and precipitate the development of non-alcoholic fatty liver disease (NAFLD)[8]. The presence of increased circulating and/or hepatic saturated fatty acids but not polyunsaturated fatty acids may exacerbate hepatic steatosis, steatohepatitis and liver cell apoptosis through activating ER stress response[9-11]. These observations implicate a crucial role for the signaling pathways from the ER in the development and progression of hepatic lipid-associated diseases.

UNFOLDED PROTEIN RESPONSE

The ER is the network of interconnected membranous structures within the cytoplasm of eukaryotic cells contiguous with the outer nuclear envelope. The ER has been primarily recognized as a compartment for protein folding and assembly, a pool of free calcium and a site for lipid and sterol biosynthesis[12]. As a protein-folding compartment, the ER provides a high-fidelity quality control system to ensure that only correctly folded proteins can be transported out of the ER while unfolded or misfolded proteins are retained in the ER and eventually degraded. Under stress conditions, such as those that disrupt protein glycosylation, disulfide bond formation, ER and calcium channels, redox/oxidative stress, nutrient deprivation or viral infections, can cause accumulation of abnormally folded proteins or unassembled subunits[13-16]. The ER has evolved a highly specific signaling pathway termed Unfolded Protein Response (UPR) to help relieve the ER from the accumulation of unfolded or misfolded proteins (Figure 1). There are three ER transmembrane proteins that function as UPR transducers: inositol-requiring 1α (IRE1α), double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6). It has been proposed that all three UPR transducers have the ER chaperone glucose regulate protein 78 (GRP78) bound to their ER luminal domains. This interaction allows GRP78 to act as a repressor of activation of the UPR transducers[17]. Under ER stress conditions, dissociation of GRP78 from the ER luminal domains of IRE1α, PERK and/or ATF6 allows them to be activated.

Within minutes to hours after ER stress, the UPR transducer PERK-mediated translation attenuation occurs to prevent newly-synthesized proteins entering into the ER[18,19]. The activated PERK cytosolic domain leads to translational attenuation by phosphorylating α subunit of eukaryotic translation-initiation factor 2α (eIF2α). This procedure also causes cell cycle arrest in G1 phase by translational attenuation of the proteins involved in running the cell cycle. Under ER stress, IRE1α is also activated by homodimerization and autophosphorylation. Activated IRE1α functions as an endoribonuclease to splice the mRNA encoding a basic leucine zipper (bZIP) transcription factor XBP1[20-22]. In addition to its endoribonuclease activity, activated IRE1α can serve as a scaffold protein that recruits tumor-necrosis factor (TNF)-receptor-associated factor 2 (TRAF2) leading to activation of Jun amino-terminal kinases (JNK)-mediated inflammatory stress signaling pathway[23]. Upon UPR activation, ATF6 is released from the ER membrane and transits into the Golgi compartment where it is cleaved by site-1 protease (S1P) and site-2 protease (S2P) to release its functional fragment[24]. This fragment then moves into the nucleus and functions as a bZIP transcription factor to activate expression of UPR target genes. The primary role of the UPR is to protect the cell from ER stress by reducing the amount of proteins translocated into the ER lumen, increasing retrotranslocation and degradation of ER-localized proteins and augmenting the protein-folding capacity of the ER. However, under prolonged ER stress the role of the UPR will change from promoting cellular survival to the pathway of programmed cell death or apoptosis[25]. In recent years, the scope and consequences of ER stress and the UPR have significantly expanded. The UPR is essential for cells to augment ER protein folding capacity and remodel their secretory pathways in response to developmental demands and physiological changes[26]. In particular, the ER stress response can be triggered by metabolic factors and intrinsic feedback effectors. Prolonged or insufficient ER stress response may turn physiological mechanisms into pathological consequences[27,28]. Indeed, the UPR has been identified as fundamental cell signaling that is critical for health and disease.

UPR IN LIPOGENESIS

Recent evidence suggests that ER stress response is closely associated with lipid-associated metabolic disease. Three UPR pathways mediated through IRE1α, PERK and ATF6 were reported to be involved in the regulation of lipid metabolism. The UPR *trans-activator* XBP-1, the downstream target of IRE1α under ER stress, can regulate expression and activities of key enzymes in phospholipid biosynthesis[29]. Under ER stress, the activated form of XBP1 can increase the activity of the cytidine diphosphocholine (CDP-choline) pathway for biosynthesis of phosphatidylcholine and thus induce ER biogenesis[29,30]. Interestingly, a recent study revealed a distinguished role of XBP1 in *de novo* fatty acid synthesis in the liver[31]. The IRE1α/XBP1 UPR branch was activated in the liver of mice under the high-carbohydrate diet and directly controlled the expression of genes involved in fatty acid biosynthesis including the genes encoding acetyl CoA carboxylase 2 (Acc2), diacylglycerol acyltransferase 2 (Dgat2) and stearoyl CoA desaturase

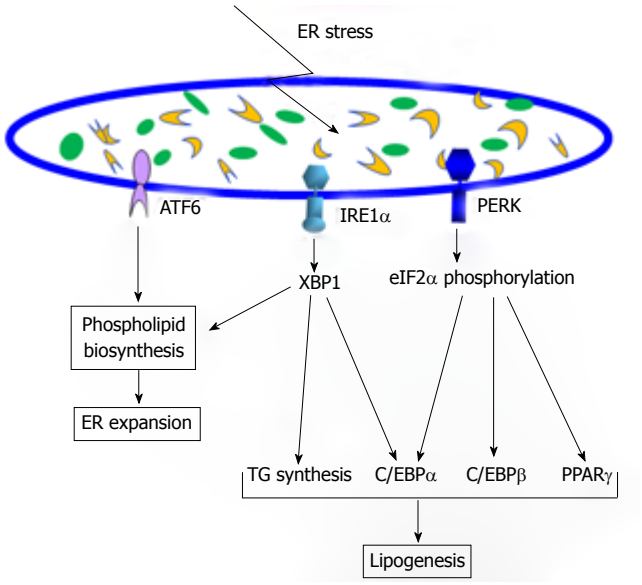

Figure 1 The involvement of the Unfolded Protein Response (UPR) signaling in lipid metabolism. The UPR pathway through inositol-requiring 1α (IRE1α)/XBP1 or activating transcription factor 6 is involved in endoplasmic reticulum (ER) expansion by enhancing phospholipid biosynthesis under ER stress conditions. The IRE1α/XBP1 pathway also regulates lipogenesis by inducing expression of the key enzymes required for triglyceride synthesis under metabolic stress. Additionally, the IRE1α/XBP1 UPR branch can drive C/EBPα expression, facilitating adipogenesis and possibly lipogenesis. The UPR pathway through PERK/eukaryotic translation-initiation factor 2α can stimulate expression of the key lipogenic regulators C/EBPα, C/EBPβ, and PPARγ, promoting lipogenesis under metabolic stress.

1 (Scd1). Deletion of XBP1 in the mouse liver caused profound hypocholesterolemia and hypotriglyceridemia which were primarily due to diminished lipogenesis. Surprisingly, the regulation of lipogenesis by the UPR component IRE1α/XBP1 was unlikely to be related to the ER stress response. The activation of XBP1 by high dietary carbohydrates and its association with other UPR components in regulating lipid metabolism remains to be further elucidated. Additionally, a recent study demonstrated that the IRE1α-XBP1 UPR pathway is indispensable for adipogenesis[32]. XBP1-deficient mouse embryonic fibroblasts and 3T3-L1 cells with XBP1 or IRE1α knockdown exhibit profound defects in adipogenesis. Intriguingly, C/EBPβ, an early adipogenic regulator, induces Xbp1 expression by directly binding to its proximal promoter region. Subsequently, spliced XBP1 binds to the promoter of C/EBPα and activates its gene expression[32]. Since C/EBPα and C/EBPβ are also key regulators of lipogenesis, the interactions between C/EBP family transcription factors and the IRE1α-XBP1 UPR pathway may play a key role in adipocyte differentiation by regulating lipid metabolism and morphological as well as functional transformations during adipogenesis.

In addition to IRE1α/XBP1, the UPR transducer ATF6 was also involved in phospholipid biosynthesis and ER expansion as well as hepatic lipid homeostasis associated with acute ER stress[33,34]. ATF6α knockout mice displayed no obvious phenotype under normal condition but showed profound hepatic steatosis under acute ER stress induced by tunicamycin challenge[34]. Acute ER Stress altered expression of genes involved in maintaining energy and lipid homeostasis. Particularly, the expression of genes encoding microsomal triglyceride transfer protein (MTTP), sterol regulatory element binding protein (SREBP)-1, peroxisome proliferator-activated receptor γ coactivator-1α (PGC1α) and peroxisome proliferator-activated receptor α (PPARα) was suppressed by ER stress in both wild type and ATF6α-null animals treated with tunicamycin, but this suppression was much greater in ATF6α-null animals. Furthermore, the cytosolic lipid droplet protein marker adipose differentiation-related protein (ADRP) was more significantly up-regulated in ATF6α knockout animals compared to the control animals. The results suggested that the increased lipid accumulation in the livers of ATF6α-deficient animals was partially due to a defect in fatty acid oxidation and possibly augmented by impaired lipoprotein secretion.

The UPR branch mediated through PERK/eIF2α was also implicated in regulating lipogenesis. In the high-fat-fed mice, PERK-mediated eIF2α phosphorylation was crucial for the expression of lipogenic genes and the development of hepatic steatosis by controlling expression of C/EBP family and PPARγ transcription factors[35]. Enforced expression of the eIF2α-specific phosphatase GADD34 can lead to lower liver glycogen levels and susceptibility to fasting hypoglycemia in lean mice and glucose tolerance and diminished hepatosteatosis in the high-fat-fed mice. Attenuated eIF2α phosphorylation resulted in lower expression of PPARγ and its upstream regulators C/EBPα and C/EBPβ suggesting that the translational control through phosphorylation of eIF2α is an important regulatory mechanism for lipogenesis under physiological ER stress. Additionally, the mammary gland lipogenesis was down-regulated in PERK-deficient mammary epithelial cells. SREBP1 expression was significantly down-regulated in the PERK-deficient mammary-gland cells[36]. Therefore, PERK-mediated UPR pathway likely regulates SREBP1-related *de novo* lipid synthesis in mammary gland. Supporting the role of ER stress response in lipogenesis, ER stress has been shown to activate the lipogenic transcription factor SREBP-1 and -2 leading to modulation of the lipogenic pathways[37,38]. Consistent with this observation, over-expression of GRP78/BiP, the master negative regulator of the UPR, in the liver of obese (*ob/ob*) mice can inhibit SREBP-1c cleavage and the expression of SREBP-1c and SREBP-2 target genes[39]. Hepatic triglyceride and cholesterol con tents were reduced and insulin sensitivity was improved in GRP78-over-expressed mice. Together, these studies confirmed crucial roles of the UPR pathways in lipogenesis and the pathogenesis of lipid-associated metabolic disease.

CONCLUSION

A growing body of evidence suggested that UPR is critically involved in lipogenesis. The UPR pathways may represent attractive targets for future therapeutic intervention in modulating lipid metabolism associated

with metabolic diseases. Indeed, recent studies demonstrated that small chemical chaperones such as 4-phenylbutyric acid (PBA) and taurine-conjugated ursodeoxycholic acid (TUDCA) can reduce ER stress in liver and adipose tissues and thus enhance insulin sensitivity and glucose tolerance in mouse models of severe obesity and type 2 diabetes (*ob/ob*)[7,40]. For future studies it is important to elucidate ER stress-associated mechanisms in regulating lipid metabolism under metabolic conditions. The resultant information will be important for designing novel strategies for the prevention and treatment of metabolic disease.

REFERENCES

1 **Jump DB**, Botolin D, Wang Y, Xu J, Christian B, Demeure O. Fatty acid regulation of hepatic gene transcription. *J Nutr* 2005; **135**: 2503-2506

2 **Xu J**, Nakamura MT, Cho HP, Clarke SD. Sterol regulatory element binding protein-1 expression is suppressed by dietary polyunsaturated fatty acids. A mechanism for the coordinate suppression of lipogenic genes by polyunsaturated fats. *J Biol Chem* 1999; **274**: 23577-23583

3 **Zelcer N**, Tontonoz P. Liver X receptors as integrators of metabolic and inflammatory signaling. *J Clin Invest* 2006; **116**: 607-614

4 **Schoonjans K**, Staels B, Auwerx J. Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. *J Lipid Res* 1996; **37**: 907-925

5 **Iizuka K**, Bruick RK, Liang G, Horton JD, Uyeda K. Deficiency of carbohydrate response element-binding protein (ChREBP) reduces lipogenesis as well as glycolysis. *Proc Natl Acad Sci USA* 2004; **101**: 7281-7286

6 **Ozcan U**, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Görgün C, Glimcher LH, Hotamisligil GS. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* 2004; **306**: 457-461

7 **Ozcan L**, Ergin AS, Lu A, Chung J, Sarkar S, Nie D, Myers MG Jr, Ozcan U. Endoplasmic reticulum stress plays a central role in development of leptin resistance. *Cell Metab* 2009; **9**: 35-51

8 **Gentile CL**, Pagliassotti MJ. The role of fatty acids in the development and progression of nonalcoholic fatty liver disease. *J Nutr Biochem* 2008; **19**: 567-576

9 **Wei Y**, Wang D, Topczewski F, Pagliassotti MJ. Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells. *Am J Physiol Endocrinol Metab* 2006; **291**: E275-E281

10 **Wang D**, Wei Y, Pagliassotti MJ. Saturated fatty acids promote endoplasmic reticulum stress and liver injury in rats with hepatic steatosis. *Endocrinology* 2006; **147**: 943-951

11 **Wei Y**, Wang D, Gentile CL, Pagliassotti MJ. Reduced endoplasmic reticulum luminal calcium links saturated fatty acid-mediated endoplasmic reticulum stress and cell death in liver cells. *Mol Cell Biochem* 2009; **331**: 31-40

12 **Gething MJ**, Sambrook J. Protein folding in the cell. *Nature* 1992; **355**: 33-45

13 **McMillan DR**, Gething MJ, Sambrook J. The cellular response to unfolded proteins: intercompartmental signaling. *Curr Opin Biotechnol* 1994; **5**: 540-545

14 **Sidrauski C**, Chapman R, Walter P. The unfolded protein response: an intracellular signalling pathway with many surprising features. *TrendsCell Biol* 1998; **8**: 245-249

15 **Mori K**. Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell* 2000; **101**: 451-454

16 **Kaufman RJ**. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *GenesDev* 1999; **13**: 1211-1233

17 **Bertolotti A**, Zhang Y, Hendershot LM, Harding HP, Ron D. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol* 2000; **2**: 326-332

18 **Shi Y**, Vattem KM, Sood R, An J, Liang J, Stramm L, Wek RC. Identification and characterization of pancreatic eukaryotic initiation factor 2 alpha-subunit kinase, PEK, involved in translational control. *Mol Cell Biol* 1998; **18**: 7499-7509

19 **Harding HP**, Zhang Y, Ron D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* 1999; **397**: 271-274

20 **Yoshida H**, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* 2001; **107**: 881-891

21 **Shen X**, Ellis RE, Lee K, Liu CY, Yang K, Solomon A, Yoshida H, Morimoto R, Kurnit DM, Mori K, Kaufman RJ. Complementary signaling pathways regulate the unfolded protein response and are required for C. elegans development. *Cell* 2001; **107**: 893-903

22 **Calfon M**, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, Clark SG, Ron D. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* 2002; **415**: 92-96

23 **Urano F**, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 2000; **287**: 664-666

24 **Haze K**, Yoshida H, Yanagi H, Yura T, Mori K. Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Mol Biol Cell* 1999; **10**: 3787-3799

25 **Zhang K**, Kaufman RJ. Identification and characterization of endoplasmic reticulum stress-induced apoptosis in vivo. *Methods Enzymol* 2008; **442**: 395-419

26 **Ron D**, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol* 2007; **8**: 519-529

27 **Zhang K**, Kaufman RJ. From endoplasmic-reticulum stress to the inflammatory response. *Nature* 2008; **454**: 455-462

28 **Ji C**. Dissection of endoplasmic reticulum stress signaling in alcoholic and non-alcoholic liver injury. *J Gastroenterol Hepatol* 2008; **23** Suppl 1: S16-S24

29 **Sriburi R**, Bommiasamy H, Buldak GL, Robbins GR, Frank M, Jackowski S, Brewer JW. Coordinate regulation of phospholipid biosynthesis and secretory pathway gene expression in XBP-1(S)-induced endoplasmic reticulum biogenesis. *J Biol Chem* 2007; **282**: 7024-7034

30 **Sriburi R**, Jackowski S, Mori K, Brewer JW. XBP1: a link between the unfolded protein response, lipid biosynthesis, and biogenesis of the endoplasmic reticulum. *J Cell Biol* 2004; **167**: 35-41

31 **Lee AH**, Scapa EF, Cohen DE, Glimcher LH. Regulation of hepatic lipogenesis by the transcription factor XBP1. *Science* 2008; **320**: 1492-1496

32 **Sha H**, He Y, Chen H, Wang C, Zenno A, Shi H, Yang X, Zhang X, Qi L. The IRE1alpha-XBP1 pathway of the unfolded protein response is required for adipogenesis. *Cell Metab* 2009; **9**: 556-564

33 **Bommiasamy H**, Back SH, Fagone P, Lee K, Meshinchi S, Vink E, Sriburi R, Frank M, Jackowski S, Kaufman RJ, Brewer JW. ATF6alpha induces XBP1-independent expansion of the endoplasmic reticulum. *J Cell Sci* 2009; **122**: 1626-1636

34 **Rutkowski DT**, Wu J, Back SH, Callaghan MU, Ferris SP, Iqbal J, Clark R, Miao H, Hassler JR, Fornek J, Katze MG, Hussain MM, Song B, Swathirajan J, Wang J, Yau GD, Kaufman RJ. UPR pathways combine to prevent hepatic steatosis caused by ER stress-mediated suppression of transcriptional master regulators. *Dev Cell* 2008; **15**: 829-840

35 **Oyadomari S**, Harding HP, Zhang Y, Oyadomari M, Ron

D. Dephosphorylation of translation initiation factor 2alpha enhances glucose tolerance and attenuates hepatosteatosis in mice. *Cell Metab* 2008; **7**: 520-532

36 **Bobrovnikova-Marjon E**, Hatzivassiliou G, Grigoriadou C, Romero M, Cavener DR, Thompson CB, Diehl JA. PERK-dependent regulation of lipogenesis during mouse mammary gland development and adipocyte differentiation. *Proc Natl Acad Sci USA* 2008; **105**: 16314-16319

37 **Wang H**, Kouri G, Wollheim CB. ER stress and SREBP-1 activation are implicated in beta-cell glucolipotoxicity. *J Cell Sci* 2005; **118**: 3905-3915

38 **Colgan SM**, Tang D, Werstuck GH, Austin RC. Endoplasmic reticulum stress causes the activation of sterol regulatory element binding protein-2. *Int J Biochem Cell Biol* 2007; **39**: 1843-1851

39 **Kammoun HL**, Chabanon H, Hainault I, Luquet S, Magnan C, Koike T, Ferré P, Foufelle F. GRP78 expression inhibits insulin and ER stress-induced SREBP-1c activation and reduces hepatic steatosis in mice. *J Clin Invest* 2009; **119**: 1201-1215

40 **Ozcan U**, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, Görgün CZ, Hotamisligil GS. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* 2006; **313**: 1137-1140

S- Editor Zhang HN **L- Editor** Roemmele A **E- Editor** Liu N

Int J Clin Exp Med 2010; 3(1):33-40
www.ijcem.com /IJCEM912003

Review Article

Integration of ER stress, oxidative stress and the inflammatory response in health and disease

Kezhong Zhang[1, 2, 3]

[1]Center for Molecular Medicine & Genetics, [2]Department of Immunology and Microbiology, [3]Karmanos Cancer Institute, The Wayne State University School of Medicine, Detroit, MI 48201, USA.

Received December 12, 2009; accepted January 4, 2010; available online January 8, 2010

Abstract: There has been much effort to define the molecular basis by which pathophysiological stimuli initiate and/or propagate the inflammatory response. Recent research endeavors on stress response from a cellular organelle called the endoplasmic reticulum (ER) shed new light on the understating of the molecular basis of the inflammatory response and its interaction with other intracellular stress signaling pathways. As a protein folding compartment and dynamic calcium store, the ER plays major roles in sensing cellular stress and mediating highly-specific signaling pathways termed Unfolded Protein Response (UPR). The UPR signaling emanating from the ER has been identified as one of the avenues leading to the inflammatory response. The integration of ER stress, oxidative stress, and the inflammatory response is critical to the pathogenesis of a variety of diseases. In this brief review, we discuss some representative evidence for the integration of ER stress, oxidative stress, and inflammation in health and disease.

Keywords: ER stress, oxidative stress, inflammation, unfolded protein response, metabolic factors, inflammatory disease

Introduction

In eukaryotic cells, the ER is an organelle responsible for folding and assembly of membrane and secreted proteins, synthesis of lipids and sterols, and storage of free calcium [1, 2]. As a protein folding compartment, the ER provides a unique oxidizing environment in which molecular chaperones and folding enzymes facilitate and promote protein post-translational modification, folding, and oligomerization [1]. The ER has evolved a high-fidelity quality control system to ensure that only correctly folded proteins are transported to the Golgi compartment and then delivered to the extracellular environment. However, physiological states that increase protein-folding demand, or stimuli that disrupt protein folding reactions, create an imbalance between the protein-folding load and the capacity of the ER. This can cause the accumulation of unfolded or misfolded proteins in the ER lumen - the condition referred to "ER stress" [2-7]. To deal with the accumulation of unfolded or misfolded protein in the ER, the cells activate a group of signal transduction pathways collectively termed Unfolded Protein Response (UPR) to alter transcriptional and translational programs. In mammalian cells, the basic UPR pathways are mediated through three ER-transmembrane protein factors, including IRE1α (inositol-requiring 1 alpha), PERK (double-strand RNA-activated protein kinase-like ER kinase), and ATF6 (activating transcription factor 6). IRE1α is a protein kinase and endoribonuclease that can splice the mRNA encoding a basic leucine zipper (bZIP) transcription factor XBP1 under the ER stress, leading to expression of the activated form of XBP1 [6, 8, 9]. The IRE1α/XBP1 arm of the UPR facilitates cell adaptation to ER stress by activating expression of a group of genes that are required for protein refolding, secretion, and degradation of misfolded proteins [10]. PERK is a protein kinase that phosphorylates alpha-subunit of eukaryotic translation initiation factor (eIF2α), leading to translational attenuation in general [11, 12]. The translational attenuation can reduce the workload of the ER by preventing production of newly-synthesized proteins. ATF6 is a bZIP transcription factor of CREB/ATF family that is acti-

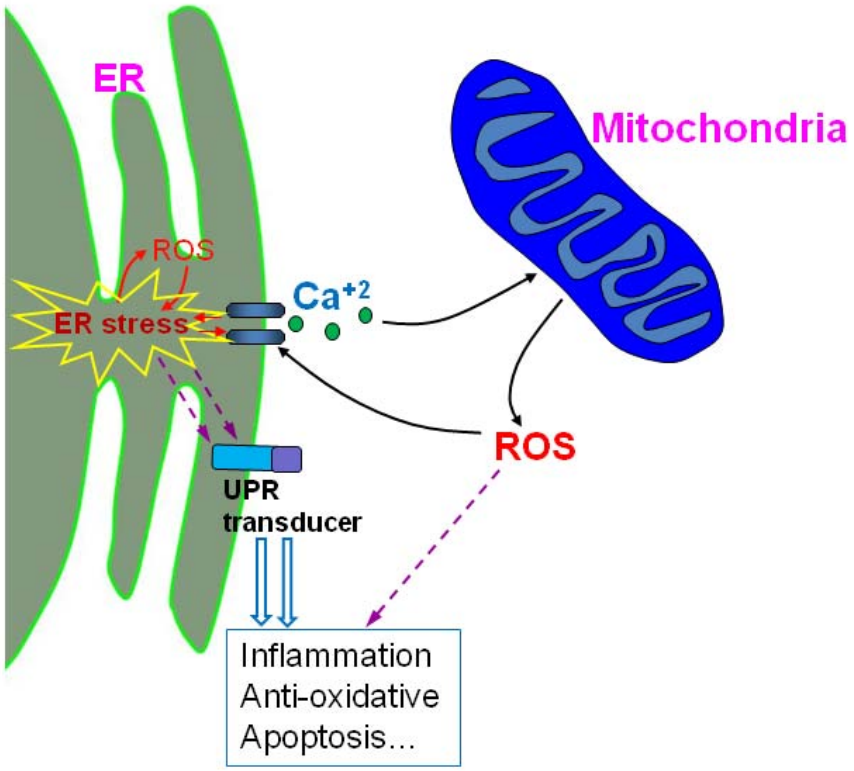

Figure 1. Crosstalk between ER stress, oxidative stress, and the inflammatory response. Many physiologic or pathologic conditions can stimulate the production of ROS. ROS can target ER-based calcium channels and chaperones, leading to release of calcium from the ER to the cytosol. Increased cytosolic calcium can stimulate mitochondria metabolism to produce more ROS. Mitochondrial ROS can further accentuate calcium release from the ER, leading to the accumulation of a toxic level of ROS. Meanwhile, perturbation of ER calcium homeostasis can disrupt protein folding process, causing ER stress and the activation of the UPR. The UPR can subsequently promote the inflammatory response, anti-oxidative stress response, apoptosis, and other stress response pathways.

vated through a regulated-intramembrane proteolysis (RIP) under ER stress [13]. Activated ATF6 activates expression of UPR target genes encoding ER chaperones and folding enzymes to help protein folding, secretion, and degradation [14]. The initial phase of the UPR is to facilitate the cell adaption to ER stress. However, if the attempt to recover from ER stress fails or the ER stress gets prolonged, the UPR will induce cell death programs to eliminate the stressed cells [15].

In recent years, the scope and consequence of ER stress and the UPR have been significantly expanded. A growing body of evidence suggests that ER stress, oxidative stress, and the inflammatory response are cross-linked and that limiting of either one will affect the others [7, 16]. Inflammation can be triggered by chronic excess of metabolic factors, such as cytokines, hormones, cholesterol, lipid, and glucose. Interestingly, in many physiological or pathological systems, those metabolic factors can also trigger ER stress and oxidative stress, which can further disrupt metabolic function, leading to more inflammation. Such vicious cycles could be accounted for many pathophysiological events leading to metabolic deterioration in specialized cell types, such as macrophage, adipocytes, and pancreatic β-cells [7]. Importantly, intracellular calcium signals and free radicals, such as reactive oxygen species (ROS) and nitric oxide (NO), are key messengers for the interaction between ER stress, oxidative stress, and inflammation. In these interactive signal transduction processes, the functions of the ER and mitochondria are closely linked. These two organelles build up a dynamic network where they generate calcium

signals and ROS to stimulate ER stress, oxidative stress, and inflammation (**Figure 1**).

Metabolic factors that trigger ER stress, oxidative stress, and the inflammatory response

Cholesterol

Cholesterol is an essential nutrient component for life but also associated with inflammation and the pathogenesis of metabolic disease if it is in pathological excess. Cholesterol, either synthesized in the ER or derived from the diet, is an essential component required to build and maintain all cell membranes. The cholesterol metabolism is closely associated with the ER function. This is well illustrated by the macrophage loaded by excessive unesterified, or "free" cholesterol [17, 18]. Upon elevation of lipids in the bloodstream, a metabolic condition referred as "hyperlipidemia", macrophages take up excessive amounts of cholesterol, resulting in formation of foam cells or inflammatory macrophages. Notably, the accumulation of cholesterol in macrophages induced synthesis of pro-inflammatory cytokines, including TNFα and IL6, by activating the nuclear factor kappa B (NF-kB) and the mitogen-activated protein kinases-mediated inflammatory pathways [19, 20]. Importantly, the activation of all these inflammatory pathways required cholesterol trafficking to ER and were modulated by PERK- and IRE1α-mediated UPR signaling. It has been demonstrated that the accumulation of cholesterol in macrophage leads to overload of unesterified, or "free" cholesterol in the ER membranes and subsequent depletion of ER calcium stores [17]. This condition causes ER stress and elicits the UPR in the macrophages as shown by the activation of three ER stress sensors, including PERK, IRE1α and ATF6, and their downstream effector molecules. Interestingly, CHOP, the target of PERK-mediated UPR pathway, is required for IL6 induction and full activation of Erk kinase induced by overload of cholesterol [19]. However, one or more other ER stress response pathways, possibly the ER-overload response, might also contribute to NF-kB activation induced by cholesterol accumulation, as the ER calcium release and ROS generation occurred with cholesterol overloading in the macrophage [19, 21]. Eventually, if the hyperlipidemia condition becomes chronic, the ER stress-induced pro-apoptotic factor CHOP would be activated to induce macrophage death that possibly contributes to necrosis and plaque disruption in atherosclerotic lesion [17, 22].

Homocysteine

Homocysteine is a sulfur-containing amino acid that is generated inside the body and required for biosynthesis of methionine and cysteine. The absence of vitamin B6, B12 or folic acid in food, or chronic alcohol consumption can affect activities of enzymes required for methionine, homocysteine and cysteine metabolisms and subsequently lead to accumulation of homocysteine in the body. Homocysteine has been considered as an independent risk factor for cardiovascular diseases. The highly reactive thiol group of homocysteine undergoes oxidization to form ROS, inducing oxidative stress and the inflammatory response [23, 24]. Homocysteine can also interrupt disulfide bond formation during protein folding, and thus causing ER stress and activation of the UPR [25, 26]. In fact, multiple cellular stress pathways including ER stress, oxidative stress, and inflammation are associated with accelerated atherosclerosis in animal models fed with high-homocysteine diets. Markers of ER stress (GRP78/94 and phospho-PERK), oxidative stress (HSP70), and inflammation (phospho-IkB) were simultaneously detected in advanced atherosclerotic lesions fed with high-homocysteine diets. ER stress-induced apoptosis, which is synergized by oxidative stress and inflammatory signaling, likely accounts for the cell injury and dysfunction during atherosclerosis induced by homocysteine [27].

Cytokines

Pro-inflammatory cytokines including TNFa, IL6, and IL1b can induce ER stress in liver hepatocytes, leading to the activation of the hepatocyte-specific stress sensor CREBH to mediate a liver acute phase response [28]. In mouse cancer fibrosarcoma cells, TNFα appears to be a strong ER stress inducer that activates three major UPR pathways as shown by PERK-mediated eIF2α phosphorylation, *Xbp1* mRNA splicing and ATF6 cleavage [29]. In the central nervous system, the presence of T cell–derived cytokine interferon-gamma (IFN-γ) was associated with the activation of the PERK branch of the UPR and ER stress-induced apoptosis in the oligodendrocyte, a cell type that produces vast amounts of myelin [30-32]. Myelin is a unique, lipid-rich, multilamellar sheath that wraps axons

of neurons. Thus, IFN-γ is believed to contribute to immune-mediated demyelinating disorders by inducing ER stress-mediated oligodendrocyte death. It has been documented that those inflammatory cytokines induce ER stress by triggering calcium signals and accumulation of ROS associated with the ER protein folding process and mitochondrial metabolisms [30]. ER stress can also arise in the inflammatory state of obese adipose tissue. In obesity, elevated levels of cytokine and hormones, such as leptin, insulin, TNFα, IL6, and IL1β, stimulate metabolisms and cell differentiation programs in the liver, adipose, and pancreas, leading to increased protein folding demands to the ER [33]. This situation would result in a physiological UPR and chronic inflammation in the specialized cell types, such as macrophages, hepatocytes, adipocytes, and pancreatic β cells [34]. Additionally, the bacterial toxin lipopolysaccharide (LPS), a strong inducer of the inflammatory response, has been shown to induce ER stress and ER stress-associated apoptosis in the liver and lung tissues [28, 35].

Glucose

Glucose is a primary source of energy and metabolic intermediate in most organisms from bacteria to humans. The metabolism of glucose is tightly controlled at the levels of synthesis and utilization through hormonal regulation [36]. The ER is exquisitely sensitive to glucose availability. The blood glucose levels influence the energy supply for the protein folding process in the ER, and thus are associated with the activation of the UPR and inflammation by stimulating ER stress and the production of free radicals. UPR signaling has been proposed to be essential to maintain glucose homeostasis [37]. This is well exemplified in pancreatic β cells, the specialized insulin-producing cells. As blood glucose declines, energy may become limiting for protein folding in the ER and therefore PERK-mediated UPR pathway may be activated, leading to translational attenuation to reduce the ER workload. As blood glucose levels rise, the UPR pathways are turned off so that translation would accelerate, allowing entry of new proinsulin into the ER. However, continual elevation of blood glucose could prolong elevated proinsulin translation and eventually activate the UPR as the secretion capacity of the ER is overwhelmed [38]. This condition could also induce inflammation as characterized by increased expression of pro-inflammatory cytokines [39, 40]. Therefore, a delicate balance between glucose levels and the UPR needs to be maintained. Disturbances in either direction may lead to β cell dysfunction.

Fatty acids

Accumulating evidence suggested that saturated fatty acids, such as palmitate and oleate, can induce ER stress and ER stress-associated apoptosis in various metabolic cells by triggering calcium signals and free radicals [34, 41]. Palmitate, the major saturated fatty acid in the "Western" style diet, was shown to induced ER stress-associated apoptosis mediated through CHOP in liver cells [41]. The presence of increased circulating and/or hepatic saturated fatty acids (palmitate and stearate), but not polyunsaturated fatty acids (oleate and linoleate) may exacerbate steatohepatitis and lipotoxicity through activating ER stress-associated apoptosis [41, 42]. These observations implicated a crucial role for the signaling pathways from the ER in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). Moreover, inhibition of ER calcium release by calcium chelators can prevent saturated fatty acid-induced ER stress and apoptosis in H4IIE liver cells and in primary rat hepatocytes, supporting that saturated fatty acids induce ER stress through disrupting ER calcium pool [43]. More recently, palmitate was shown to induce ER stress and apoptosis in hypothalamic neurons through a c-Jun N-terminal kinase (JNK)-dependent pathway [44]. Palmitate can also induce lipotoxic ER stress in pancreatic β cells via the effect on intracellular calcium homeostasis [45, 46].

In addition to those described above, many other metabolic factors can also induce both ER stress and inflammatory pathways in a variety of cell types when they are in pathological excess. For example, neurotransmitters including 6-hydroxydopamine and glutamine can induce the UPR and inflammation in cellular models of Parkinson's disease by disrupting calcium homeostasis and/or stimulating oxidative stress in the neuron cells [47] [48]. Notably, most of those effects are generated by the similar mechanism involving production of calcium signals and reactive oxygen intermediates.

Calcium and free radicals as the messengers triggering ER stress, oxidative stress, and the inflammatory response

Calcium and free radicals including ROS and NO are intracellular signaling messengers that play critical roles in cell physiology and disease pathogenesis. In fact, calcium signals and free radicals are major inflammatory mediators. Over the past few years, it has become increasingly apparent that calcium and free radicals are essential mediators that link ER stress to inflammation during metabolic processes. As an intracellular calcium store, the ER possesses calcium at a concentration thousands of times greater than that in the cytosol. The calcium levels inside the ER are modulated by the ER-located calcium release channels, including inositol-1,4,5-trisphosphate receptor (IP_3R) and the ryanodine receptor, the calcium uptake mechanism mediated through Sarco/Endoplasmic Reticulum Calcium ATPase (SERCA), and numerous ER-associated enzymatic cascades [49-52]. ER stress-inducing pharmaceutical agents and many metabolic factors, such as cytokines, hormones, neurotransmitters, and lipids, can target on ER-based calcium channels and affect the activities of calcium-dependent protein folding enzymes, leading to the release of Ca^{2+} from the ER lumen [7, 16, 53]. This will increase the concentration of cytosolic Ca^{2+} influx, which subsequently stimulates the mitochondria metabolism to generate more ROS. ROS can further target the ER calcium channels and protein folding enzymes to exacerbate ER calcium release and ER stress. As a consequence, unfolded or misfolded proteins accumulate in the ER, followed by the activation of the UPR pathways to promote the inflammatory response, anti-oxidative response, apoptosis, and other stress response pathways [6, 7] (**Figure 1**). This forward vicious cycle may account for many pathological processes that are triggered by inflammatory stimuli, oxidative stress, and/or ER stress. Notably, recent studies suggested that mitochondria can form junctions with the sarco/ER, which provides a platform for the local calcium-ROS interaction [52, 54].

NO is a free radical gas produced during a variety of metabolic processes, such as regulation of blood vessel dilatation, immune response, and neurotransmissions. Accumulation of a large amount of NO is toxic to the host and known to cause many deterious diseases including hypotension, septic shock, neurodegenerative disease, and diabetes. Excessive NO can target on ER calcium stores and mitochondrial electron transfer chains, causing ER stress and production of ROS. This mechanism accounts for most NO-induced inflammation and metabolic disorders. In addition, recent findings suggest that NO can inhibit the activity of protein disulfide isomerase (PDI) that is required for protein disulfide bond formation, thereby hampering proper protein folding and aggravating ER stress in neuronal cells [55].

Specialized cell types in which ER stress, oxidative stress, and the inflammatory response are integrated.

The cross-talks between ER stress, oxidative stress and the inflammatory response are well demonstrated at the functional interface of professional cells of primarily immune or metabolic nature. Those cells, including liver hepatocytes, macrophages, adipocytes, pancreatic β cells, and neuronal oligodendrocytes, are specialized for a high secretory capacity in cope with the increased demand of protein synthesis [7, 56, 57]. Therefore, they are exquisitely sensitive to ER stress or changes of metabolic status. In those cells, nutrients such as glucose, lipids, and cytokines can activate both the UPR and the inflammatory pathways by triggering calcium signals, ROS, and/or NO. The inflammatory stress signaling regulates the production of various cytokines including TNFα and IL6, and promotes production of other metabolic factors classified as hormones, growth factors, and neurotransmitters to mount a broader inflammatory response. For example, adipocyte is the most abundant cell type composing the adipose tissue. In addition to its inherent properties of fat cells in energy storage, adipocytes play a crucial role in the generation of a variety of pro-inflammatory cytokines, chemokines and hormones [57]. Metabolic conditions, such as lipid accumulation, elevated glucose or excessive cytokines, can stimulate increased demands for protein synthesis and production of ROS in adipocytes, which subsequently induce the UPR and inflammatory response to accelerate production of cytokines and hormones [57, 58]. Integrated UPR and inflammatory signaling in adipocytes may significantly contribute to the inflammatory state of obesity that is associated with insulin resistance. A similar "stress-

inflammation" interactive loop also occur to macrophages and pancreatic β cells where ER stress, oxidative stress, and inflammation interact and amplify by attacking the ER and mitochondria as well as various other metabolic pathways [7, 19, 33, 59].

Concluding remark

We discussed recent evidence of the integration of ER stress, oxidative stress, and inflammation in health and disease. Depending on the cell type and physiological process, either ER stress, or oxidative stress, or the inflammatory response may be more prominent or upstream of the others. However, these signaling pathways interact and are ultimately integrated in the pathogenesis of a variety of diseases. The related information is particularly important for the design of effective therapeutics for the inflammatory diseases by modulating ER stress, oxidative stress, and/or the inflammatory response. Given the complexity of stress signaling network, targeting a signal inflammatory stress mediator may not be effective or beneficial in controlling disease pathogenesis. Therefore, the research work in delineating the interface of stress signaling and the inflammatory response will definitely contribute to our better understanding of the pathogenesis of inflammatory diseases as well as pharmaceutical interventions toward controlling inflammation and stress.

Acknowledgement

The research in the Zhang laboratory is partially supported by the American Heart Association (AHA) Scientist Development Award (0635423Z), the AHA Grant-in-Aid (09GRNT2280479), the Department of Defense Breast Cancer Research Program (BC095179P1), and the Karmanos Cancer Institute pilot grant.

Please address correspondence to: Kezhong Zhang, PhD, 540 E. Canfield Avenue, Detroit, MI 48201, USA. Tel: 313-577-2669; Fax: 313-577-5218; Email: kzhang@med.wayne.edu

References

[1] Gething MJ and Sambrook J. Protein folding in the cell. Nature 1992; 355: 33-45.
[2] Kaufman RJ. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes.Dev. 1999; 13: 1211-1233.
[3] McMillian DR, Gething MJ and Sambrook J. The cellular response to unfolded proteins: Intercompartmental Signaling. Current Opinions in Biotechnology 1994; 5: 540-545.
[4] Sidrauski C, Chapman R and Walter P. The unfolded protein response: an intracellular signalling pathway with many surprising features. Trends.Cell Biol 1998; 8: 245-249.
[5] Mori K. Tripartite management of unfolded proteins in the endoplasmic reticulum. Cell 2000; 101: 451-454.
[6] Ron D and Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol 2007; 8: 519-529.
[7] Zhang K and Kaufman RJ. From endoplasmic-reticulum stress to the inflammatory response. Nature 2008; 454: 455-462.
[8] Mori K, Ma W, Gething MJ and Sambrook J. A transmembrane protein with a cdc2+/CDC28-related kinase activity is required for signaling from the ER to the nucleus. Cell 1993; 74: 743-756.
[9] Cox JS, Shamu CE and Walter P. Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. Cell 1993; 73: 1197-1206.
[10] Lee AH, Iwakoshi NN and Glimcher LH. XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. Mol Cell Biol 2003; 23: 7448-7459.
[11] Shi Y, Vattem KM, Sood R, An J, Liang J, Stramm L and Wek RC. Identification and characterization of pancreatic eukaryotic initiation factor 2 alpha-subunit kinase, PEK, involved in translational control. Mol.Cell Biol 1998; 18: 7499-7509.
[12] Harding HP, Zhang Y and Ron D. Protein translation and folding are coupled by an endoplasmic-reticulum- resident kinase [In Process Citation]. Nature 1999; 397: 271-274.
[13] Haze K, Yoshida H, Yanagi H, Yura T and Mori K. Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol.Biol Cell 1999; 10: 3787-3799.
[14] Okada T, Yoshida H, Akazawa R, Negishi M and Mori K. Distinct roles of activating transcription factor 6 (ATF6) and double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) in transcription during the mammalian unfolded protein response. Biochem J 2002; 366: 585-594.
[15] Zhang K and Kaufman RJ. Identification and characterization of endoplasmic reticulum stress -induced apoptosis in vivo. Methods Enzymol 2008; 442: 395-419.
[16] Malhotra JD, Miao H, Zhang K, Wolfson A, Pennathur S, Pipe SW and Kaufman RJ. Antioxidants reduce endoplasmic reticulum stress and im-

prove protein secretion. Proc Natl Acad Sci U S A 2008; 105: 18525-18530.
[17] Feng B, Yao PM, Li Y, Devlin CM, Zhang D, Harding HP, Sweeney M, Rong JX, Kuriakose G, Fisher EA, Marks AR, Ron D and Tabas I. The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages. Nat Cell Biol 2003; 5: 781-792.
[18] Tabas I, Seimon T, Timmins J, Li G and Lim W. Macrophage apoptosis in advanced atherosclerosis. Ann N Y Acad Sci 2009; 1173 Suppl 1: E40-45.
[19] Li Y, Schwabe RF, DeVries-Seimon T, Yao PM, Gerbod-Giannone MC, Tall AR, Davis RJ, Flavell R, Brenner DA and Tabas I. Free cholesterol-loaded macrophages are an abundant source of tumor necrosis factor-alpha and interleukin-6: model of NF-kappaB- and map kinase-dependent inflammation in advanced atherosclerosis. J Biol Chem 2005; 280: 21763-21772.
[20] Hu P, Han Z, Couvillon AD, Kaufman RJ and Exton JH. Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1alpha-mediated NF-kappaB activation and down-regulation of TRAF2 expression. Mol Cell Biol 2006; 26: 3071-3084.
[21] Pahl HL and Baeuerle PA. The ER-overload response: activation of NF-kappa B. Trends Biochem Sci 1997; 22: 63-67.
[22] Devries-Seimon T, Li Y, Yao PM, Stone E, Wang Y, Davis RJ, Flavell R and Tabas I. Cholesterol-induced macrophage apoptosis requires ER stress pathways and engagement of the type A scavenger receptor. J Cell Biol 2005; 171: 61-73.
[23] Starkebaum G and Harlan JM. Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. J Clin Invest 1986; 77: 1370-1376.
[24] Welch GN and Loscalzo J. Homocysteine and atherothrombosis. N Engl J Med 1998; 338: 1042-1050.
[25] Lentz SR and Sadler JE. Homocysteine inhibits von Willebrand factor processing and secretion by preventing transport from the endoplasmic reticulum. Blood 1993; 81: 683-689.
[26] Werstuck GH, Lentz SR, Dayal S, Hossain GS, Sood SK, Shi YY, Zhou J, Maeda N, Krisans SK, Malinow MR and Austin RC. Homocysteine-induced endoplasmic reticulum stress causes dysregulation of the cholesterol and triglyceride biosynthetic pathways. J Clin Invest 2001; 107: 1263-1273.
[27] Zhou J, Werstuck GH, Lhotak S, de Koning AB, Sood SK, Hossain GS, Moller J, Ritskes-Hoitinga M, Falk E, Dayal S, Lentz SR and Austin RC. Association of multiple cellular stress pathways with accelerated atherosclerosis in hyperhomocysteinemic apolipoprotein E-deficient mice. Circulation 2004; 110: 207-213.
[28] Zhang K, Shen X, Wu J, Sakaki K, Saunders T, Rutkowski DT, Back SH and Kaufman RJ. Endoplasmic reticulum stress activates cleavage of CREBH to induce a systemic inflammatory response. Cell 2006; 124: 587-599.
[29] Xue X, Piao JH, Nakajima A, Sakon-Komazawa S, Kojima Y, Mori K, Yagita H, Okumura K, Harding H and Nakano H. Tumor necrosis factor alpha (TNFalpha) induces the unfolded protein response (UPR) in a reactive oxygen species (ROS)-dependent fashion, and the UPR counteracts ROS accumulation by TNFalpha. J Biol Chem 2005; 280: 33917-33925.
[30] Lin W, Harding HP, Ron D and Popko B. Endoplasmic reticulum stress modulates the response of myelinating oligodendrocytes to the immune cytokine interferon-gamma. J Cell Biol 2005; 169: 603-612.
[31] Lin W, Kemper A, Dupree JL, Harding HP, Ron D and Popko B. Interferon-gamma inhibits central nervous system remyelination through a process modulated by endoplasmic reticulum stress. Brain 2006; 129: 1306-1318.
[32] Lin W, Bailey SL, Ho H, Harding HP, Ron D, Miller SD and Popko B. The integrated stress response prevents demyelination by protecting oligodendrocytes against immune-mediated damage. J Clin Invest 2007; 117: 448-456.
[33] Hotamisligil GS. Inflammation and metabolic disorders. Nature 2006; 444: 860-867.
[34] Kharroubi I, Ladriere L, Cardozo AK, Dogusan Z, Cnop M and Eizirik DL. Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: role of nuclear factor-kappaB and endoplasmic reticulum stress. Endocrinology 2004; 145: 5087-5096.
[35] Endo M, Oyadomari S, Suga M, Mori M and Gotoh T. The ER stress pathway involving CHOP is activated in the lungs of LPS-treated mice. J Biochem 2005; 138: 501-507.
[36] Itoh N and Okamoto H. Translational control of proinsulin synthesis by glucose. Nature 1980; 283: 100-102.
[37] Kaufman RJ, Scheuner D, Schroder M, Shen X, Lee K, Liu CY and Arnold SM. The unfolded protein response in nutrient sensing and differentiation. Nat Rev Mol Cell Biol 2002; 3: 411-421.
[38] Scheuner D, Vander Mierde D, Song B, Flamez D, Creemers JW, Tsukamoto K, Ribick M, Schuit FC and Kaufman RJ. Control of mRNA translation preserves endoplasmic reticulum function in beta cells and maintains glucose homeostasis. Nat Med 2005; 11: 757-764.
[39] Patel C, Ghanim H, Ravishankar S, Sia CL, Viswanathan P, Mohanty P and Dandona P. Prolonged reactive oxygen species generation and nuclear factor-kappaB activation after a high-fat, high-carbohydrate meal in the obese. J Clin Endocrinol Metab 2007; 92: 4476-4479.
[40] Boni-Schnetzler M, Thorne J, Parnaud G, Marselli L, Ehses JA, Kerr-Conte J, Pattou F, Halban PA, Weir GC and Donath MY. Increased interleukin

(IL)-1beta messenger ribonucleic acid expression in beta -cells of individuals with type 2 diabetes and regulation of IL-1beta in human islets by glucose and autostimulation. J Clin Endocrinol Metab 2008; 93: 4065-4074.
[41] Wei Y, Wang D, Topczewski F and Pagliassotti MJ. Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells. Am J Physiol Endocrinol Metab 2006; 291: E275-281.
[42] Wang D, Wei Y and Pagliassotti MJ. Saturated fatty acids promote endoplasmic reticulum stress and liver injury in rats with hepatic steatosis. Endocrinology 2006; 147: 943-951.
[43] Wei Y, Wang D, Gentile CL and Pagliassotti MJ. Reduced endoplasmic reticulum luminal calcium links saturated fatty acid-mediated endoplasmic reticulum stress and cell death in liver cells. Mol Cell Biochem 2009; 331: 31-40.
[44] Mayer CM and Belsham DD. Palmitate Attenuates Insulin Signaling and Induces Endoplasmic Reticulum Stress and Apoptosis in Hypothalamic Neurons: Rescue of Resistance and Apoptosis through Adenosine 5' Monophosphate-Activated Protein Kinase Activation. Endocrinology 2009; Epub ahead of print, PMID 19952270.
[45] Lai E, Bikopoulos G, Wheeler MB, Rozakis-Adcock M and Volchuk A. Differential activation of ER stress and apoptosis in response to chronically elevated free fatty acids in pancreatic beta-cells. Am J Physiol Endocrinol Metab 2008; 294: E540-550.
[46] Gwiazda KS, Yang TL, Lin Y and Johnson JD. Effects of palmitate on ER and cytosolic Ca2+ homeostasis in beta-cells. Am J Physiol Endocrinol Metab 2009; 296: E690-701.
[47] Ryu EJ, Harding HP, Angelastro JM, Vitolo OV, Ron D and Greene LA. Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. J Neurosci 2002; 22: 10690-10698.
[48] Yamamuro A, Yoshioka Y, Ogita K and Maeda S. Involvement of endoplasmic reticulum stress on the cell death induced by 6-hydroxydopamine in human neuroblastoma SH-SY5Y cells. Neurochem Res 2006; 31: 657-664.
[49] Luo X, He Q, Huang Y and Sheikh MS. Transcriptional upregulation of PUMA modulates endoplasmic reticulum calcium pool depletion-induced apoptosis via Bax activation. Cell Death Differ 2005; 12: 1310-1318.
[50] Oakes SA, Scorrano L, Opferman JT, Bassik MC, Nishino M, Pozzan T and Korsmeyer SJ. Proapoptotic BAX and BAK regulate the type 1 inositol trisphosphate receptor and calcium leak from the endoplasmic reticulum. Proc Natl Acad Sci U S A 2005; 102: 105-110.
[51] Hajnoczky G, Csordas G, Krishnamurthy R and Szalai G. Mitochondrial calcium signaling driven by the IP3 receptor. J Bioenerg Biomembr 2000; 32: 15-25.
[52] Csordas G and Hajnoczky G. SR/ER-mitochondrial local communication: calcium and ROS. Biochim Biophys Acta 2009; 1787: 1352-1362.
[53] Deniaud A, Sharaf el dein O, Maillier E, Poncet D, Kroemer G, Lemaire C and Brenner C. Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis. Oncogene 2008; 27: 285-299.
[54] Yan Y, Liu J, Wei C, Li K, Xie W, Wang Y and Cheng H. Bidirectional regulation of Ca2+ sparks by mitochondria-derived reactive oxygen species in cardiac myocytes. Cardiovasc Res 2008; 77: 432-441.
[55] Uehara T, Nakamura T, Yao D, Shi ZQ, Gu Z, Ma Y, Masliah E, Nomura Y and Lipton SA. S-nitrosylated protein-disulphide isomerase links protein misfolding to neurodegeneration. Nature 2006; 441: 513-517.
[56] Schenk S, Saberi M and Olefsky JM. Insulin sensitivity: modulation by nutrients and inflammation. J Clin Invest 2008; 118: 2992-3002.
[57] Gregor MG and Hotamisligil GS. Adipocyte stress: The endoplasmic reticulum and metabolic disease. J Lipid Res 2007; 48:1905-1914.
[58] Lionetti L, Mollica MP, Lombardi A, Cavaliere G, Gifuni G and Barletta A. From chronic overnutrition to insulin resistance: the role of fat-storing capacity and inflammation. Nutr Metab Cardiovasc Dis 2009; 19: 146-152.
[59] Gurzov EN, Ortis F, Cunha DA, Gosset G, Li M, Cardozo AK and Eizirik DL. Signaling by IL-1beta+IFN-gamma and ER stress converge on DP5/Hrk activation: a novel mechanism for pancreatic beta-cell apoptosis. Cell Death Differ 2009; 16: 1539-1550.

Am J Transl Res 2010;2(1):65-74
www.ajtr.org /AJTR912002

Review Article

Endoplasmic reticulum stress response in cancer: molecular mechanism and therapeutic potential

Guohui Wang [1], Zeng-Quan Yang [3, 4], Kezhong Zhang [1, 2, 3]

[1]Center for Molecular Medicine & Genetics, [2]Department of Immunology and Microbiology, [3]Karmanos Cancer Institute, [4]Department of Pathology, The Wayne State University School of Medicine, Detroit, MI 48201, USA.

Received December 9, 2009, accepted December 12, 2009, available online January 1, 2010

Abstract: In eukaryotic cells, the endoplasmic reticulum (ER) is an organelle that is responsible for protein folding and assembly, lipid and sterol biosynthesis, and free calcium storage. In the past decade, intensive research effort has been focused on intracellular stress signaling pathways from the ER that lead to transcriptional and translational reprogramming of stressed cells. These signaling pathways, which are collectively termed Unfolded Protein Response (UPR), are critical for the cell to make survival or death decision under ER stress conditions. In recent years, research in the cancer field has revealed that ER stress and the UPR are highly induced in various tumors and are closely associated with cancer cell survival and resistance to anti-cancer treatments. Identifying the UPR components that are activated or suppressed in malignancy and exploring cancer therapeutic potentials by targeting the UPR are hot research spots. In this review, we summarize the recent progress in understating UPR signaling in cancer and its related therapeutic potential.

Keywords: Endoplasmic reticulum, ER stress, unfolded protein response, cancer, malignancy, cancer therapy

Introduction

Inside a eukaryotic cell, there are numerous organelles that play essential roles in cell survival and functions. Besides the nucleus, the largest organelle is endoplasmic reticulum (ER), an extensive membranous labyrinth network of tubules, vesicles and sac that surrounds the nucleus and expands to the cytosol [1]. The ER has been primarily recognized as a compartment for protein folding and assembly, a pool of free calcium, and a site for lipid and sterol biosynthesis [2, 3]. Approximately one-third of newly synthesized proteins are translocated into the ER where they fold and assemble with the help of a series of molecular chaperones and folding catalysts. Inside the ER, co- and post-translational modifications, including disulfide bond formation and N-linked glycosylation, play important roles in the folding and oligomeric assembly of proteins. The ER provides a high-fidelity quality control system to ensure that only correctly folded proteins can be transported out of the ER while unfolded or misfolded proteins are retained in the ER and eventually degraded [2]. As a protein-folding compartment, the ER is exquisitely sensitive to alterations in homeostasis. A number of biochemical, physiologic to pathologic stimuli, such as those that cause ER calcium depletion, altered glycosylation, nutrient deprivation, oxidative stress, DNA damage, or energy perturbation/ fluctuations, can interrupt the protein folding process and subsequently cause accumulation of unfolded or misfolded proteins in the ER - a condition referred to "ER stress" [3-8]. To ensure the fidelity of protein folding and to handle the accumulation of unfolded or misfolded proteins, the ER has evolved a group of signal transduction pathways, the unfolded protein response (UPR) signaling pathways, to alter transcriptional and translational programs [3, 7]. The basic UPR pathways in mammalian cells consist of three main signaling cascades initiated by three primary ER-localized protein stress sensors: IRE1α (inositol-requiring 1 alpha), PERK (double-strand RNA-activated protein kinase-like ER kinase), and ATF6 (activating transcription factor 6).

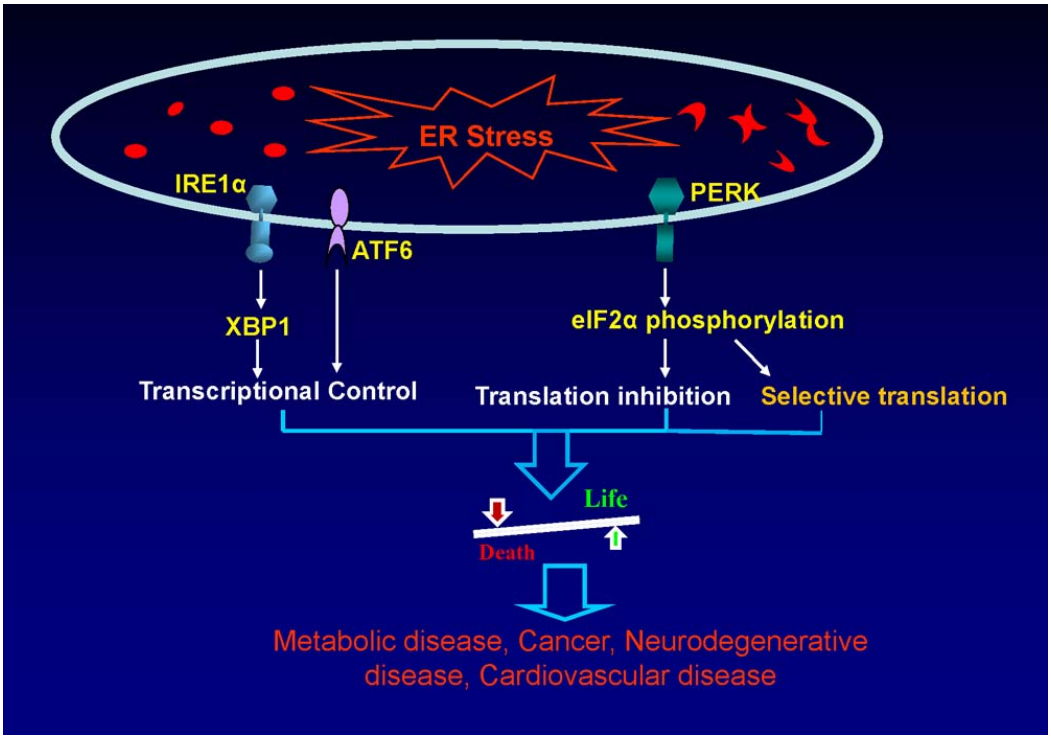

Figure 1. Role of UPR signaling in health and disease. Under ER stress, three ER stress sensors IRE1α, PERK and ATF6, are activated to alter transcriptional and translational programs to protect the cell from stress caused by the accumulation of unfolded or misfolded proteins. The UPR is critical for the cell to make survival or death decisions under ER stress conditions by altering translational and transcriptional programs. Regulation through UPR signaling is crucial for the development of a variety of diseases, including metabolic disease, cancer, neurodegenerative disease, and cardiovascular disease.

IRE1α is a protein kinase and endoribonuclease [9, 10], PERK is a protein kinase that is known to phosphorylate alpha-subunit of eukaryotic translation initiation factor (eIF2α) [11, 12], and ATF6 is a basic leucine zipper (bZIP) transcription factor of CREB/ATF family [13]. The primary role of the UPR is to prevent the cell from ER stress by reducing the amount of proteins translocated into the ER lumen, increasing retro-translocation and degradation of ER-localized proteins, and augmenting the protein-folding capacity of the ER (**Figure 1**). However, if the attempt to recover from ER stress fails, the UPR will induce cell death programs to eliminate the stressed cells [14].

During tumorgenesis, the high proliferation rate of cancer cells requires increased activities of ER protein folding, assembly, and transport, a condition that can induce physiological ER stress [15]. Moreover, as the tumor grows, cancer cells experience increasing nutrient starvation and hypoxia, which are strong inducers for the accumulation of unfolded or misfolded proteins in the ER and the activation of the UPR pathways [15, 16]. Indeed, accumulating evidence has demonstrated that the UPR is an important mechanism required for cancer cells to maintain malignancy and therapy resistance. Additionally, the possibility of targeting the UPR signaling as a novel therapeutic strategy has greatly inspired the cancer research community and pharmaceutical industry.

The UPR pathways

When cells encounter ER stress, an immediate response will be the activation of ER stress sensor PERK through its homo-dimerization and auto-phosphorylation [17]. Activated PERK phosphorylates translation initiation factor eIF2α, leading to protein translational attenuation in general. PERK-mediated translation attenuation provides a survival signal, as this can reduce the ER workload by preventing newly-synthesized proteins from entering into the ER which is saturated by unfolded or misfolded proteins. This is evidenced by the fact that the

inactivation of PERK-mediated UPR pathway reduces cells' ability to survive ER stress [18, 19]. However, while general protein translation is inhibited, PERK-mediated eIF2α phosphorylation can lead to preferential translation of specific mRNAs that contain multiple upstream open reading frames in their 5'-untranslated regions (ORFs). These upstream ORFs are bypassed only when eIF2α is phosphorylated, thus allowing translation of the mRNA [20]. One of those mRNAs is known to encode the transcription factor 4 (ATF4). Under ER stress, phosphorylated eIF2α selectively initiates translation of *atf4* mRNA [21]. ATF4 subsequently activates expression of genes involved in cell metabolism, anti-oxidative response, and ER stress-associated apoptosis [18, 22].

Along with PERK-mediated translational repression, IRE1α- and ATF6-mediated UPR pathways are also activated to increase protein folding capacity and ER-associated protein degradation. Under ER stress, IRE1α is activated through its homo-dimerization and auto-phosphorylation. Activated IRE1α can function as an endoribonuclease to initiate removal of a 26 nucleotide intron from the mRNA encoding X-box binding protein 1 (XBP1) [17]. This unconventional mRNA splicing generates a translation frameshift that enables the spliced *Xbp1* mRNA to encode a functional potent bZIP transcription factor. The spliced XBP1 can activate expression of a group of ER chaperones and enzymes to help protein folding, maturation, secretion, as well as degradation of misfolded proteins [23]. In addition to its endoribonuclease activity, phosphorylated IRE1α can also serve as a scaffold protein that recruits tumour-necrosis factor (TNF)-receptor-associated factor 2 (TRAF2), leading to activation of JUN N-terminal kinase (JNK)-mediated signaling pathways [24]. Notably, the pro-apoptotic B-cell lymphoma 2 (BCL-2) family members BAX and BAK can directly bind to the cytosolic domain of IRE1α and augment its kinase and endoribonuclease activities [25, 26]. The interaction of BCL-2 family members with IRE1α may provide a molecular link between ER stress and apoptosis pathways. On activation of the UPR, ATF6 is also released from the ER membrane, and transits to the Golgi compartment where it is processed by proteases to produce an activated bZIP transcription factor that activates expression of UPR target genes [13]. Similar to spliced XBP1, cleaved ATF6 also activates expression of a group of genes involved in protein folding, secretion, and degradation in the ER [23, 27]. However, recent evidence suggests that ATF6, but not XBP1, is dispensable for the differentiation, function, or survival of specialized cell types where the UPR signaling is required [28, 29].

If the stressed cells fail to adapt to and recover from ER stress through the UPR-mediated survival programs, the UPR will initiate apoptotic pathways to remove the stressed cells. The well-defined pathway involved in the transition from ER stress to apoptosis is mediated by a transcription factor called GADD153/CHOP that is downstream of the PERK/eIF2α UPR pathway [14, 30-32]. Under prolonged ER stress, activated PERK phosphorylates eIF2α, which can selectively induce translation of the mRNA encoding ATF4. ATF4 induces a pro-apoptotic factor GADD153/CHOP to mediate ER stress-induced apoptosis. This is probably a case in some viral infections in which the organism utilizes ER stress-induced apoptosis to eliminate the infected, stressed cells in order to limit viral replication [33, 34]. Additionally, as part of the UPR program, ER-associated Protein Degradation (ERAD) is responsible for the degradation of aberrant or misfolded proteins in the ER and, in addition to this "quality control" function, also accounts for the degradation of several metabolically regulated, active ER proteins [35, 36]. During the process of ERAD, molecular chaperones and associated factors recognize and target substrates for retrotranslocation to the cytoplasm, where they are polyubiquitinated and degraded by 26S proteasome. ERAD is essential for maintaining ER homeostasis, and disruption of ERAD is closely associated with ER stress-induced apoptosis [37].

The UPR in malignancy

Cancer cells possess rapid glucose metabolism and fast growth rate, which leads to poor vascularisation of tumor mass, low oxygen supply, nutrient deprivation, and pH changes [16, 38]. On the other hand, cancer cells can express mutant proteins that cannot be correctly folded, and experience insufficient ER energy supply, alteration of the redox environment, and viral infection [39]. All of these can cause ER stress and activation of the UPR. Increasing evidence suggests that the UPR provides survival signaling pathways required for tumor growth. Indeed, increased expression of the UPR components, including the UPR *trans*-activators XBP1 and

ATF6, ER stress-associated pro-apoptotic factor CHOP, as well as ER chaperones GRP78/BIP, GRP94, and GRP170, have been detected in breast cancer, hepatocellular carcinomas, gastric tumors, and esophageal adenocarcinomas [40]. Cancer cells may adapt to ER stress and evade stress-induced apoptotic pathways by differentially activating the UPR branches [41-43]. Here, we discuss recent advances in understating the roles of different UPR components in malignancy.

ER chaperone GRP78/BiP

GRP78/BiP (glucose-regulated protein of 78 kDa) is an abundant ER chaperone that uses ATP/ADP cycling to regulate the protein folding process [44, 45]. It has been proposed that the initial activation of three ER stress sensors, including IRE1α, PERK and ATF6, depends on the dissociation of GRP78 in response to ER stress [7]. Recent studies suggested that GRP78 plays critical cytoprotective roles in oncogenesis [38, 44]. Increased expression of GRP78 has been observed in a variety of cancers [46-48]. GRP78 over-expression was shown to provide important survival signals for cancer cells during oncogenesis and confers drug resistance in both proliferating and dormant cancer cells [15].

The evidence that GRP78 is required for cancer cell survival came from the observation that suppression of GRP78 in fibrosarcoma cells inhibited their ability to form tumors *in vivo* [49]. GRP78 has also been implicated in promoting tumor cell proliferation. Over-expression of GRP78 correlated with increased proliferation rates of a range of glioma cells, while the knockdown of GRP78 resulted in decreased proliferation rates of glioma cells [41]. Dong et al showed that *Grp78* heterozygosity prolonged the latency period and significantly impeded tumor growth in a genetic mouse model of breast cancer where GRP78 expression level was reduced by half [50]. Their results suggested that GRP78 regulates cancer progression through three mechanisms, including enhancement of tumor cell proliferation, protection against apoptosis, and promotion of tumor angiogenesis. Recently, in a large series of breast cancer cases, expression of GRP78 and XBP-1 was observed in 76% and 90% of the breast cancers [47]. The results suggested that the UPR is activated in the majority of breast cancers and probably confers resistance to chemotherapy.

Additionally, a link between the high GRP78 expression level and poor clinical outcome of cancer therapy has been observed. For example, high levels of GRP78 expression correlate with increasing tumor grade in hepatocellular carcinoma, poor clinical outcome in breast cancer, high rates of recurrence and mortality in prostate cancer, and high rates of nodal metastasis and reduced survival in gastric cancer[48, 51-53].

UPR signaling through IRE1α/XBP1

The UPR signaling through ER stress sensor IRE1α and *trans*-activator XBP1 controls the upregulation of a broad spectrum of UPR-related genes involved in protein folding, transport, and ERAD [23]. In addition to classical UPR -related genes, the IRE1α/XBP1 arm of the UPR also regulates expression of the genes involved in cell differentiation, inflammation, lipogenesis, and apoptotic pathways [54]. A number of recent studies suggested that the IRE1/XBP1 arm of the UPR is essential for malignancy maintenance under oncogenic stress. Transformed mouse embryonic fibroblasts or human fibrosarcoma tumor cells (HT1080) that lack XBP1 displayed the inability to grow as tumor xenografts in SCID mice [55, 56]. Instead, XBP1-deficient cells showed increased apoptosis and decreased clonogenic survival under ER stress or hypoxia condition. Furthermore, expression of the dominant-negative form of IRE1α or inhibition of XBP1 by RNAi reduced blood vessel formation during tumorgenesis in an intradermal angiogenesis model or a human tumor xenograft model [57]. However, expression of spliced XBP-1 restored angiogenesis in IRE1α dominant -negative expressing cells, suggesting that the UPR signaling through IRE1α/XBP1 is crucial for angiogenesis in the early stage of tumor development. Interestingly, the un-spliced form of *Xbp1* mRNA was shown to encode a rapid-turnover protein that can function as a dominant negative factor to inhibit spliced XBP1 activities [58, 59]. While high expression levels of spliced XBP1 were associated with increased tumor survival, high levels of the unspliced form of XBP1 caused increased apoptosis of tumor cells [60].

UPR signaling through PERK/eIF2α

During tumorgenesis, cancer cells need to tolerate a subset of oncogenesis-associated cellular stresses including DNA damage, hypoxia, pro-

teotoxic, mitotic, and oxidative stress [16]. In order to adapt to and overcome the stress, tumor cells remodel the transcriptional and translational programs by activating pro-survival signaling pathways. The UPR signaling through PERK/eIF2α has been demonstrated to confer a survival advantage for tumor cells under hypoxic stress [61]. Hypoxic stress can activate PERK, leading to phosphorylation of eIF2α in tumor cells [61, 62]. Transformed mouse embryonic fibroblasts from the PERK-deficient animals and HT29 colorectal carcinoma cells expressing dominant-negative PERK exhibited lower survival rates under hypoxic conditions, compared to wild type cells [62]. These cells formed smaller tumors and displayed higher levels of apoptotic activity in hypoxic areas than the wild-type control cells [63]. Additionally, tumors derived from PERK-deficient mouse embryonic fibroblasts exhibited limited ability to stimulate angiogenesis [64]. Furthermore, the critical role of PERK/eIF2α-mediated UPR signaling in hypoxia survival is supported by a study with mouse embryonic fibroblasts expressing a "knock-in" mutant of eIF2α (S51A) that cannot be phosphorylated by PERK [63]. These cells displayed an increased susceptibility to hypoxia with virtually no survival under prolonged hypoxia conditions.

The therapeutic potential of targeting the UPR components

The importance of the UPR in malignancy maintenance has inspired great interest in exploring therapeutic potentials by targeting the UPR components. Tumor cells grow under oncogenic stress caused by hypoxia, nutrient deprivation, DNA damage, metabolic and oxidative stress and therefore rely on an activated UPR for survival [15, 47]. However, most normal cells are not subjected to stress, and the UPR pathways remain inactive state in these cells. This discrepancy between tumor cells and normal cells may offer an advantage for the agents that target the UPR to achieve the specificity in cancer therapy. In the following, we provide some representative evidence for cancer therapeutic applications by targeting UPR components.

GRP78/BiP as a cancer therapeutic target and biomarker

Expression of GRP78 protein correlated with both the rate of patient survival and the depth of tumor invasion. In human cancers, elevated GRP78 level generally indicates the higher pathologic grade, recurrence, and poor patient survival in breast, liver, prostate, colon, and gastric cancers, although lung cancer is an exception [15]. Additionally, GRP78 expression was positively correlated with increasing tumor thickness and with increasing dermal tumor mitotic index [65]. These observations have inspired the idea of targeting GRP78 for cancer therapy. Indeed, recent studies supported that knockdown of GRP78 can suppress cancer cell growth and improve the sensitivity of cancer cells to the treatments. Knockdown of GRP78 by siRNA could slow down the growth of glioma cells and increase their sensitivity to chemotherapeutic agents, including temozolomide, 5-fluorouracil and CPT-11 [41]. The cytotoxic effect of GRP78 knockdown has been confirmed in many cancer cell lines [66, 67], although one study suggested that the pro-survival role of GRP78 in tumorgenesis is possibly cell-line specific [68]. Researchers have been actively screening for a specific GRP78 inhibitor as an anticancer agent [69-71].

GRP78 is an abundant molecular chaperone that localizes to the ER lumen. However, recent evidence suggested that a sub-fraction of GRP78 localized to the surface of specific cell types, including malignant cells [72]. Preferential expression of GRP78 on the surface of tumor cells but not in normal organs suggests that surface GRP78 can serve as a biomarker for cancer-specific therapy. Indeed, some of recent studies supported that ER chaperones GRP78 and GRP94 are effective biomarkers for indicating aggressive behavior and poor prognosis in cancer [51, 53, 73, 74], although there is evidence that GRP78 as a cancer biomarker might be tumor-type specific [15, 52, 75].

Proteasome inhibitors

The ubiquitin-proteasome pathway is one of central players in the regulation of several diverse cellular processes. Proteasome inhibitors can block the action of proteasomes, inhibit the degradation of proteins critically involved in regulation of cell proliferation and survival, and eventually lead to growth inhibition and apoptosis. Proteasome inhibitors have been intensively studied in the treatment of cancers. Bortezomib (Velcade; PS-341) is a highly selective and reversible proteasome inhibitor that has been approved for clinical use against multiple myeloma and is in clinical trials as a single

agent or in combination with chemotherapeutics against other solid tumor malignancies [76, 77]. The *in vitro* studies have confirmed the cytotoxic effects of bortezomib on a broad range of cancer cell types, including prostate, lung, breast, colon, and non-Hodgkin's lymphoma [78-81]. It can induce additive or synergistic cytotoxic activities against cancer cells when combined with other antineoplastic agents [81-83]. Although the mechanisms involved in its anticancer activity are still being elucidated, bortezomib was recently shown to cause the accumulation of misfolded proteins in the ER and ER stress-associated apoptosis by inhibiting 26S proteasome activity and subsequent ERAD machinery [58, 84-86]. Moreover, bortezomib was shown to suppress the IRE1α/XBP1 arm of the UPR by inhibiting IRE1α endoribonuclease/kinase activity and by stabilizing the unspliced form of XBP1, a dominant negative for the functional XBP1 protein [58, 59]. In addition to bortezomib, therapeutic potentials of other proteasome inhibitors were also investigated. For example, BU-32 (NSC D750499-S), a highly selective proteasome inhibitor, was effective in suppressing *in vitro* and *in vivo* breast cancer cells, on which bortezomib has limited effect [87].

ERAD inhibitor

Under ER stress, ERAD removes aberrant or misfolded proteins from the ER through protein retrotranslocation and ubiquitin-proteasome degradation systems [36, 88]. Defects in ERAD cause the accumulation of misfolded proteins in the ER and thus trigger ER stress-induced apoptosis [37]. In the process of ERAD, a cytosolic ATPase named p97 plays key roles in extracting misfolded proteins that are polyubiquitinated and transporting them to the proteasome for degradation. Recently, Eeyarestatin I (EerI), a chemical inhibitor that can block ERAD, has been shown to have preferential cytotoxic activity against cancer cells [89, 90]. EerI can target p97 complex to inhibit deubiquitination of p97-associated ERAD substrates, which is required for the degradation process [90]. Like bortezomib, EerI induces an integrated stress response in the ER as well as apoptosis via the Bcl-2 homology3 (BH3)-only pro-apoptotic protein NOXA. EerI activates the CREB/ATF transcription factors ATF3 and ATF4, which form a complex capable of binding to the NOXA promoter and activate NOXA expression [89]. Interestingly, EerI was found to be able to block ubiquitination of histone H2A to relieve its inhibition on NOXA transcription [89]. These studies suggested that the ERAD inhibitor EerI may represent a novel class of anticancer drugs that integrate ER stress response with epigenetic mechanisms to induce cell death.

Other therapeutic potential associated with ER stress

Several other distinct agents have been reported to have anti-cancer potentials by modulating ER stress response. Versipelostatin, a novel macrocyclic compound, showed highly selective cytotoxicity to glucose-deprived tumor cells and *in vivo* tumors by inhibiting GRP78 induction and expression of the UPR transactivators XBP1 and ATF4 [69, 91]. An engineered fusion protein, epidermal growth factor-SubA (EGF-SubA), was reported to be highly toxic to growing and confluent epidermal growth factor receptor-expressing cancer cells, and its cytotoxicity is thought to be mediated by rapid cleavage of GRP78 [70]. Systemic delivery of EGF-SubA resulted in a significant inhibition of human breast and prostate tumor xenografts in mouse models. Additionally, Salazar et al reported that δ-tetrahydrocannabinol, the main active component of marijuana, induces human glioma cancer cell death through stimulation of ER stress-associated autophagy [92]. δ-tetrahydrocannabinol can induce ceramide accumulation and the ER stress response that triggers autophagy through inhibition of the Akt/mammalian target of rapamycin complex 1 axis. The δ-tetrahydrocannabinol-induced autophagic death of human and mouse cancer cells suggested that cannabinoid administration may be an effective strategy for cancer therapy.

Concluding remarks

Significant progress has been made in elucidating the mechanism and role of the ER stress response in oncogenesis and cancer therapy resistance. The related findings have raised an exciting possibility of targeting the UPR components as an effective strategy for cancer therapy and overcoming drug resistance. For future research, it is important to delineate the distinct roles of the UPR branches that may provide survival or death signal in tumorgenesis or cancer therapy. The related information will be essential for pharmaceutical design toward controlling cancer through modulating UPR signaling. Research in this topic will significantly advance our understanding of cancer biology and be infor-

mative to its therapeutic application against cancer.

Acknowledgements

The research in the Zhang laboratory is partially supported by the American Heart Association (AHA) Scientist Development Award (0635423Z), the AHA Grant-in-Aid (09GRNT2280479), the Department of Defense Idea Grant (BC095179P1), and the Karmanos Cancer Institute pilot grant. The research in the Yang laboratory is partially supported by the Department of Defense Breast Cancer Program grants (BC083945 and BC083954) and the Karmanos Cancer Institute pilot grant.

Please address correspondence to: Kezhong Zhang, Ph.D., 540 E. Canfield Avenue, Wayne State University School of Medicine, Detroit, MI 48201, USA. Tel: 313-577-2669; Fax: 313-577-5218; Email: kzhang@med.wayne.edu

References

[1] Porter KR, Claude A and Fullam EF. A Study of Tissue Culture Cells by Electron Microscopy : Methods and Preliminary Observations. J Exp Med 1945; 81: 233-246.
[2] Gething MJ and Sambrook J. Protein folding in the cell. Nature 1992; 355: 33-45.
[3] Kaufman RJ. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev. 1999; 13: 1211-1233.
[4] McMillian DR, Gething MJ and Sambrook J. The cellular response to unfolded proteins: Intercompartmental Signaling. Current Opinions in Biotechnology 1994; 5: 540-545.
[5] Sidrauski C, Chapman R and Walter P. The unfolded protein response: an intracellular signalling pathway with many surprising features. Trends.Cell Biol 1998; 8: 245-249.
[6] Mori K. Tripartite management of unfolded proteins in the endoplasmic reticulum. Cell 2000; 101: 451-454.
[7] Ron D and Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol 2007; 8: 519-529.
[8] Zhang K and Kaufman RJ. From endoplasmic-reticulum stress to the inflammatory response. Nature 2008; 454: 455-462.
[9] Mori K, Ma W, Gething MJ and Sambrook J. A transmembrane protein with a cdc2+/CDC28-related kinase activity is required for signaling from the ER to the nucleus. Cell 1993; 74: 743-756.
[10] Cox JS, Shamu CE and Walter P. Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. Cell 1993; 73: 1197-1206.
[11] Shi Y, Vattem KM, Sood R, An J, Liang J, Stramm L and Wek RC. Identification and characterization of pancreatic eukaryotic initiation factor 2 alpha-subunit kinase, PEK, involved in translational control. Mol.Cell Biol 1998; 18: 7499-7509.
[12] Harding HP, Zhang Y and Ron D. Protein translation and folding are coupled by an endoplasmic -reticulum- resident kinase. Nature 1999; 397: 271-274.
[13] Haze K, Yoshida H, Yanagi H, Yura T and Mori K. Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol Biol Cell 1999; 10: 3787-3799.
[14] Zhang K and Kaufman RJ. Identification and characterization of endoplasmic reticulum stress-induced apoptosis in vivo. Methods Enzymol 2008; 442: 395-419.
[15] Lee AS. GRP78 induction in cancer: therapeutic and prognostic implications. Cancer Res 2007; 67: 3496-3499.
[16] Luo J, Solimini NL and Elledge SJ. Principles of cancer therapy: oncogene and non-oncogene addiction. Cell 2009; 136: 823-837.
[17] Schroder M and Kaufman RJ. The Mammalian unfolded protein response. Annu Rev Biochem 2005; 74: 739-789.
[18] Scheuner D, Song B, McEwen E, Liu C, Laybutt R, Gillespie P, Saunders T, Bonner-Weir S and Kaufman RJ. Translational control is required for the unfolded protein response and in vivo glucose homeostasis. Mol Cell 2001; 7: 1165-1176.
[19] Harding HP, Zeng H, Zhang Y, Jungries R, Chung P, Plesken H, Sabatini DD and Ron D. Diabetes mellitus and exocrine pancreatic dysfunction in perk-/- mice reveals a role for translational control in secretory cell survival. Mol Cell 2001; 7: 1153-1163.
[20] Kaufman RJ. Regulation of mRNA translation by protein folding in the endoplasmic reticulum. Trends Biochem Sci 2004; 29: 152-158.
[21] Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M and Ron D. Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol Cell 2000; 6: 1099-1108.
[22] Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, Sadri N, Yun C, Popko B, Paules R, Stojdl DF, Bell JC, Hettmann T, Leiden JM and Ron D. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol Cell 2003; 11: 619-633.
[23] Lee AH, Iwakoshi NN and Glimcher LH. XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. Mol Cell Biol 2003; 23: 7448-7459.

[24] Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP and Ron D. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science 2000; 287: 664-666.
[25] Hetz C, Bernasconi P, Fisher J, Lee AH, Bassik MC, Antonsson B, Brandt GS, Iwakoshi NN, Schinzel A, Glimcher LH and Korsmeyer SJ. Proapoptotic BAX and BAK modulate the unfolded protein response by a direct interaction with IRE1alpha. Science 2006; 312: 572-576.
[26] Todd DJ, Lee AH and Glimcher LH. The endoplasmic reticulum stress response in immunity and autoimmunity. Nat Rev Immunol 2008; 8: 663-674.
[27] Okada T, Yoshida H, Akazawa R, Negishi M and Mori K. Distinct roles of activating transcription factor 6 (ATF6) and double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) in transcription during the mammalian unfolded protein response. Biochem J 2002; 366: 585-594.
[28] Yamamoto K, Sato T, Matsui T, Sato M, Okada T, Yoshida H, Harada A and Mori K. Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6alpha and XBP1. Dev Cell 2007; 13: 365-376.
[29] Wu J, Rutkowski DT, Dubois M, Swathirajan J, Saunders T, Wang J, Song B, Yau GD and Kaufman RJ. ATF6alpha optimizes long-term endoplasmic reticulum function to protect cells from chronic stress. Dev Cell 2007; 13: 351-364.
[30] Marciniak SJ, Yun CY, Oyadomari S, Novoa I, Zhang Y, Jungreis R, Nagata K, Harding HP and Ron D. CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. Genes Dev 2004; 18: 3066-3077.
[31] Ohoka N, Yoshii S, Hattori T, Onozaki K and Hayashi H. TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. Embo J 2005; 24: 1243-1255.
[32] Yamaguchi H and Wang HG. CHOP is involved in endoplasmic reticulum stress-induced apoptosis by enhancing DR5 expression in human carcinoma cells. J Biol Chem 2004; 279: 45495-45502.
[33] He B. Viruses, endoplasmic reticulum stress, and interferon responses. Cell Death Differ 2006; 13: 393-403.
[34] Jordan R, Wang L, Graczyk TM, Block TM and Romano PR. Replication of a cytopathic strain of bovine viral diarrhea virus activates PERK and induces endoplasmic reticulum stress-mediated apoptosis of MDBK cells. J Virol 2002; 76: 9588-9599.
[35] Schroder M and Kaufman RJ. ER stress and the unfolded protein response. Mutat Res 2005; 569: 29-63.
[36] Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS and Walter P. Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. Cell 2000; 101: 249-258.
[37] Vembar SS and Brodsky JL. One step at a time: endoplasmic reticulum-associated degradation. Nat Rev Mol Cell Biol 2008; 9: 944-957.
[38] Healy SJ, Gorman AM, Mousavi-Shafaei P, Gupta S and Samali A. Targeting the endoplasmic reticulum-stress response as an anticancer strategy. Eur J Pharmacol 2009; 625: 234-246.
[39] Hsiao JR, Chang KC, Chen CW, Wu SY, Su IJ, Hsu MC, Jin YT, Tsai ST, Takada K and Chang Y. Endoplasmic reticulum stress triggers XBP-1-mediated up-regulation of an EBV oncoprotein in nasopharyngeal carcinoma. Cancer Res 2009; 69: 4461-4467.
[40] Boelens J, Lust S, Offner F, Bracke ME and Vanhoecke BW. Review. The endoplasmic reticulum: a target for new anticancer drugs. In Vivo 2007; 21: 215-226.
[41] Pyrko P, Schonthal AH, Hofman FM, Chen TC and Lee AS. The unfolded protein response regulator GRP78/BiP as a novel target for increasing chemosensitivity in malignant gliomas. Cancer Res 2007; 67: 9809-9816.
[42] Rutkowski DT and Kaufman RJ. That which does not kill me makes me stronger: adapting to chronic ER stress. Trends Biochem Sci 2007; 32: 469-476.
[43] Hersey P and Zhang XD. Adaptation to ER stress as a driver of malignancy and resistance to therapy in human melanoma. Pigment Cell Melanoma Res 2008; 21: 358-367.
[44] Li J and Lee AS. Stress induction of GRP78/BiP and its role in cancer. Curr Mol Med 2006; 6: 45-54.
[45] Kleizen B and Braakman I. Protein folding and quality control in the endoplasmic reticulum. Curr Opin Cell Biol 2004; 16: 343-349.
[46] Uramoto H, Sugio K, Oyama T, Nakata S, Ono K, Yoshimastu T, Morita M and Yasumoto K. Expression of endoplasmic reticulum molecular chaperone Grp78 in human lung cancer and its clinical significance. Lung Cancer 2005; 49: 55-62.
[47] Scriven P, Coulson S, Haines R, Balasubramanian S, Cross S and Wyld L. Activation and clinical significance of the unfolded protein response in breast cancer. Br J Cancer 2009; 101: 1692-1698.
[48] Shuda M, Kondoh N, Imazeki N, Tanaka K, Okada T, Mori K, Hada A, Arai M, Wakatsuki T, Matsubara O, Yamamoto N and Yamamoto M. Activation of the ATF6, XBP1 and grp78 genes in human hepatocellular carcinoma: a possible involvement of the ER stress pathway in hepatocarcinogenesis. J Hepatol 2003; 38: 605-614.
[49] Jamora C, Dennert G and Lee AS. Inhibition of tumor progression by suppression of stress protein GRP78/BiP induction in fibrosarcoma B/

C10ME. Proc Natl Acad Sci U S A 1996; 93: 7690-7694.
[50] Dong D, Ni M, Li J, Xiong S, Ye W, Virrey JJ, Mao C, Ye R, Wang M, Pen L, Dubeau L, Groshen S, Hofman FM and Lee AS. Critical role of the stress chaperone GRP78/BiP in tumor proliferation, survival, and tumor angiogenesis in transgene-induced mammary tumor development. Cancer Res 2008; 68: 498-505.
[51] Lee E, Nichols P, Spicer D, Groshen S, Yu MC and Lee AS. GRP78 as a novel predictor of responsiveness to chemotherapy in breast cancer. Cancer Res 2006; 66: 7849-7853.
[52] Zhang J, Jiang Y, Jia Z, Li Q, Gong W, Wang L, Wei D, Yao J, Fang S and Xie K. Association of elevated GRP78 expression with increased lymph node metastasis and poor prognosis in patients with gastric cancer. Clin Exp Metastasis 2006; 23: 401-410.
[53] Daneshmand S, Quek ML, Lin E, Lee C, Cote RJ, Hawes D, Cai J, Groshen S, Lieskovsky G, Skinner DG, Lee AS and Pinski J. Glucose-regulated protein GRP78 is up-regulated in prostate cancer and correlates with recurrence and survival. Hum Pathol 2007; 38: 1547-1552.
[54] Acosta-Alvear D, Zhou Y, Blais A, Tsikitis M, Lents NH, Arias C, Lennon CJ, Kluger Y and Dynlacht BD. XBP1 controls diverse cell type- and condition-specific transcriptional regulatory networks. Mol Cell 2007; 27: 53-66.
[55] Romero-Ramirez L, Cao H, Nelson D, Hammond E, Lee AH, Yoshida H, Mori K, Glimcher LH, Denko NC, Giaccia AJ, Le QT and Koong AC. XBP1 is essential for survival under hypoxic conditions and is required for tumor growth. Cancer Res 2004; 64: 5943-5947.
[56] Chen Y, Feldman DE, Deng C, Brown JA, De Giacomo AF, Gaw AF, Shi G, Le QT, Brown JM and Koong AC. Identification of mitogen-activated protein kinase signaling pathways that confer resistance to endoplasmic reticulum stress in Saccharomyces cerevisiae. Mol Cancer Res 2005; 3: 669-677.
[57] Romero-Ramirez L, Cao H, Regalado MP, Kambham N, Siemann D, Kim JJ, Le QT and Koong AC. X box-binding protein 1 regulates angiogenesis in human pancreatic adenocarcinomas. Transl Oncol 2009; 2: 31-38.
[58] Lee AH, Iwakoshi NN, Anderson KC and Glimcher LH. Proteasome inhibitors disrupt the unfolded protein response in myeloma cells. Proc Natl Acad Sci U S A 2003; 100: 9946-9951.
[59] Tirosh B, Iwakoshi NN, Glimcher LH and Ploegh HL. Rapid turnover of unspliced Xbp-1 as a factor that modulates the unfolded protein response. J Biol Chem 2006; 281: 5852-5860.
[60] Davies MP, Barraclough DL, Stewart C, Joyce KA, Eccles RM, Barraclough R, Rudland PS and Sibson DR. Expression and splicing of the unfolded protein response gene XBP-1 are significantly associated with clinical outcome of endocrine-treated breast cancer. Int J Cancer 2008; 123: 85-88.
[61] Fels DR and Koumenis C. The PERK/eIF2alpha/ATF4 module of the UPR in hypoxia resistance and tumor growth. Cancer Biol Ther 2006; 5: 723-728.
[62] Koumenis C, Naczki C, Koritzinsky M, Rastani S, Diehl A, Sonenberg N, Koromilas A and Wouters BG. Regulation of protein synthesis by hypoxia via activation of the endoplasmic reticulum kinase PERK and phosphorylation of the translation initiation factor eIF2alpha. Mol Cell Biol 2002; 22: 7405-7416.
[63] Bi M, Naczki C, Koritzinsky M, Fels D, Blais J, Hu N, Harding H, Novoa I, Varia M, Raleigh J, Scheuner D, Kaufman RJ, Bell J, Ron D, Wouters BG and Koumenis C. ER stress-regulated translation increases tolerance to extreme hypoxia and promotes tumor growth. EMBO J 2005; 24: 3470-3481.
[64] Blais JD, Addison CL, Edge R, Falls T, Zhao H, Wary K, Koumenis C, Harding HP, Ron D, Holcik M and Bell JC. Perk-dependent translational regulation promotes tumor cell adaptation and angiogenesis in response to hypoxic stress. Mol Cell Biol 2006; 26: 9517-9532.
[65] Zhuang L, Scolyer RA, Lee CS, McCarthy SW, Cooper WA, Zhang XD, Thompson JF and Hersey P. Expression of glucose-regulated stress protein GRP78 is related to progression of melanoma. Histopathology 2009; 54: 462-470.
[66] Tsutsumi S, Namba T, Tanaka KI, Arai Y, Ishihara T, Aburaya M, Mima S, Hoshino T and Mizushima T. Celecoxib upregulates endoplasmic reticulum chaperones that inhibit celecoxib-induced apoptosis in human gastric cells. Oncogene 2006; 25: 1018-1029.
[67] Zu K, Bihani T, Lin A, Park YM, Mori K and Ip C. Enhanced selenium effect on growth arrest by BiP/GRP78 knockdown in p53-null human prostate cancer cells. Oncogene 2006; 25: 546-554.
[68] Suzuki T, Lu J, Zahed M, Kita K and Suzuki N. Reduction of GRP78 expression with siRNA activates unfolded protein response leading to apoptosis in HeLa cells. Arch Biochem Biophys 2007; 468: 1-14.
[69] Saito S, Furuno A, Sakurai J, Sakamoto A, Park HR, Shin-Ya K, Tsuruo T and Tomida A. Chemical genomics identifies the unfolded protein response as a target for selective cancer cell killing during glucose deprivation. Cancer Res 2009; 69: 4225-4234.
[70] Backer JM, Krivoshein AV, Hamby CV, Pizzonia J, Gilbert KS, Ray YS, Brand H, Paton AW, Paton JC and Backer MV. Chaperone-targeting cytotoxin and endoplasmic reticulum stress-inducing drug synergize to kill cancer cells. Neoplasia 2009; 11: 1165-1173.
[71] Paton AW, Beddoe T, Thorpe CM, Whisstock JC, Wilce MC, Rossjohn J, Talbot UM and Paton JC. AB5 subtilase cytotoxin inactivates the endoplasmic reticulum chaperone BiP. Nature 2006; 443:

548-552.
[72] Papalas JA, Vollmer RT, Gonzalez-Gronow M, Pizzo SV, Burchette J, Youens KE, Johnson KB and Selim MA. Patterns of GRP78 and MTJ1 expression in primary cutaneous malignant melanoma. Mod Pathol 2009; doi:10.1038/modpathol.2009.152 (in press)
[73] Zheng HC, Takahashi H, Li XH, Hara T, Masuda S, Guan YF and Takano Y. Overexpression of GRP78 and GRP94 are markers for aggressive behavior and poor prognosis in gastric carcinomas. Hum Pathol 2008; 39: 1042-1049.
[74] Pootrakul L, Datar RH, Shi SR, Cai J, Hawes D, Groshen SG, Lee AS and Cote RJ. Expression of stress response protein Grp78 is associated with the development of castration-resistant prostate cancer. Clin Cancer Res 2006; 12: 5987-5993.
[75] Fu Y and Lee AS. Glucose regulated proteins in cancer progression, drug resistance and immunotherapy. Cancer Biol Ther 2006; 5: 741-744.
[76] Richardson PG, Mitsiades C, Hideshima T and Anderson KC. Bortezomib: proteasome inhibition as an effective anticancer therapy. Annu Rev Med 2006; 57: 33-47.
[77] Richardson PG, Barlogie B, Berenson J, Singhal S, Jagannath S, Irwin D, Rajkumar SV, Srkalovic G, Alsina M, Alexanian R, Siegel D, Orlowski RZ, Kuter D, Limentani SA, Lee S, Hideshima T, Esseltine DL, Kauffman M, Adams J, Schenkein DP and Anderson KC. A phase 2 study of bortezomib in relapsed, refractory myeloma. N Engl J Med 2003; 348: 2609-2617.
[78] Adams J, Palombella VJ, Sausville EA, Johnson J, Destree A, Lazarus DD, Maas J, Pien CS, Prakash S and Elliott PJ. Proteasome inhibitors: a novel class of potent and effective antitumor agents. Cancer Res 1999; 59: 2615-2622.
[79] Ling YH, Liebes L, Jiang JD, Holland JF, Elliott PJ, Adams J, Muggia FM and Perez-Soler R. Mechanisms of proteasome inhibitor PS-341-induced G(2)-M-phase arrest and apoptosis in human non-small cell lung cancer cell lines. Clin Cancer Res 2003; 9: 1145-1154.
[80] Pham LV, Tamayo AT, Yoshimura LC, Lo P and Ford RJ. Inhibition of constitutive NF-kappa B activation in mantle cell lymphoma B cells leads to induction of cell cycle arrest and apoptosis. J Immunol 2003; 171: 88-95.
[81] Cusack JC, Jr., Liu R, Houston M, Abendroth K, Elliott PJ, Adams J and Baldwin AS, Jr. Enhanced chemosensitivity to CPT-11 with proteasome inhibitor PS-341: implications for systemic nuclear factor-kappaB inhibition. Cancer Res 2001; 61: 3535-3540.
[82] Hideshima T, Richardson P, Chauhan D, Palombella VJ, Elliott PJ, Adams J and Anderson KC. The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. Cancer Res 2001; 61: 3071-3076.
[83] Mitsiades N, Mitsiades CS, Richardson PG, Poulaki V, Tai YT, Chauhan D, Fanourakis G, Gu X, Bailey C, Joseph M, Libermann TA, Schlossman R, Munshi NC, Hideshima T and Anderson KC. The proteasome inhibitor PS-341 potentiates sensitivity of multiple myeloma cells to conventional chemotherapeutic agents: therapeutic applications. Blood 2003; 101: 2377-2380.
[84] Fels DR, Ye J, Segan AT, Kridel SJ, Spiotto M, Olson M, Koong AC and Koumenis C. Preferential cytotoxicity of bortezomib toward hypoxic tumor cells via overactivation of endoplasmic reticulum stress pathways. Cancer Res 2008; 68: 9323-9330.
[85] Fribley A, Zeng Q and Wang CY. Proteasome inhibitor PS-341 induces apoptosis through induction of endoplasmic reticulum stress-reactive oxygen species in head and neck squamous cell carcinoma cells. Mol Cell Biol 2004; 24: 9695-9704.
[86] Nawrocki ST, Carew JS, Dunner K, Jr., Boise LH, Chiao PJ, Huang P, Abbruzzese JL and McConkey DJ. Bortezomib inhibits PKR-like endoplasmic reticulum (ER) kinase and induces apoptosis via ER stress in human pancreatic cancer cells. Cancer Res 2005; 65: 11510-11519.
[87] Agyin JK, Santhamma B, Nair HB, Roy SS and Tekmal RR. BU-32: a novel proteasome inhibitor for breast cancer. Breast Cancer Res 2009; 11: R74.
[88] McCracken AA and Brodsky JL. Evolving questions and paradigm shifts in endoplasmic-reticulum-associated degradation (ERAD). Bioessays 2003; 25: 868-877.
[89] Wang Q, Mora-Jensen H, Weniger MA, Perez-Galan P, Wolford C, Hai T, Ron D, Chen W, Trenkle W, Wiestner A and Ye Y. ERAD inhibitors integrate ER stress with an epigenetic mechanism to activate BH3-only protein NOXA in cancer cells. Proc Natl Acad Sci U S A 2009; 106: 2200-2205.
[90] Wang Q, Li L and Ye Y. Inhibition of p97-dependent protein degradation by Eeyarestatin I. J Biol Chem 2008; 283: 7445-7454.
[91] Shin-Ya K. Novel antitumor and neuroprotective substances discovered by characteristic screenings based on specific molecular targets. Biosci Biotechnol Biochem 2005; 69: 867-872.
[92] Salazar M, Carracedo A, Salanueva IJ, Hernandez-Tiedra S, Lorente M, Egia A, Vazquez P, Blazquez C, Torres S, Garcia S, Nowak J, Fimia GM, Piacentini M, Cecconi F, Pandolfi PP, Gonzalez-Feria L, Iovanna JL, Guzman M, Boya P and Velasco G. Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells. J Clin Invest 2009; 119: 1359-1372.

BC095179P1-3098

ENDOPLASMIC RETICULUM FACTOR ERLIN2 PRESERVES ONCOGENESIS BY REGULATING DE NOVO LIPOGENESIS

Kezhong Zhang, Zeng-Quan Yang, Guohui Wang, Xiaogang Wang, and Xuebao Zhang

Wayne State University

Increased de novo lipogenesis has been recognized as a hallmark of aggressive cancers and is implicated in maintaining rapid cancer cell proliferation. Recently, we identified an endoplasmic reticulum (ER) lipid raft-associated 2 *(*ERLIN2) gene that is amplified and overexpressed in a subset of aggressive breast cancers. We have demonstrated that ERLIN2 plays a supporting oncogenic role by facilitating cancer cell adaption to oncogenesis-associated cellular stress. In this study, we showed that ERLIN2 is required to maintain malignancy of human breast cancer cells by promoting de novo lipogenesis. Gain- and loss-of-function approaches demonstrated that ERLIN2 is required for expression of lipogenic transcriptional activators and key enzymes involved in cholesterol and triglyceride biosynthesis. Correlating with the upregulation of lipogenic gene expression, forced expression of ERLIN2 in liver and breast cancer cells increased accumulation of cytosolic lipid droplets in response to metabolic stress, including depletion or enhancement of insulin signals as well as elevation of the saturated fatty acid palmitate. Consistently, human breast cancers of both luminal and Her2 subtypes, in which the ERLIN2 gene is amplified and overexpressed, maintain high expression levels of lipogenic transcriptional activators and lipid biosynthesis enzymes. As a consequence of ERLIN2 depletion, the ERLIN2-knockdown breast cancer cells displayed reduced cytosolic lipid deposition concomitant with reduced cell proliferation rates and increased apoptotic activity. In summary, our study implicates an unprecedented role for ERLIN2 in preserving oncogenesis by promoting de novo lipogenesis.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-10-1-0153.

Poster P36-18

BC095179-2796

ENDOPLASMIC RETICULUM FACTOR ERLIN2 PLAYS AN ONCOGENIC ROLE BY MODULATING ER STRESS RESPONSE IN BREAST CANCER

Zeng-Quan Yang, Stephen Ethier, and Kezhong Zhang
Wayne State University

Amplification of the 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, particularly in luminal subtype. ERLIN2 is an ER membrane protein and has recently been identified as a novel mediator of ER-associated degradation. In this study, we found that amplification of the ERLIN2 gene and overexpression of the ERLIN2 protein are present in both luminal and Her2 subtypes of breast cancer. Gain- and loss-of-function approaches demonstrated that ERLIN2 is a novel oncogenic factor that is associated with the ER stress response pathway. We demonstrated that ERLIN2 expression at protein level was modulated by the ER stress pathway through the IRE1α/XBP1 axis in breast cancer cells. We show that overexpression 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 a selective growth advantage for breast cancer cells by facilitating a cytoprotective response to various cellular stresses associated with oncogenesis. The information provided in this report sheds new light on understanding the mechanism of breast cancer malignancy.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-10-1-0152.