Chaperone Hsp47 drives malignant growth and invasion by modulating an ECM gene network

Jieqing Zhu¹,²§, Gaofeng Xiong¹,²§, Hanjiang Fu³, B. Mark Evers¹,⁴, Binhua P. Zhou¹,⁵, Ren Xu¹,²*

¹Markey Cancer Center, ²Department of Pharmacology and Nutritional Sciences, University of Kentucky, Lexington, KY 40536, USA; ³Beijing Institute of Radiation Medicine, 27 Taiping Road, Beijing 100850, People's Republic of China; ⁴Department of Surgery, ⁵Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY 40536, USA

§ These authors contribute equally to this work.

*Corresponding author: Ren Xu, Ph.D.

email: ren.xu2010@uky.edu

Phone: 859-323-7889

Running title: Hsp47 regulates ECM transcription network in breast cancer

Key words: Tumor microenvironment, chaperon protein, co-expression network analysis

Financial support: AHA (12SDG8600000 to R. Xu), ACS (IRG 85-001-22 to R. Xu), COBRE Pilot Award (201407231052 to R. Xu)

Conflict of interest: The authors disclose no potential conflicts of interest.

The manuscript contains 4982 words and 7 figures.
ABSTRACT

The extracellular matrix (ECM) is a determining factor in the tumor microenvironment that restrains or promotes malignant growth. In this report, we show how the molecular chaperone protein Hsp47 functions as a nodal hub in regulating an ECM gene transcription network. A transcription network analysis showed that Hsp47 expression was activated during breast cancer development and progression. Hsp47 silencing reprogrammed human breast cancer cells to form growth-arrested and/or non-invasive structures in 3D cultures, and to limit tumor growth in xenograft assays by reducing deposition of collagen and fibronectin. Co-expression network analysis also showed that levels of microRNA-29b and 29c were inversely correlated with expression of Hsp47 and ECM network genes in human breast cancer tissues. We found that miR-29 repressed expression of Hsp47 along with multiple ECM network genes. Ectopic expression of miR-29b suppressed malignant phenotypes of breast cancer cells in 3D culture. Clinically, increased expression of Hsp47 and reduced levels of miR-29b and 29c were associated with poor survival outcomes in breast cancer patients. Our results show that Hsp47 is regulated by miR-29 during breast cancer development and progression, and that increased Hsp47 expression promotes cancer progression in part by enhancing deposition of ECM proteins.
INTRODUCTION

Extracellular matrix (ECM) molecules, including collagen proteins, fibronectin, proteoglycans, and glycoproteins provide essential physical scaffolds to maintain tissue structure and various biochemical signals to modulate cellular function (1, 2). Around 300 ECM proteins have been identified from the human genome using bioinformatics tools and proteomics techniques (3). Expression and deposition of collagen and fibronectin are significantly greater in breast cancer tissue than in non-malignant mammary tissue (4). Enhanced deposition and cross-linking of collagen and other ECM proteins increase tissue stiffness, disrupt normal tissue architecture and enhance both tumor growth and invasion (5, 6). Moreover, collagen and fibronectin have been identified as prognostic markers and are associated with cancer recurrence (7, 8). Taken together, these results indicate that increased expression and deposition of these ECM proteins are crucial for cancer development and progression. But how these ECM proteins are coordinately regulated during breast cancer progression remains unknown.

Gene co-expression network analysis is a systems biology approach using correlation statistics as pairwise similarity measurements between gene expression profiles, followed by either direct correlation thresholding or a combination of significance level tests with correlation thresholding (9). This approach identifies strong relationships that connect transcripts’ regulatory patterns to the functional organization of the cell, and this method has been successfully used to identify gene networks associated with skin tumor susceptibility (10). Using microarray data generated from hundreds of human breast cancer tissues, gene co-expression network analysis identified two microenvironment gene networks (11). One of the networks represents the coordinated action of ECM remodeling in breast cancer tissue, and is associated with activation of the TGF-β pathway (11). The TGF-β pathway regulates a broad range of responses including
microenvironment remodeling (12). In the canonical TGF-β signaling pathway, binding of TGF-β to its receptor activates the transcription factor Smad and modulates gene transcription (13). A major effect of TGF-β on the microenvironment is the deposition of ECM via induction of collagen types I, III, IV, VII and X, fibronectin, and proteoglycans (14).

Expression and deposition of ECM components are subject to tight spatiotemporal regulation during normal development and cancer progression (15), and the importance of cancer cells in ECM deposition and remodeling has recently been demonstrated (16, 17). Dr. Hynes’s laboratory showed that human cancer cells generate a variety of human ECM proteins in a xenograft model system (18). Inhibition of collagen production in breast cancer cells by blocking P4HAs significantly reduced tumor growth and metastasis in the mouse xenograft model (19, 20). These studies indicate that cancer cells are an active and important component in ECM reorganization. Therefore, an understanding of how ECM proteins are coordinately regulated in cancer tissues, and identifying the key factors that integrate the ECM network are vital in order to target and inhibit ECM-dependent cancer progression.

The SerpinH1 gene encodes a heat-inducible protein (Hsp47) and locates at chromosome 11q13.5, one of the most frequently amplified regions in human cancer (21). Enhanced expression of Hsp47 has been detected in cancer tissue (22, 23). Hsp47 has been identified as a molecular chaperon that is required for the proper folding and secretion of collagen proteins. Hsp47 transiently interacts with the triple helix region of newly synthesized procollagen in the endoplasmic reticulum, and this interaction is required for the proper folding and secretion of collagen proteins (24-26). Inhibition of Hsp47 binding is thought to be an efficient strategy for blocking collagen deposition and ECM remodeling (27). Deletion of Hsp47 in mice severely impairs maturation of collagen fibers and basement membrane formation, and also causes
embryonic lethality (28). These data indicate that Hsp47-regulated collagen maturation is crucial for normal embryonic development. However, the function and regulation of Hsp47 during breast cancer development and progression remains unknown.

Here, we show that expression of Hsp47, a hub of the ECM transcription network, is associated with cancer progression and poor clinical outcome in human breast cancer patients. Silencing Hsp47 expression reprogrammed breast cancer cells to form polarized and/or non-invasive structures in 3D culture and significantly inhibited tumor growth in vivo, accompanied by reduced deposition of collagen and fibronectin. Expression of Hsp47 and the ECM network genes is inversely correlated with miR-29b and 29c in human breast cancer tissues. We further demonstrate that Hsp47 and multiple genes in the ECM transcription network are repressed by miR-29b and 29c in mammary epithelial cells. These results indicate that Hsp47, as a downstream target of miR29, promotes breast cancer progression by modulating ECM deposition.
MATERIALS AND METHODS

Antibodies and reagents. 5-ethynyl-2’-deoxyuridine (Edu) staining kit was obtained from Invitrogen. Matrigel® and type I collagen were purchased from BD Bioscience. ShRNA constructs selectively targeting HSP47, collagen IV or fibronectin were purchased from Sigma (MISSION shRNA library). Antibodies to the following proteins were obtained as indicated: Hsp47 and lamin A/C (Santa Cruz); tubulin and α6 integrin (Millipore); Flag (Sigma); collagen I, collagen IV and fibronectin (Abcam).

Cell culture. HMT-3522, S1, and T4-2 cells (a kind gift from Dr. Mina J Bissell) were maintained as previously described (29). MDA-MB-231 and BT549 (ATCC) cells were cultured in DMEM/F12 (Sigma), Hs578T in DMEM (Sigma), both supplemented with 10% fetal bovine serum, 10 units/ml of penicillin and 0.1 mg/ml of streptomycin (Invitrogen). In 3D culture, cells were plated on Matrigel® and maintained in the culture medium containing 5% Matrigel. T4 cells were seeded at a density of 2.1×10^4 cells per cm^2; while MDA-MB-231 and Hs578T (ATCC) were seeded at 1.4×10^4 cells per cm^2. For production of lentivirus, 293FT cells were transfected with shRNA vector plus packaging vectors using FuGENE (Promega). Culture supernatants containing viral particles were collected 48h after transfection. Cells were infected with lentivirus and selected using puromycin for at least three days. Cell migration and invasion assay were performed as previously described (30).

Quantitative RT-PCR. Quantitative RT-PCR was carried out as previously described (30). The relative quantification of gene expression for each sample was analyzed by the ΔCt method. The following primers were used to amplify HSP47: 5’-TGAAGATCTGGATGGGGAAG-3’ AND 5’-CCGCACTAGGAAGATGAAGG-3’; COL1A1: 5’-ATGGATTCCAGTTCGAGTATGGC-3’ AND 5’-ATGGATTCCAGTTCGAGTATGGC-3’; COL3A1: 5’-
AGGTCCCTCGGGTAACACT-3’ AND 5’ ACTTTTCACCCTGACACCCTG-3’; 18S rRNA: 5’-ACCTGGTTGATCCTGCCAGT-3’ AND 5’-CTGACCAGGTTGTTTTGAT-3’. For quantification of miRNA expression, TaqMan probes were used according to the manufacture’s protocol (Applied Biosystems). Ct values were normalized to RNU44.

Transfection and lentivirus infection. The miRNA mimics and inhibitors were designed and synthesized by GenePharma. Cells were plated in 6-well plate to 70% confluence. For each well, 20 μM miRNA were added into 250 μl Opti-MEM, with 5 μl of Lipofectamine 2000 (Invitrogen). The mixture was added to cells and incubated for 6 h before replacing with normal culture medium. Total RNA was prepared 48 h after transfection and used for RT–PCR.

Western blot analysis and immunofluorescence. Western blots were performed as previously described (30). Exposures were acquired and quantified using a FluroChem HD2 (Alpha Innotech).

Cells in Matrigel® were smeared on slides, dried briefly, then fixed with 4% paraformaldehyde (PFA)/PBS and permeabilized in 0.5 % Triton X-100/PBS. Samples were blocked with 1% BSA and 5% goat serum/PBS for 1h, followed by incubation with the primary antibody in blocking buffer for 2h at room temperature and the secondary antibody for 50min at room temperature. Images were acquired with an Olympus FV1000 microscope using a 1.3 NA 40x objective with oil immersion.

Xenograft experiment. Six-week-old female SCID mice were randomly grouped and subcutaneously injected with 5×10⁶ shCtrl (control) or shHSP47 T4-2 cells. Tumors were measured with a caliper every three days for three weeks to analyze tumor growth. Tumor volume was also monitored using an in vivo imaging system (IVIS®). The experiment was terminated with the sacrifice of all mice, and tumor fragments obtained at necropsy were
weighed, imaged, and fixed with 4% PFA for histology. For the orthotopic mammary tumor experiments, female SCID mice (six weeks old) were injected with $1 \times 10^6$ sh-control or shHsp47 MDA-MB-231/Luc cells into mammary fat pad. Tumor volume was measured using IVIS. All procedures were performed within the guidelines of the Division of Laboratory Animal Resources at the University of Kentucky.

*Masson’s trichrome staining and immunohistochemistry analysis.* Xenograft tumor sections were de-paraffinized and rehydrated. Samples were re-fixed with Bouin’s solution at 60°C for 60 minutes, stained in Weigert's working hematoxylin for 10 minutes, and then stained in Biebrich scarlet-acid fuchsin solution for 5 minutes. Sections were incubated in phosphomolybdic-phosphotungstic acid solution for 10 minutes, and then were transferred to aniline blue solution and incubated for 5 minutes. Images were taken with a Nikon microscope. The percentage of collagen was quantified by calculating the ratio of the blue staining (collagen) area to the total area of the tumor section using Imagescope analysis software (19). Immunohistochemistry analysis was performed as described previously (20).

*Co-expression network analysis.* The gene co-expression network analyses were performed with Cytoscape as previous described (11). The expression data of microRNAs and the ECM network genes were obtained from microRNA and gene microarray profiles generated from 97 human breast cancer tissues (GSE19536).

*Kaplan-Meier survival analysis and other statistical analysis.* We examined Hsp47 expression in 404 breast tumor expression arrays taken from studies by van de Vijver *et al.* (31) (n=295) and Chin *et al.* (32) (n=118). In each dataset, the tumor samples were evenly divided into Hsp47 low, Hsp47 high, and Hsp47 medium based on the Hsp47 mRNA level. This method allowed us to compare relative Hsp47 expression levels across both data sets fused as a single group of
patients. Significant differences in survival time were assessed using the Cox proportional hazard (log-rank) test.

Analysis of Hsp47mRNA levels in normal and malignant tissues was performed in the TCGA breast cancer dataset downloaded from Oncomine. The association between mRNA levels of Hsp47 with other genes and microRNAs was evaluated with Spearman correlation analysis. All experiments were repeated at least twice. Results are reported as mean ± the standard error of the mean; the significance of difference was assessed by independent Student’s t-test. P<0.05 represents statistical significance and P<0.01 represents sufficiently statistical significance. All reported P-values were two-tailed. Statistical analysis was conducted with SigmaPlot (Systat Software, Inc.) and SAS (version 9.2; SAS Institute Inc.).

RESULTS

*Hsp47 is associated with expression of the ECM network genes during breast cancer development and progression.*

Remodeling of the ECM microenvironment is a necessary event for breast cancer development and progression. Using gene co-expression network analysis, we discovered a co-expression network that integrates the coordinated action of ECM remodeling from hundreds of human breast cancer tissues (11), and identified Hsp47 (*SerpinH1*) as a node of this network (Figure 1A). Hsp47 mRNA levels significantly correlated with the expression of collagen I (*Col1A1*), IV (*Col4A1*), and fibronectin (*FN1*) in human breast cancer tissues (Figure 1B, C, D) as well as in 24 human breast cancer cell lines (Supplemental Table 1). These correlations suggest a functional connection and/or co-regulation between Hsp47 and these ECM proteins. Using the TCGA breast cancer dataset, we determined that the expression of the ECM network
genes and Hsp47 was significantly increased in cancer samples compared with normal mammary tissue (Figure 1E, F). The increased mRNA levels of Hsp47 were also detected in other types of cancers (Supplemental Figure 1A). The protein levels of Hsp47 in breast cancer cell lines were higher than the levels in non-malignant cell lines (Figure 1G). Among breast cancer cell lines, Hsp47 levels were higher in basal-like breast cancer when compared with the luminal cancer cells (Figure 1H) by quantitative RT-PCR analysis. These results indicate that expression of Hsp47 and the ECM transcription network is activated during breast cancer development. However, roles of Hsp47 in regulating breast cancer development and progression remain to be addressed.

Hsp47 regulates the malignant phenotypes of breast cancer cells in 3D culture.

To determine function of enhanced Hsp47 expression in breast tumor progression, we silenced Hsp47 in four breast cancer cell lines, T4-2, MDA-MB 231, BT549 and Hs-578T, with two shRNAs (Figure 2A). The 3D tissue culture model provides a physiologically-relevant microenvironment for monitoring tissue morphogenesis of non-malignant and malignant cells (29, 33). In this model, malignant T4-2 cells form disorganized and proliferative structures (29). Knockdown of Hsp47 reprogrammed the malignant T4-2 cells to form polarized structures with reduced colony size in 3D culture (Figure 2B, C). MDA-MB 231, BT549 and Hs-578T cells form stellate structures in 3D culture, reflecting decreased cell-cell interactions and enhanced cell invasiveness (Figure 2D) (34). Silencing Hsp47 in MDA-MB 231, BT549, and Hs-578T cells significantly reduced branching in 3D culture (Figure 2D, E). Knockdown of Hsp47 also significantly reduced proliferation of T4-2 and MDA-MB 231 cells in 3D culture and inhibited cell invasion in the Transwell assay (Figure 2F, G). Non-malignant S1 cells contain low levels of Hsp47 compared with their malignant T4-2 counterpart (Figure 1E), and form polarized acini in
3D culture. Ectopic expression of Hsp47 disrupted the polarized acinus structures and increased colony size (Figure 2H), but Hsp47 expression failed to induce colony formation in non-malignant cells (Supplemental Figure 1B). The results from these gain and loss of function experiments indicate that Hsp47 promotes cancer progression in 3D culture by enhancing cell proliferation and invasion.

Since Hsp47 levels are associated with expression of collagen and fibronectin, we investigated whether Hsp47 modulates 3D malignant phenotypes of breast cancer cells via enhanced secretion and deposition of ECM proteins. Silencing Hsp47 expression in breast cancer cells reduced the levels of collagen I, IV, and fibronectin in the conditioned medium, but had little effect on the cellular protein levels (Figure 3A). It has been reported that Hsp47-deficiency induces procollagen aggregates in the endoplasmic reticulum, and the arrogated protein is subsequently degraded by the autophagy-lysosome pathway (35). This degradation may explain why we did not detect accumulation of ECM protein in Hsp47-silenced cells. Deposition of collagen IV and fibronectin was also down-regulated in Hsp47-silenced T4-2 cells in 3D culture (Figure 3B). In addition, multiple components of the ECM transcription network, such as collagen III and V have been identified as targets of Hsp47 (25). Thus, Hsp47 may serve as a functional hub to facilitate maturation of multiple proteins in the ECM network.

Increased fibronectin production is associated with and promotes breast cancer progression (17), but the functional roles of collagen IV in breast cancer have not been investigated. We showed that secretion of collagen IV and fibronectin was enhanced in malignant T4-2 cells compared to non-malignant S1 cells (Figure 3C). By analyzing the published microarray datasets in the Oncomine, we found that mRNA levels of Col4A1 and FN1 were significantly upregulated in many solid tumors (Supplemental Figure 1A). To determine
whether enhanced production of collagen IV and fibronectin is crucial for the malignant 3D phenotypes of T4-2, we silenced *Col4A1* or *FN1* expression with shRNA. Knockdown of *Col4A1* or *FN1* significantly reduced colony size of T4-2 cells in 3D culture and increased number of polarized colonies (Figure 3D, E, F, G, H, I). However, reduction of collagen I expression in T4-2 cells had little effect on the malignant phenotypes in 3D culture (data not shown). Knockdown of fibronectin in MDA-MB 231 cells significantly reduced invasive branching in 3D culture (Figure 3J), but silencing collagen IV in MDA-MB 231 cells is not sufficient to suppress invasive branching in 3D culture (data not shown). To determine whether exogenous fibronectin can restore invasive behavior in Hsp47-silenced tumor cells, we performed a number of rescue experiments. We found that exogenous fibronectin restored cell migration and partially rescued invasive behavior in Hsp47-silenced MDA-MB 231 cells (Figure 3K, L, M). These results suggest that Hsp47 promotes breast cancer progression in part by enhancing secretion and deposition fibronectin and collagens.

*MiR-29b and 29c repress expression of Hsp47 and the ECM network genes.*

MicroRNA regulates gene expression by targeting the three prime untranslated regions (3'UTR) of mRNA. Given the small sequence match required, an individual microRNA can regulate hundreds of genes (36, 37). Thus, we hypothesized that microRNA was involved in the activation of Hsp47 and the ECM network during breast cancer progression. We found that the seed complementary sites for miR-29, let-7, miR-200, miR-181 and miR-300 were enriched in the 3'UTR region of the ECM network genes. Using co-expression network analysis, we identified a number of microRNAs, including miR-29b and 29c that were negatively correlated with expression of the ECM network genes in human breast cancer tissues (Figure 4A). MiR-29b has recently been identified as a metastasis suppressor and functions by altering tumor
microenvironment (38). A potential binding site for miR-29 was also identified in the 3'UTR region of the Hsp47 gene (Figure 4B), and this binding site is conserved among species (Figure 4B). Moreover, the 3'UTR regions from about half of ECM network genes (19/41) contain miR-29 binding sites (Supplemental Figure 2A).

To examine function of miR-29b and 29c in regulating Hsp47 and the ECM network genes, we introduced microRNA mimics and inhibitors into malignant and non-malignant mammary epithelial cell lines. Transfection of miR-29 mimics reduced the protein levels of Hsp47 in breast cancer cells (Figure 4C), while introduction of miR-29b and 29c inhibitors enhanced the expression of Hsp47 in the non-malignant mammary epithelial cell line MCF10A (Figure 4D). Quantitative RT-PCR demonstrated that miR-29b and 29c mimics also inhibited the expression of the ECM network genes that contain miR-29 binding sites in the 3'UTR region (Figure 4E), suggesting that the ECM microenvironment is regulated by miR-29 at the transcriptional network level. To determine whether miR-29 regulates Hsp47 expression by directly targeting the 3' UTR, we ligated the Hsp47 3' UTR region with putative miR-29 binding sites downstream of the coding sequence of luciferase. Ectopic expression of miR-29b significantly reduced the reporter activity (Figure 4F). Mutation in the seed complementary site reduced miR-29b-mediated repression for Hsp47 (Figure 4F). Thus, miR-29 inhibits Hsp47 expression by directly targeting to the 3' UTR region.

To determine function of miR-29b in suppressing breast cancer progression, the miR-29b expression vector was stably transfected in breast cancer cells. Ectopic expression of miR-29b in T4-2 cells significantly reduced colony size in 3D culture (Figure 4G, H). Introduction of miR-29b in MDA-MB 231 cells suppressed invasive branching (Figure 4I, J), which replicates the phenotype of Hsp47-silenced cells in 3D culture. To examine the clinical association between
miR-29 and Hsp47 expression during breast cancer progression, we performed Spearman correlation analysis using the microRNA and mRNA expression profiles generated from 97 human breast cancer tissues (39). We found that miR-29b (Figure 4K) and 29c (Figure 4L) negatively correlated with mRNA levels of Hsp47 in human breast cancer tissues. These results suggest that downregulation of miR-29b and 29c induces Hsp47 expression during breast cancer progression.

*MiR-29 mediates TGF-β-induced Hsp47 expression.*

Co-expression network analysis revealed that expression of the ECM network was associated with activation of the TGF-β pathway (11). Treatment with TGF-β induced Hsp47 transcription in non-malignant S1 and MCF-10A cells (Figure 5B), while blocking the TGF-β pathway with SB431542 (a TGFBR inhibitor) reduced Hsp47 expression in MDA-MB 231 cells (Figure 5C). We also found that mRNA levels of Hsp47 and TGF-β were significantly correlated in human breast cancer tissues (Figure 5A) and cell lines (Supplemental Figure 2B). These results indicate that Hsp47 is regulated by the TGF-β pathway in breast cancer cells.

Next, we examined whether miR-29 is a downstream target of the TGF-β signaling during breast cancer progression. TGF-β treatment significantly suppressed miR-29b expression in non-malignant MCF-10A cells (Figure 5D), while incubation with SB431542 enhanced miR-29b expression in MDA-MB 231 cells (Figure 5E, F). Importantly, transfection of miR-29b or 29c inhibitors rescued Hsp47 expression in SB431542-treated breast cancer cells (Figure 5G). We also found that expression of miR-29b and the TGF-β ligand were negatively correlated in human breast cancer tissues (Figure 5H). Therefore, miR-29b at least partially mediates the TGF-β-induced Hsp47 expression and ECM remodeling during breast cancer development and progression.
Enhanced Hsp47 expression promotes tumor growth in vivo and correlates with poor prognosis.

To determine the in vivo function of Hsp47 during breast cancer progression, control and Hsp47-silenced T4-2 cells were subcutaneously injected into the flanks of female mice. Tumor growth was significantly reduced in the Hsp47-silenced group compared to the control group (Figure 6A, B). Cancer cells in the Hsp47-silenced tumors were less proliferative compared to the cells in the control group (Figure 6C), which is consistent with reduced tumor growth in Hsp47-silenced tumors. Masson's trichrome staining demonstrated significantly less collagen deposition in the Hsp47-silenced tumors when compared to the control T4-2 tumors (Figure 6D). The function of Hsp47 in regulating tumor growth was also verified in an orthotopic mammary tumor model. Control and Hsp47-silenced MDA-MB 231 cells were transplanted into the mammary fat pads of female SCID mice. We found that silencing Hsp47 expression also inhibited tumor growth in the mammary fat pads (Figure 6E). Reduced collagen and fibronectin deposition was also observed in orthotopically placed Hsp47-silenced tumors (Supplemental Figure 3A).

To address the clinical relevance of enhanced Hsp47 expression in human breast cancer, we assessed the association between mRNA levels of Hsp47 and patient survival using the published microarray data generated from more than 400 breast cancer tissue samples (31, 32). Breast cancer patients were evenly divided into three groups based on the Hsp47 mRNA levels (low, moderate and high). Kaplan-Meier log rank analysis showed that patients whose tumor had higher Hsp47 expression levels had a significantly shorter overall and recurrent-free survival periods (Figure 7A, B). Similar results were also obtained in a large cohort microarray dataset containing 4142 breast cancer samples (Supplemental Figure 3B). Moreover, high grade tumors expressed more Hsp47 compared to low grade tumors (Figure 7C). These results indicate that
activation of Hsp47 correlates with breast cancer progression. Since miR-29b and 29c are inversely correlated with Hsp47, we further analyzed the association between these two microRNAs and clinical outcomes. We found that downregulation of miR-29b and 29c was associated with poor prognosis in human breast cancer patients (Figure 7D, E). These results suggested that activation of miR-29/Hsp47 axis is associated with a poor clinical outcome.

DISCUSSION

Discovery of the ECM transcription network suggests that expression of the ECM proteins is coordinately regulated during breast cancer development and progression (11). In the present study, we demonstrate that Hsp47 is a hub of the ECM network and modulates tumor microenvironment by regulating deposition of multiple ECM proteins. We have shown that upregulation of Hsp47 is associated with development of solid tumors, including colon, brain, esophageal, and head and neck cancer. Increased Hsp47 mRNA levels correlated with a poor clinical outcome in breast cancer patients. Silencing Hsp47 reprogrammed breast cancer cells to form polarized or non-invasive structures in 3D culture and inhibited tumor growth in the xenograft mammary tumor model. These results indicate that the increased Hsp47 expression is crucial for tumor progression.

Knockdown of Hsp47 in breast cancer cells reduced secretion and deposition of ECM proteins, including fibronectin and collagens. Increased expression and deposition of fibronectin in breast cancer tissue are associated with poor prognosis, and it has also been detected in metastatic niches (40, 41). Fibronectin promotes cancer progression by enhancing cell proliferation, invasion and colonization (17, 41). We found that exogenous fibronectin partially restored invasive behavior in Hsp47-silenced breast cancer cells. These results indicate that
fibronectin is an important downstream target of Hsp47 in promoting breast cancer progression. However, fibronectin could not completely restore malignant phenotypes in Hsp47-silenced cells, suggesting that collagen and other Hsp47 targeted proteins also contribute to this process. Thus, it is crucial to characterize the Hsp47-targeted proteins using unbiased high throughput proteomic analysis in the future. The information obtained from the high throughput assay may be essential for a comprehensive understanding of how Hsp47 promotes cancer progression.

Hsp47 has been identified as a collagen-binding protein, and is considered a collagen-specific chaperon (24). Our study confirmed the function of Hsp47 in regulating collagen deposition and identified fibronectin as a new target in breast cancer cells. We showed that mRNA levels of Hsp47 and fibronectin were significantly correlated in human breast cancer tissue and cells lines. Hsp47 silencing reduced secretion and deposition of fibronectin in breast cancer cells. Fibronectin does not contain Gly-X-Y repeats that form triple helix structure; it would be interesting to determine whether Hsp47 binds to fibronectin in order to facilitate its assembly and deposition. It has been shown that assembly and deposition of fibronectin depend on fibronectin-integrin interaction and are induced by cell contractility and Rho activation (42-44). Fibronectin assembly is impaired in FAK-null cells, indicating FAK is a critical regulator of fibronectin deposition (45). Our unpublished data showed that knockdown of Hsp47 reduced the number and size of focal adhesion in breast cancer cells. It is plausible that Hsp47 regulates fibronectin deposition indirectly by enhancing cell contractility and FAK activity.

Cancer cells are an important source of ECM in cancer tissue and deposit a significant amount collagen, fibronectin and tenascin-C (4, 16). However, roles of cancer cell-produced ECM in the progression of breast cancer have not been appreciated until recently. We found that breast cancer cell lines express multiple ECM protein genes and their mRNA levels correlated
with Hsp47 expression. Collagen IV and fibronectin expression was enhanced during breast cancer development. Silencing Col4A1 or FN1 reprogrammed the malignant T4-2 cells to form polarized spheroid structures. Interestingly, the exogenous collagen IV in Matrigel® cannot rescue the malignant phenotypes of Col4A1-silenced T4-2 cells in the 3D culture. These results suggest that cancer cell-deposited collagen IV is crucial for disruption of polarized acinus structures. Although cancer cells and stromal cells in breast cancer tissue both generate significant amount of ECM proteins, the roles of these proteins in cancer progression may be different. The fibrillar collagen produced by cancer-associated fibroblast enhances fibrosis and cancer stiffness, and subsequently promotes cancer progression (46, 47). The ECM molecules deposited by stromal cells also provide physical and biochemical cues to direct cancer cell migration and invasion. We found that silencing Hsp47 reduced production of collagen and fibronectin in cancer cells and inhibited tumor growth in xenograft model. Thordur Oskarsson et al have shown that cancer cell-produced tenascin C enhances dissemination and survival of tumor cells during the early phases of metastasis by generating a niche that is susceptible to metastasis (16). These results suggest that ECM molecules generated by cancer cell are crucial for cell survival and proliferation as essential components of the cancer cell niche.

We showed that miR-29b and 29c were negatively correlated with expression of the ECM network genes in human breast cancer tissues. Introduction of miR-29 mimics significantly reduced expression of multiple ECM network genes. In addition, it has been shown that collagen types I, III, and IV, and ELN expression are regulated by miR-29 during the development of murine abdominal aortic aneurysms (48). These results indicate that miR-29b and 29c regulate ECM remodeling through repression of the transcription network. We identified Hsp47 as a direct target of miR-29b and 29c. A recent study also shows that miR-29a represses Hsp47
expression in glioma (49). Given the important function of Hsp47 in ECM protein maturation, miR-29 can also modulate secretion and deposition of ECM proteins by suppressing Hsp47 expression, which provides an alternative pathway for miR-29 to regulate ECM microenvironment. It has been shown that miR-29 is inhibited by TGF-β in systemic sclerosis and fibrosis (50). Jonathan Chou et al. demonstrate that TGF-β is a miR-29 target gene (38). Together these results suggest a feedback loop between the TGF-β pathway and miR-29. We showed that expression of TGF-β, miR-29b, and Hsp47 are significantly correlated in human breast cancer tissues. Therefore, miR-29b may serve as an important mediator of the TGF-β pathway to regulate expression of Hsp47 and the ECM network genes. Transcription factor GATA3 maintains luminal mammary epithelial cell differentiation, induces miR-29b expression in luminal-type breast cancers and inhibits cancer metastasis (38). We found that GATA3 and Hsp47 expression is inversely correlated in human breast cancer tissues (Supplemental Figure 4A), and that Hsp47 is highly expressed in basal-like breast cell lines (Figure 1H) and ER negative breast cancer tissues (Supplemental Figure 4B). These results suggest that Hsp47 is upregulated in basal-like cancer and may contribute to cancer metastasis.

In summary, reduced miR-29 levels and increased Hsp47 expression are crucial for activation of the ECM network and remodeling of the ECM microenvironment in breast cancer tissue. Given the important function of ECM microenvironment at multiple stages of cancer progression, these findings provide insights into novel strategies to suppress ECM-dependent cancer progression.

ACKNOWLEDGEMENTS
We are grateful to Dr. Mina J. Bissell for her support in this project and providing T4-2 and S1 cells lines. We thank the pathology core facility at Markey Cancer Center for assistance in tissue processing. This study was supported by start-up fund from Markey Cancer Center and funding support from AHA (12SDG8600000 to R. Xu), ACS (IRG 85-001-22 to R. Xu). This study was supported in part by NIH Grant Number P30GM110787 from the National Institute of General Medical Sciences, its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH or the NIGMS.
References

Figure legends

Figure 1. Hsp47 expression is upregulated during breast cancer development and correlated with mRNA levels of multiple ECM protein genes. A) An ECM transcription network was identified by co-expression network analysis. Hsp47 (Serpinh1) is a hub of the ECM network. (B-D) Scatter plot of correlated mRNA levels between Hsp47 and (B) CollA1, (C) Col4A1, and (D) FN1 in normal and malignant breast tissues (n=593). Expression data were obtained from the TCGA microarray dataset (Oncomine). (E) Box plots showed the mRNA levels of the ECM network genes in breast cancer tissues (n=532) and normal tissues (n=61), p<0.001. (F) Box plots showed the mRNA levels of Hsp47 in breast cancer tissues (n=532) and normal tissues (n=61), p<0.001. Data were obtained from the TCGA microarray dataset. (G) Western blot showed expression of Hsp47 in breast cancer cells (BT549, MDA-MB 231, T4-2, and SUM-140) and non-malignant cells (S1 and MCF10A). (H) Quantitative RT-PCR analyzing expression of Hsp47 gene in basal type of breast cancer (red, n=7) and luminal cancer cells (gray, n=8).

Figure 2. Silencing Hsp47 suppresses malignant phenotypes of breast cancer cells in 3D culture. (A) T4-2 cells were infected with lentivirus containing control shRNA and Hsp47 shRNA. The knockdown efficiency was assessed by Western blot. (B) Phase and confocal images showed the morphology and α6-integrin staining (basal marker, green) of control and Hsp47-silenced T4-2 cells. (C) Colony size of the control and Hsp47-silenced T4-2 cells in 3D culture, n=50, ** p<0.01. (D, E) Control and Hsp47-silenced MDA-MB-231, BT549, and Hs-578T cells were cultured in 3D for 4 days. (D) Phase images showed the organoid morphology in 3D culture. (E) Quantitative data showed that the branching structures was significantly reduced in Hsp47-silenced MDA-Mb 231 cells, n=35, ** p<0.01. (F) Cell proliferation was examined by
the 5-ethynyl-2′-deoxyuridine (EdU) labeling assay. Bar graph represents the ratio of EdU positive cells in control and Hsp47-silenced T4-2 and MDA-MB 231 cells, n=3; * p<0.05. (G) Cell invasion was assessed by the Transwell assay. Results indicate that silencing Hsp47 significantly inhibits cell invasion in T4-2 and MDA-MB 231 cells, n=4; * p<0.05, ** p<0.01. (H) Phase and confocal images showed the morphology and α6-integrin staining (basal marker, green) of the control and Hsp47-expressing S1 cells. The cells were cultured in 3D Matrigel™ for 4 days.

**Figure 3. Knockdown of Hsp47 inhibits secretion and deposition of collagen and fibronectin.** (A) The secreted and cellular levels of collagen I (Col I), IV (Col IV), fibronectin (FN) and were analyzed by Western blot in control and Hsp47-silenced T4-2 cells. (B) Confocal images showed the deposition of collagen IV and fibronectin in control and Hsp47-silenced T4-2 cells. (C) Expression of collagen IV and fibronectin (FN) in S1 and T4-2 cells were analyzed by Western blot. (D, E) Phase images (D) and quantification (E) of the colony size of control and Col4A1-silenced T4-2 cells in 3D culture, n=100; ** p<0.01. (F) Basal polarity in control and Col4A1-silenced T4-2 cells was assessed by α6-integrin staining; the percentage of polarized colonies was determined, n=40; ** p<0.01. (G) Bar graph showing the percentage of EdU positive cells in control and Col4A1-silenced T4-2 cells, n=3; ** p<0.01. (H, I) Phase images (H) and quantification (I) of the colony size of control and FN1-silenced T4-2 cells in 3D culture, n=100, ** p<0.01. (J) Phase images and quantification of invasive branch structures in control and FN1-silenced MDA-MB 231 cells. (K, L) Cell migration (K) and invasion (L) of Hsp47-silenced MDA-MB 231 cells were analyzed in the presence and absence of exogenous fibronectin (10 μg/ml) (FN), *p<0.05; ** p<0.01. (M) Phase images of invasive phenotypes of...
Hsp47-silenced MDA-MB 231 cells in 3D culture. Matrigel was mixed with fibronectin (12.5 μg/ml) (FN) or same volume of PBS.

**Figure 4.** MiR-29b and 29c repress expression of Hsp47 and ECM network genes during breast cancer progression. (A) Co-expression network analysis showed that miR-29b and 29c were associated with the ECM transcription network. (B) Sequence alignment showed the miR-29 binding site in the 3’UTR region of Hsp47 gene (SerpinH1) (Upper panel). The target site of miR-29 in Hsp47 3’-UTR is highly conserved among mammalian species (Lower panel). (C) Hsp47 protein levels were assessed by Western blot in control and miR-29 mimic-transfected T4-2 cells. (D) Hsp47 protein levels were assessed by Western blot in control and miR-29 inhibitor-transfected MCF10A cells. (E) Quantitative RT-PCR analysis of the expression of Hsp47 and the ECM network genes in control and miR-29 mimic-transfected cells; n=4, ** p<0.01; * p<0.05. (F) Luciferase reporter analysis showed that miR-29b inhibited Hsp47 expression by targeting the seed complementary sequence in the 3’UTR region. pGL3-Hsp47-3’-UTR reporter plasmid in which the luciferase-coding sequence had been fused to the 3’-UTR of firefly luciferase was cotransfected into HEK293 cells with negative control (white columns) or miR-122 duplex (red columns). Hsp47-Mu indicates the introduction of alterations into the seed complementary sites; n=4, *p< 0.05. (G, H) Phase images (G) and quantification (H) of the colony size of the control and miR-29b-expressing T4-2 cells in 3D culture. (I, J) Phase images (I) and quantification (J) of the invasive branch structures in control and miR-29b-expressing MDA-MB 231 cells. (K, L) Spearman correlation analysis showed the negatively correlated expression of Hsp47 and miR-29b, 29c in human breast cancer tissues, n=97.

**Figure 5.** MiR-29b/Hsp47 axis is regulated by the TGF-β pathway during breast cancer progression. (A) Spearman correlation analysis showed the correlated expression of Hsp47 and
TGFB3 in human breast cancer tissues, n=97. (B) Hsp47 expression was measured by quantitative RT-PCR in control and TGF-β treated S1 and MCF10A cells. The cells were treated with TGF-β (5ng/ml) for 48 hours, n=4, *, p<0.05. (C) Hsp47 expression was assessed by Western blot in control and SB431542 treated MDA-MB 231 cells. Cells were treated with SB431542, a TGFBR inhibitor, for 48 hours. (D) MiR-29b expression in control and TGF-β treated MCF10A cells was analyzed by quantitative PCR analysis, n=4, ** p<0.01. (E) Quantitative PCR analysis of miR-29b expression in the control and SB431542 treated MDA-MB 231 cells, n=4, * p<0.1. (G) MDA-MB 231 cells were treated with SB431452 for 12 hours, and then transfected with miR-29b or 29c inhibitors. Hsp47 expression was assessed by Western blot. (H) Spearman correlation analysis showed a negative correlation between miR-29b and TGFB3 levels in human breast cancer tissues, n=97.

**Figure 6. Silencing Hsp47 inhibits tumor growth in vivo.** (A) Control or Hsp47-silenced T4-2/Luciferase cells were subcutaneously injected into SCID mice. IVIS images showed the tumor size in each group after 6 weeks. (B) The curves illustrated tumor growth of control and Hsp47-silenced T4-2 cells in SCID mice over time; n=6, * p<0.05. (C) Quantification of Ki67 staining, indicative of cell proliferation, in control and ShHsp47-expressing tumors; ** p<0.01. (D) Masson’s trichrome staining of tumor sections (blue, collagen; black, nuclei; red, cytoplasm); scale bar, 200µm. The bar graph illustrated the collagen staining- area in tumors formed by control and Hsp47-silenced T4-2 cells. (E) Control or Hsp47-silenced MDA-MB 231/Luciferase cells were injected into the fat pads of SCID mice. IVIS images showed tumors in representative mice from each group. The bar graph showed that tumor volume formed by Hsp47-silenced cells was significantly reduced compared with control cells; n=6, ** p<0.01.
Figure 7. Increased Hsp47 expression is associated with poor prognosis. (A, B) Kaplan-Meier survival analysis showed the association of Hsp47 expression with the overall survival (A) and the recurrent-free survival in human breast cancer patients (B). The tumor samples were classified as low (n=137), high (n=138), and moderate (n=138) based on the mRNA levels of Hsp47 in the published microarray datasets. Significant differences in survival time were calculated using the Cox proportional hazard (log-rank) test. (C) Bar graph showing the mRNA levels of Hsp47 in different stages of breast cancer. Data were from Dr. Chin’s microarray dataset. (D, E) Kaplan-Meier survival analysis showed the association of miR-29b (D) and 29c (E) expression with the overall survival in human breast cancer patients. The tumor samples were classified as low (n=33) and high (n=66) based on the mRNA levels of miR-29b and 29c in the published microarray datasets. Significant differences in survival time were calculated using the Cox proportional hazard (log-rank) test.
Figure 1.
Figure 2.

A

<table>
<thead>
<tr>
<th></th>
<th>Sh Ctrl</th>
<th>Sh Hsp47 A</th>
<th>Sh Hsp47 B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubulin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

T4-2 cells

ShCtrl

ShHsp47

Green (α6 integrin)

Blue (Nuclei)

C

Diameter of colony (μm)

<table>
<thead>
<tr>
<th></th>
<th>ShCtrl</th>
<th>ShHsp47A</th>
<th>ShHsp47B</th>
</tr>
</thead>
</table>

D

MDA-MB 231    Hs-578T    BT549

Sh Ctrl

ShHsp47

E

Invasive branch

<table>
<thead>
<tr>
<th></th>
<th>ShCtrl</th>
<th>ShHsp47A</th>
<th>ShHsp47B</th>
</tr>
</thead>
</table>

F

Positive EDU

<table>
<thead>
<tr>
<th></th>
<th>Sh Ctrl</th>
<th>Sh Hsp47 A</th>
<th>Sh Hsp47 B</th>
</tr>
</thead>
</table>

G

Arbitrary value of invasion

<table>
<thead>
<tr>
<th></th>
<th>Sh Ctrl</th>
<th>Sh Hsp47 A</th>
<th>Sh Hsp47 B</th>
</tr>
</thead>
</table>

H

S1 Ctrl

S1 HSP47

Green (α6 integrin)

Blue (Nuclei)
Figure 4.

A

B

3' uuguGACUAAAGLUAA-GCCACGAGu 5' hsa-miR-29b
5' gcucUGA-CACACAGGGGGUGCu 3' SERPINH1

Homo sapiens
Mus musculus
Rattus norvegicus
Canis lupus
Bos taurus
Gallus gallus

gttcgtgacacagctgtgta
gttcgtgacacagctgtgta
gttcgtgacacagctgtgta
gttcgtgacacagctgtgta
gttcgtgacacagctgtgta
ccttcgccatacagctgtgta

C

ctrl 29a 29b 29c
Hsp47
LAM A/C

D

Ctrl 29a inh 29c inh
Hsp47
Tubulin

E

F

Relative luciferase activity

pcDNA3
pcDNA-miR-29b
pGLO Hsp47
pGLO-Hsp47-Mu

G

T4-ctrl
T4-miR-29b

H

Diameter of colony (μm)

I

MDA231-ctrl
MDA231-miR-29b

J

Invasive branches

K

p=0.000023
r=-0.418

L

p=0.00000000000717
r=-0.518

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 2015 American Association for Cancer Research.
Figure 5.

A. Scatter plot showing the relationship between Hsp47 and TGF-β3 with a linear regression line. The correlation coefficient is p=0.000221 and r=0.369.

B. Bar graph comparing Hsp47/18S expression in Ctrl and TGF-β treated samples. An asterisk denotes a significant difference.

C. Western blot images showing Hsp47 and Lam A/C expression in S1 and MCF10A cells treated with different concentrations of SB431542 (Ctrl, 1 μM, 10 μM).

D. Bar graph showing miR-29b expression in Ctrl and TGF-β treated samples. Asterisks indicate significant differences.

E. Bar graph showing miR-29b expression after treatment with SB431542.

F. Graph illustrating the concentration-dependent effect of SB431542 on miR-29b expression.

G. Western blot images showing Hsp47 and Lam A/C expression in Ctrl, miR-29b Inh, and miR-29c Inh treated samples with SB431543.

H. Scatter plot showing the relationship between miR-29b and TGF-β3 with a linear regression line. The correlation coefficient is p=0.00163 and r=-0.317.
Figure 6.
Figure 7.

A. Overall Survival

B. Recurrent Free Survival

C. mRNA levels of Hsp47

D. Overall Survival

E. Overall Survival
Chaperone Hsp47 drives malignant growth and invasion by modulating an ECM gene network

Jieqing Zhu, Gaofeng Xiong, Hanjiang Fu, et al.

Cancer Res  Published OnlineFirst March 5, 2015.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-1027

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2015/03/07/0008-5472.CAN-14-1027.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.