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, and Ren Xu1,2

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 noninvasive structures in 3D cultures, and to limit tumor growth in xenograft assays by reducing deposition of collagen and fibronectin. Coexpression network analysis also showed that levels of microRNA(miR)-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. Cancer Res; 75(8); 1–12. ©2015 AACR.

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 nonmalignant 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. However, how these ECM proteins are coordinately regulated during breast cancer progression remains unknown.

Gene coexpression 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 coexpression 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 TGFβ pathway (11). The TGFβ pathway regulates a broad range of responses, including microenvironment remodeling (12). In the canonical TGFβ signaling pathway, binding of TGFβ to its receptor activates the transcription factor Smad and modulates gene transcription (13). A major effect of TGFβ 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 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 remain 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 noninvasive 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 miR-29, promotes breast cancer progression by modulating ECM deposition.

**Materials and Methods**

**Antibodies and reagents**

The 5-ethyl-2'-deoxyuridine (Edu) Staining Kit was obtained from Invitrogen. Matrigel and type I collagen were purchased from BD Biosciences. 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 Biotechnology); 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, Life Science Division, Lawrence Berkeley National Laboratory, Berkeley, CA) 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% FBS, 10 U/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, whereas 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 48 hours after transfection. Cells were infected with lentivirus and selected using puromycin for at least 3 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 ΔΔCt method. The following primers were used for amplifying HSP47: 5'-TTAGATCGTGTCCAGCCAG-3' and 5'-TGGCCTACTGAAGTGAAGG-3'; COL1A1: 5'-ATG-GATTCCAGTGGATGATGGG-3' and 5'-CATTGACAGCTACGCTTAGG-3'; COL3A1: 5'-AGTTCCCTGGACTGATAC-3' and 5'-ACATTTTCACCCTTGACACCCTG-3'; 18S rRNA: 5'-ACCTGGTT-GATCCTGGCCAGT-3' and 5'-CTGACCCGGGTGTTTGTGATG-3'. For quantification of miRNA expression, TaqMan probes were used according to the manufacturer's protocol (Applied Biosystems). Cq 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 μmol/L 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 hours before replacing with normal culture medium. Total RNA was prepared 48 hours 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 FluoroChem HD2 (Alpha Innotech). Cells in Matrigel were smeared on slides, dried briefly, then fixed with 4% paraformaldehyde/PBS and permeabilized in 0.5% Triton X-100/PBS. Samples were blocked with 1% BSA and 5% goat serum/PBS for 1 hour, followed by incubation with the primary antibody in blocking buffer for 2 hours at room temperature and the secondary antibody for 50 minutes at room temperature. Images were acquired with an Olympus FV1000 microscope using a 1.3 NA 40× objective with oil immersion.

**Xenograft experiment**

Six-week-old female SCID mice were randomly grouped and s.c. injected with 5 × 10^6 shCtrl (control) or shHsp47 T4-2 cells. Tumors were measured with a caliper every 3 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% paraformaldehyde for histology. For the orthotopic mammary tumor experiments, female SCID mice (6-weeks-old) were injected with 1 × 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 deparaffinized and rehydrated. Samples were re-fixed with Bouin's solution at 60°C for 60
Hsp47 Regulates ECM Transcription Network in Breast Cancer

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 coexpression network analysis, we discovered a coexpression network that integrates the coordinated action of ECM remodeling from hundreds of human breast cancer tissues (11), and identified Hsp47 (Serpint1H) as a node of this network (Fig. 1A). Hsp47 mRNA levels significantly correlated with the expression of collagen I (Col1A1), IV (Col4A1), and fibronectin (FN1) in human breast cancer tissues (Fig. 1B–D) as well as in 24 human breast cancer cell lines (Supplementary Table S1). These correlations suggest a functional connection and/or coregulation 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 (Fig. 1E and F). The increased mRNA levels of Hsp47 were also detected in other types of cancers (Supplementary Fig. S1A). The protein levels of Hsp47 in breast cancer cell lines were higher than the levels in nonmalignant cell lines (Fig. 1G). Among breast cancer cell lines, Hsp47 levels were higher in basa-like breast cancer when compared with the luminal cancer cells (Fig. 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 (Fig. 2A). The 3D tissue culture model provides a physiologically relevant microenvironment for monitoring tissue morphogenesis of nonmalignant 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 (Fig. 2B and C). MDA-MB-231, BT549, and Hs-578T cells form stellate structures in 3D culture, reflecting decreased cell–cell interactions and enhanced cell invasiveness (Fig. 2D and E). Silencing Hsp47 in MDA-MB-231, BT549, and Hs-578T cells significantly reduced branching in 3D culture (Fig. 2D and 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 (Fig. 2F and G). Nonmalignant S1 cells contain low levels of Hsp47 compared with their malignant T4-2 counterpart (Fig. 1E), and form polarized acini in 3D culture. Ectopic expression of Hsp47 disrupted the polarized acinus structures and increased colony size (Fig. 2H), but Hsp47 expression failed to induce colony formation in nonmalignant cells (Supplementary Fig. S1B). 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.

Because 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 (Fig. 3A). It has been reported that Hsp47 deficiency induces procollagen aggregates in the endoplasmic reticulum, and the argorated 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 downregulated in Hsp47-silenced T4-2 cells in 3D culture (Fig. 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 with nonmalignant S1 cells (Fig. 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 (Supplementary Fig. S1A). 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 (Fig. 3D–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 MDA-MB-231 cells significantly reduced invasive branching in 3D culture (Fig. 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 (Fig. 3K–M). These results suggest that Hsp47 promotes breast cancer progression in part by enhancing secretion and deposition of 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 coexpression 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 (Fig. 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 (Fig. 4B), and this binding site is conserved among species (Fig. 4B). Moreover, the 3′UTR regions from about half of ECM network genes (19/41) contain miR-29–binding sites (Supplementary Fig. S2A).

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 nonmalignant mammary epithelial cell lines. Transfection of miR-29 mimics reduced the protein levels of Hsp47 in breast cancer cells (Fig. 4C), whereas introduction of miR-29b and -29c inhibitors enhanced the expression of Hsp47 in the nonmalignant mammary epithelial cell line MCF10A (Fig. 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 (Fig. 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 (Fig. 4F). Mutation in the seed complementary site reduced miR-29b–mediated repression for Hsp47 (Fig. 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 (Fig. 4G and H). Introduction of miR-29b in MDA-MB-231 cells suppressed invasive branching (Fig. 4I and 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 (Fig. 4K) and -29c (Fig. 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 TGFβ-induced Hsp47 expression

Coexpression network analysis revealed that expression of the ECM network was associated with activation of the TGFβ pathway (11). Treatment with TGFβ-induced Hsp47 transcription in non-malignant S1 and MCF-10A cells (Fig. 5B), while blocking the TGFβ pathway with SB431542 (a TGFBR inhibitor) reduced Hsp47 expression in MDA-MB-231 cells (Fig. 5C). We also found that mRNA levels of Hsp47 and TGFβ were significantly correlated in human breast cancer tissues (Fig. 5A) and cell lines (Supplementary Fig. S2B). These results indicate that Hsp47 is regulated by the TGFβ pathway in breast cancer cells.

Next, we examined whether miR-29 is a downstream target of the TGFβ signaling during breast cancer progression. TGFβ treatment significantly suppressed miR-29b expression in nonmalignant MCF-10A cells (Fig. 5D), whereas incubation with SB431542 enhanced miR-29b expression in MDA-MB-231 cells (Fig. 5E and F). Importantly, transfection of miR-29b or -29c inhibitors rescued Hsp47 expression in SB431542-treated breast cancer cells (Fig. 5G). We also found that expression of miR-29b and the TGFβ ligand were negatively correlated in human breast cancer tissues (Fig. 5H). Therefore, miR-29b at least partially mediates the TGFβ-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 s.c. injected into the flanks of female mice. Tumor growth was significantly reduced in the Hsp47-silenced group compared with the control group (Fig. 6A and B). Cancer cells in the Hsp47-silenced tumors were less proliferative compared with the cells in...
the control group (Fig. 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 with the control T4-2 tumors (Fig. 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 (Fig. 6E). Reduced collagen and fibronectin deposition was also observed in orthotopically placed Hsp47-silenced tumors (Supplementary Fig. S3A).

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 patients. Spearman correlation analysis shows the negatively correlated expression of Hsp47 and miR-29b, miR-29c in human breast cancer tissues, n = 97. Figure 4.

miR-29b and -29c repress expression of Hsp47 and ECM network genes during breast cancer progression. A, coexpression network analysis shows that miR-29b and -29c were associated with the ECM transcription network. B, sequence alignment shows the miR-29–binding site in the 3’UTR region of Hsp47 gene (SerpinH1; top). The target site of miR-29 in Hsp47 3’UTR is highly conserved among mammalian species (bottom). C, Hsp47 protein levels were assessed by Western blot analysis in control and miR-29 mimic-transfected T4-2 cells. D, Hsp47 protein levels were assessed by Western blot analysis 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 MCF10A 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-29b duplex (red columns). Hsp47-Mu indicates the introduction of alterations into the seed complementary sites; n = 4; *, P < 0.05. G and 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 and J, phase images (I) and quantification (J) of the invasive branch structures in control and miR-29b–expressing MDA-MB-231 cells. ***, P < 0.001. K and L, Spearman correlation analysis shows the negatively correlated expression of Hsp47 and miR-29b, miR-29c in human breast cancer tissues, n = 97.

the control group (Fig. 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 with the control T4-2 tumors (Fig. 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 (Fig. 6E). Reduced collagen and fibronectin deposition was also observed in orthotopically placed Hsp47-silenced tumors (Supplementary Fig. S3A).

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 patients.
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 (Fig. 7A and B). Similar results were also obtained in a large cohort microarray dataset containing 4,142 breast cancer samples (Supplementary Fig. S3B). Moreover, high grade tumors expressed more Hsp47 compared to low grade tumors (Fig. 7C). These results indicate that activation of Hsp47 correlates with breast cancer progression. Because 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 (Fig. 7D and E). These results suggested that activation of the 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 noninvasive 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 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 that 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 tenasin-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 breast cancer cells and inhibited tumor growth in xenograft model. Oskarsson and colleagues (16) have shown that cancer cell–produced tenasin C enhances dissemination and survival of tumor cells during the early phases of metastasis by generating a niche that is susceptible to metastasis. 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 TGFβ in systemic sclerosis and fibrosis (50). Chou and colleagues (38) demonstrate that TGFβ is a miR-29 target gene. Together these results suggest a feedback loop between the TGFβ pathway and miR-29. We showed that expressions of TGFβ, miR-29b, and Hsp47 are significantly correlated in human breast cancer tissues. Therefore, miR-29b may serve as an important mediator of the TGFβ 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 expressions are inversely correlated in human breast cancer tissues (Supplementary Fig. S4A), and that Hsp47 is highly expressed in basal-like breast cancer tissues (Fig. 1H) and ER-negative breast cancer tissues (Supplementary Fig. S4B). 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.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Disclaimer**

The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the NIH or the NIGMS.

**Authors’ Contributions**

Conception and design: R. Xu

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Zhu, G. Xiong, R. Xu

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Zhu, G. Xiong, B.P. Zhou, R. Xu

Writing, review, and/or revision of the manuscript: J. Zhu, G. Xiong, B.M. Evers, B.P. Zhou, R. Xu

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Fu, B.M. Evers

Study supervision: B.P. Zhou, R. Xu

**Acknowledgments**

The authors thank Dr. Mina J. Bissell for her support in this project and providing T4-2 and S1 cells lines. The authors thank the pathology core facility at Markey Cancer Center for assistance in tissue processing.

**Grant Support**

This study was supported by a start-up fund from the Markey Cancer Center and grants from AHA (12SDG8600000 to R. Xu), ACS (IRG 85-001-22 to R. Xu), and a COBRE Pilot Award (201407231052 to R. Xu). This study was supported in part by NIH grant number P50GM110787 from the National Institute of General Medical Sciences.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 7, 2014; revised December 30, 2014; accepted January 21, 2015; published OnlineFirst March 5, 2015.
References


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

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.