Gadd45a Functions as a Promoter or Suppressor of Breast Cancer Dependent on the Oncogenic Stress

Jennifer S. Tront, Yajue Huang, Albert A. Fornace Jr., Barbara Hoffman, and Dan A. Liebermann

Abstract

Gadd45a plays a pivotal role as a stress sensor that modulates cellular responses to various stress stimuli including oncogenic stress. We reported that the stress sensor Gadd45a gene functions as a tumor suppressor in Ras-driven breast tumorigenesis via increasing JNK-mediated apoptosis and p38-mediated senescence. In contrast, here, we show that Gadd45a promotes Myc-driven breast cancer by negatively regulating MMP10 via GSK3 β/β-catenin signaling, resulting in increased tumor vascularization and growth. These novel findings indicate that Gadd45a functions as either a tumor promoter or suppressor, is dependent on the oncogenic stress, and is mediated via distinct signaling pathways. Collectively, these novel findings highlight the significance of the type of oncogenic alteration on how stress response genes function during initiation and progression of tumorigenesis. Because Gadd45a is a target for BRCA1 and p53, these findings have implications regarding BRCA1/p53 tumor suppressor functions. Cancer Res; 70(23); 9671-81. ©2010 AACR.

Introduction

Gadd45a plays a pivotal role as a stress sensor that modulates the cellular response to a variety of stress conditions, including genotoxic and oncogenic stresses (1–3). It mediates stress responses via interacting with and modulating the function of partner proteins that play key roles in cell cycle control (4–6), DNA repair (7, 8), and cell survival (9–12).

We have recently reported that loss of Gadd45a significantly accelerated the onset of Ras-driven breast tumorigenesis, providing a novel model for Gadd45a in the suppression of breast tumor development (13). Gadd45a suppressed Ras-driven tumor growth by increasing both JNK-mediated tumor cell apoptosis and p38-mediated tumor cell senescence.

c-Myc is a key regulator of cell proliferation, in which deregulation of Myc expression contributes to the initiation and progression of tumorigenesis (14). For nearly all cases of solid human tumors, the relative amount of Myc protein is increased in the tumor tissue compared with the surrounding normal tissue, which suggests that deregulated and/or elevated levels of Myc contributes to the tumorigenic phenotype (14). Nearly 50% of all breast cancer tumors display significantly elevated levels of Myc protein (14–16). In light of these observations, we sought to extend our investigation of the role Gadd45a plays in modulating breast carcinogenesis to ask whether Gadd45a also modulates Myc-driven breast tumorigenesis. To this end, we took advantage of the established breast cancer–prone MMTV-c-Myc transgenic mouse model, in which deregulated Myc is under the control of the mouse mammary tumor virus (MMTV) promoter (17). Gadd45a-deficient mice and MMTV-c-Myc transgenic mice were interbred to generate MMTV-c-Myc transgenic mice that are either wildtype or null for Gadd45a. Using these mice in conjunction with MMTV-Ras mice that are either wildtype or null for Gadd45a, we asked whether Gadd45a modulates Myc-driven tumorigenesis and how this compares with its involvement in Ras-driven breast carcinogenesis.

Intriguingly, in contrast to what was observed with oncogenic Ras, we showed that Gadd45a promoted Myc-driven breast tumorigenesis. Furthermore, it is shown that the mechanism by which Gadd45a promotes Myc-driven breast tumorigenesis differs radically from the mechanism employed to suppress Ras-driven breast carcinogenicity. Collectively, these novel observations provide a novel paradigm indicating that depending on the nature of the oncogenic stress, Gadd45a may function as either a tumor suppressor or a tumor promoter by employing different effector pathways.

Materials and Methods

Mice

MMTV-Myc transgenic mice (FVB genetic background) were obtained from Charles River Laboratories (Wilmington, MA). Gadd45a−/− mice (C57Bl6 × 129Sv background) were graciously provided by Albert Fornace. Offspring from interbreeding Gadd45a−/− and MMTV-Myc mice were generated.
as littermates from common matings, so all animals were maintained in a mixed genetic background. Offspring from crosses between MMTV-Myc and Gadd45a−/− mice were screened by PCR for their Myc and Gadd45a status. At the same time, MMTV-Ras mice that differed in their Gadd45a status were also generated as published in Tront et al. (13). At the time of weaning, genomic DNA was isolated from a tail clipping by standard procedures for PCR analysis. Primers for the detection of MMTV-Myc were 5′-CCAGGTTGATGTCC-CTTCCACATC-3′ (5′-sense) and 5′-GAAAAGTGGCCTGACG-TCTAAGA-3′ (3′-antisense). To assess Gadd45a status, PCR reactions using 3 primers allowed for simultaneous detection of the wild-type and mutant Gadd45a allele. These primers consisted of a 5′-upstream primer (5′-CACCCTCTGGTACCCCTGCACAA-3′), a common 3′-antisense primer (5′-CCA-GAGACCTAGACACGCCTT-3′), and a neospecific primer (5′-AAGCCTATGCTCCAGACTCC-3′). Reactions were run for 37 cycles of 94°C for 1 minute, 63°C for 14 seconds, and 72°C for 12 seconds.

Tumor formation and onset

Four-week-old female mice from all genotypes were observed twice weekly for the formation of visible tumor masses. Upon detection of a mass, the tumor growth properties were monitored every other day for 15 days or until the general health of the animal was compromised, at which time the mouse was sacrificed in accordance with Temple University and National Institute of Health guidelines. Tumor measurements were taken with hand calipers to measure tumor volume (calculated tumor volume (mm³) = (W² × L) where W is width and L is length). Tumor growth curves were generated by plotting the average daily tumor growth against time. Tumor onset was plotted using a Kaplan-Meier survival curve. Differences between Kaplan-Meier survival curves were determined using the Student t test.

Western blotting

Seventy-five micrograms of protein extract was resolved on an sodium dodecyl sulfate polyacrylamide gel, followed by transfer to an Immobilon-P membrane. Following blocking, the membrane was incubated with primary antibody overnight at 4°C [phospho-JNK (9251), phospho-p38 (4631), and GSK3β (9315)—Cell Signalning Technology; β-galactosidase (ab616), MMP10 (ab4045), CD31 (28365), CD105 (27422), Gadd45a (ab33173)—Abcam; and β-catenin (610154)—BD Biosciences]. Sections were incubated with a peroxidase-conjugated secondary antibody for 30 minutes at room temperature, followed by treatment with ABC reagent (Vector Laboratories) for 30 minutes. Sections were stained with 3,3′-diaminobenzidine substrate and counterstained with hematoxylin. For CD31 and CD105 double staining, equal amounts of both antibodies were mixed and used for the overnight staining. For all immunohistochemical (IHC) analysis, a minimum of 5 samples from each genotype were analyzed blindly for each analysis. Samples were analyzed in triplicate. The number of positive stained cells and the total number cells were determined with Image J photo software, using a 10 × 450 field range. Differences between genotypes were evaluated using the Student t test.

Microarray analysis

Five micrograms of total RNA from tumor samples from the various genotypes was used as a template for cDNA synthesis. cDNA was labeled with biotin-dUTP, using AmpoLabeling-LRP kit (SuperArray Bioscience). The cDNA probe was applied to prehybridized Mouse Signal Transduction in Cancer Gene Array (MM-044) membranes. The hybridization was done at 60°C for 12 hours. After washing, the membranes were blocked and treated with alkaline phosphatase-conjugated streptavidin and exposed to alkaline phosphatase chemiluminescent substrate. The membranes were exposed to X-ray film and the spots were analyzed using the GEArray Expression Analysis Suite Software (SA Bioscience).

Electromobility shift assay

Searching GenBank, we identified a potential T-cell factor (TCF) binding site in the promoter of matrix metalloproteinase 10 (MMP10) that matched the consensus sequence 5′-AATTCAAGAG-3′. The following double-stranded oligonucleotide was used as a probe: TCF: 5′-ATA TAT TCA AAG GAC CCA GGT; TCF: 5′-ATA TAG CCA AAG GAC CCA GGT. The probe was end-labeled and used in a reaction with nuclear cells were analyzed using light microscopy. Necrotic regions of the tumor were avoided. Using a 10 × 450 field range, the number of TUNEL-positive stained cells and the total number of propidium iodide stained cells was determined with Image J photo program (NIH). Percentage apoptosis was calculated by dividing the total number of positive cells by the total number of cells. A minimum of 5 samples per genotype were analyzed. Differences in percentage apoptosis between genotypes were evaluated using the Student t test.

Immunohistochemistry

Paraffin-embedded tissue slides were deparaffinized, rehydrated, and subjected to antigen unmasking by sodium citrate (pH 6.0) for 30 minutes at a sub-boiling temperature. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 10 minutes. Sections were blocked with 5% serum for 1 hour at room temperature, followed by incubation with primary antibody overnight at 4°C [phospho-JNK (9251), phospho-p38 (4631), and GSK3β (9315)—Cell Signalning Technology; β-galactosidase (ab616), MMP10 (ab4045), CD31 (28365), CD105 (27422), Gadd45a (ab33173)—Abcam; and β-catenin (610154)—BD Biosciences]. Sections were incubated with a peroxidase-conjugated secondary antibody for 30 minutes at room temperature, followed by treatment with ABC reagent (Vector Laboratories) for 30 minutes. Sections were stained with 3,3′-diaminobenzidine substrate and counterstained with hematoxylin. For CD31 and CD105 double staining, equal amounts of both antibodies were mixed and used for the overnight staining. For all immunohistochemical (IHC) analysis, a minimum of 5 samples from each genotype were analyzed blindly for each analysis. Samples were analyzed in triplicate. The number of positive stained cells and the total number cells were determined with Image J photo software, using a 10 × 450 field range. Differences between genotypes were evaluated using the Student t test.
Results

Gadd45a is upregulated during Myc-driven breast carcinogenesis, resulting in an acceleration of Myc-driven mammary tumorigenesis

Our working hypothesis was that Gadd45a is a stress sensor protein that is upregulated by oncogenic stress during breast carcinogenesis and modulates tumor development. To assess the validity of our hypothesis, Gadd45a expression was examined in normal and tumor mammary tissue obtained from both MMTV-Myc and MMTV-Ras mice that are either wild-type or null for Gadd45a expression. For comparison, Gadd45a expression was also assessed in nonmammary tissue (i.e., spleen tissue).

Gadd45a expression was undetectable in normal mammary and spleen tissues obtained from all genotypes (Fig. 1), whereas Gadd45a expression was observed in comparable breast tumor tissue obtained from MMTV-Myc and MMTV-Ras (Fig. 1A) and MMTV-Ras (Fig. 1B) mice. Taken together, these data, in conjunction with our previous findings (13), support the hypothesis that Gadd45a expression is upregulated during breast carcinogenesis driven by either of the 2 oncogenes.

To assess the effect of Gadd45a deficiency on Myc-driven breast carcinogenesis compared with the Ras-driven carcinogenesis, female animals from MMTV-Myc mice that are either wildtype or null for Gadd45a expression were monitored twice weekly for the development of tumors. Tumorigenesis was decelerated in Myc/Gadd45a−/− mice compared with Myc/Gadd45a+/+ control animals (P < 0.05; Fig. 1C). The median tumor onset, measured as the time in which 50% of animals develop tumors, was 6 months for Myc/Gadd45a−/− mice, whereas the loss of Gadd45a delayed the median tumor onset to 10 months. Myc-expressing Gadd45a heterozygous mice display an intermediate phenotype with a median tumor onset of 8 months. The loss of Gadd45a resulted in an overall decrease in tumor incidence, for which only 67% of mice developed tumors within 12 months compared with 88% for Myc/Gadd45a+/+ mice. This was in sharp contrast to the Ras breast cancer model, in which the loss of Gadd45a resulted in an increase in tumor incidence (Fig. 1E).

To determine the effect of loss of Gadd45a on the overall rate of tumor growth, tumor volume was monitored every 2 days by caliper measurements, starting upon first tumor visualization and continuing for approximately 2 weeks or until the general well-being of the animal was compromised. Mammary tumors arising from Myc-expressing Gadd45a-deficient mice displayed significantly decreased rates of growth compared with those mice that express Gadd45a (P < 0.05; Fig. 1D). For example, at 14 days, the average Myc/Gadd45a−/− tumor had a volume of 3,166 ± 881 mm3 (n = 8), whereas the Gadd45a-expressing tumors had an average tumor volume of 4,476 ± 996 mm3 (n = 6). These observations are in stark contrast to results for MMTV-Ras mice, for which the loss of Gadd45a resulted in increased tumor size (Fig. 1E). Myc/Gadd45a−/− tumors displayed an intermediate phenotype.

Taken together, these data indicate that Gadd45a functions to promote Myc-driven tumor growth, which is in contrast to its tumor-suppressive function in the context of Ras-driven tumorigenesis.

Histologic examination reveals massive cell death in Myc-expressing Gadd45a-deficient tumors

To elaborate on the differences in the rate of formation and growth between Myc- and Ras-driven breast carcinogenesis, the histopathology of tumor tissue sections from the different genotypes was determined. Higher histologic tumor grades are associated with loss of cellular shape and size uniformity, increased nuclear size, hyperchromatic nuclei, and the presence of multinucleated cells. As seen in Figure 2 and Supplementary Figure 1, the presence of Gadd45a in MMTV-Myc mice is associated with a higher histologic grade than Gadd45a-deficient tumors. Myc-expressing Gadd45a wild-type tumors displayed a loss of cellular shape and size uniformity, increased nuclear size, and hyperchromatic nuclei. Importantly, Myc-expressing Gadd45a-deficient tumor tissue samples displayed massive cellular death, consisting of cellular debris and nuclei fragments (Fig. 2A). Myc/Gadd45a−/− tumors displayed less aggressive phenotypes than wild-type tumors, which is reflected in the tumor growth rates. It should be noted that only in the complete absence of Gadd45a do we find massive levels of cell death. In sharp contrast, in MMTV-Ras mice, the Gadd45a-deficient tumors had a more aggressive histologic phenotype than Gadd45a-expressing tumors, without massive cell death (Fig. 2B) (13).

These observations suggest that for Myc-driven breast cancer, Gadd45a promotes the development of aggressive tumors whereas in Ras-driven breast cancer Gadd45a suppresses the development of aggressive tumors.

Gadd45a has opposing effects on apoptosis and senescence in Myc-driven compared with Ras-driven breast carcinogenesis

Because histologic examination detected massive cell death in Myc-expressing Gadd45a-deficient tumors, the level of...
apoptosis for the different tumors was ascertained using TUNEL analysis. In the presence of Myc, loss of Gadd45a resulted in a significant increase in apoptosis compared with wild-type controls ($P < 0.05$) (Fig. 3A). The opposite effect was observed for Ras-driven breast carcinogenesis, in which the loss of Gadd45a resulted in decreased apoptosis (Fig. 3E).

Oncogene-induced senescence has been reported to play a role in retarding tumor progression (18–20). Therefore, we investigated the levels of senescence by immunohistochemistry using anti-β-galactosidase antibody. It was observed that the loss of Gadd45a significantly increased the level of cellular senescence in the presence of Myc ($P < 0.05$) (Fig. 3B). In contrast, loss of Gadd45a significantly decreased cellular senescence in the context of Ras-driven breast tumorigenesis (Fig. 3E).

In the case of Ras-driven breast tumors, Gadd45a-mediated activation of JNK and p38 was correlated with Ras-induced apoptosis and senescence, respectively (Fig. 3E) (13). It was of interest, therefore, to analyze the levels of both activated JNK and p38 in Myc-driven breast tumors. Intriguingly, it was observed that in breast tumors from MMTV-Myc mice there was no significant difference between the levels of either activated JNK or activated p38 in the presence or absence of Gadd45a (Fig. 3C–E).

Taken together, these findings show that Gadd45a employs an alternate mechanism to promote Myc-driven breast carcinogenesis, as opposed to suppressing Ras-mediated breast cancer.

**Gadd45a suppresses MMP10 expression, resulting in increased capillary vascularization in Myc-driven breast tumors**

To explore possible mechanisms by which Gadd45a promotes Myc-driven breast carcinogenesis, a gene expression
A profiling study was conducted using DNA microarray membranes designed to screen for signal transduction pathways in cancer (GEArray Q Series Mouse Signal Transduction in Cancer Gene Array, SuperArray) (Fig. 4A). The expression profiles of Myc-generated breast tumors from Gadd45a wild-type, heterozygous, and null mice were evaluated using the appropriate software (Materials and Methods). A list of genes that displayed significant changes in expression between Myc+/Gadd45a+/+ and Myc+/Gadd45a–/– tumors is provided in Supplementary Figure 2. The MMP10 gene showed more than 6-fold higher expression in Myc-expressing Gadd45a-deficient breast tumors than in Gadd45a wild-type tumors. The significance of this finding became the focus of additional experiments.

Immunohistochemistry employing MMP10 antibody confirmed the array induction data; there was increased MMP10 expression in Myc-expressing Gadd45a-deficient tumor samples compared with wild-type tumor samples (P < 0.05) (Fig. 4B). Levels of MMP10 were examined in Ras-driven breast tumors, either wildtype or null for Gadd45a, to determine whether MMP10 expression is altered in Ras-driven breast tumors; however, no significant difference was detected (Fig. 4D and Supplementary Fig. 3A). These results indicate that Gadd45a elicits distinct signaling profiles depending on the nature of the activated oncogene.

MMP10 (also known as Stromelysin-2) plays a role in capillary tubular network collapse and regression because of its ability to degrade various components of the basement membrane matrix, such as collagen type IV, laminin, and proteoglycans (21). Thus, we examined the capillary tubular network in breast tumors from the various genotypes to determine how the level of MMP10 correlated with the status of the capillary networks. Immunohistochemistry was performed with antibodies to CD31 and CD105, marker proteins known to play a role in capillary network maintenance, angiogenesis, and vascularization (22, 23). It can be seen that the loss of Gadd45a was associated with significantly decreased levels of vascularization in Myc-driven breast tumors, either wildtype or null for Gadd45a, to determine whether MMP10 expression is altered in Ras-driven breast tumors; however, no significant difference was detected (Fig. 4D and Supplementary Fig. 3A). These results indicate that Gadd45a elicits distinct signaling profiles depending on the nature of the activated oncogene.

MMP10 (also known as Stromelysin-2) plays a role in capillary tubular network collapse and regression because of its ability to degrade various components of the basement membrane matrix, such as collagen type IV, laminin, and proteoglycans (21). Thus, we examined the capillary tubular network in breast tumors from the various genotypes to determine how the level of MMP10 correlated with the status of the capillary networks. Immunohistochemistry was performed with antibodies to CD31 and CD105, marker proteins known to play a role in capillary network maintenance, angiogenesis, and vascularization (22, 23). It can be seen that the loss of Gadd45a was associated with significantly decreased levels of vascularization in Myc-driven breast tumors, either wildtype or null for Gadd45a, to determine whether MMP10 expression is altered in Ras-driven breast tumors; however, no significant difference was detected (Fig. 4D and Supplementary Fig. 3A). These results indicate that Gadd45a elicits distinct signaling profiles depending on the nature of the activated oncogene.

MMP10 (also known as Stromelysin-2) plays a role in capillary tubular network collapse and regression because of its ability to degrade various components of the basement membrane matrix, such as collagen type IV, laminin, and proteoglycans (21). Thus, we examined the capillary tubular network in breast tumors from the various genotypes to determine how the level of MMP10 correlated with the status of the capillary networks. Immunohistochemistry was performed with antibodies to CD31 and CD105, marker proteins known to play a role in capillary network maintenance, angiogenesis, and vascularization (22, 23). It can be seen that the loss of Gadd45a was associated with significantly decreased levels of vascularization in Myc-driven breast tumors, either wildtype or null for Gadd45a, to determine whether MMP10 expression is altered in Ras-driven breast tumors; however, no significant difference was detected (Fig. 4D and Supplementary Fig. 3A). These results indicate that Gadd45a elicits distinct signaling profiles depending on the nature of the activated oncogene.

MMP10 (also known as Stromelysin-2) plays a role in capillary tubular network collapse and regression because of its ability to degrade various components of the basement membrane matrix, such as collagen type IV, laminin, and proteoglycans (21). Thus, we examined the capillary tubular network in breast tumors from the various genotypes to determine how the level of MMP10 correlated with the status of the capillary networks. Immunohistochemistry was performed with antibodies to CD31 and CD105, marker proteins known to play a role in capillary network maintenance, angiogenesis, and vascularization (22, 23). It can be seen that the loss of Gadd45a was associated with significantly decreased levels of vascularization in Myc-driven breast tumors, either wildtype or null for Gadd45a, to determine whether MMP10 expression is altered in Ras-driven breast tumors; however, no significant difference was detected (Fig. 4D and Supplementary Fig. 3A). These results indicate that Gadd45a elicits distinct signaling profiles depending on the nature of the activated oncogene.

MMP10 (also known as Stromelysin-2) plays a role in capillary tubular network collapse and regression because of its ability to degrade various components of the basement membrane matrix, such as collagen type IV, laminin, and proteoglycans (21). Thus, we examined the capillary tubular network in breast tumors from the various genotypes to determine how the level of MMP10 correlated with the status of the capillary networks. Immunohistochemistry was performed with antibodies to CD31 and CD105, marker proteins known to play a role in capillary network maintenance, angiogenesis, and vascularization (22, 23). It can be seen that the loss of Gadd45a was associated with significantly decreased levels of vascularization in Myc-driven breast tumors, either wildtype or null for Gadd45a, to determine whether MMP10 expression is altered in Ras-driven breast tumors; however, no significant difference was detected (Fig. 4D and Supplementary Fig. 3A). These results indicate that Gadd45a elicits distinct signaling profiles depending on the nature of the activated oncogene.

MMP10 (also known as Stromelysin-2) plays a role in capillary tubular network collapse and regression because of its ability to degrade various components of the basement membrane matrix, such as collagen type IV, laminin, and proteoglycans (21). Thus, we examined the capillary tubular network in breast tumors from the various genotypes to determine how the level of MMP10 correlated with the status of the capillary networks. Immunohistochemistry was performed with antibodies to CD31 and CD105, marker proteins known to play a role in capillary network maintenance, angiogenesis, and vascularization (22, 23). It can be seen that the loss of Gadd45a was associated with significantly decreased levels of vascularization in Myc-driven breast tumors, either wildtype or null for Gadd45a, to determine whether MMP10 expression is altered in Ras-driven breast tumors; however, no significant difference was detected (Fig. 4D and Supplementary Fig. 3A). These results indicate that Gadd45a elicits distinct signaling profiles depending on the nature of the activated oncogene.

MMP10 (also known as Stromelysin-2) plays a role in capillary tubular network collapse and regression because of its ability to degrade various components of the basement membrane matrix, such as collagen type IV, laminin, and proteoglycans (21). Thus, we examined the capillary tubular network in breast tumors from the various genotypes to determine how the level of MMP10 correlated with the status of the capillary networks. Immunohistochemistry was performed with antibodies to CD31 and CD105, marker proteins known to play a role in capillary network maintenance, angiogenesis, and vascularization (22, 23). It can be seen that the loss of Gadd45a was associated with significantly decreased levels of vascularization in Myc-driven breast tumors, either wildtype or null for Gadd45a, to determine whether MMP10 expression is altered in Ras-driven breast tumors; however, no significant difference was detected (Fig. 4D and Supplementary Fig. 3A). These results indicate that Gadd45a elicits distinct signaling profiles depending on the nature of the activated oncogene.

MMP10 (also known as Stromelysin-2) plays a role in capillary tubular network collapse and regression because of its ability to degrade various components of the basement membrane matrix, such as collagen type IV, laminin, and proteoglycans (21). Thus, we examined the capillary tubular network in breast tumors from the various genotypes to determine how the level of MMP10 correlated with the status of the capillary networks. Immunohistochemistry was performed with antibodies to CD31 and CD105, marker proteins known to play a role in capillary network maintenance, angiogenesis, and vascularization (22, 23). It can be seen that the loss of Gadd45a was associated with significantly decreased levels of vascularization in Myc-driven breast tumors, either wildtype or null for Gadd45a, to determine whether MMP10 expression is altered in Ras-driven breast tumors; however, no significant difference was detected (Fig. 4D and Supplementary Fig. 3A). These results indicate that Gadd45a elicits distinct signaling profiles depending on the nature of the activated oncogene.

MMP10 (also known as Stromelysin-2) plays a role in capillary tubular network collapse and regression because of its ability to degrade various components of the basement membrane matrix, such as collagen type IV, laminin, and proteoglycans (21). Thus, we examined the capillary tubular network in breast tumors from the various genotypes to determine how the level of MMP10 correlated with the status of the capillary networks. Immunohistochemistry was performed with antibodies to CD31 and CD105, marker proteins known to play a role in capillary network maintenance, angiogenesis, and vascularization (22, 23). It can be seen that the loss of Gadd45a was associated with significantly decreased levels of vascularization in Myc-driven breast tumors, either wildtype or null for Gadd45a, to determine whether MMP10 expression is altered in Ras-driven breast tumors; however, no significant difference was detected (Fig. 4D and Supplementary Fig. 3A). These results indicate that Gadd45a elicits distinct signaling profiles depending on the nature of the activated oncogene.
tumors \( P < 0.05 \) (Fig. 4C), whereas no significant differences in vascularization were detected between Gadd45a-wildtype and Gadd45a-deficient tumors in Ras-driven breast tumors (Fig. 4D and Supplementary Fig. 3B).

Collectively, the data suggest a novel mechanism for Gadd45a-mediated promotion of Myc-driven tumorigenesis via suppression of MMP10 expression.

**MMP10 expression is regulated by GSK3β/β-catenin signaling in Myc-driven breast tumors**

Evidence has implicated the transcriptional activator β-catenin in regulating expression of MMPs (24). While the majority of β-catenin is associated with cell–cell adhesion complexes, a small fraction is present in the cytoplasm, its level regulated by glycogen synthase kinase 3β (GSK3β) phosphorylation and subsequent proteasome degradation (25). Phosphorylated GSK3β is inactive, whereas dephosphorylation renders it active contributing to the degradation of β-catenin. Hence, inactivation of GSK3β via phosphorylation results in the accumulation of hypophosphorylated β-catenin, which, in turn, leads to its nuclear translocation and binding to and activation of the TCF family of transcription factors (24).

While direct β-catenin transcriptional regulation of several MMPs has been identified, the role of β-catenin in MMP10 regulation had not been established (24, 26). Therefore, it was first determined whether β-catenin could bind to MMP10 promoter sequences. Searching GenBank, a potential TCF-binding element was identified as the promoter of MMP10 (26). Electromobility shift assays were performed using the identified binding sequence in the MMP10 promoter as the probe and nuclear extracts from SW480 colon cancer cells, which are known to express high levels of β-catenin (24). β-Catenin bound to the probe, whereas excess cold probe competed for the binding (Fig. 5A). The addition of β-catenin antibody resulted in a specific supershift of the band, indicating that β-catenin was bound to the probe. Taken together, these
results implicate β-catenin in transcriptional activation of the MMP10 gene.

Next, we asked whether Gadd45a regulation of MMP10 expression was mediated by the GSK3β/β-catenin signaling pathway. To this end, we determined the phosphorylation status of GSK3β and the level of β-catenin in our breast tumor tissue samples by immunohistochemistry. As shown in Figure 5B, there was a high level of phosphorylated GSK3β in Myc-expressing Gadd45a-deficient tumor samples compared with Gadd45a wild-type samples (P < 0.05). Consistent with this observation, loss of Gadd45a also was correlated with significantly increased levels of nuclear β-catenin (P < 0.05) (Fig. 5C). In contrast, loss of Gadd45a had no effect on phospho-GSK3β or β-catenin in Ras-driven breast tumors (Fig. 5D and Supplementary Fig. 4A and B).

Although it is known that Gadd45a promotes GSK3β dephosphorylation in keratinocytes, we chose to further our investigation of the role of Gadd45a on the regulation of the GSK3β pathway in the mammary tissue (26). Using total cell lysates from Gadd45a-null mammary tissue, we performed a phosphatase assay with increasing amounts of purified Gadd45a protein. Our results show that under this in vitro experimental setting, Gadd45a can promote the dephosphorylation of GSK3β in a dose-dependent manner (Supplementary Fig. 5). It should be noted, however, that under physiologic in vivo setting, the effect of Gadd45a on the phosphorylation status of GSK3β appears to be manifested in the presence of deregulated Myc but not in the presence of oncogenic Ras because Gadd45a loss had no effect on the phosphorylation status of GSK3β or β-catenin in Ras-driven breast tumors.

Taken together, these novel findings implicate Gadd45a as a negative regulator of MMP10 expression via GSK3β/β-catenin signaling, which promotes Myc-driven breast tumor vascularization and tumor development.
**Discussion**

Generation and side-by-side analysis of MMTV-Myc versus MMTV-Ras mice strains highlight a unique role for Gadd45α as either a suppressor or a promoter of breast cancer development, employing distinct signaling pathways in response to distinct oncogenic stress stimuli (Fig. 7 and Supplementary Fig. 7). Our data indicate that the Gadd45α tumor suppressor function, mediated through the activation of JNK and p38 stress kinases, contributes to Ras-induced apoptosis and senescence, respectively, and is a unique response to Ras oncogenic stress (Fig. 7B). In contrast, the tumor promoter function of Gadd45α, mediated through negative regulation of MMP10 expression via the GSK3β/β-catenin signaling cascade, resulting in increased tumor vascularization, is a unique response to oncogenic Myc (Fig. 7A). These novel results indicate that Gadd45α can function to either promote or suppress breast tumor development through the engagement of different signaling pathways, depending on the molecular nature of the activated oncogene.

In the case of Myc-driven breast carcinogenesis, we have shown that the loss of Gadd45α results in increased levels of MMP10 through the inactivation of the GSK3β/β-catenin signaling pathway (Fig. 7). MMP10 was identified as a direct target of β-catenin. Loss of Gadd45α increased expression of MMP10 and, in turn, contributed to increased tumor vascularization. Together with our previous findings (39), these results suggest that Gadd45α may have important therapeutic implications for breast cancer treatment, with inhibition of MMP10 as a potential target for therapeutic intervention.
phosphorylation of the GSK3β kinase, rendering it inactive, and dramatically increased the levels of β-catenin. Inactivation of GSK3β by phosphorylation is known to result in the accumulation of hypophosphorylated β-catenin, which, in turn, translocates to the nucleus where it binds to TCF and functions as a transcriptional activator (24). It is noteworthy that while our work was in progress, evidence was obtained that in keratinocytes, UV-induced Gadd45a directly associates with GSK3β to promote GSK3β dephosphorylation and activation (26). These findings are supported by our data providing direct evidence that in normal mammary tissue, Gadd45a promotes dephosphorylation of GSK3β.

MMP10 is known to play a role in capillary tubular network collapse and regression because of its ability to degrade various components of basement membrane matrix, such as collagen type IV, laminin, and proteoglycans (21). Thus, it is logical to assume that the decrease in vascularization that is observed in Myc-expressing tumors deficient for Gadd45a is a direct consequence of MMP10 activation. In turn, the massive levels of cellular death, associated with the accumulation of cellular debris and increased apoptosis observed in Myc-expressing tumors deficient for Gadd45a, are likely to be the consequence of the decrease in vascularization. It is notable that the massive cell death, as evident by large areas of cellular debris and nuclei fragments, observed upon histologic analysis of Myc/Gadd45a−/− tumor sections (Fig. 2A), by far exceeds the percentage of apoptotic cell death detected by the TUNEL assay (Fig. 3A). Keeping in mind that the TUNEL IHC data in essence is a snapshot of cells undergoing apoptosis at the time of tumor fixation, the histologic data reflect accumulative apoptotic cell death during tumor development. However, it is also possible that in addition to apoptosis, necrosis contributes to Myc/Gadd45a−/− tumor cell death. The increase in cell senescence in Myc-expressing tumor tissue deficient for Gadd45a may also be due at least partially to the decrease in vascularization. Alternatively, or in addition,
it may also be the consequence of the role implicated for MMP10 as a regulator of cellular senescence (27). Whatever may be the case, the in vivo RNAi-mediated suppression of MMP10 expression, correlating with increases in Myc-driven tumor growth, provides direct evidence for a novel role of MMP10 in suppression of breast carcinogenesis.

The notion that the response of Gadd45a to oncogenic stress signals depends on the nature of the activated oncogenes is novel and intriguing. These results stress the significance of the type of oncogenic alterations found in the target cell on how stress response genes, such as Gadd45a, influence the initiation and progression of tumors. Because Gadd45a is a transcriptional target for both BRCA1 and p53 as tumor suppressors in the context of distinct oncogenic stressors. It is noteworthy that deficiency of the CDK1 p21 was observed to differentially affect Ras- versus Myc-driven mammary tumor properties, promoting either growth arrest or proliferation, depending on the specific cellular context, although an exact mechanism was not identified (28).

Finally, we would like to point out that our findings raise several interesting questions that warrant further investigation: 1) How does Gadd45a interface with different signaling cascades in response to distinct oncogenic stress? 2) How does Gadd45a influence breast carcinogenesis driven by oncogenes other than Ras and Myc? 3) How does Gadd45a modulate the development of other tumor types driven by distinct oncogenic stress? 4) What role does Gadd45a expression or the lack of it play in human breast carcinogenicity? and finally, 5) Do other Gadd45 proteins (i.e., Gadd45b and Gadd45g),
either separately or in combination with Gadd45a, modulate breast tumor development? Current research is targeted at addressing these interesting issues.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

References


Grant Support

This work was funded by U.S. Army Breast Cancer Idea Award (D.A. Liebermann) and NIH 1 R01 CA122376-01A1 (D.A. Liebermann).

Received 06/17/2010; revised 07/30/2010; accepted 08/23/2010; published OnlineFirst 11/23/2010.
Correction: Gadd45a Functions as a Promoter or Suppressor of Breast Cancer Dependent on the Oncogenic Stress

In this article (Cancer Res 2010;70:9671–81), which was published in the December 1, 2010 issue of Cancer Research (1), there is a typographical error in the name of the third author. The correct name should be Albert J. Fornace, Jr.

Reference

Published online First January 18, 2011.
©2011 American Association for Cancer Research.
doi: 10.1158/0008-5472.CAN-10-4479
Cancer Research

Gadd45a Functions as a Promoter or Suppressor of Breast Cancer Dependent on the Oncogenic Stress

Jennifer S. Tront, Yajue Huang, Albert A. Fornace, Jr., et al.

Cancer Res 2010;70:9671-9681. Published OnlineFirst November 23, 2010.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-2177

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/11/23/0008-5472.CAN-10-2177.DC1

Cited articles
This article cites 28 articles, 5 of which you can access for free at:
http://cancerres.aacrjournals.org/content/70/23/9671.full.html#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
/content/70/23/9671.full.html#related-urls

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.