mda-7/IL-24 Expression Inhibits Breast Cancer Through Up-regulation of Growth Arrest-Specific Gene 3 (gas3) and Disruption of β1 Integrin Function

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Li et al., mda-7/IL-24 suppresses breast cancer through up-regulation of GAS3

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Abstract

MDA-7/IL-24, a member of the IL10 family of cytokines, inhibits growth of various human cancer cells, yet the underlying mechanism is largely unknown. Here we report that mda-7/IL-24 efficiently suppresses the development of rat mammary tumors in vivo. Microarray analysis for genes differentially expressed in rat mammary tumor cells over-expressing MDA-7/IL-24 compared to those that do not express this cytokine identified growth arrest-specific gene-3 (gas3) as a target for mda-7/IL-24. Up-regulation of gas3 by mda-7/IL-24 was STAT3-dependent. Induction of gas3 inhibited attachment and proliferation of tumor cells in vitro and in vivo by inhibiting the interaction of β1 integrin with fibronectin. A mutated GAS3, which is unable to bind β1 integrin, was also unable to inhibit fibronectin-mediated attachment and cell growth both in adherent and suspension cultures, suggesting that GAS3 exerts its effects through interaction with and regulation of β1 integrin. Thus, mda-7/IL-24 inhibits breast cancer growth, at least in part, through up-regulation of GAS3 and disruption of β1 integrin function. Importantly, the expression of the mda-7/IL-24 receptor, IL-20R1, is highly correlated with GAS3 expression in human breast cancer (p=1.02x10^-9), and the incidence of metastases is significantly reduced in HER2+ breast cancer patients expressing high levels IL-20R1. Together, our results identify a novel MDA-7/IL-24-GAS3-β1integrin-fibronectin signaling pathway that suppresses breast cancer growth and can be targeted for therapy.
Introduction

Breast cancers are highly heterogeneous and include luminal-like tumors expressing the estrogen receptor (ER), HER2-like overexpressing HER2 receptor, and basal-like subtypes that do not express these receptors (triple negative) (1, 2). While chemotherapy plus hormone therapy (tamoxifen, aromatase inhibitors) or therapies that target HER2 (trastuzumab/Herceptin) significantly reduce the incidence of relapse, most women who develop metastases eventually succumb to the disease. There is, therefore, a great need for novel drugs that can inhibit tumor growth by attacking different vulnerabilities of breast cancer cells. Melanoma differentiation-associated gene-7, (mda-7) initially identified by differentiation induction subtraction hybridization (DISH) from human melanoma cells, is a member of the IL-10 family of cytokines, now known as mda-7/IL-24 (3). The activity of MDA-7/IL-24 as a growth suppressor in melanoma and other cancer cells was first demonstrated in 1996 (4). Subsequently, ectopic expression of this cytokine was shown to lead to irreversible growth inhibition, reversal of the malignant phenotype, and terminal differentiation in a wide variety of solid tumors including breast cancer (4, 5). Interestingly, these effects are not elicited in the normal cellular counterparts of these tumors (6). Moreover, the ability of MDA-7/IL-24 to induce bystander cancer-specific cell killing has been confirmed in vivo in a range of human xenograft studies in nude mice and in phase I clinical trials involving patients with melanomas and other advanced solid cancers (3, 7, 8). Although apoptosis induced by MDA-7/IL-24 involves binding of the cytokine to the IL-20R1/IL-20R2 heterodimeric receptor complex, it can also occur through direct intracellular pathways involving binding to BiP/GRP78 and translocation to the endoplasmic reticulum resulting in an ‘unfolded protein stress response’ (8-10). The exact mechanism underlying the
wide-spectrum, anti-cancer activity of mda-7/IL-24 is not known and may be cancer-specific, depending on pre-existing genetic and epigenetic alterations (3).

Most strains of rats used experimentally such as Wistar-Furth (WF), develop multiple mammary adenocarcinomas following initiation with a mammary carcinogen, whereas a few strains such as the Copenhagen (Cop) are resistant to the development of mammary tumors (11). To investigate these differences in susceptibility, we previously isolated cell lines from tumors induced in resistant Cop x Wistar-Furth F1 rats by infusion of a retrovirus harboring the v-Has-ras gene directly into the main mammary ducts (12). Some of these cell lines grew in soft agar, while others display anchorage-dependent growth. Through microarray analysis, we found that expression of MDA-7/IL-24 and β4 integrin were inversely correlated with the ability to grow in soft agar (13). Ectopic expression of mda-7/IL-24 in anchorage-independent cells inhibited growth in monolayer culture and in soft agar, their ability to migrate and invade in vitro, and the development of tumors in nude mice. We showed that while β4 integrin did not play a direct role in regulating cell growth, it was a downstream target of MDA-7/IL-24, acting in concert with the latter to suppress cell proliferation. Moreover, we showed that growth suppression by MDA-7/IL-24 was associated with up-regulation of p27Kip1 via activation of STAT3 (13).

Here we show that mammary tumorigenesis in rats can be blocked by infection with an adenovirus expressing mda-7/IL-24. We demonstrate that growth arrest-specific gene 3 (GAS3/PMP22), is induced by mda-7/IL-24, and that GAS3 inhibits the attachment and proliferation of tumor cells, at least in part, by blocking the interaction of β1 integrin with fibronectin. Moreover, we show that primary human breast cancers exhibit a positive correlation between expression of the IL-24 receptor and GAS3 and that receptor expression correlates with metastasis free survival. Thus, our data identify a novel inhibitory pathway downstream of mda-7/IL-24, which is important in breast cancer spread and can be targeted for therapy.
Materials and Methods

Tumor induction

Twenty-four 49 day-old Wistar-Furth female rats were intraperitoneally injected with 75 mg/kg methylnitrosurea (MNU) in 0.05% acetic acid. Thirty days post-injection we infused via the nipples, the main ducts of thoracic, abdominal or inguinal mammary glands of 12 rats (5-7 glands infused/rat) with 15 μl of a solution containing ~10^8 pfu of adenovirus containing the human mda-7/IL-24 gene, 8 mg/kg polybrene and 2 g/ml indigo carmine according to our previously published protocol (12). Glands of 12 control rats were similarly infused with ~10^8 pfu of empty adenovirus. The rats were palpated weekly and tumors were harvested when they were >20 mm in diameter or when the animals appeared moribund. A portion of each tumor was fixed in formalin for histological analysis.

Growth of cells in nude mice

Four groups of six female athymic nude mice (CD1 – Nu/Nu), 7 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA, USA). After a week of acclimatization, 2x10^6 FE1.2 cells expressing GAS3 or vector alone, in a volume of 50 μl, were injected subcutaneously. Tumor size was measured twice weekly with vernier calipers by a single-blinded observer. Tumor growth was measured and plotted according to the non-parametric Kaplan-Meier analysis. Tumors were harvested 2 weeks post-injection.

Cell culture

The establishment of the rat breast cancer cell lines was described previously (12). All cell lines were maintained in α-minimum essential medium (α-MEM), supplemented with 10% fetal bovine serum (FBS), 10 ng/ml epithelial growth factor, 1 μg/ml hydrocortisone and 1μg/ml 17-β-estradiol (Sigma-Aldrich, St Louis, MO). All culture experiments were repeated 3 times. To examine growth rates, 1x10^4 cells were seeded per well on 24-well plates and counted for 4
consecutive days. To examine the regulation of GAS3 by MDA-7/IL-24, FE1.2-IL-24 cells treated with 10 or 20 μM of the STAT3 inhibitor AG9, as previously described (13).

**Attachment assays**

Attachment assays were performed on tissue culture plates or on petri dishes pre-coated with fibronectin (20μg/ml) or laminin (20μg/ml). Cells (1x10⁴/ml) were plated in triplicate and counted and photographed 2 or 24 h post-culture. In some experiments, the STAT3 inhibitor AG9 (Calbiochem, Millipore, Ontario) was added to the culture medium of the attached cells and they were photographed 4 h later. The mean percentage of the attached versus the round cells was determined for at least 20 fields of view.

**Growth of cells in soft agar**

To monitor growth of cells in soft agar, two layers of agarose were used. The bottom layer contained 0.5% agarose, the top layer 0.3% agarose, both in α-MEM. Triplicate samples of 5000 cells were seeded on 6 cm plates and incubated for 5-7 days. At the end of the incubation period, colonies with > 25 cells were counted.

**Western blotting**

Western blotting was performed either as we have described previously (13), or for GAS3, as described (32). Polyclonal rabbit anti-rat antibodies were obtained from the following sources: rabbit anti-rat GAS3 (Abcam, Cambridge, MA) used at a dilution of 1:500; rabbit anti-rat IL-24 (GenHunter, Nashville, TN) used at a dilution of 1:1000; β-actin (Sigma, St Louis, MO) used at a dilution of 1/50,000; pan-cadherin (Cell Signaling Technology, Danvers, MA) used at a dilution of 1:1000. Protein band densities were determined with the ChemiGenius² Bio Imaging System using the Gene Tools Software v4.02 (Syngene, Frederick, MD).

**Immunoprecipitation**
Li et al., mda-7/IL-24 suppresses breast cancer through up-regulation of GAS3

Semi-confluent FE1.2, FE1.2+GAS3 or FE1.2+GAS3GM cells were lysed with NP-40 lysis buffer (1% Nonidet P-40, 2mM phenylmethlsulfonyl, fluoride, 10μg/mL aprotinin, 2μg/mL pepstatin, 10mM iodoacetamide, 0.1mM EDTA, 0.1mM EGTA, 10mM HEPES, and 10mM KCl). Two μg of anti-β1 integrin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to 0.5 mg of cell lysates in 1ml and shaken at 4C overnight. Protein-A beads were then added to the mixture and shaking continued for additional 2 h. The beads were then washed 3 times with NP-40 buffer, boiled and proteins separated on 10% SDS-PAGE gel and Western blots prepared using GAS3 and β1 antibodies. To show loading and input controls, Western blots were also prepared from the total extracts (20 μg) using β-actin and GAS3 antibodies.

**Immunohistochemistry**

Immunohistochemistry was performed on cells grown on cover slips overnight. Cells were fixed in 4% paraformaldehyde (PFA) and blocked using 10% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA). Triplicate cover slips were incubated with 1:500 polyclonal rabbit GAS3 antibody (Novus Biologicals, Littleton, CO) overnight at 4°C. Control cells were incubated with buffer only. The next day, the cover slips were incubated with 1:1000 biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA), for 1 h at room temperature. ABC complex (Vector Laboratories) was then applied to the cover slips for 30 min at room temperature, followed by DAB for 2 min (Vector Laboratories) and counter-staining with Hematoxylin. Cells were dehydrated prior to mounting using xylene base mounting medium.

**Infection and transfection**

A gas3-producing retrovirus was generated by inserting the entire 658bp coding sequence of rat gas3 in either sense or anti-sense orientations into a unique EcoR1 site in the retroviral
expression vector MSCV2.1 (13). Replication-defective viruses were prepared by transfecting the viral plasmid into the helper-free packaging cell line GP+A (B8) as described previously (13). For viral infection, supernatants from the virus-producing cells were used to infect FE1.2 cells, plated at a density of 2x10^6. After 48 h, G418-resistant cells were pooled and subjected to subcloning by limited dilution. Transfection was performed using Lipoctamine 2000 according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA).

**Fibronectin siRNA transfection**

Cells were transiently transfected with 2ug of siRNA against fibronectin (Gene Pharma, Shanghai, China) or control oligonucleotides for 6h according to the manufacturer’s instructions, as previously described (33). Fresh medium containing 10% FBS was then added to the transfected cultures and they were maintained overnight, spun down then seeded onto 6-well plates and incubated for 30 min. Attached cells were counted.

**Primers**

*mda-7/IL-24* primers: Forward; ACG GCC AGG TCA TCA CTA TTG, Reverse; AGG TCT TTA CGG ATG TCA ACG, β-actin primers: Forward; GCA ACC CAG TGT GAG TAG CA, Reverse; GAT GGT GTC CCC TGC ATT AC. *Gas3* primers and controls were supplied by Applied Biosystems (Foster City, CA; Rn005668351-AI PMP22, Lot # 282134).

**Statistical analysis**

Statistical analyses were performed using the two-tailed Student’s t-test or ANOVA with significance considered at p<0.05 using Origin 3.5 software (Microcal Software, Northampton, MA). Values are reported as mean ± standard error (SE). The correlation between IL-20R1 and GAS3 genes was assessed by Pearson's Correlation method with median-centered, log-2 transformed and RMA normalized microarray gene expression values. Kaplan-Meier curves and Hazard ratios was generated as described (34).
Li et al., *mda-7/IL-24 suppresses breast cancer through up-regulation of GAS3*

## Results

*Inhibition of rat mammary carcinogenesis by an adenovirus harboring mda-7/IL-24*

We have previously shown that *mda-7/IL-24* expression blocks the proliferation of rat mammary tumor cells *in vitro* (13). To determine if *mda-7/IL-24* can inhibit tumor development *in vivo*, 24 female Wistar-Furth rats were intraperitoneally injected with the mammary carcinogen methylnitrosourea (MNU; 75 mg/kg) (12). Thirty days later, when we have shown that the mammary glands of the carcinogen-treated rats contain a significant number of preneoplastic lesions (14), 12 rats were infused with a recombinant adenovirus expressing the human *mda-7/IL-24* gene into the main ducts of thoracic, abdominal or inguinal mammary glands (~10^8 plaque-forming units (pfu), 5-7 glands injected/rat). The other 12 control rats were infused with empty adenovirus (~10^8 pfu). Tumors were first palpated in the control rats at about 6 weeks following MNU administration and were harvested over the following 20 weeks when tumors reached ~2.5 cm in diameter. Six of the control rats developed tumors in this time period (Supplemental Table I). Histological analysis showed that all tumors were typical adenocarcinomas (12). In contrast, during the same time period, no mammary tumors developed in the glands of rats infused with the *mda-7/IL-24*-containing adenovirus (significantly different from controls, *p*<0.005 by \chi^2 analysis). Interestingly, two tumors developed in these rats in glands that had not been infused with adenovirus-*mda-7/IL-24*. These results demonstrate that *mda-7/IL-24* expression inhibits breast cancer development *in vivo*.

*mda-7/IL-24 induces GAS3 expression*

We previously showed that proliferation and anchorage-independent growth of FE1.2 tumor cells were readily suppressed by MDA-7/IL-24 (12). To begin to address the mechanism of this growth inhibition, four independent RNA isolates of FE1.2 cells expressing the *mda-7/IL-
Li et al., mda-7/IL-24 suppresses breast cancer through up-regulation of GAS3

24 gene in the sense (FE1.2+IL-24S) or antisense (FE1.2+IL-24AS) orientation (13) were subjected to microarray analysis (44k Whole Rat Genome arrays, Agilent Technologies). A number of genes were consistently upregulated or down-regulated in the cells expressing the sense mda-7/IL-24 versus the anti-sense controls (Supplemental Table II). Of particular interest was the 27.8-fold up-regulation of gas3, a gene initially identified in quiescent, serum-starved NIH-3T3 mouse fibroblasts (15). We also observed a 10.2-fold increase in expression of β4 integrin, which we previously showed to be upregulated by mda-7/IL-24 (13). We confirmed MDA-7/IL-24-induced expression of gas3 in FE1.2 cells by quantitative real-time PCR (Fig. 1A) and immunoblotting (Fig. 1B). Furthermore, we demonstrated that the levels of gas3 RNA and protein were significantly higher in anchorage-dependent FE1.3 cells, which expressed high levels of mda-7/IL-24 (13), than in FE1.2 cells, which express negligible amounts of this cytokine (Fig. 1). Thus, these studies identify GAS3 as a target of mda-7/IL-24 both at the RNA and protein levels in mammary tumor cells.

GAS3 expression suppresses the growth of rat mammary tumor cells in monolayer culture and in soft agar

We first showed that growth arrest induced by serum starvation induced GAS3 expression in both FE1.2 (28-fold) and FE1.3 (10-fold) cells (Supplemental Fig. 1). To determine directly the effect of gas3 expression on FE1.2 cell growth, we infected these cells with a retrovirus (MSCV2.1) expressing gas3 (FE1.2+GAS3) or vector control (FE1.2+Vector). Western blot analysis confirmed high expression of GAS3 only in FE1.2+GAS3 cells (Fig. 2A). When cultured in vitro, FE1.2+GAS3 cells displayed a reduced ability to proliferate in adherent, monolayer cultures compared to FE1.2+Vector cells (Fig. 2B). GAS3 expression also significantly suppressed the number of colonies that grew in soft agar compared to control cells.
Li et al.,  mda-7/IL-24 suppresses breast cancer through up-regulation of GAS3

(Fig. 2C). Importantly, gas3-mediated growth inhibition of FE1.2 cells was also observed in vivo when FE1.2+GAS3 or FE1.2+Vector cells were transplanted subcutaneously into nude mice (Fig. 2D).

To test for dependency of mda-7/IL-24-induced suppression of cell proliferation on gas3, we knocked-down gas3 expression by siRNA in FE1.2+IL-24S cells which overexpress GAS3 (Supplemental Fig. 2A). Stable expression of gas3 siRNA led to a 50% knockdown of GAS3 expression (Supplemental Fig. 2A/B). This partial knockdown of GAS3 resulted in a significant increase in the number of colonies formed in soft agar compared to parental cells (Supplemental Fig. 2C).

To identify the mechanism by which MDA-7/IL-24 induces GAS3, we treated FE1-2+IL-24 cells with an inhibitor of STAT3, which we previously showed to mediate growth inhibition by MDA-7/IL-24 through up-regulation of p27Kip1 (13). The FE1.2+IL-24 cells treated with 10 or 20 μM of the STAT3 inhibitor AG9 exhibited a 43% and 59% reduction, respectively, in GAS3 expression (Fig. 2E). Addition of AG9 to the culture of the GAS3 over-expressing FE1.2+GAS3 cells also significantly inhibited attachment to the plates (Supplemental Figures 3A and B). Thus, MDA-7/IL-24 induces GAS3 and promotes attachment through a STAT3-dependent pathway. Together, these results define GAS3 as a critical mediator of mda-7/IL-24-induced growth suppression of rat mammary tumor cells and indicate that GAS3 expression alone suffices to inhibit cell proliferation both in vitro and in vivo.

GAS3 growth inhibition is mediated through its binding to β1 integrin and disruption of interaction with the β1 ligand fibronectin

Next, we sought to determine the downstream target of the MDA-7/IL-24/GAS3 pathway. We noticed that under normal growth conditions, FE1.2+vector control cells displayed
Li et al.,  mda-7/IL-24 suppresses breast cancer through up-regulation of GAS3

a flattened morphology (Fig. 3) and remained alive after becoming fully confluent. In contrast, FE1.2+GAS3 cells proliferated slowly (Fig. 2B), displayed rounded morphology (Fig. 3) and rapidly died when they reached semi-confluent growth (Supplemental Fig. 4), suggesting that GAS3 expression may alter cell attachment. Indeed, GAS3 directly binds β1 integrin as revealed by immunoprecipitation (Supplemental Fig. 5). The interaction was specific to β1 integrin since we observed no binding of GAS3 to β4 integrin (data not shown), even though we previously demonstrated induction of β4 integrin by MDA-7/IL-24 (13). Our observation of GAS3-β1 integrin interaction is consistent with a previous report suggesting that GAS3 might affect cell-cell interaction through this integrin (16).

To examine whether GAS3 expression alters cellular attachment, FE1.2+GAS3 or control cells were seeded at the same density onto Petri dishes coated with fibronectin, the ligand for β1 integrin (17). In control uncoated dishes, minimal attachment was observed by either FE1.2+GAS3 or control cells (Fig. 3). Fibronectin coating resulted in a dramatic increase in cell attachment for FE1.2+vector cells (86% ± 4) compared to FE1.2+GAS3 cells (56% ± 4) two hours after plating (Supplemental Fig. 6A). Due to the shorter doubling time of control cells, there was a further increase in their number compared to the FE1.2+GAS3 cells 24 hours post-plating (Supplemental Fig. 6B). FE1.2 cells expressing a glycosylated mutant of GAS3 (designated GAS3GM and described in more detail below) had attachment and proliferation levels similar to FE1.2+Vector cells (Supplemental Fig. 6A and B). In contrast to the differential growth on fibronectin-coated plates, FE1.2+GAS3 cells attached as well or even better than control cells to plates coated with laminin, the ligand for the β4 integrin (Fig. 3; Supplemental Fig. 7). These results suggest that GAS3 expression in mammary cancer cells suppresses cell proliferation and cell adhesion by binding to and blocking the interaction of β1 integrin with fibronectin. Interestingly, Western blot analysis revealed a significant upregulation of
endogenous fibronectin expression in FE1.2+GAS3 cells compared to FE1.2+vector cells (Fig. 4A). However, the level of fibronectin released into the medium by GAS3 overexpressing cells was much lower than control cells (Fig. 4B), suggesting that GAS3 interaction with β1 integrin inhibits secretion of fibronectin, resulting in intracellular accumulation of this factor. To test the effect of controlled fibronectin downregulation on the growth of FE1.2 cells, we knocked down fibronectin expression using three independent siRNA treatments (Fig. 4C). These siRNAs significantly inhibited attachment of cells overexpressing GAS3 as well as control cells in proportion to the knockdown efficiency of fibronectin expression (Fig. 4D). Our results suggest that GAS3 inhibits cell attachment through two complementary mechanisms: reduction in endogenous fibronectin secretion and reduction in β1 integrin interaction with exogenous fibronectin.

*Mutation within an N-linked glycosylated site of GAS3 restores fibronectin-mediated attachment and growth retardation in culture*

The GAS3 protein has been previously reported to contain three consensus motifs for N-linked glycosylation (18). To determine the role of GAS3 glycosylation on cell growth inhibitory activity and the interaction of fibronectin with β1 integrin, we generated a GAS3 mutant allele carrying NCT to EEE substitutions at amino acid positions 41-43 (GAS3GM). This allele was cloned into a retroviral vector and transduced into FE1.2 cells. As shown in Fig. 5A, the GAS3GM protein migrated slightly faster than native GAS3, presumably because glycosylation retards migration through PAGE. Immunohistochemical analysis demonstrated that GAS3 expression was similar in both FE1.3 and FE1.2+GAS3GM cells and was substantially higher than in FE1.2 cells (Supplemental Fig. 8). FE1.2+GAS3GM, FE1.2+GAS3
and FE1.2+vector cells were then plated on uncoated and fibronectin-coated dishes and cell attachment was assessed 24 h later. As shown in Fig. 5B and Supplemental Fig. 6B, FE1.2+GAS3GM and FE1.2+vector cells attached with similar efficiency to fibronectin-coated dishes whereas FE1.2+GAS3 cells failed to attach. Moreover, the glycosylation mutant allele failed to suppress cell growth *in vitro* or in soft agar and cells did not die upon reaching confluency (Fig. 6A-C and Supplemental Fig. 6A). Consistent with these results, we showed that glycosylation defective GAS3 failed to bind \( \beta_1 \) integrin (Fig. 5A). These results suggest that glycosylation of GAS3 is critical for its binding to \( \beta_1 \) integrin and its ability to inhibit attachment to fibronectin, tumor cell growth and survival.

*Expression of mda-7/IL-24 receptor IL-20R1 correlates with GAS3 expression and predicts clinical outcome of breast cancer patients*

To extend our studies to human cancer, we first tested the effect of *mda-7/IL-24* on GAS3 expression and growth of the human breast cancer cell line MD-MBA-231 and MD-MBA-435 (19). After transducing these cells with the retroviral vector encoding *mda-7/IL-24*, GAS3 expression was strongly induced (Fig. 7A, left). Furthermore, growth of both cell lines was significantly reduced (Fig. 7A, right), in agreement with other reports using human melanoma cells (4, 5, 20) and our results with rat mammary tumor cells (Fig. 2).

Based on these results, we predicted that expression of MDA-7/IL-24 or its receptor IL-20R1/IL-20R2 (type 1 IL-20 receptor) (10) may correlate with expression of GAS3 and may lead to better clinical outcome for breast cancer patients. To test this notion, we examined several publicly available breast cancer cohorts with microarray and metastasis-free survival data (GSE2603, GSE5327, GSE6532, and GSE11121; PubMed, GEO-DataSets). Although
expression of MDA-7/IL-24 was not correlated with GAS3 (data not shown), expression of IL-20R1 and GAS3 were highly correlated, with R=0.24 ($p=10^{-9}$) for all breast cancer patients and R=0.26 ($p=0.002$) for HER2+ breast cancer patients (Fig. 7B). Next, we grouped the tumors on the basis of IL-20R1 expression. Approximately 27% (174 of 633) of patients expressed 2-fold or more IL-20R1 in these cohorts. A Kaplan-Meier analysis revealed that the incidence of metastasis was significantly reduced for patients expressing high levels of IL-20R1 compared to those with lower IL-20R1 levels ($p=0.023$, 95% CI: 1.07 - 2.38) with hazard ratio (HR) of 1.59 (Fig. 7C, left). Since Ha-ras is activated in MNU-induced rat mammary tumors (21, 22), we asked whether the risk of metastasis further increased in the low IL-20R1 subgroup in HER2+ breast cancers in which ras is activated. Indeed, patients with 2-fold higher HER2 expression and low IL-20R1 expression had a significantly worse prognosis, with an HR of 2.22, compared to those with low HER2 and high IL-20R1 expression ($p=0.0485$) (Fig. 7C, right). Thus, expression of the mda-7/IL-24 receptor correlates with GAS3 expression and reduced incidence of metastasis.

Discussion

Cancer development is a multistep process associated with the accumulation of mutations in various oncogenes and tumor suppressor genes. In addition to these genetic events, epigenetic and microenvironmental factors contribute significantly to this process (23). Resistance to cancer development has been reported for a number of different tissues in rats and is linked to distinct loci (11). We previously showed that mda-7/IL-24 suppresses the growth of rat mammary carcinoma cells and may play a role in the resistance of some strains of rats to mammary carcinogenesis (13). In the present study, we provide direct in vivo evidence to support
Li et al.,  

*mda-7/IL-24 suppresses breast cancer through up-regulation of GAS3*  

these observations through administration of an adenovirus transducing *mda-7/IL-24* into the main mammary duct of Wistar-Furth rats that are highly susceptible to MNU-induced mammary carcinogenesis. We observed a total inhibition of breast cancer development in these rats. Since we treated the rats with the adenovirus at a time when the mammary glands contain significant numbers of preneoplastic lesions, our results suggest that *mda-7/IL-24* suppresses the progression of preneoplastic cells into more advanced lesions and, ultimately, adenocarcinomas. This suggests that *mda-7/IL-24* is a cancer resistance gene that may have preventive/therapeutic potential.

The inhibitory effect of *mda-7/IL-24* on the growth of various human cancers including melanoma and breast cancer has been previously documented (3). Moreover, a phase I clinical trial has recently validated the potential utility of this cytokine as a therapeutic agent (7, 24). By analyzing metastasis-free survival data in a large cohort of breast cancer patients, we have now identified a two-fold survival advantage in tumor biopsies expressing high levels of the MDA-7/IL-24 receptor IL-20R1. While this study did not reveal a correlation with MDA-7/IL-24 expression itself, higher levels of IL-20R1 expression in cancer cells could activate more efficient signaling downstream of the receptor leading to the significant survival benefit we observed. This finding was supported by a higher expression of the downstream effector GAS3 in patients expressing higher levels of IL-20R1. Moreover, the ability of exogenously administered His-MDA-7/IL-24 protein to induce stabilization of *mda-7/IL-24* mRNA and production of MDA-7/IL-24 protein in both cancer and normal cells, followed by secretion of the protein, supports its ‘bystander anti-tumor’ functions and may contribute to promotion of enhanced local and systemic anti-tumor effects *in vivo* (8).
Li et al., mda-7/IL-24 suppresses breast cancer through up-regulation of GAS3

To understand how mda-7/IL-24 signaling inhibits the growth of breast cancer, we performed a microarray analysis of genes induced when mda-7/IL-24 was over-expressed in our anchorage-independent rat mammary cell line FE1.2. We showed that mda-7/IL-24 expression led to an almost 30-fold upregulation of the gas3 gene. We confirmed this induction at the protein level and showed that it was STAT3-dependent. GAS3 was originally shown to be associated with growth arrest in serum-starved NIH-3T3 fibroblasts (15). Gas3 expression is driven by two alternate promoters P1 and P2, which drive transcription of two transcripts 1A and 1B respectively, containing different non-coding exons (25). Both transcripts yield identical proteins, but the two promoters confer tissue-specific control of expression (26). P1 leads to expression in the peripheral and central nervous systems and is thought to be important for myelin formation (27, 28). Amplification of the gas3/PMP22 locus is associated with the autosomal dominant Charcot-Marie-Tooth disease (29). Our results suggest it is important to test whether GAS3 and the downstream pathway described here are also involved in this progressive neuropathic syndrome.

We demonstrated that over-expression of GAS3 in rat mammary cancer cells that normally express negligible amounts of this protein, inhibits their proliferation in culture and growth in nude mice. Thus, we have demonstrated for the first time that the inhibition of cancer cell growth by mda-7/IL-24 is mediated, at least in part, through up-regulation of GAS3. Consistent with a previous study (16), we have shown that GAS3 binds to β1 integrin, and demonstrated that this interaction depends on N-glycosylation of GAS3. Moreover, we showed that this binding blocks the interaction of β1 integrin with its ligand fibronectin. We also demonstrated that siRNA-mediated down-regulation of fibronectin reduced survival of breast cancer cells, suggesting that binding of GAS3 to β1 integrin plays a critical role in its ability to inhibit cancer cell growth. Our results are consistent with previous observations in which
peptides containing the integrin recognition sequence RGD, can inhibit metastasis of mouse melanoma cells by blocking α4-β1 interaction (18) and that the tumor-suppressive activity of mda-7/IL-24 can be significantly increased when the RGD sequence is fused in frame with the mda-7/IL-24 gene (30).

Surprisingly, we showed that mammary cancer cells expressing a glycosylation mutant form of GAS3 bind better to fibronectin coated plates and exhibit a higher growth rate in soft agar than cells expressing native GAS3. This additional growth potential may stem from the ability of mutant GAS3 to compete out inhibitory effects of the remaining wild-type GAS3. Drugs that inhibit N-linked glycosylation have been investigated for the treatment of cancers because of their ability to block the function of genes that promote tumor growth (31). Our results, however, showed unexpectedly, an opposite effect on tumor cell growth after mutating the glycosylation site of GAS3, suggesting that specific activation of GAS3 glycosylation may promote mammary tumor cell growth.

In summary, we have demonstrated that signaling mediated through MDA-7/IL-24 and its receptors inhibits mammary tumor growth. We provide evidence that growth inhibition by MDA-7/IL-24 is mediated, at least in part, through activation of GAS3. The association of GAS3 and β1 integrin results in the disruption of the binding of β1 to fibronectin, thereby inhibiting cell attachment and proliferation. Our results strengthen the notion that mda-7/IL-24 has cancer preventive/therapeutic potential and elucidate downstream tumor suppressor signaling involving GAS3, β1 integrin and fibronectin. Finally, our observation that expression of the MDA-7/IL-24 receptor correlates with reduced metastasis suggests that therapeutic activation of components of this pathway may improve clinical outcome for breast cancer patients.

Disclosure of Potential Conflicts of interest:
No potential conflicts of interest were disclosed.

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References


Li et al., mda-7/IL-24 suppresses breast cancer through up-regulation of GAS3


FIGURE LEGENDS

FIGURE 1. GAS3 expression in rat mammary tumor cells. (A) Real-time PCR analysis of FE1.2 and FE1.3 cells and FE1.2 cells infected with retrovirus containing mda-7/IL-24 in the sense (FE1.2+IL-24S) or antisense (FE1.2+IL-24AS) orientation, * p<0.001. (B) GAS3 protein expression in the indicated cells.

FIGURE 2. GAS3 expression suppresses growth of FE1.2 cells in monolayer culture, in soft agar and in vivo in nude mice. (A) Western blot analysis of GAS3 expression in FE1.2 cells infected with retrovirus containing GAS3 (FE1.2+GAS3) or vector control (FE1.2+vector). (B) Growth analysis of transduced FEI.2 cells in monolayer culture. (C) Number of colonies formed in soft agar. (D) Growth of cells in nude mice, * p<0.05. (E) Transduced FE1.2 cells were treated with the indicated doses of the STAT3 inhibitor AG9 for 24 hours then subjected to Western blot analysis. Band density was determined using the ChemiGenius2 Bio Imaging System and Gene Tools Software.

FIGURE 3. GAS3 suppresses binding of cells to fibronectin. FE1.2+GAS3 or FE1.2+vector cells (1x10^4) were plated on tissue culture plates or petri dishes with and without fibronectin or laminin coating. Cells were incubated for 24 hours then photographed as indicated.
FIGURE 4. Expression of GAS3 blocks secretion of fibronectin and inhibits attachment. (A) Western blot of fibronectin (FN) expression in FE1.2 vector control and GAS3 transduced cells. (B) Western blot of fibronectin (FN) in the culture medium of FE1.2 vector control and GAS3 transduced cells. (C) Western blots of fibronectin in FE1.2 vector control and GAS3 transduced cells transfected with three different fibronectin siRNA oligonucleotides. (D) Attachment of vector control and GAS3 transduced FE1.2 cells transfected with the indicated fibronectin siRNAs, to tissue culture plates, * $p<0.01$.

FIGURE 5. Mutations within the glycosylation motif of GAS3 (GAS3GM) abolish immunoprecipitation with β1 integrin and suppression of binding of FE1.2+GAS3 cells to fibronectin. (A) Extracts (1mg) from the indicated cells immunoprecipitated (IP) using β1 integrin antibody were subjected to Western blot analysis using GAS3 antibody; total extracts (20 μg) from these cells were also subject to Western blot analysis using GAS3 or β-actin antibodies. (B) FE1.2+GAS3, FE1.2+GAS3GM or FE1.2+vector cells (1x10⁴) were plated on petri dishes with no coating or coated with fibronectin. Cells were incubated for 24 h and photographed at 10 or 20X magnification.

FIGURE 6. N-linked glycosylation mutant of GAS3 (GAS3GM) abolishes its growth suppressing activity and interaction with integrin β1. (A) Triplicate cultures (1x10⁴) of FE1.2+GAS3, FE1.2+GAS3GM and FE1.2+vector cells grown for the indicated days and counted after trypsinization. (B) Colony numbers of FE1.2+vector, FE1.2+GAS3GM and FE1.2+GAS3 cells grown in soft agar, * $p<0.01$ (C) Cells (1x10³) were grown in soft agar and photographed with or without Coomassie-blue staining after a one week incubation. Magnification 5X.
**FIGURE 7.** MDA-7/IL-24 suppresses growth of human breast cancer and melanoma cell lines in culture, and expression of IL-20R1 correlates with expression of GAS3 and affects clinical outcome of breast cancer patients. (A) Left: Western blotting analysis of MDA-7/IL-24 and GAS3 expression in human breast cancer MDA-MB-231 and melanoma MDA-MB-435 cell lines, β-actin was used as loading control. Right: Growth of MDA-MB-231+MDA-7/IL-24, MDA-MB-231+vector, MDA-MB-435+MDA-7/IL-24 and MDA-MB-435+vector cells during the indicated time period in culture. (B) Positive and significant correlations between expression of IL-20R1 and GAS3 in breast cancer patients. (C) Patients with 2 fold or more IL-20R1 expression above median (IL-20R1+) were selected and compared to the rest of the population (IL-20R1−). Significant increase in metastasis-free survival (MFS) was observed for all IL-20R1+ breast cancer patients (*left*, HR=1.59; IL-20R1+, n=174, IL-20R1−, n=459) or the HER2+ population (*right*, HR=2.22; IL-20R1+, n=29, IL-20R1−, n=104).
Figure 1

A

Gas3 Expression (Fold Change)

FE 1.2  FE 1.3  FE1.2+ IL-24 AS  FE1.2+ IL-24 S

B

FE1.2  FE1.3  FE1.2+IL-24 AS  FE1.2+IL-24 S

GAS3

Pan-Cadherin
Figure 2

A) Western blot analysis of GAS3 and Pan-Cadherin expression in Vector and GAS3 transfected cells.

B) Cell count over days for GAS3 and Vector transfected cells.

C) Colony numbers for FE1.2+Vector and FE1.2+GAS3 transfected cells.

D) Tumor volume for FE1.2+Vector and FE1.2+GAS3 transfected cells.

E) Western blot analysis of GAS3 and β-actin expression under different conditions.

* indicates statistical significance.
Figure 3

<table>
<thead>
<tr>
<th>Cells plated</th>
<th>Petri dishes</th>
<th>Tissue culture dishes</th>
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<tbody>
<tr>
<td></td>
<td>No coating</td>
<td>fibronectin</td>
</tr>
<tr>
<td>FE1.2+GAS3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10X</td>
<td></td>
<td></td>
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<tr>
<td>FE1.2+Vector</td>
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<td></td>
</tr>
<tr>
<td>10X</td>
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<tr>
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</tr>
</tbody>
</table>
Figure 4

(A) Western blot of fibronectin (FN) and β-actin in cells treated with Vector or GAS3.

(B) Western blot of fibronectin (FN) and β-actin in FE1.2+GAS3 and FE1.2+Vector cells.

(C) Western blot showing the effect of fibronectin siRNA on FN expression in FE1.2+GAS3 and FE1.2+Vector cells.

(D) Graph showing the number of cells (x10^4 cells/ml) for FE1.2+Vector and FE1.2+GAS3 treated with control or fibronectin siRNA.
Figure 5

A

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<tr>
<th>FE1.2+Vector</th>
<th>FE1.2+GAS3GM</th>
<th>FE1.2+GAS3</th>
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<tbody>
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<td>IP/β1</td>
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<tr>
<td>GAS3</td>
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<tr>
<td>Total extract</td>
<td>GAS3</td>
<td>β-actin</td>
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</table>

B

<table>
<thead>
<tr>
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<th>fibronectin</th>
<th>No coating</th>
<th>fibronectin</th>
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<tbody>
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</tr>
<tr>
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<td></td>
<td></td>
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</tr>
<tr>
<td>FE1.2+GAS3GM</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

10x 20x
Figure 6

A

Cell Number (x10^4)

Days

0 1 2 3

- FE1.2+Vector
- FE1.2+GAS3GM
- FE1.2+GAS3

B

Colony number

FE1.2+ Vector
FE1.2+ GAS3GM
FE1.2+ GAS3

C

Colonies 5x

FE1.2+GAS3
FE1.2+Vector
FE1.2+GAS3GM

Coomassie Blue

FE1.2+GAS3
FE1.2+Vector
FE1.2+GAS3GM
Figure 7

A

MDA-MB-435  MDA-MB-231

FE1.3  vector  MDA-7/IL-24  vector  MDA-7/IL-24

IL-24

GAS3

β-actin

B

All Patients

HER2+

GAS3

r = 0.24

p = 1.02 x 10^{-9}

IL20R1

HER2+

GAS3

r = 0.26

p = 0.00226

IL20R1

C

All Patients

HER2+

95% CI: 1.07 - 2.38

p = 0.0228

Accessibility:

Metastasis-Free Survival (%)

95% CI: 0.86 - 5.88

p = 0.0485

Months

30  90  150  270

IL20R1-

IL20R1+
Molecular Cancer Research

mda-7/IL-24 Expression Inhibits Breast Cancer Through Up-regulation of Growth Arrest-Specific Gene 3 (gas3) and Disruption of β1 Integrin Function

You-Jun Li, Guodong Liu, Yanmei Li, et al.

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