**Abstract**

Melanoma differentiation-associated gene (MDA)-7/interleukin (IL)-24, a member of the IL-10 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 overexpressing MDA-7/IL-24 compared with those that do not express this cytokine identified growth arrest-specific gene-3 (gas3) as a target for mda-7/IL-24. Upregulation 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 upregulation 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 = 1.02 × 10^{-13}), and the incidence of metastases is significantly reduced in patients with HER2+ breast cancer expressing high-levels of IL-20R1. Together, our results identify a novel MDA-7/IL-24-GAS3-β1 integrin–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, HER2-like overexpressing HER2 receptor, and basal-like subtypes that do not express these receptors (triple negative; refs. 1, 2). Although 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 from human melanoma cells, is a member of the interleukin (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 shown 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 cancerspecific 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 preexisting genetic and epigenetic alterations (3).

Most strains of rats used experimentally such as Wistar-Furth 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 × 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, whereas others display anchorage-dependent growth. Through microarray analysis, we found that the 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 although 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 upregulation 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 show 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 (MFS). 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 methylnitrosourea (MNU) in 0.05% acetic acid. Thirty days after 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 approximately 10^8 plaque-forming units (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 approximately 10^8 pfu of empty adenovirus. The rats were palpated weekly and tumors were harvested when they were more than 20 mm in diameter or when the animals appeared moribund. A portion of each tumor was fixed in formalin for histologic analysis.

Growth of cells in nude mice

Four groups of 6 female athymic nude mice (CD1–Nu/Nu), 7 weeks of age, were purchased from Charles River Laboratories. After a week of acclimatization, 2 × 10^6 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 nonparametric Kaplan–Meier analysis. Tumors were harvested 2 weeks after 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% FBS, 10 ng/mL EGF, 1 μg/mL hydrocortisone, and 1 μg/ml 17-β-estradiol (Sigma-Aldrich). All culture experiments were repeated 3 times. To examine growth rates, 1 × 10^5 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 were treated with 10 or 20 μmol/L of the STAT3 inhibitor AG9, as previously described (13).

Attachment assays

Attachment assays were conducted on tissue culture plates or on Petri dishes precoated with fibronectin (20 μg/mL) or laminin (20 μg/mL). Cells (1 × 10^4/mL) were plated in triplicate and counted and photographed 2 or 24 hours after culture. In some experiments, the STAT3 inhibitor AG9 (Calbiochem) was added to the culture medium of the attached cells and they were photographed 4 hours 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 the growth of cells in soft agar, 2 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 5,000 cells were seeded on 6 cm plates and incubated for 5 to 7 days. At the end of the incubation period, colonies with more than 25 cells were counted.

Western blotting

Western blotting was conducted either as we have described previously (13), or for GAS3, as described in ref. (14). Polyclonal rabbit anti-rat antibodies were obtained from the following sources: rabbit anti-rat GAS3 (Abcam) used at a dilution of 1:500; rabbit anti-rat IL-24 (GenHunter) used at a dilution of 1:1,000; β-actin (Sigma) used at a dilution of 1/50,000; and pan-cadherin (Cell Signaling Technology) used at a dilution of 1:1,000. Protein band densities were determined with the ChemiGenius2 Bio Imaging System using the Gene Tools Software v4.02 (Syngene).
Immunoprecipitation
Semiconfluent FE1.2, FE1.2+GAS3, or FE1.2+GAS3-GM cells were lysed with NP-40 lysis buffer (1% Nonidet P-40, 2 mmol/L phenylmethylsulfonylefluoride, 10 μg/mL aprotinin, 2 μg/mL pepstatin, 10 mmol/L iodoacetamide, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 10 mmol/L HEPES, and 10 mmol/L KCl). Two micrograms of anti-β1 integrin antibody (Santa Cruz Biotechnology Inc.) was added to 0.5 mg of cell lysates in 1 mL and shaken at 4°C overnight. Protein-A beads were then added to the mixture and shaking was continued for additional 2 hours. The beads were then washed 3 times with NP-40 buffer, boiled, and proteins separated on 10% SDS-PAGE gel, and Western blot analyses prepared using GAS3 and β1 antibodies. To show loading and input controls, Western blot analyses were also prepared from the total extracts (20 μg) using β-actin and GAS3 antibodies.

Immunohistochemistry
Immunohistochemistry was conducted on cells grown on cover slips overnight. Cells were fixed in 4% paraformaldehyde and blocked using 10% normal goat serum (Jackson ImmunoResearch Laboratories). Triplicate cover slips were incubated with 1:500 polyclonal rabbit GAS3 antibody (Novus Biologicals) overnight at 4°C. Control cells were incubated with buffer only. The next day, the cover slips were incubated with 1:1,000 biotinylated goat anti-rabbit secondary antibody (Vector Laboratories), for 1 hour at room temperature. ABC complex (Vector Laboratories) was then applied to the cover slips for 30 minutes at room temperature, followed by 3,3-diaminobenzidine for 2 minutes (Vector Laboratories) and counterstaining with hematoxylin. Cells were dehydrated before mounting using xylene base mounting medium.

Infection and transfection
A gas3–producing retrovirus was generated by inserting the entire 658 bp coding sequence of rat gas3 in either sense or antisense 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+4 (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 2 × 105. After 48 hours, G418-resistant cells were pooled and subjected to subcloning by limited dilution. Transfection was conducted using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen).

Fibronectin siRNA transfection
Cells were transiently transfected with 2 μg of siRNA against fibronectin (Gene Pharma) or control oligonucleotides for 6 hours according to the manufacturer’s instructions, as previously described (15). 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 minutes. 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 ACC, β-actin primers: Forward; GCA ACC CAG TGT GAG TAG CÀ, Reverse; GAT GGT GTG CCC TGC ATT AC. Gas3 primers and controls were supplied by Applied Biosystems (Rn00568351-A1 PMP22, Lot # 282134).

Statistical analysis
Statistical analyses were conducted using the 2-tailed Student t test or ANOVA with significance considered at P ≤ 0.05 using Origin 3.5 software (Microcal Software). Values are reported as mean ± SE. The correlation between IL-20R1 and GAS3 genes was assessed by Pearson Correlation method with median-centered, log-2-transformed, and Robust Multi array Average normalized microarray gene expression values. Kaplan–Meier curves and HRs were generated as described (16).

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 whether mda-7/IL-24 can inhibit tumor development in vivo, 24 female Wistar-Furth rats were intraperitoneally injected with the mammary carcinogen MNU (75 mg/kg; ref. 12). Thirty days later, when we showed that the mammary glands of the carcinogen-treated rats contain a significant number of preneoplastic lesions (17), 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 (~108 pfu, 5–7 glands injected/rat). The other 12 control rats were infused with empty adenovirus (~108 pfu). The other 12 control rats were infused with empty adenovirus (~108 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 approximately 2.5 cm in diameter. Six of the control rats developed tumors in this time period (Supplementary Table S1). Histologic 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 χ2 analysis). Interestingly, 2 tumors developed in these rats in glands that had not been infused with adenovirus mda-7/IL-24. These results show 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
mRNA expression of GAS3 only in FE1.2 and FE1.3 (10-fold) cells (Supplementary Fig. S1). To starvation induced GAS3 expression in both FE1.2 (28-fold) tumor cells in monolayer culture and in soft agar GAS3 expression suppresses the growth of rat mammary and protein levels in mammary tumor cells.

in vitro When cultured reduced ability to proliferate in adherent, monolayer cultures we infected these cells with a retrovirus to identify GAS3 as a target of ligible amounts of this cytokine (Fig. 1). Thus, these studies of gas3 expression in FE1.2 cells by quantitative real-time PCR (qRT-PCR; Fig. 1A) and immunoblotting (Fig. 1B). Furthermore, we showed 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 (Supplementary Fig. S1). 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 with FE1.2+vector cells (Fig. 2B). GAS3 expres-

Figure 1. GAS3 expression in rat mammary tumor cells. A, RT-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 FE1.2 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 ChemiGenius® Bio Imaging System and Gene Tools Software.

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expression by siRNA in FE1.2+IL-24S cells, which over-express GAS3 (Supplementary Fig. S2A). Stable expression of gas3 siRNA led to a 50% knockdown of GAS3 expression (Supplementary Fig. S2A and S2B). This partial knockdown of GAS3 resulted in a significant increase in the number of colonies formed in soft agar compared with parental cells (Supplementary Fig. S2C).

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 upregulation of p27kip1 (13). The FE1.2+IL-24 cells treated with 10 or 20 μmol/L 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 (Supplementary Fig. S3A and S3B). 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 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 semiconfluent growth (Supplementary Fig. S4), suggesting that GAS3 expression may alter cell attachment. Indeed, GAS3 directly binds β1 integrin as revealed by immunoprecipitation (Supplementary Fig. S5). The interaction was specific to β1 integrin because we observed no binding of GAS3 to β4 integrin (data not shown), even though we previously showed 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 (19).

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 (20). 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 with FE1.2+GAS3 cells (56% ± 4) 2 hours after plating (Supplementary Fig. S6A). Because of the shorter doubling time of control cells, there was a further increase in their number compared with the FE1.2+GAS3 cells 24 hours after plating (Supplementary Fig. S6B). 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 (Supplementary Fig. S6A and S6B). 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 and Supplementary Fig. S7). 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 with 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 3 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 2 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 3 consensus motifs for N-linked glycosylation (21). 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 substitutions at amino acid positions 41 to 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 showed that GAS3 expression was similar in both FE1.3 and FE1.2 cells and was substantially higher than in FE1.2 cells (Supplementary Fig. S8). 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 hours later. As shown in Fig. 5B and Supplementary Fig. S6B, 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

**Figure 4.** Expression of GAS3 blocks secretion of fibronectin and inhibits attachment. A, Western blot analysis 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 blot analysis of fibronectin in FE1.2 vector control and GAS3 transduced cells transfected with 3 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.
soft agar, and cells did not die upon reaching confluence (Fig. 6A–C and Supplementary Fig. S6A). 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 patients with breast cancer

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 lines MD-MBA-231 and MD-MBA-435 (22). 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, 23) and our results with rat mammary tumor cells (Fig. 2).

On the basis of these results, we predicted that expression of MDA-7/IL-24 or its receptor IL-20R1/IL-20R2 (type 1 IL-20R; ref. 10) may correlate with the expression of GAS3 and may lead to better clinical outcome for patients with breast cancer. To test this notion, we examined several
publicly available breast cancer cohorts with microarray and MFS 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 $R = 0.24$ ($P = 10^{-5}$) for all patients with breast cancer and $R = 0.26$ ($P = 0.002$) for patients with HER2$^+$ breast cancer (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 with those with lower IL-20R1 levels ($P = 0.023$, 95% confidence interval, 1.07–2.38) with HR of 1.59 (Fig. 7C, left). Because Ha-ras is activated in MNU-induced rat mammary tumors (24, 25), 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 with those with low HER2 and high IL-20R1 expression ($P = 0.0485$; Fig. 7C, right). Thus, expression of the mda-7/IL-24R 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 (26). 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 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. Because 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 use of this cytokine as a therapeutic agent (7, 27). By analyzing MFS data in a large cohort of patients with breast cancer, we have now identified a 2-fold survival advantage in tumor biopsies expressing high levels of the MDA-7/IL-24 receptor IL-20R1. Although 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
to be associated with growth arrest in serum-starved NIH-3T3 to support its “bystander antitumor” functions and may contribute to promotion of enhanced local and systemic effects in vivo (8).

To understand how mda-7/IL-24 signaling inhibits the growth of breast cancer, we conducted a microarray analysis of genes induced when mda-7/IL-24 was overexpressed 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 (18). GAS3 expression is driven by 2 alternate promoters P1 and P2, which drive transcription of 2 transcripts 1A and 1B, respectively, containing different noncoding exons (28). Both transcripts yield identical proteins, but the 2 promoters confer tissue-specific control of expression (29). P1 leads to expression in the peripheral and central nervous systems and is thought to be important for myelin formation (30, 31). Amplification of the gas3/PMP22 locus is associated with the autosomal dominant Charcot–Marie– Tooth disease (32). 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 showed that overexpression 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 shown for the first time that the inhibition of cancer cell growth by mda-7/IL-24 is mediated, at least in part, through upregulation of GAS3. Consistent with a previous study (19), we have shown that GAS3 binds to β1 integrin, and showed 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 showed that siRNA-mediated
downregulation 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 (Arg-Asp-Ser) can inhibit metastasis of mouse melanoma cells by blocking α4–β1 interaction (21) 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 (33).

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 (34). Our results, however, showed unexpectedly, an opposite effect on tumor cell growth after tumor growth (34). Our results, however, showed unexpectedly, an opposite effect on tumor cell growth after

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 patients with breast cancer.

Disclosure of Potential Conflicts of interest

P. Fisher has ownership interest (including patents) in the Columbia University. No potential conflicts of interest were disclosed by the other authors.

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In summary, we have shown 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 patients with breast cancer.

Disclosure of Potential Conflicts of interest

P. Fisher has ownership interest (including patents) in the Columbia University. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.) G. Liu, J.C. Liu, S. Shan, R. Dass, P.B. Fisher, M.C. Archer, Y. Ben-David
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