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Metastasis Tumor Associated Protein 2 Enhances Metastatic Behavior and is Associated with Poor Outcomes in Estrogen Receptor-Negative Breast Cancer

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Abstract

Metastasis remains a major clinical problem in breast cancer. One family of genes previously linked with metastasis is the metastasis tumor associated (MTA) family, with members MTA1 enhancing and MTA3 inhibiting cancer metastasis. We have previously found that MTA2 enhances anchorage-independent growth in estrogen receptor α (ERα) breast cancers, and, in combination with other genes, performed as a predictive biomarker in ERα-positive breast cancer. We therefore hypothesized that MTA2 enhances breast cancer progression. To test this, cell growth, soft-agar colony formation, migration, and *in vivo* metastasis were examined in MTA2-overexpressing and vector control transfected ERα-negative breast cancer cells. Pathways regulating cell-cell interaction, adhesion, and signaling through the Rho pathway were also investigated. Effects of the inhibition of the Rho pathway using a Rho Kinase (ROCK) inhibitor were assessed in soft agar colony formation and motility assays in MTA2-overexpressing cells. MTA2 expression was associated with poor prognostic markers, and levels of MTA2 were associated with increased risk of early recurrence in retrospective analyses. MTA2 overexpression was associated with enhanced metastasis, and pathways regulating cell-cell interactions *in vitro* and *in vivo*. Most critically, MTA2-enhanced motility could be blocked by inhibiting Rho pathway signaling. We present the novel finding that MTA2 defined a subset of ERα-negative patients with a particularly poor outcome.

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Conflict of Interest

None of the authors have any competing financial interests in relation to the work described.

Keywords

Metastasis; Breast Cancer; MTA2; Rho; Cytoskeleton

Introduction

The majority of breast cancer patients succumb to complications from distant metastases rather than the primary tumor. [1] Genes from many signaling pathways and regulatory networks have been implicated in metastasis formation including regulation of gene expression in the nucleus [2] and activation of cytoskeletal reorganization. [3–6] Metastasis is a complex process involving invasion, anchorage-independent growth, survival, and growth at distant sites, where each step is rate limiting for metastasis. [1] Thus, there is a great clinical need for agents that can prevent or target the process of metastatic dissemination and formation.

The metastasis tumor associated (MTA) family of proteins has been implicated in the metastatic process. [7–11] MTA's are members of the nucleosome remodeling and histone deacetylation (NuRD) complex directing associated histone deacetylase (HDAC) to both histone and non-histone proteins, thus regulating chromatin condensation and protein function. [7–13] The MTA family is comprised of three genes MTA1, MTA2, and MTA3. Though MTA proteins are each members of the NuRD complex, they appear to mediate distinct cellular functions and to play different roles in the metastatic process. [7,9,10]

MTA1 was identified by cDNA screening of metastatic versus non-metastatic rat adenocarcinoma, [14,15] and has been linked with metastasis in several cancer types. [10] Conversely, MTA3 is a repressor of snail [16] and is lost in the polyoma virus middle-T mammary oncogene model of mammary cancer, while MTA1 and MTA2 expression is retained. [11] MTA2 is critical for twist-mediated enhancement of migration of mouse 4T1 mammary tumors, [13] and overexpression of MTA2 in ERα-positive breast cancer cells resulted in an estrogen-independent phenotype with enhanced anchorage-independent growth, [12] a phenotype associated with metastasis. Thus, we hypothesize that MTA2 may have a role in metastasis in breast cancer.

Materials and Methods

Plasmids and chemicals

The MTA2 plasmid (a kind gift from Wei Gu, Columbia University, New York, NY) [12] and the YFP-tagged Rho GDIα plasmid have been previously described. [17] Fasudil and H-1152 were obtained from Tochris Biosciences (Ellisville, MO) and suspended in H2O.

Cell culture and stable transfection

ERα-negative MDA-MB-231 cells were obtained from American Type Tissue Culture Collection (Manassas, VA). Cells were maintained at 37C in 5% CO in minimal essential medium (MEM; Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS, GemCell, West Sacramento, CA), 200 units/ml penicillin, 200 μg/ml streptomycin, and non-

essential amino acids (Invitrogen). Stably transfected MTA2 and Rho GDIα-overexpressing cell lines were generated as previously described. [12] For maintaining stable cell lines 1 g/L geneticin (Invitrogen) and/or 200 μg/mL zeocin was added.

Immunoblot analysis

Cell extracts were prepared using RIPA buffer (5mM Tris HCl pH 8, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), or MOPS buffer (25mM Hepes pH 7.5, 150mM NaCl, 1% igepal ca-630, 10% glycerol, 25mM NaF, 10mM $MgCl_2$, 1mM EDTA, 10mg/ml each of leupeptin and aprotinin) both supplemented with protease inhibitor cocktail III and phosphatase inhibitor cocktail I/II (Calbiochem, Darmstadt, Germany). Cell lysates were resolved by SDS-PAGE and assayed as previously described. [12] Membranes were probed with anti-MTA2 (Sigma, St. Louis, MO), anti-Rho GDIα (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-RhoC (Cell Signaling Technology, Beverly, MA). Antibodies to GAPDH (Santa Cruz Biotechnology) or p190 (BD Biosciences) were used as loading controls.

Cell growth assays

Cells were cultured then treated with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetazolium bromide (MTT, Sigma) for two hours at the specified time points. The resulting reaction was suspended in dimethly sulfoxide and absorbance was measured at 550 nm and 655 nm.

Soft agar colony formation assay

Soft agar assays were performed as previously described [12] with some modifications. Briefly, 6-wells were coated with 2 ml pre-warmed (50C) 0.7% SeaPlaque agarose (FMC, Rockland, ME) and solidified at 4C for one hour. Cells were suspended in 4 ml of pre-warmed (37C) 0.35% SeaPlaque agarose and plated at a density of 5000 cells per well. Plates were cooled for 2h at 4C and then transferred to a 37C humidified incubator. After three weeks, colonies were quantified using a GelCount colony counter (Oxford Optronix, Oxford, UK) following the manufacturer's instructions.

Wound healing assays

Cells were seeded onto 6-well plates at a density of 250,000 cells per well, and allowed to grow to confluence. Cell layers were washed two times with MEM, scrapped using a pipette tip, and washed two additional times with MEM. Growth medium (supplemented with 10 μM H-1152, 100 μM Fasudil, 200 μM Fasudil, or H_2O) was then placed on the cells and images were taken at times 0 and 24 hours. Wound width was measured in pixels using ImageJ. [18]

Tumor xenograft experiments

Cells were cultured to 80% confluence and harvested using Versene (Invitrogen). Cells were then injected into the number four mammary fat pad of athymic nude female mice at a density of five million cells in 200 μl of 50% Matrigel (BD Biosciences, San Jose, CA) and 50% growth medium (v/v). Tumors were allowed to grow to a volume of approximately 1000 mm^3 after which the primary tumors were removed. Mice were then observed for the

formation of distant metastases, after which, the mice were sacrificed and tissues processed for pathological evaluation. All immunohistochemistry was performed by the Lester and Sue Smith Breast Center Pathology Core facility at Baylor College of Medicine. Thin (5μm) sections were stained with hematoxylin and eosin (H&E) to detect metastases. Animals were housed and maintained in accordance with the animal care regulations of Baylor College of Medicine. All of the animal experiments were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee pursuant to NIH guidelines.

Rho activity assays

Rho activity assays were performed as previously described. [17] Briefly, cells were cultured to 80% confluence and harvested in RIPA buffer at 4C. Lysates were clarified by centrifugation at maximum speed in a microcentrifuge for five minutes. Lysates were then incubated with rhotekin-bound beads for 2h. The bead/lysate slurry was then washed 3 times with RIPA buffer, suspended in protein loading buffer, boiled, and subjected to immunoblot analysis.

Gene expression array hybridization

RNA was extracted as previously described. [19] Briefly, Affymetrix (Santa Clara, CA) gene expression array analysis was performed by collecting RNA from cells using the RNeasy Mini Kit from Qiagen (Valencia, CA). RNA was processed to generate labeled-cRNA which was hybridized onto Affymetrix GeneChip human genome U133 plus 2.0 arrays using manufacturer-recommended procedures for hybridization, washing, and staining with streptavidin-phycoerythrin. GeneChips were scanned, and feature quantitation was done using Affymetrix protocols.

Statistical analysis

Oncomine [20] analysis was performed using the Oncomine tool (http://www.oncomine.org). For gene expression analysis of cell lines, data were normalized and differential gene expression was assessed using the implementation of the Limma [21] and RMA [22] in Red-R. [23] Differentially expressed genes were filtered using a false discovery rate of 0.1.

For retrospective ERα-negative clinical gene expression analysis, data previously published by Wang *et al.* [GSE2034 [24] and Sabatier *et al.* [GSE21653 [25] were downloaded from Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo). [26] Samples classified as being ERα-negative were used for all analyses. Both of these datasets were analyzed separately.

To assess genes that correlated with MTA2 expression genes were filtered to select those with variability greater than the 50 percentile of inter quartile range and compared with the MTA2 signal. Significantly correlated genes were submitted to DAVID [27] for pathway analysis.

For evaluation of MTA2 as a prognostic marker in the Sabatier *et al.* dataset, samples were classified as MTA2-high if they expressed MTA2 above the 75 percentile, and MTA2-low if

at or below the 75 percentile. For evaluating MTA2 and Rho GDIα together as a prognostic marker, the Wang *et al.* dataset was used and patients were classified as MTA2-high if above the 25 percentile of expression of MTA2 and Rho GDIα-low if less than the 75 percentile of expression for Rho GDIα. Cox proportional hazards models were calculated using the Survival [28] package in R. [29]

Other statistical analyses were applied to experimental data where appropriate. Cell growth assays were analyzed using linear models. Colony forming, Rho activity assays, and wound healing assays were analyzed using ANOVA. Growth assays were analyzed using.

All statistical analyses were performed in the statistical programming language R [29] or Red-R. [23]

Results

MTA2 expression is associated with poor outcomes in ERα-negative patients

We hypothesized that MTA2 expression may be linked with metastatic spread in breast cancer. We searched for clinical features associated with MTA2 expression using Oncomine. [20] We found high MTA2 levels to be associated with poorer differentiation, greater clinical stage, higher clinical grade, and ERα-negative status (Table 1), all features associated with poor outcomes. [30,31] These data indicate that MTA2 is more highly expressed in breast cancers that have worse outcomes or have formed distant metastases.

We extended these analyses by investigating the role of MTA2 on disease free survival using a dataset previously published by Sabatier *et al.* [25] As MTA2 was associated with ERα-negative status, we limited our study to ERα-negative tumors. Based on the Oncomine analyses, we focused on patients with higher level expression of MTA2, thus we pre-selected the 75 percentile of MTA2 expression to represent high expression of MTA2. Over the entire length of follow-up, we observed a trend to poorer recurrence-free survival in the MTA2-high group (Figure 1, $P<0.08$). Analysis of the survival curves indicated a violation of the assumption of proportional hazards ($p < 0.05$) with an inflection point at 24 months (data not shown), indicating that the risk of recurrence between the MTA2-high and MTA2-low populations changes after 24 months. We had found that MTA2 was higher in Stage IV tumors, indicating that tumors with high levels of MTA2 may quickly develop clinically detectable metastases. We censored the survival data to 24 months, and found that MTA2-high patients showed significantly poorer recurrence-free survival up to 24 months (Figure 1, $P<0.006$). These data indicate that MTA2 is associated with early recurrence in ERα-negative breast cancers.

MTA2 overexpression enhances anchorage-independent growth and metastasis

After finding that MTA2 was associated with early metastasis in retrospective studies, we sought to verify these findings in ERα-negative breast cancer cell lines. We engineered the ERα-negative, metastatic, breast cancer cell line, MDA-MB-231, to overexpress MTA2 (designated MTA2.2 and MTA2.3) or control (designated Vector). Based on analysis of MTA2 mRNA expression (published by Neve *et al.* [32] MDA-MB-231 cells have intermediate levels of MTA2 expression relative to other breast cancer cell lines (data not

shown). Overexpression of MTA2 was validated using immunoblot assay (Figure 2a). The MTA2.2 and MTA2.3 clones consistently showed between 1.5 to 2 fold the level of MTA2 protein relative to Vector cells. We observed no difference (p=0.998) in growth using MTT growth assays between the Vector control and MTA2.2 and MTA2.3 cells (Figure 2b). These data indicate that MTA2 overexpression did not affect cell growth.

The ability for cancer cells to migrate and survive in the circulation is required for metastasis dessimination. [1] We used soft agar growth assays to mimic growth and survival in an anchorage-independent environment, and wound healing assays to assess motility. We found that MTA2.3 and MTA2.2 cells demonstrated enhanced soft agar growth compared to Vector by 5–15 fold, respectively (Figure 2c, Vector v. MTA2.2; p=6.3*E*–4, Vector v. MTA2.3; p=7.8*E*–4). Additionally, these clones also demonstrated a 1.5–2 fold increase, respectively, in migration speed relative to Vector cells (Figure 2d, Vector v. MTA2.2; p<2*E*–16, Vector v. MTA2.3; p<2*E*–16). These data indicate that MTA2 overexpression can significantly enhance motility and anchorage-independent growth of ERα-negative breast cancer cells, consistent with a pro-metastatic phenotype. These data are consistent with previous observations in ERα-positive T47D cells. [12]

We next hypothesized that MTA2 could directly enhance metastasis *in vivo*. We injected MTA2.2 or Vector control cells into the number four mammary fat pads of female athymic nude mice, and allowed the cells to form primary tumor xenografts. Primary xenografts developed in 100% of the mice with both cell lines. Similar to growth in culture, the growth rate of the primary tumors was equivalent between the two groups (Figure 2e, p>0.08, n=10 mice per group). Thus, MTA2 overexpression did not significantly enhance primary tumor formation or growth.

We allowed primary tumors to grow to 1000 mm^3 in volume, at which point the primary tumor was surgically removed. The mice were monitored for the formation of distant metastases at this point. The vector control did not form metastasis during the experimental time of 6 weeks (Figure 2f–g). However, within two weeks of surgery, large metastatic lesions were observed in MTA2.2 injected mice in multiple organs (Figures 2h–k). Thus, MTA2 overexpression in MDA-MB-231 cells enhanced their ability to form systemic and spontaneous metastases.

MTA2 increases expression of cytoskeletal remodeling genes

MTA2 is a component of the NuRD complex, [7,8,10,11,13] an essential regulator of gene expression. We hypothesized that MTA2 overexpression could exert a substantial impact on global gene regulation, potentially by activating a concerted metastatic program in breast cancer. To address this, we used Affymetrix microarray expression profiling to determine which cellular pathways were altered coincident with MTA2 overexpression. We found that 8189 probes were differentially expressed between the MTA2.2 and the Vector cell lines (FDR < 0.1, n=3 per group, supplemental table 1). We used DAVID [27] to identify cellular signaling pathways that were significantly enriched in genes regulated by MTA2. We found that many pathways differentially regulated between Vector and MTA2-overexpressing cells were involved in interactions between cells and the extracellular matrix (Table 2). These pathways are known to be essential for tumor motility and metastasis. [33]

We next sought to confirm our observations of MTA2-induced gene expression changes in clinical breast cancer samples. We used data from the Sabatier *et al.* [25] and the Wang *et al.* [24] datasets to identify genes significantly correlated in expression with MTA2 in ERα-negative samples. We identified 12,527 probes (Supplemental Table 2) in the Sabatier *et al.* dataset and 382 probes (Supplemental Table 3) in the Wang *et al.* dataset to be correlated with MTA2 at $p<0.01$. The combined cell line and clinical data revealed several overlapping pathways (Table 2). Changes in gene expression in the Rho pathway, such as RhoA-C, focal adhesion kinase, and Rho Kinase (ROCK), were significantly correlated with MTA2 expression. These data demonstrate that MTA2 overexpression activated a transcriptional profile that may induce a motile and metastatic phenotype, potentially through Rho pathway activation.

MTA2 overexpression increased activation of the Rho pathway

Signaling through the Rho pathway is essential for cell motility. [34,35,36] Increased activation of the Rho pathway has been associated with increased metastasis. [37] We previously reported that Rho GDIα, a negative regulator of the Rho pathway, downregulates the expression of MTA2 in ERα-positive cells. [17] Therefore, we performed immunoblot analysis to compare the levels of Rho GDIα in Vector, MTA2.2, and MTA2.3 cells. We found that endogenous expression of Rho GDIα was decreased in MTA2-overexpressing cells relative to Vector control cells (Figure 3a). We also found that the levels of Rho GDIα were lower in MTA2-overexpressing T47D cell lines compared with control. Knockdown of MTA2 in MDAMB321 cells using two independent siRNA constructs resulted in increased expression of Rho GDIα (Figure 3b). These findings further reinforce the hypothesis that MTA2 and Rho GDIα form a regulatory signature, as we have previously suggested. [17]

To determine if the Rho pathway was indeed activated in MTA2-overexpressing cells, we performed Rho activity assays. We found that levels of GTP-bound (active) RhoC were increased in MTA2.2 and MTA2.3 by approximately 1.75 fold compared with Vector control cells (Figure 3c, Vector v. MTA2.2; $p=0.046$, Vector v. MTA2.3; $p=0.032$). These data confirm our gene expression data indicating that MTA2 expression promoted Rho/cytoskeletal remodeling pathway activation.

To further confirm that the mechanism of MTA2 enhanced anchorage-independent growth was via Rho pathway activation, we overexpred a yellow fluorescent protein (YFP)-tagged Rho GDIα in MTA2.2 cells to inhibit the Rho pathway. We used immunoblot analysis to examine the expression of MTA2 and Rho GDIα in these cells (Figure 3d). Surprisingly, MTA2 protein levels were decreased, while endogenous Rho GDIα protein levels were increased in YFP-Rho GDIα cells. These data reinforce our previous observations that Rho GDIα and MTA2 are inversely regulated. [17]

We then subjected the YFP-Rho GDIα-overexpressing cells to soft agar growth assays. We found that Rho GDIα-overexpression reduced the ability to form colonies in soft agar relative to control (Figure 3e, MTA2-Vector+Rho GDIα-Vector v. MTA2.2+Rho GDIα-Vector; $p=6.3E-4$, MTA2.2+Rho GDIα-Vector v. MTA2.2+Rho GDIα-YFP, $p=0.0021$). These data lead us to conclude that MTA2 enhanced metastasis in breast cancer cells by

activating the Rho/cytoskeletal remodeling pathway at multiple key signaling nodes including Rho activation and downregulation of Rho GDIα.

We next hypothesized that blocking the Rho pathway by inhibiting ROCK, could inhibit anchorage-independent growth and motility in MTA2-overexpressing cells. We performed soft-agar colony formation assays and wound healing assays with the addition of H-1152, a potent ROCK inhibitor. We found that H-1152 inhibited soft agar colony formation (Figure 3f, Vector v. MTA2.2; p=0.037, Vector v. MTA2.3; p=0.016) and wound healing (Figure 3g, Vector v. MTA2.2; p=0.0089, Vector v. MTA2.3; p<1.8*E*–15) in both MTA2.2 and MTA2.3 cells. We also used a second, less specific, yet clinically tested, ROCK inhibitor, Fasudil [38,39] and found that Fasudil was able to block motility of MTA2-overexpressing cells (Figure 3g, Vector v. MTA2.2; p<9*E*–9, Vector v. MTA2.3; p<1*E*–7). Interestingly, Vector cells demonstrated enhanced colony formation in the presence of H-1152 10μM (p=0.045) and increased motility in the presence of Fasudil 100 μM (p=0.0037) or H-1152 10μM (p=0.0012) as compared to vehicle. ROCK functions are dependent on cell context, [40] and these data indicate that ROCK function may be regulated by MTA2 levels. These findings support the model that the effects of MTA2 on cytoskeletal organization are dependent on activation of the Rho/ROCK pathway.

MTA2 and Rho GDI α form a prognostic signature in ERα-negative breast cancer

To determine if MTA2 and Rho GDIα levels in combination form a prognostic signature in ERα-negative breast tumors, we examined these two factors in the ERα-negative subset of the Wang *et al.* dataset [24] The Wang *et al.* dataset contains only lymph node-negative tumor samples, which have better outcomes compared with lymph-node positive samples. [41,42,43] While MTA2 alone was not prognostic in the Wang *et al.* dataset (data not shown), MTA2 expression in combination with Rho GDIα expression (MTA2-high/Rho GDIα-low versus all others) formed a signature that was prognostic for early recurrence at 24 months as in the Sabatier *et al.* dataset (Figure 4, p=0.018, p=0.62 overall). These data indicate that a combination of MTA2 and Rho GDIa is required to predict outcome in lymph-node negative breast cancer populations.

Discussion

We demonstrated that MTA2 is a metastasis enhancer in ERα-negative MDA-MB-231 breast cancer cells. These results are consistent with those reported for MTA1 overexpression. [9] However, MTA1 action was dependent on HIF1α, [9] where MTA2 appears operate through Rho signaling activation. We found that MTA2 is prognostic for cancer recurrence in two previously published ER-negative cohorts, as well as being associated with clinical determinants of poor outcomes. While we had previously demonstrated the prognostic value of MTA2 and Rho GDIα in ERα-positive breast cancer, [17] this is the first report to demonstrate their significance in ERα-negative tumors. This novel finding indicates that the effects of MTA2 on metastasis may be independent of its effects on hormonal resistance.

We found that MTA2 regulates the expression of many regulators of Rho signaling, potentially via NuRD activity. The effects of MTA2 are critically linked with Rho pathway

activity, as inhibitors (Rho GDIα-add-back, Fasudil, or H-1152) could block the enhanced MTA2-associated motility and anchorage-independent growth phenotypes.

Metastases can form within 20 weeks after injection of parental MDA-MB-231 cell lines. [44] However, in MTA2-overexpressing models, metastases appeared within six weeks of initial tumor formation. In clinical data, MTA2 was increased in expression in patients presenting with Stage IV breast cancer, [45] and was associated with earlier clinical metastases either alone or in combination with Rho GDIα. [25,24] It is thought that breast cancer metastases arise from disseminated cells present at the time of surgery to remove the primary tumor which may remain undetectable for years. [46] While we have demonstrated that MTA2 can enhance the motile and anchorage-independent growth phenotypes of ERα-negative breast cancer cells, our *in vivo* and clinical data indicate that MTA2 may enhance colonization or growth at the distant site as well.

Our results demonstrate a complex regulatory relationship between MTA2 and Rho GDIα. Rho GDIα was also reported to participate in a feed-forward regulatory loop via c-jun kinase, [47] similar to our findings. Further studies are warranted to better understand the highly dynamic regulation of the Rho pathway influenced by MTA2 overexpression.

Our studies have identified two targetable pathways in breast cancer progression. MTA2 is known to regulate gene expression as a member of the NuRD complex. [7–13] HDAC inhibitors are being tested in clinical trials of metastatic cancer, and have shown preclinical success in cell line models. [48] Fasudil has been approved for the treatment of cerebral vasospasm in Japan, and has been studied in models of breast cancer metastasis with success. [38] While studies of the effects of Fasudil clinically are ongoing, our data raise the possibility that ROCK inhibitors could be effective breast cancer agents, particularly in those patients that overexpress MTA2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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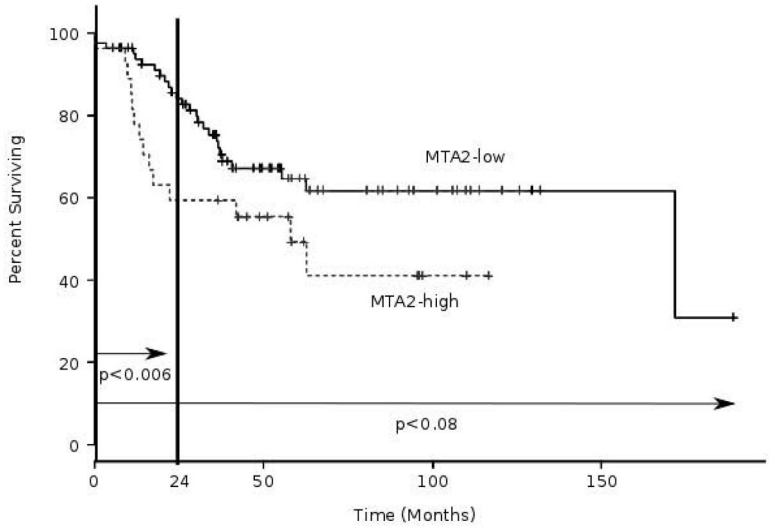

Figure 1.

Analysis of MTA2 expression in the Sabatier *et al.* [25] dataset. Tumor samples were subset based on expression of MTA2, assessed by the MTA2-probe within the microarray data, above and below the 75 percentile. Comparisons are made at twenty-four months (p =0.00534, n = 113) and the entirety of clinical follow-up (p =0.0791, n = 113).

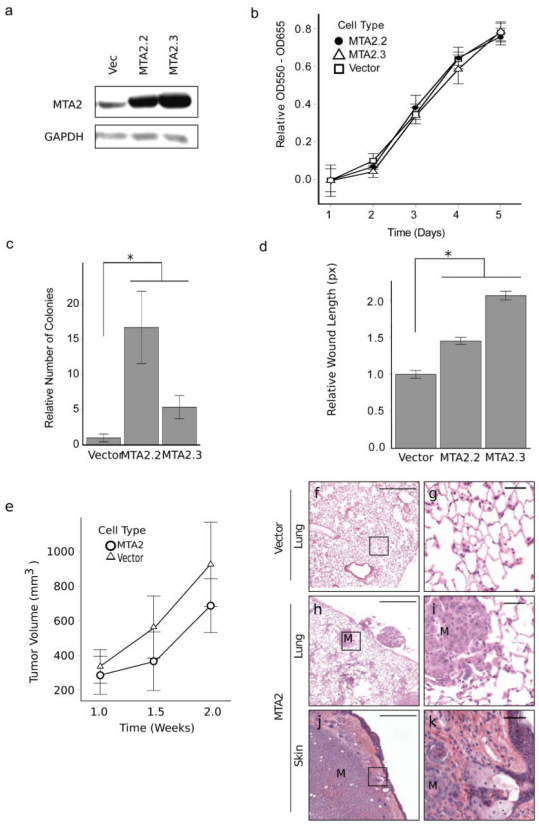

Figure 2.

MTA2-overexpression enhances the metastatic efficiency of MDA-MB-231 breast cancer cells. **(a)** Control and MTA2-overexpressing cell lysates were immunoblotted using antibodies to MTA2 and GAPDH. **(b)** MTA2-overexpression does not increase primary tumor growth. Control and MTA2-overexpressing cells were grown over time and stained with MTT. Values were normalized to the first reading for each group. **(c)** MTA2-overexpression increases anchorage-independent growth in MDA-MB-231 cells. Soft agar colony formation assay of Vector control and MTA2-overexpressing cells, values were normalized to the Vector group. Cells were seeded into soft-agar and allowed to form colonies for three weeks after which colonies were counted using the GelCount system. **(d)** MTA2-overexpression increases motility of MDA-MB-231 cells. Cells were seeded into wells and wounded with the tip of a pipette. Images were taken at time points zero and twenty-four hours and distance migrated was calculated in pixels from the images. **(e)** MTA2-overexpression does not increase tumor xenograft growth. MDA-MB-231 cells expressing either vector control or MTA2 were grown as tumor xenografts in the mammary fat pad of female athymic nude mice. Growth rates of tumors from vector and MTA2-overexpressing cells, tumor volumes were measured twice weekly. **(f–k)** MTA2-overexpression enhances metastasis of MDA-MB-231 cells. Mouse lung **(f–i)** or skin **(j,k)** from mice receiving vector control cells **(f,g)** or MTA2-overexpressing cells **(h–k)** was stained using IHC. *, $p < 0.05$, exact p-values are indicated in the text. Error-bars represent standard error of the mean. M, metastasis. Scale bar **(f,h,j)**, 500μM. Scale bar **(g,i,k)**, 50μM.

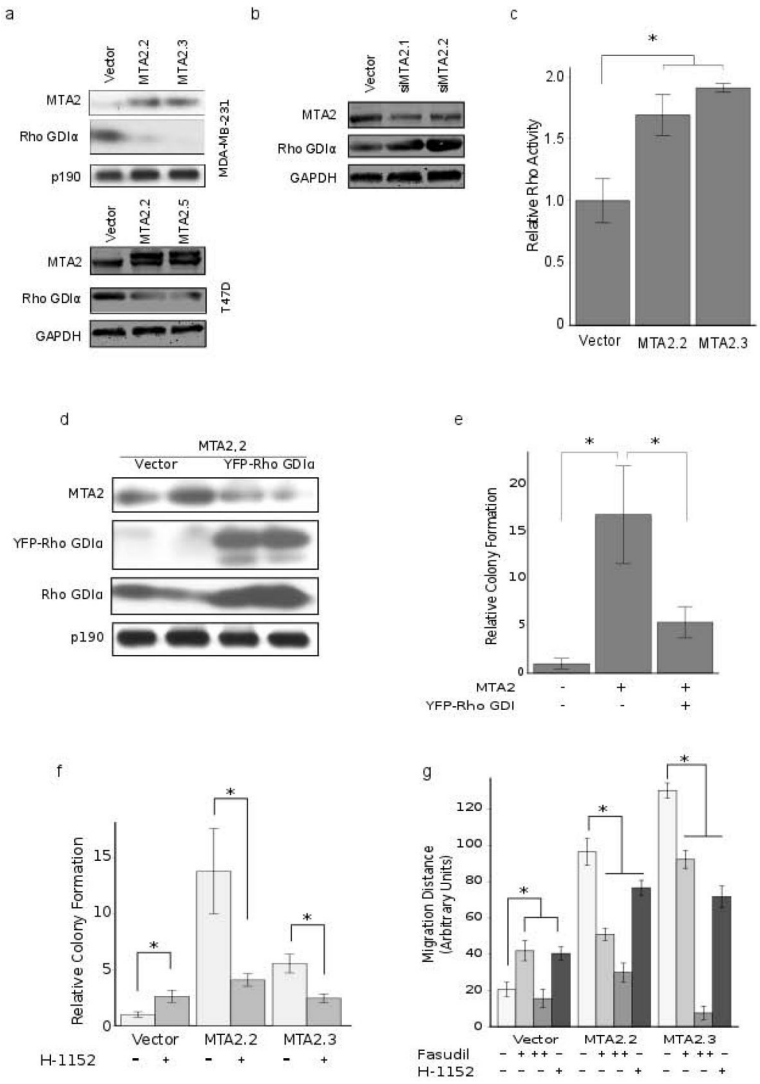

Figure 3.

MTA2's pro-metastatic activities are dependent on Rho/ROCK signaling. **(a)** MTA2-overexpression decreases expression of Rho GDIα. Cell lysates from control and MTA2-overexpressing MDA-MB-231 (top) and T47D (bottom) cells were immunobloted using antibodies to MTA2, Rho GDIα. Antibodies to p190 or GAPDH are used as loading control. **(b)** MTA2-knockdown decreases Rho GDIα expression. Immunoblot of MDA-MB-231 cells transfected with independent siRNA constructs targeting MTA2. **(c)** MTA2-overexpression enhances Rho pathway activity. Control or MTA2-overexpressing cell lysates were used in rhotekin-binding assays to assess the level of Rho activation. Normalized GTP-bound Rho levels are shown (A.U.) **(d)** Immunoblot of MTA2.2 cells transfected with either vector control or YFP-tagged Rho GDIα. **(e)** Rho GDIα re-expression reduces anchorage-independent growth. Soft-agar colony forming assays of compound vector, MTA2.2/YFP vector, and MTA2.2/YFP-Rho GDIα cells were used to assess anchorage-independent growth. **(f)** Inhibition of Rho activity blocks MTA2-associated anchorage-independent growth. Soft-agar colony formation assay of vector control or MTA2-overexpressing cells

treated with 10μM H-1152 or vehicle are shown. Counts are normalized to the Vector group. **(g)** Inhibition of Rho activity blocks MTA2-associated motility. Wound healing assays comparing vector control or MTA2-overexpressing cells treated with vehicle, Fasudil 100μM, Fasudil 200μM, or 10μM H-1152 are shown. *, $p < 0.05$, exact p-values indicated in the text. Error-bars indicate standard error of the mean.

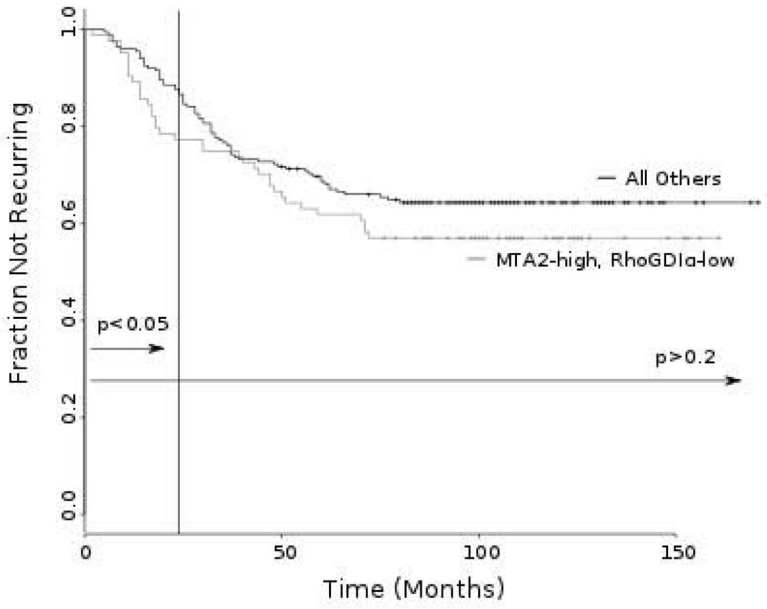

Figure 4.

MTA2 and Rho GDIα collaborate to enhance rapid metastasis in ERα-negative breast cancer. A gene signature was constructed using MTA2 and Rho GDIα. Patients from the Wang *et al.* dataset [24] were classified as MTA2-high and Rho GDIα-low showed significantly more recurrence events when data were censored to 24 months compared with all other patients (p=0.018). No significant difference in outcomes was detected when the entire dataset was evaluated (p=0.62).

Table 1

Comparison of clinical features differentiated by MTA2 expression[a].

Clinical Feature[a]	P-value	Study Reference
Grade: 3 > 1	0.00039	Ginestier et al. [49]
Differentiation: Poor > Well	0.05	Desmedt et al. [50]
Stage: IV > I	0.015	Chin et al. [45]
Clinical Risk High > Low	0.002	Desmedt et al. [50]

[a]> indicate the group that showed the higher expression of MTA2.

Table 2

Pathways associated with MTA2 expression.

Pathway	MDA-MB-231 Vector v. MTA2[a]	Wang *et al.* Study[a]	Sabatier *et al.* Study[a]	p-value product	Benjamini product
Focal adhesion	74 (1.43)	10 (1.8602)	120 (1.4659)	2.065E-12	7.871E-8
Regulation of actin cytoskeleton	70 (1.26)	11 (1.9130)	114 (1.3019)	2.515E-7	2.609E-4
MAPK signaling pathway	98 (1.43)	14 (1.96)	134 (1.23)	1.601E-9	9.358E-6

[a]Number of genes (fold enrichment)