Breast Cancer Metastasis Suppressor-1 Differentially Modulates Growth Factor Signaling*

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That metastatic tumor cells grow in selective non-native environments suggests an ability to differentially respond to local microenvironments. BRMS1, like other metastasis suppressors, halts ectopic growth (metastasis) without blocking orthotopic tumor formation. BRMS1-expressing tumor cells reach secondary sites but do not colonize distant tissues, compelling the hypothesis that BRMS1 selectively restricts the ability of tumor cells to respond to exogenous regulators in different tissues. Here we report that BRMS1 expression in metastatic human breast cancer cells leads to a selective reduction in epidermal growth factor receptor expression and downstream (AKT) signaling. Signaling through another receptor tyrosine kinase, hepatocyte growth factor receptor (c-Met), remains unaltered despite reduced levels of the signaling intermediate phosphatidylinositol (4,5)-bisphosphate. Interestingly, reduced downstream calcium signaling is observed following treatment with platelet-derived growth factor, consistent with decreased phosphatidylinositol (4,5)-bisphosphate. However, platelet-derived growth factor receptor expression is unaltered. Thus, BRMS1 differentially attenuates cellular responses to mitogenic signals, not only dependent upon the specific signal received, but at varying steps within the same signaling cascade. Specific modulation of signaling responses received from the microenvironment may ultimately dictate which environments are permissive/restrictive for tumor cell growth and provide insights into the biology underlying metastasis.

Metastasis begins with dissemination of cells from the primary tumor and entails survival in the bloodstream and subsequent arrival and proliferation at secondary sites (1). However, following seeding at different organ sites, growth of tumor cells is nonrandom, depending on the cancer type. Organotropism of metastasis was first characterized by Sir Stephen Paget in his “seed and soil” hypothesis. Paget noted that breast cancer metastases were more frequently observed in bone and lymph nodes than at other sites in the body. Recent findings extend his observations with the concept of a “pre-metastatic niche” created by tumor cells for their eventual arrival at secondary sites (2). To ensure survival, the tumor cell must be able to respond to microenvironmental cues at every step of the metastatic cascade, especially at secondary sites. Secondary tumor growth likely parallels growth at the primary site, the major distinction being that tumor cells find themselves in non-native ectopic environments. Interfering with a tumor cell’s ability to interact with microenvironmental factors would therefore be expected to limit its capacity to proliferate. Importantly, arrival and seeding at particular sites do not guarantee growth.

Metastasis suppressors are a growing family of more than 20 proteins that, when re-expressed in metastatic cells, block metastasis without preventing tumor growth at orthotopic sites (3). Their mechanisms of action have not been fully elucidated, but many appear to block growth at secondary sites by interfering with the tumor cell’s ability to respond to its microenvironment (1). BRMS1 (breast cancer metastasis suppressor-1), expressing tumor cells maintain the ability to form primary tumors and, despite maintaining the ability to seed secondary sites, do not colonize distant tissues (4). Because BRMS1 selectively determines where cells grow or not, our objective in this study was to begin testing the hypothesis that BRMS1 differentially regulates growth factor responses.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—MDA-MB-231 and MDA-MB-435 human breast carcinoma cells were transfected with a lentiviral vector construct expressing BRMS1 under the control of a cytomegalovirus promoter (4). The origin of MDA-MB-435 cells has recently been called into question. However, MDA-MB-435 cells have been demonstrated to express milk proteins and metastasize from mammary fat pad but not subcutaneous tissue, consistent with the cell line being a breast carcinoma (5, 6). Three single-cell clones were chosen from each cell line:
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231BRMS1.9, 231BRMS1.10, 231BRMS1.12, 435BRMS1.10, 435BRMS1.13, and 435BRMS1.14. MDA-MB-231/435 vector transfectants (231/435) and 231BRMS1/435BRMS1 clones were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (DMEM/F-12) supplemented with 1% nonessential amino acids and glutamine (Invitrogen) and containing 5% fetal bovine serum. Parental 231 and 231BRMS1 cells were passaged with 0.125% trypsin and 2 mM EDTA (Invitrogen) in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free phosphate-buffered saline, and parental 435 and 435BRMS1 cells were passaged using 2 mM EDTA. Cell lines were confirmed to be free of Mycoplasma contamination. No antibiotics or antimitotics were used during routine culture. Whenever growth factor treatment was required, cells were cultured under serum-free conditions (DMEM/F-12) for 24 h prior to treatment.

**Immunoblotting and Immunoprecipitation**—Cells were rinsed twice with ice-cold phosphate-buffered saline and lysed in a buffer containing 25 mM Tris-HCl (pH 7.4), 50 mM β-glycerol phosphate, 0.5 mM EDTA, 5% glycerol, 0.1% Triton X-100, 1 mM sodium orthovanadate, 1 mM benzamidine, and protease inhibitor mixture containing aprotonin, leupeptin, and phenylmethylsulfonyl fluoride (Roche Applied Science). Protein concentration was determined using a Bradford colorimetric assay (Pierce). Protein was denatured with Laemmli buffer at 95 °C for 5 min, and lysate equal to 50 μg was loaded into each well. Proteins were separated using either 8 or 12% SDS-polyacrylamide gels, and resolved proteins were transferred to polyvinylidene difluoride membranes. Membranes were incubated in Tris-buffered saline containing 0.05% Tween 20 and 5% fat-free dry milk for 1 h at room temperature. Membranes were incubated with primary antibodies to epidermal growth factor receptor (EGFR; 2232), p42/p44 mitogen-activated protein kinase (MAPK; 9102), or phospho-p42/p44 MAPK (9101) (Cell Signaling, Danvers, MA) or phospho-AKT-Ser473 (P4112) or AKT (P2482) (Sigma) overnight at 4 °C and subsequently with horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h. Signals were visualized using ECL (Amerham Biosciences) following the manufacturer’s instructions.

For immunoprecipitation, cell lysate (500 μg) was incubated with 1 μg of primary antibody for 1 h, followed by incubation with 15 μl of Protein A/G beads (IP05, Calbiochem) for 1 h at 4 °C in a total volume of 500 μl with cell lysis buffer. Beads were pelleted at 13,000 × g for 1 min and washed with buffer containing 300 mM NaCl, 50 mM Tris-HCl for a total of three washes. Protein was denatured by addition of 25 μl of Laemmli buffer to the bead pellet and boiling at 95 °C for 5 min. Immunoprecipitated proteins in the supernatant were resolved by SDS-PAGE, followed by immunoblotting as described above. shRNA for BRMS1—435 or 435BRMS1 cells were transected with a shRNA oligonucleotide against BRMS1 or a nonspecific control shRNA (V2HS_72900, Open Biosystems) using RNA-intro (RHS3600, Open Biosystems) according to the manufacturer’s instructions. Medium was replaced 6 h following transfection with complete DMEM. Following transfection, cells were harvested for RNA with TRIzol (Invitrogen) reagent after 5 days, and mRNA levels were determined by real-time quantitative PCR.

**Tagman Quantitative Real-time PCR**—mRNA levels of BRMS1, EGFR, and endogenous ribosomal S9, as an internal reference (7), were quantified using real-time PCR analysis (Taqman) on an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). Total cellular RNA was extracted using TRIzol, and ethanol-precipitated RNA was resuspended in 60 μl of diethyl pyrocarbonate-treated water. Amplification of specific PCR products was detected using fluorescent probes labeled with 6-carboxyfluorescein at the 5′ end. PCR was performed in a total reaction volume of 50 μl containing 4 mM MgCl<sub>2</sub>, 200 μM each dNTP, 1.25 units of Taq polymerase, and 3 μl of total RNA. The relative mRNA expression of the target genes was quantified using the comparative cycle time method. Each sample was assayed in triplicate.

**Immunocytochemistry**—Cells were plated on glass coverslips and serum-starved for 24 h. Cells were subsequently treated with and without EGF (50 ng/ml) for 30–60 min, fixed using 4% paraformaldehyde for 20 min, and permeabilized using 0.5% Triton X-100 for 8 min. F-actin was stained using rhodamine-conjugated phalloidin; nuclei were counterstained using 4′,6-diamidino-2-phenylindole, and cells were viewed under a Olympus IX-70 inverted epifluorescence microscope. Representative images were obtained, combined, and processed using IPLab (v3.7) deconvolution software (Scanalytics, Rockville, MD) at the University of Alabama at Birmingham High Resolution Imaging Core facility.

**Ratiometric Calcium Imaging**—Cells grown on coverslips were incubated in medium containing 5 μM Fura-2/AM (Molecular Probes) from a 5 mM dimethyl sulfoxide stock for 60 min. After incubation, coverslips were washed twice and transferred to a glass plate with an 8-mm hole, which was sealed with vacuum grease, forming a well to which 50 μl of medium was added. Cells were illuminated with a Lambda DG-4 light, and images were collected with a CoolSnap HQ camera controlled by Metafluor imaging software (Molecular Devices, Sunnyvale, CA). Multiple cells within the field of view were tracked as individual data sets; images were collected (one image/s), and ratio values were saved. Excitation wavelengths were 340 and 380 nm, and data were collected at 510 nm; the ratio was a result of the 340/380 image. For collection of ratiometric data from growth factor-stimulated cells, the experiments were carried out as above, except cells grown on coverslips were incubated in serum-free medium for 3 h prior to loading with Fura-2/AM. Ratiometric data were collected for 6–10 min to confirm a stable base line. EGF was then added to the medium, and data collection was continued. Ionophore A23187 (Calbiochem) was added at the end of the experiment to demonstrate that all cells still mobilized calcium. For inhibitor studies in EGF-stimulated cells, the cells were treated with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (10 μM, Calbiochem) for 45 min prior to EGF stimulation.
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**Immunohistochemistry**—BRMS1 expression was assessed using a monoclonal antibody (3a1.21) as described previously (8). Briefly, 4-μm-thick unstained sections were placed onto electrostatically charged glass slides and baked overnight. Primary antibody (1:50) and antigen detection was performed using a peroxidase-conjugated secondary antibody/3-diaminobenzidine chromogen. Costaining was done using anti-EGFR antibody 31G7 (Zymed Laboratories Inc.) at a dilution of 1:50 using alkaline phosphatase to activate the chromogen.

**Luciferase Reporter Assays**—Briefly, 30,000 HEK293 cells were plated in 24-well plates and allowed to attach for 24 h. Combinations of pcDNA3-BRMS1, phRL-SV40-Renilla luciferase (Promega), truncation mutants of EGFR pEGFR1/4/6/7/9/10-Luc (firefly) (9), or empty vectors were transfected in HEK293 cells in triplicate wells to a total concentration of 2 μg/ml using Lipofectamine reagent (Invitrogen) in Opti-MEM (Invitrogen). Medium was replaced with serum-containing medium after 6 h. Following transfection, cells were lysed after 48 h, and lysates were frozen immediately at −80 °C and used the next day. Luciferase activity was measured using Dual-Luciferase activity buffers (Promega) using a Berthold luminometer. Firefly luciferase readings were normalized to Renilla luciferase readings, and data were expressed as relative luciferase activity, where control was set at 100%. 435BRMS1 cells were also seeded in 6-well plates at a final seeding density of 200,000 cells/well and allowed to attach for 24 h. Cells were transfected with pEGFR1-Luc (0.9 μg) alone or in combination with BRMS1 small interfering RNA (s24634, s24632; Ambion) or negative control small interfering RNA (4390849, Ambion) with Lipofectamine reagent. phRL-SV40-Renilla (0.1 μg) was used as a transfection control. Small interfering RNAs were transfected at a final concentration of 5 nM. Medium was replaced 24 h later, and cell lysates were collected and frozen at −80 °C. Luciferase activity was measured as described above.

**RESULTS**

**BRMS1 Selectively Regulates Growth Factor Receptor Expression**—We and others have previously shown that BRMS1-expressing cells grow in orthotopic sites, but not ectopic sites (10–12). Re-expression of BRMS1 increased susceptibility to in vitro anoikis (4). Metastasis suppression can then be explained by fewer BRMS1-expressing cells reaching secondary sites. Nonetheless, some tumor cells still seeded various ectopic sites, but failed to proliferate and establish overt metastases. We reasoned that survival in non-native environments (i.e. during circulation and at ectopic sites) requires tumor cells to interact with and respond to their immediate microenvironments through cell-surface receptors and activate downstream survival signals.

We examined receptor expression of EGFR, platelet-derived growth factor receptor (PDGFR), and hepatocyte growth factor (c-Met) receptor following BRMS1 re-expression. These receptor tyrosine kinases are known to promote individual events in the multistep metastatic cascade, including proliferation, invasion, and angiogenesis. Receptor expression was measured by immunoblotting in multiple BRMS1-expressing clones derived from 231 and 435 cells and compared with vector-transduced controls. EGFR protein expression was substantially reduced in 231BRMS1 cells, whereas EGFR protein was undetectable in 435BRMS1 cells. Immunoblots revealed no changes in PDGFR-β protein expression in vector-transduced versus BRMS1-expressing cells. PDGFR-α was not detected in either 231/435 cells or their BRMS1-expressing counterparts (data not shown). Expression of the pro-form of c-Met appeared to be slightly elevated in 231BRMS1 and 435BRMS1 cells compared with controls (Fig. 1).

EGFR mRNA expression was measured by real-time quantitative PCR in multiple BRMS1-expressing clones in 231 and 435 cells. EGFR mRNA decreased in BRMS1-expressing cells, following a trend similar to that of protein expression (Fig. 2A). shRNA to BRMS1 in 435BRMS1 cells partially restored EGFR expression, confirming the specificity of BRMS1 in modulating EGFR expression (Fig. 2C). Because BRMS1 is a component of Sin3-histone deacetylase complexes that regulate transcription (13), we examined whether BRMS1 expression affected EGFR promoter activity. Cotransfection of BRMS1 along with the construct for the proximal EGFR promoter region (~16 to ~1109) tagged to a luciferase reporter (9) in HEK293 cells reduced full-length EGFR promoter activity by more than 90% (Fig. 2B). Furthermore, shRNA targeting BRMS1 in 435BRMS1 cells increased EGFR promoter activity in a luciferase reporter assay (supplemental Fig. 2). To validate data from our cell line models, we performed immunohistochemical analysis of primary human breast carcinomas for EGFR and BRMS1. EGFR (red stain, Fig. 2D) and BRMS1 (brown stain, Fig. 2D) were generally mutually exclusive. As expected, BRMS1 staining was localized mainly to the nucleus, whereas EGFR staining was localized mostly to the cell membrane.

**Re-expression of BRMS1 Modulates Growth Factor-induced Downstream Signaling**—Expression of BRMS1 leads to a selective down-regulation of the second messenger phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P2) (14). The conversion of PtdIns(4,5)P2 to PtdIns(3,4,5)P3 is essential for activation of molecules downstream of PI3K (reviewed in Ref. 15), and therefore, phosphoinositide-dependent signaling was
first examined in response to different growth factors. Because we also observed selective changes in receptor expression (decreased EGFR, minimally increased c-Met, or unchanged PDGFR expression), we asked whether downstream signaling mirrored changes in upstream signaling molecules.

We specifically examined phosphorylation of AKT in response to EGF, hepatocyte growth factor (HGF), and PDGF. Dose- and time-response studies were performed to determine optimal response, and defined parameters were used for all further studies. 231/231BRMS1 and 435/435BRMS1 cells were treated with EGF (0–50 ng/ml), HGF (50 ng/ml), or PDGF (50 ng/ml) for 15 min, and cell lysates were analyzed for AKT (protein kinase B) Ser473 phosphorylation by immunoblotting using phosphorylation-specific antibodies. Results were verified for consistency in both cell lines, and representative data are shown. EGF induced AKT phosphorylation in a dose-dependent manner in 231 cells, but decreased it in 231BRMS1 cells. Consistent with the loss of EGFR expression, 435BRMS1 cells completely failed to respond to EGF stimulation (Fig. 3A).

However, AKT phosphorylation in response to HGF was maintained in BRMS1-expressing cells in the presence of the decreased upstream second messenger PtdIns(4,5)P₂.

p42/p44 MAPK phosphorylation in response to EGF, HGF, and PDGF treatments was also examined. Because 231 cells have a Gly → Asp mutation in the Ki-Ras at codon 13, making it constitutively active (16, 17), it was not surprising that EGF or HGF treatment failed to further stimulate MAPK phosphorylation (Fig. 3B). Immunoblotting proved to have insufficient sensitivity to detect changes in MAPK phosphorylation in 231BRMS1 cells. MAPK phosphorylation was also assessed in response to EGF and HGF treatments. Again, we found no significant differences between 231 and 231BRMS1 cells in response to these growth factors (Fig. 3C).

FIGURE 3. BRMS1 decreases AKT but not MAPK phosphorylation. 231/435 and 231BRMS1/435BRMS1 cells were treated with 0–50 ng/ml EGF or HGF for 15 min, and whole cell lysates were immunoblotted for phospho-AKT (pAKT; A) or phospho-MAPK (B) phosphorylation. Total AKT and MAPK were used as loading controls. C, 231 and 231BRMS1 cells were treated with and without 50 ng/ml EGF for 15 min, and whole cell lysates were obtained. EGFR was immunoprecipitated (IP) with an anti-EGFR antibody, and the proteins were separated by SDS-PAGE. The blot was probed with an anti-phosphotyrosine antibody to determine receptor phosphorylation status.
sensitivity to detect changes in AKT or MAPK phosphorylation following PDGF treatment, and therefore, data for PDGF treatment are not shown.

To evaluate whether BRMS1 affected the ability of EGFR to autophosphorylate after EGF treatment, EGFR was immunoprecipitated from whole cell lysates of 231/231BRMS1 cells, and receptor phosphotyrosine status was determined after EGF treatment (50 ng/ml, 15 min). Although EGFR tyrosine phosphorylation was lower in the 231BRMS1 cells, the level of phosphorylation was approximately proportional to the reduced EGFR levels (Figs. 1A and 3C). To further assess downstream effects, actin cytoskeletal organization was measured in response to EGF. Cells treated with and without EGF (50 ng/ml) were stained with rhodamine-conjugated phalloidin. Whereas vector transfectants responded to EGF stimulation over time (0–60 min) with lamellipodial formation, 231BRMS1 cells showed reduced reorganization and lamellipodial formation; both were completely absent in 435BRMS1 cells (supplemental Fig. 1).

BRMS1 Decreases Intracellular Mobilization Independent of Receptor Expression Status—One of the major pathways affecting intracellular calcium release involves activation of phosphoinositide signaling. PI3K converts PtdIns(4,5)P2 to PtdIns(3,4,5)P3, and levels of both can increase rapidly in response to growth factor stimulation. Furthermore, PtdIns(4,5)P2 serves as a precursor for inositol 1,4,5-triphosphate, which mobilizes intracellular calcium (15, 18, 19). Release of calcium from intracellular stores is important in modulation of the actin cytoskeleton for lamellipodial and filopodial formation during active processes, including motility and invasion (20, 21).

We have previously demonstrated that re-expression of BRMS1 leads to a selective reduction (>90%) in levels of the second messengers PtdIns(4,5)P2 and inositol 1,4,5-triphosphate levels (14). Both EGF and PDGF treatment (50 ng/ml) induced intracellular calcium release in parental breast carcinoma cells that was abrogated when BRMS1 was re-expressed (Fig. 4). Previous demonstration that PDGF (50 ng/ml) treatment of 435BRMS1 cells failed to elicit a calcium mobilization response (14) was confirmed. Furthermore, consistent with the lack of decrease in upstream signaling, no decrease in intracellular calcium mobilization was observed following HGF treatment (data not shown).

DISCUSSION

With improved imaging and diagnostic procedures, cancers are being diagnosed earlier and cure rates are improving, yet 25–30% of women with breast cancer develop metastases months to years following primary tumor removal (22, 23). Tumor cells that had already disseminated had lain dormant during the interim, only to recommence growth because either the tumor cell itself or its microenvironment had changed. It is well established that local microenvironments influence disseminated tumor cell fate. Tumor cell responses to each microenvironment encountered during the metastatic cascade also determine survival and subsequent growth. This cross-talk is most intuitive for tumor cells seeding various tissues following dissemination.

The focus of the current study was borne from the following observations. 1) BRMS1-expressing cells grow progressively at the orthotopic site (i.e. mammary fat pad), whereas they fail to grow at ectopic sites (e.g. lung and bone) (10–12). 2) BRMS1 induces anoikis in breast carcinoma (4) and in non-small cell lung cancer cells (24), and fewer BRMS1-expressing cells reach secondary sites compared with the parental cells. Activation of the PI3K-AKT axis is important in mediating anchorage-independent survival (25, 26). Our data with decreased activation of
downstream mediators of PI3K support the observation of increased anoikis in BRMS1-expressing cells (4). 3) BRMS1-expressing cells reaching the secondary site do not form overt metastases (4). 4) In human patients, BRMS1 expression has been inversely correlated with growth factor receptor (HER2) expression (8). These observations led to the hypothesis that BRMS1 expression regulates selective growth factor responsiveness. These data also raise the possibility that selective regulation of growth factor signaling may influence where BRMS1-expressing cells do or do not grow.

Interaction of a tumor cell with its microenvironment involves outside-in as well as inside-out signaling, and these interactions are critical for eventual growth and proliferation (27). In this study, we have focused on the modulation of outside-in signaling by BRMS1. Although not an exhaustive panel, responses to three distinct growth factors (EGF, PDGF, and HGF, all of which have been implicated in breast tumor and metastatic progression) were analyzed (28, 29). The mutated signaling response to EGF in BRMS1-expressing cells can be attributed, in part, to reduction (231BRMS1) or complete loss (435BRMS1) of EGFR expression, whereas decreased calcium mobilization through PDGF-R may be a consequence of reduced PtdIns(4,5)P2 levels. The loss of upstream PtdIns(4,5)P2 does not explain, however, the maintenance of AKT phosphorylation in BRMS1-expressing cells following HGF treatment. Alternate routes of AKT phosphorylation are known (30), and activation through other pathways cannot be ruled out and is beyond the scope of this study. Furthermore, consistent with the lack of decrease in upstream signaling, no decrease in calcium mobilization was observed following HGF treatment (data not shown). Previous reports by us and others have also demonstrated decreased NF-kB activity (which is downstream of AKT) in the presence of BRMS1 (24, 31). Differential activation of downstream pathways is expected with different growth factors. It is interesting that BRMS1 modulates signaling through the same pathway (PI3K-AKT) at different levels of the signaling cascade. Notwithstanding cross-talk between different signaling cascades, components of the PI3K-AKT pathway appears to be more susceptible to inhibition than the MAPK cascade.

The exact mechanism of BRMS1 repressing EGFR transcription remains unclear. BRMS1 may affect (reduce either expression or activity) transcription factors or another signaling intermediate that determines EGFR expression. Alternatively, as part of the Sin3-histone deacetylase complexes, BRMS1 could directly repress EGFR transcription. However, the current lack of a chromatin immunoprecipitation-grade BRMS1 antibody precludes testing this hypothesis. We have recently shown that substitution of Leu174 with Asp (L174D) of BRMS1 decreases osteopontin expression comparable with wild-type BRMS1; however, deletion of the first coiled-coil domain of BRMS1 restores osteopontin expression. EGFR expression is still down-regulated in all BRMS1 mutants. Furthermore, BRMS1 mutants still maintain the ability to suppress metastasis, suggesting that selective gene regulation may be a function of the composition of the BRMS1 complex (12).

**In vitro** experiments are limited in their reconstruction of the complex milieu of tissue microenvironments. Our experiments do not recapitulate the multiple effects of different factors acting on the tumor cell in tandem. Furthermore, the relative expression and the spectrum of growth factors present in the individual tissue microenvironment are not defined, and therefore, manipulating expression of any single growth factor in a particular tissue, although theoretically feasible, is not practical experimentally, considering that cancer cells colonize multiple organs. Nonetheless, a surrogate, as demonstrated in our experiments, is to test individual growth factors *in vitro* by analysis of receptor levels concomitant with signaling responses in tumor cells. Furthermore, BRMS1-expressing cells do not show major changes in *in vitro* assays that measure different steps in the metastatic cascade (11), limiting our use of *in vitro* assays to measure experimental end points.

Given the redundancy and cross-talk that occur between multiple signaling pathways, our data imply that a multi-pronged therapeutic approach that targets multiple cascades or multiple levels within the same cascade could be beneficial for the treatment of metastases. Preliminarily, our data also begin to characterize the phenotype required for metastasis suppression. Although we cannot yet fully explain the requirements for growth at the orthotopic *versus* ectopic sites, native differences in tissue microenvironments, in addition to the tumor cell phenotype, may be major contributors in determining outcome. Differential signaling within the tumor cell (as demonstrated in our experiments) may mirror a global change that reflects stringent growth/survival factor dependence, which determines microenvironments where tumor cells can thrive.

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