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Ubiquitous *Brms1* expression is critical for mammary carcinoma metastasis suppression via promotion of apoptosis

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Abstract

Morbidity and mortality of breast cancer patients are drastically increased when primary tumor cells are able to spread to distant sites and proliferate to become secondary lesions. Effective treatment of metastatic disease has been limited; therefore, an increased molecular understanding to identify biomarkers and therapeutic targets is needed. Breast cancer metastasis suppressor 1 (BRMS1) suppresses development of pulmonary metastases when expressed in a variety of cancer types, including metastatic mammary carcinoma. Little is known of Brms1 function throughout the initiation and progression of mammary carcinoma. The goal of this study was to investigate mechanisms of *Brms1*-mediated metastasis suppression in transgenic mice that express *Brms1* using polyoma middle T oncogene-induced models. *Brms1* expression did not significantly alter growth of the primary tumors. When expressed ubiquitously using a β-actin promoter, *Brms1* suppressed pulmonary metastasis and promoted apoptosis of tumor cells located in the lungs but not in the mammary glands. Surprisingly, selective expression of *Brms1* in the mammary gland using the MMTV promoter did not significantly block metastasis nor did it promote apoptosis in the mammary glands or lung, despite MMTV-induced expression within the lungs. These results strongly suggest that cell type-specific over-expression of *Brms1* is important for *Brms1*-mediated metastasis suppression.

Keywords

Metastasis; BRMS1; PyMT; Transgenic; MMTV; Ubiquitous; Apoptosis; Breast cancer; Mouse; Mammary; Tumor microenvironment

Introduction

Metastasis is the most lethal attribute of breast cancers. Metastases are typically inoperable, difficult to target for treatment, and directly contribute to increased morbidity and mortality of breast cancer. Continuous improvements in early detection methods and treatments have contributed to 5-year breast cancer survival rates approaching 100%. However, survival rates are drastically reduced below 25% upon development of distant metastases [1]. An improved understanding of the molecular mechanisms of metastasis is necessary for identification of novel treatment strategies.

In recent years there has been an increasing appreciation for metastasis suppressors which have provided necessary tools to dissect the molecular underpinnings of metastasis [2, 3]. Breast cancer metastasis suppressor 1 (*BRMS1*) significantly reduces lung metastases in athymic mice when expression is ectopically restored in human metastatic cell lines from breast [4], melanoma [5], ovarian [6], and non-small cell lung carcinomas [7]. BRMS1 alters multiple cellular pathways involved in metastasis including: gap junctional intercellular communication [7-10], phosphoinositide pools and signaling [11, 12], modification of nuclear factor kappa B (NFκB) signaling [13-15], and cellular motility and invasion [5, 6,

15, 16]. In addition to these changes, Phadke et al. [17] demonstrated that *BRMS1* expression decreased disseminated tumor cell viability and cell seeding in vivo, phenomena consistent with in vitro activation of pro-apoptotic signaling via caspase 3 and PARP cleavage.

Mediation of these pathways is likely attributed to BRMS1-regulated gene transcription via BRMS1 interactions with SIN3: histone deacetylase (HDAC) chromatin remodeling complexes through direct interaction with AT-rich interacting domain 4A (ARID4A) and suppressor of defective silencing 3 (SUDS3) [18, 19]. Although there is no evidence of BRMS1 functioning as a transcription factor, BRMS1 has been shown to recruit HDAC1, and presumably SIN3 complexes, to NFκB consensus regions leading to inhibition of NFκB activity [15, 20].

BRMS1 orthologs are highly conserved across many species. Especially pertinent to this study, the amino acid sequence of murine Brms1 is 95% homologous with human BRMS1 [21]. When re-expressed in 4T1 or 66cl4 metastatic mammary carcinoma cells, *Brms1* significantly reduced metastases to the lungs in syngeneic BALB/c mice [22]. Furthermore, murine Brms1 associates with HDAC1 affirming functional similarity to human BRMS1, warranting its use in genetically-engineered mice to further investigate mechanisms of action in metastasis suppression.

Although restoration-of-expression experiments have been useful in xenograft and syngeneic models, these experimental systems do not fully recapitulate initiation and progression of malignancy and host-tumor interactions that occur throughout tumor progression [23, 24]. Development of *Brms1* metastasis models in which tumors arise de novo and spontaneously metastasize (by utilizing genetically-engineered mice) provides new insight into mechanisms of metastasis suppression. We developed two transgenic mouse models which over-express *Brms1*. The first, Brms1^{MMTV}, selectively expresses *Brms1* in mammary glands using the mouse mammary tumor virus (MMTV) promoter. In the second, Brms1^{Ubqs}, *Brms1* is expressed ubiquitously using the cytomegalovirus (CMV)-enhanced chicken beta actin promoter. Both models were used to test the impact of *Brms1* over-expression on primary tumor development and metastatic potential by crossing them with the well-characterized polyoma middle T (PyMT) oncogene mammary tumor mouse model [25, 26]. As predicted, Brms1 function as a metastasis suppressor was affirmed in the Brms1^{Ubqs} × MMTV-PyMT F_1 mice. Surprisingly, F_1 generation mice from Brms1^{MMTV} × MMTV-PyMT still developed metastases at similar levels to PyMT mice. These results reveal a more complex mode of action for Brms1-mediated metastasis suppression than was previously recognized.

Materials and methods

Mouse husbandry and analysis

All animal studies were carried out under the approval of the University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee (IACUC). For mammary-selective expression of *Brms1* cDNA, the 8.4 kb pMAMneo mammalian expression vector which contains the RSV-LTR enhancer linked to the MMTV-LTR promoter was utilized. The vector contains the RSV-LTR enhancer linked to the MMTV-LTR promoter. The ubiquitous Brms1 expresser (Brms1^{Ubqs}) expresses *Brms1* in most tissues by the CMV-enhanced chicken β-actin promoter (CAG promoter). To generate Brms1^{Ubqs} mice, the pCX expression vector carrying the CAG promoter (a combination of the CMV early enhancer element and chicken β-actin promoter) was utilized. All pronuclear injections were performed by the UAB Transgenic Core and resulting chimera were mated

with wild-type C57BL/6 mice resulting in heterozygous Brms1 transgenic mice. PyMT oncogene transgenic mice were obtained from Jackson Laboratories (Bar Harbor, ME).

All mice were tail-clipped, weaned (at ~3 weeks), and genotyped. Transgenic *Brms1* mice were genotyped by PCR using the following primers: GCCAAACTGAGTCAGAGGAG and GTGTTGTTTGGCTCCCTG. Transgene presence was identified by the presence of two genetic bands, one 720 bp endogenous *Brms1* band and a 275 bp *Brms1* cDNA band. PyMT mice were genotyped by PCR using the following primers: AACGGCGGAGCGAGGAACTG and ATCGGGCTCAGCAACACAAG. Transgenic mice were identified by the presence of a 556 bp genetic band. To validate increased *Brms1* expression, normal mammary gland and lung tissues from transgenic Brms1 mice were collected, flash-frozen using liquid nitrogen, and expression was analyzed using real-time quantitative reverse transcription PCR (RT-qPCR).

Because heterozygous $PyMT^{+/-}$ females develop benign hyperplastic lesions at puberty and cannot effectively nurse pups, heterozygous males were crossed with heterozygous $Brms1^{tg/wt}$ (transgenic) females to yield an F1 generation. $PyMT^{+/-}/Brms1^{wt}$ mice expressed endogenous levels of wild-type *Brms1*, $PyMT^{+/-}/Brms1^{tg/wt}$ mice expressed the *Brms1* transgene expression, and $PyMT^{-/-}/Brms1^{wt}$ and $PyMT^{-/-}/Brms1^{tg/wt}$ mice expressed wild-type and transgenic *Brms1*, respectively, in the absence of the *PyMT* oncogene. When possible, $PyMT^{+/-}/Brms1^{wt}$ and $PyMT^{+/-}/Brms1^{tg/wt}$ littermates were compared for tumorigenicity and metastatic potential.

Mice were monitored daily and incidence of the first tumor appearance was recorded. Body weight and total tumor burden were determined at necropsy. Normal mammary glands and mammary tumors were flash-frozen using liquid nitrogen for gene and protein analyses. In addition, aliquots of tumor tissue were formalin-fixed and paraffin-embedded for histological analyses. Tissues were processed, sectioned and either stained with hematoxylin and eosin or utilized for immunohistochemical assays. Lungs were dissected and fixed in either Bouin's fixative or formalin-fixed and paraffin-embedded for histology. The number of lung metastases was counted by visual inspection of fixed lungs and by histological analysis. The number of macroscopic metastases in $PyMT^{+/-}/Brms1^{tg/wt}$ was compared to control mice ($PyMT^{+/-}/Brms1^{wt}$) to determine changes in metastatic potential that occurred due to *Brms1* over-expression. Liver, spleen, and kidney were also collected and analyzed for the presence of metastases in other organs in addition to the lungs. When possible, littermates were compared as described above.

Real-time quantitative reverse-transcription PCR

Flash-frozen tissue was homogenized by mortar and pestle and lysed with Qiazol Lysis Reagent (Qiagen, Valencia, CA). RNA was isolated using the miRNEasy extraction kit (Qiagen) according to the manufacturer's protocol, reverse-transcribed and quantified using the miScript SYBR Green PCR kit (Qiagen) and *Brms1*-specific Quantitect Assay primers (Qiagen). Relative RNA expression in all samples was normalized to 18s rRNA expression levels and fold-change was calculated as previously described [27].

Immunohistochemistry

For immunohistochemical analyses of proliferation and microvessel density, 5 μm thick sections of formalin-fixed, paraffin-embedded mammary tumor tissue sections were deparaffinized in xylene and rehydrated in graded alcohols. For antigen retrieval, slides were immersed and boiled for 20 min in a diluted (1:30), pH 9.0, antigen unmasking solution (Vector Laboratories, Burlingame, CA). Slides were incubated in a horse serum blocking solution (ImmPRESS system, Vector Laboratories) for 1 h followed by incubation with

either Ki67 primary monoclonal antibody (Clone TEC3 Antigen, 1:100 dilution, Dako, Carpinteria, CA) or CD31 primary polyclonal antibody (Ab28364, 1:100 dilution, Ab-Cam, Cambridge, MA) in phosphate-buffered saline solution containing 1% bovine serum albumin. Appropriate secondary antibody (ImmPRESS, Vector Laboratories) was applied and slides were incubated in DAB (3,3′-diaminobenzidine) peroxidase substrate solution (Dako). Each slide was then incubated with Harris hematoxylin counterstain (Fisher Scientific, Waltham, MA). Nuclei of cells positive for Ki67 stained brown and the percentage of positive cells per high powered microscopic field was determined. A minimum of 1,000 cells were counted for each mammary tumor sample. Intratumoral microvascular density (iMVD) was determined by averaging the number of CD31-positive vessels in representative areas with the highest microvessel density (designated “hot spots”) as previously described [28]. A minimum of three “hot spots” were counted per sample. To assess iMVD in metastases, the total number of CD31-positive vessels was counted per metastatic lesion per sample. Only lesions containing more than 500 cells were assessed for iMVD to analyze changes in angiogenesis due to potentially hypoxic conditions.

For analyses of apoptosis in tumor and lung tissue, the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA) was utilized according to the manufacturer’s protocol. Apoptotic bodies/nuclei were stained brown with DAB peroxidase substrate and the percentage of positive cells within the primary tumor per high powered microscopic field was determined as described above. The percentage of total positive cells per metastatic lesion was determined.

Statistical analysis

Continuous parameters were compared by the student’s *t* test for body weight, age at necropsy, and number of metastases when controlling for age. The primary endpoint of interest was the number of visible lung macroscopic metastases in each genotype group. Due to a non-normal distribution, the median test was used to compare the total number of lung metastases per group. Because metastatic potential correlates with total tumor burden in PyMT mice, a multivariable analysis for number of lung metastases was conducted using generalized Poisson regression. Age was used as an offset variable in the multivariable model because some of the animals were euthanized early due to high tumor burden which may have affected the number of metastases. Tumor latency was calculated as the percentage of tumor-free mice throughout the study using Kaplan–Meier analyses. Data from tumor latency studies were analyzed by non-parametric Wilcoxon rank sum test. Tumor burden, proliferation, and apoptosis studies were analyzed by two-sample paired student’s *t* test assuming equal variance. All analyses were performed using SAS (Ver. 9.2®) statistical analysis software. A P value <0.05 was deemed statistically significant.

Results

Brms1 expression is increased in transgenic mice

To validate increased *Brms1* expression, RT-qPCR analysis was performed on normal mammary gland and lung tissues from ubiquitous and mammary-selective transgenic *Brms1* mice. Both $Brms1^{MMTV}$ ($P = 0.31$) and $Brms1^{Ubqs}$ mice ($P = 0.01$) exhibited an increase of *Brms1* expression in mammary gland compared to wild-type C57BL/6 mice (Fig. 1a). Although *Brms1* should be over-expressed in all tissues in $Brms1^{Ubqs}$ mice, we focused on expression in the major tissues involved in the PyMT mammary tumor model—mammary glands and lungs. As expected, *Brms1* expression in lungs of $Brms1^{Ubqs}$ mice was five times greater than observed in wild-type mice ($P = 0.02$) (Fig. 1b). *Brms1* was also increased in lung tissue of $Brms1^{MMTV}$ mice ($P = 0.04$), confirming previous reports of “leaky” expression of the MMTV promoter [29].

Increased *Brms1* expression does not significantly alter tumor latency or growth

Female F_1 crossed mice (litter of $PyMT^{+/-} \times Brms1^{tg/wt}$) were compared for analysis of tumor and metastasis development. As previously demonstrated [25, 26], all mice expressing the PyMT oncogene developed mammary carcinoma in multiple (sometimes all 10) mammary glands. As expected, $PyMT^{-/-} \times Brms1^{Ubqs}$ and $PyMT^{-/-} \times Brms1^{MMTV}$ littermates did not spontaneously develop tumors. There were no significant differences in tumor latency when comparing control mice ($PyMT^{+/-}/Brms1^{wt}$) to tumor-bearing transgenic Brms1 mice ($PyMT^{+/-}/Brms1^{MMTV}$ or $PyMT^{+/-}/Brms1^{Ubqs}$) (Fig. 2a, b). All mice developed palpable tumors at approximately 12–14 weeks of age. There were no differences in tumor or lung pathology when comparing controls to either *Brms1* over-expressing mice (Supplemental Fig. 1). Analysis of primary mammary tumors isolated from $PyMT^{+/-}/Brms1^{Ubqs}$ or $PyMT^{+/-}/Brms1^{MMTV}$ revealed increased *Brms1* expression in tumors from transgenic mice compared to tumors from $PyMT^{+/-}/Brms1^{wt}$ controls (Fig. 2c, d). There were no significant differences in the total primary tumor burden per mouse (i.e. mass of all mammary tumors combined) when comparing $PyMT^{+/-}/Brms1^{Ubqs}$ or $PyMT^{+/-}/Brms1^{MMTV}$ mice with respective controls (Fig. 2e, f; Table 1).

Ubiquitous *Brms1* expression significantly suppresses lung metastases

To assess the impact of *Brms1* over-expression on metastasis of PyMT-induced mammary carcinoma, lungs were collected from $PyMT^{+/-}/Brms1^{wt}$, $PyMT^{+/-}/Brms1^{MMTV}$, and $PyMT^{+/-}/Brms1^{Ubqs}$ mice and macroscopic metastases were counted with a dissecting microscope. Over-expression of *Brms1* predominantly in mammary tissue, $PyMT^{+/-}/Brms1^{MMTV}$, did not significantly alter the median number of macroscopic lung metastases ($P = 0.78$; Fig. 3a; Table 1) when compared to $PyMT^{+/-}/Brms1^{wt}$ controls. There was no significant difference in the number of metastases in $PyMT^{+/-}/Brms1^{MMTV}$ mice when controlled for overall tumor mass and age at necropsy ($P = 0.63$) in a multivariable analysis.

However, ubiquitous over-expression of *Brms1*, $PyMT^{+/-}/Brms1^{Ubqs}$, reduced the number of visible lung metastases compared to $PyMT^{+/-}/Brms1^{wt}$ controls. The median number of metastases was higher in the control mice (median = 31) compared to the $Brms1^{Ubqs}$ mice (median = 8; $P = 0.06$) (Fig. 3b, c; Table 1). Subsequently, $Brms1^{Ubqs}$ mice had significantly fewer metastases at necropsy in a multivariable analysis controlling for total tumor burden and age at necropsy ($P < 0.0001$). H&E stained lung sections revealed no gross morphological differences when comparing metastatic lesions from $Brms1^{Ubqs}$ and control mice (Supplemental Fig. 1). As in the control mice, $PyMT^{+/-}/Brms1^{MMTV}$ and $PyMT^{+/-}/Brms1^{Ubqs}$ mice had no macroscopic nor microscopic metastases in liver, spleen, or kidney (Supplemental Fig. 2). There were no differences in the size of metastatic lesions in the lungs of $PyMT^{+/-}/Brms1^{MMTV}$ or $PyMT^{+/-}/Brms1^{Ubqs}$ when compared to respective control mice. To determine whether metastasis suppression was related to alterations that occurred in the primary tumor, proliferation, apoptosis and microvascular density were measured. No significant changes were found (Fig. 4a–c).

Ubiquitous *Brms1* expression induces apoptosis in lung metastases

Apoptosis was analyzed by TUNEL staining to determine whether ubiquitous expression of *Brms1* altered apoptosis within metastatic lung lesions. Metastatic foci from $PyMT^{+/-}/Brms1^{Ubqs}$ mice exhibited a 3-fold higher apoptosis compared to $PyMT^{+/-}/Brms1^{wt}$ littermates (Fig. 5a, c). There was no significant change in microvessel density within the lung metastases of $PyMT^{+/-}/Brms1^{Ubqs}$ mice (data not shown), suggesting that induction of apoptosis was independent of angiogenesis. Despite increased *Brms1* expression in $PyMT^{+/-}/Brms1^{MMTV}$ lungs, there was no significant difference in apoptosis of $PyMT^{+/-}/Brms1^{MMTV}$ lung lesions when compared to control $PyMT^{+/-}/Brms1^{wt}$ mice (Fig. 5b).

Discussion

For decades, researchers have sought to fully understand the genetic complexities involved in the development and progression of breast carcinoma, including how the host-tumor interactions can potentiate or inhibit tumor progression. Understanding how tumor cells adapt to changing environments, including their ability to survive and proliferate in foreign tissues (i.e. ectopic sites), is important for identification of relevant targets for metastatic disease. These are the first studies to reveal that *Brms1* function, as an important mediator of apoptosis and metastasis, may be context-dependent and rely on specific cell-type expression.

Consistent with the definition of a metastasis suppressor, *Brms1* over-expression did not discernibly affect primary tumor incidence or growth. When expressed ubiquitously, *Brms1* suppressed metastasis and induced apoptosis when tumor cells seeded the lungs. The pro-apoptotic effect occurred independently of angiogenic properties and was not reflected in similar analyses of corresponding primary tumors. However, it had been previously shown that BRMS1 could potentiate apoptosis in vitro, sensitize tumor cells to anoikis in vivo, and correlate with expression of pro-apoptotic genes in clinical samples of breast cancer patients [15, 17, 30]. The data presented in this report suggest that BRMS1 over-expression within the microenvironment where metastases form (i.e. lung parenchyma) could be just as important for induction of tumor cell apoptosis. In support of this notion, several studies have reported that conditioned media from cultured normal lung fragments induced anti-apoptotic signaling [31] or increased the survival of metastatic mammary carcinoma cells [32], implying that the lung microenvironment can facilitate tumor cell survival. Over-expression of *Brms1* in certain environments may reverse those effects. However, the situation may be more complex and depend on specific cell-type expression since *Brms1* was also over-expressed in the lungs of Brms1^{MMTV} mice.

Taken together, our findings suggest a potential role for stromal *Brms1* expression as a modulator of metastasis. Inhibition of stromal cell signaling appears to be essential for suppression of metastasis by PyMT-derived tumors which rely, in part, on stromal-associated promotion of tumor cell migration, invasion, and metastasis [33-37]. The MMTV promoter (utilized in PyMT and Brms1^{MMTV} mice) drives expression within epithelial cells whereas the CMV-enhanced chicken β-actin promoter (utilized in Brms1^{Ubqs} mice) drives expression in both stromal and epithelial cells). Because MMTV-induced *Brms1* expression neither suppressed metastasis nor induced apoptosis within metastatic lesions, it appears that *Brms1* expression within both stromal and epithelial cells is necessary for both phenotypes. But, additional studies will be necessary to test this hypothesis directly.

It remains unclear why *Brms1* expression in mammary epithelium was not sufficient for metastasis suppression. Prior investigations utilizing xenograft and syngeneic models involved restoration of *BRMS1* expression in metastatic cells, i.e. cells which have already overcome outstanding limitations in progression. The autochthonous models utilized here include initiation and progression of mammary carcinomas in a continuously evolving tumor microenvironment and constitutively expressed *PyMT* and *Brms1*. Although the findings reported here seemingly contradict transfection and over-expression data, one must consider the dynamics of mammary stromal-epithelial interactions that occur throughout mammary carcinoma progression [38]. The stroma regulates mammary epithelial growth, glandular development and homeostasis. Upon transformation and disease progression, tumor cells bypass stromal regulation and recruit new stromal constituents that modulate the microenvironment to support continuous growth and development [39]. While tumor-stromal interactions are undoubtedly relevant in the injection models, the developmental time line is attenuated and does not fully recapitulate the impact of an evolving

microenvironment throughout progression. Our findings suggest that the tumor microenvironment and host-tumor interactions play pivotal roles in Brms1-mediated metastasis suppression. Collectively, these studies highlight the importance of using multiple model systems to gain a more complete understanding of the mechanisms of metastasis suppression. For example, transplantation of mammary tumors representing different breast cancer molecular subtypes onto transgenic mice could be done. Although planned, those studies are beyond the scope of this initial report.

Another potential explanation for failure of MMTV-driven *Brms1* to suppress metastasis may relate to the expression levels in the transgenic mice. *Brms1* expression in mammary glands of Brms1 transgenic mice was analyzed in virgin mice (i.e. when the fat pad is still relatively under-developed and has few epithelial cells compared to the total mass). The normal mammary gland of virginal Brms1^{MMTV} mice did not exhibit a significant increase in *Brms1* expression. Expression was markedly increased in primary tumors of PyMT$^{+/-}$/Brms1^{MMTV} mice (and was comparable to expression in PyMT$^{+/-}$/Brms1^{Ubqs} tumors), which have a greater proportion of epithelium-derived cells compared to the normal mammary gland and which is consistent with the MMTV promoter being expressed only in the epithelial compartment. Perhaps a spatiotemporal (before oncogenesis and throughout progression) or a threshold level of BRMS1 expression is required to suppress metastasis. Generating a panel of mice with a range of Brms1 expression under temporal control would need to be done in order to evaluate this possibility, but is beyond the scope of this initial report.

Host genetic background is another important factor involved in the development of mammary carcinoma metastasis [40, 41]. The data presented here represent the F_1 generation of the *PyMT* oncogene on an FVB strain crossed with the *Brms1* transgene on the C57BL/6 strain. This is important because Hunter and colleagues showed that the F_1 generation MMTV-PyMT × C57BL/6 cross were metastasis suppressed [40, 41]. When this project began, congenic MMTV-PyMT on a C57BL/6 background did not exist. They recently became available, allowing assessment of the impact of *Brms1* over-expression on MMTV-PyMT independent of background genetic complications. We have initiated these studies but require larger numbers of animals before we can accurately assess or interpret the findings.

Our results provide a potential explanation for inconsistencies reported for *BRMS1* mRNA and protein expression in clinical samples [42-46]. Interpretation of the clinical studies is affected by the purity of sample (i.e. contamination by stromal cells), discordance between mRNA and BRMS1 protein levels and BRMS1 localization in the nucleus versus cytoplasm. The finding that *Brms1*-mediated metastasis suppression may be influenced by expression within the stromal compartment strongly suggests that expression in specific cell types may be more important than expression within primary tumor cells as an indicator of metastatic disease than previously appreciated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BRMS1	Breast cancer metastasis suppressor 1
PyMT	Polyoma middle T antigen
MMTV	Mouse mammary tumor virus
CMV	Cytomegalovirus
Ubqs	Ubiquitous
DAB	3,3′-Diaminobenzidine
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TdT	Terminal deoxynucleotidyl transferase
NFkB	Nuclear factor kappa B
HDAC	Histone deacetylase
Tg	Transgenic
Wt	Wild-type

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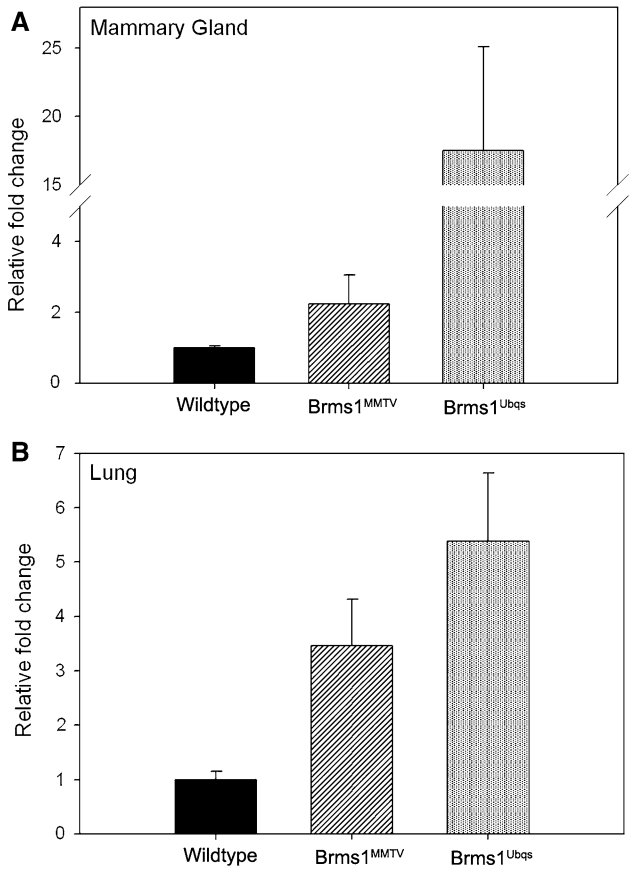

Fig. 1.

Brms1 expression is increased in transgenic mice. Two-step RT-qPCR using SYBR Green was utilized to detect *Brms1* in normal mouse tissues. Relative expression is graphed as fold change compared to wild-type control. All samples were normalized to ribosomal 18S RNA. **a** Relative expression of *Brms1* in normal mammary gland tissue from wild-type ($Brms1^{wt}$), mammary-selective ($Brms1^{MMTV}$; $P = 0.31$), and ubiquitous Brms1 mice ($Brms1^{Ubqs}$; $P = 0.01$). **b** Relative expression of *Brms1* in normal lung from wild-type ($Brms1^{wt}$), mammary-selective ($Brms1^{MMTV}$; $P = 0.04$), and ubiquitous Brms1 mice ($Brms1^{Ubqs}$; $P = 0.02$). Statistical analysis was performed using student's two sample *t* test assuming equal variance. *Error bars* standard error of the mean for biological replicates (triplicate)

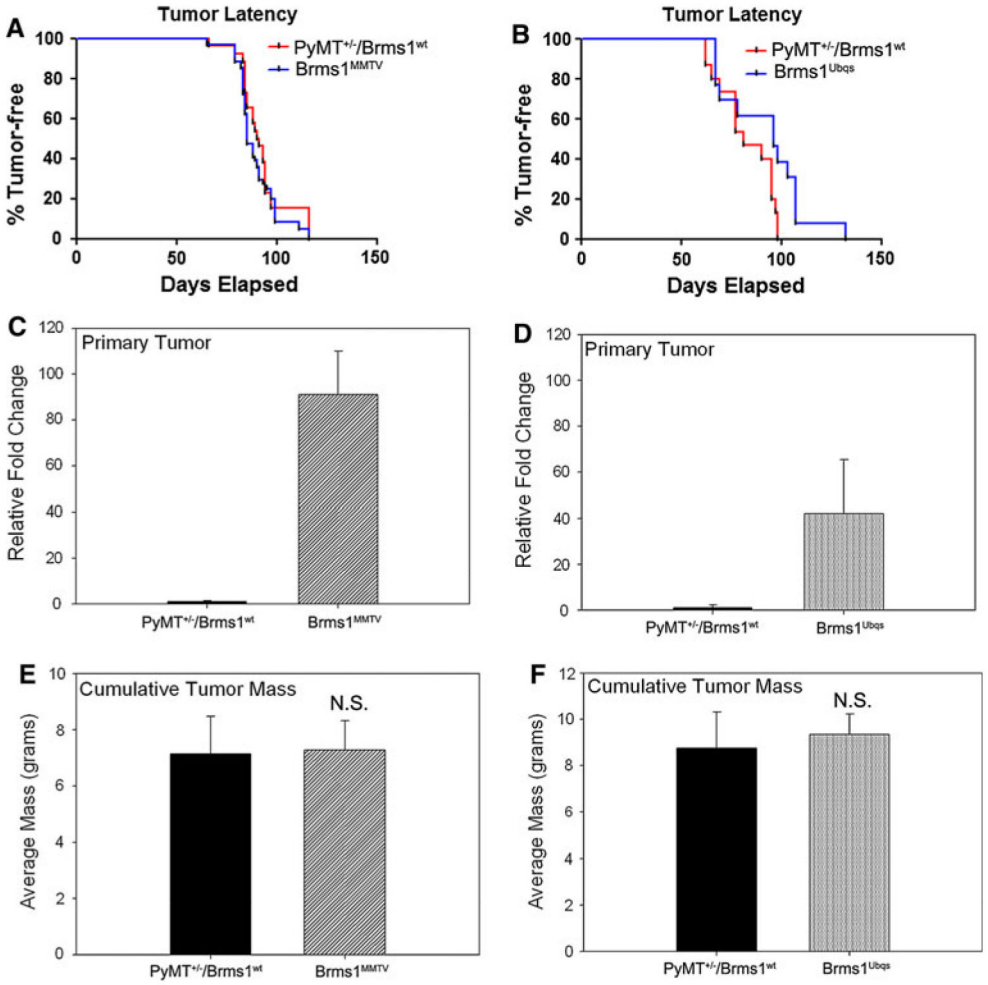

Fig. 2.

Tumor latency and mass are not significantly altered by *Brms1* over-expression in autochthonous primary tumors. Mice were palpated weekly and date of first palpable tumor was recorded as tumor latency. **a** and **b** survival analyses of tumor-bearing mice comparing controls ($PyMT^{+/-}/Brms1^{wt}$) and transgenic Brms1 mice ($PyMT^{+/-}/Brms1^{MMTV}$ and $PyMT^{+/-}/Brms1^{Ubqs}$). Tumor onset is represented by percentage of tumor-free mice throughout the study. Non-parametric Wilcoxon rank sum analysis revealed no significant change in tumor latency. $Brms1^{MMTV}$ and $Brms1^{Ubqs}$ denote F_1 crossed mice of $PyMT^{+/-}$ × $Brms1^{MMTV}$ and $PyMT^{+/-}$ × $Brm\text{-}s1^{Ubqs}$, respectively. **c** and **d** Two-step RT-qPCR (as described in Materials and methods) comparing expression of *Brms1* in primary tumors from $PyMT^{+/-}/Brms1^{wt}$, $PyMT^{+/-}/Brms1^{MMTV}$, and $PyMT^{+/-}/Brms1^{Ubqs}$. Relative expression is graphed as fold change compared to control mice, $PyMT^{+/-}/Brms1^{wt}$. All samples were normalized to ribosomal 18S RNA. *Error bars* standard error of the mean for biological replicates (triplicate). **e** and **f** Tumors were removed and collectively weighed to determine overall tumor burden. *Graphs* average total tumor mass per group. Statistical analysis using two-sample student's *t* test revealed no significant differences in total primary tumor burden per mouse when comparing transgenic $PyMT^{+/-}/Brms1^{MMTV}$ ($P = 0.94$) and $PyMT^{+/-}/Brms1^{Ubqs}$ ($P = 0.80$) with respective controls. *Error bars* standard error of the mean for biological replicates

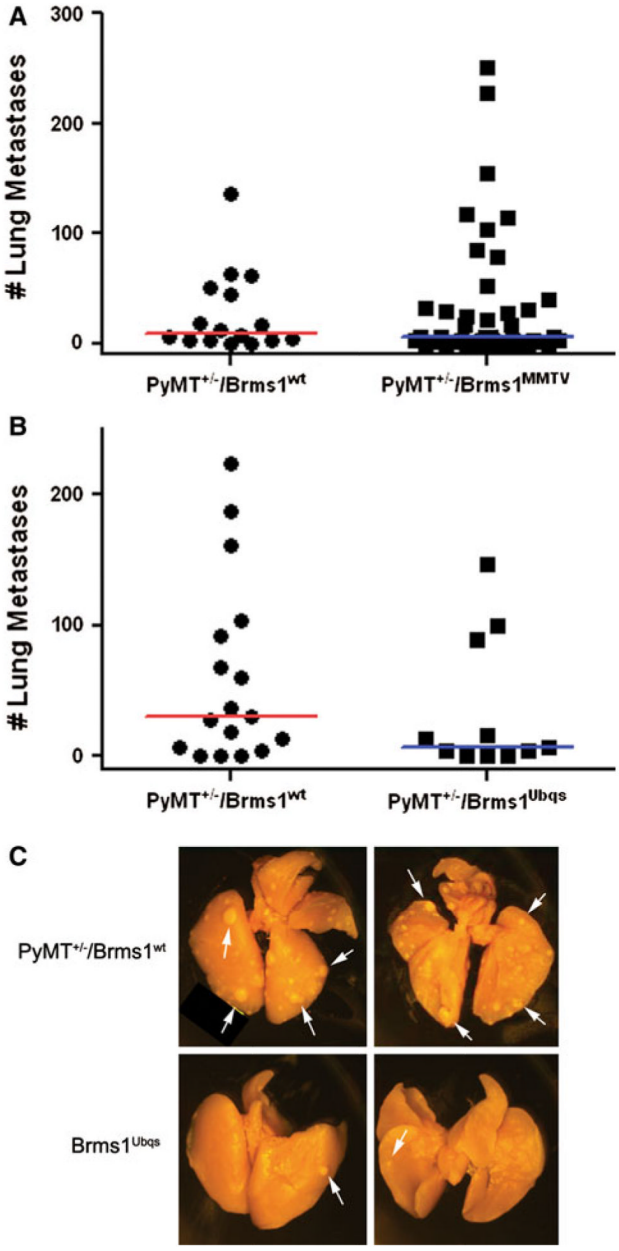

Fig. 3.

Ubiquitous expression of *Brms1* significantly suppresses lung metastases. Lungs were removed from each mouse and fixed in Bouin's fixative. Visible metastases were counted using a dissecting microscope. **a** *Scatter plot* number of metastases per mouse. *The red line* median number of visible metastases in control mice, $PyMT^{+/-}/Brms1^{wt}$ ($n = 19$) (median = 6) and $PyMT^{+/-}/Brms1^{MMTV}$ ($n = 47$) (median = 5), respectively. There was no significant difference in the median number of lung metastases. **b** *Scatter plot* number of metastases per mouse. *Red* and *blue line* represents median number of visible metastases in control mice, $PyMT^{+/-}/Brms1^{wt}$ ($n = 17$) (median = 31) and $PyMT^{+/-}/Brms1^{MMTV}$ ($n = 11$) (median = 8), respectively. $PyMT^{+/-}/Brms1^{Ubqs}$ exhibited fewer metastases when compared to controls, $PyMT^{+/-}/Brms1^{wt}$. Poisson regression controlling for age and total tumor burden at necropsy revealed a significant reduction in $PyMT^{+/-}/Brms1^{Ubqs}$ mice ($P < 0.0001$). **c**

Representative images of lungs collected from control and PyMT$^{+/-}$/Brms1^{Ubqs} mice (dorsal and ventral views of same lung). *White arrows* highlight visible metastases

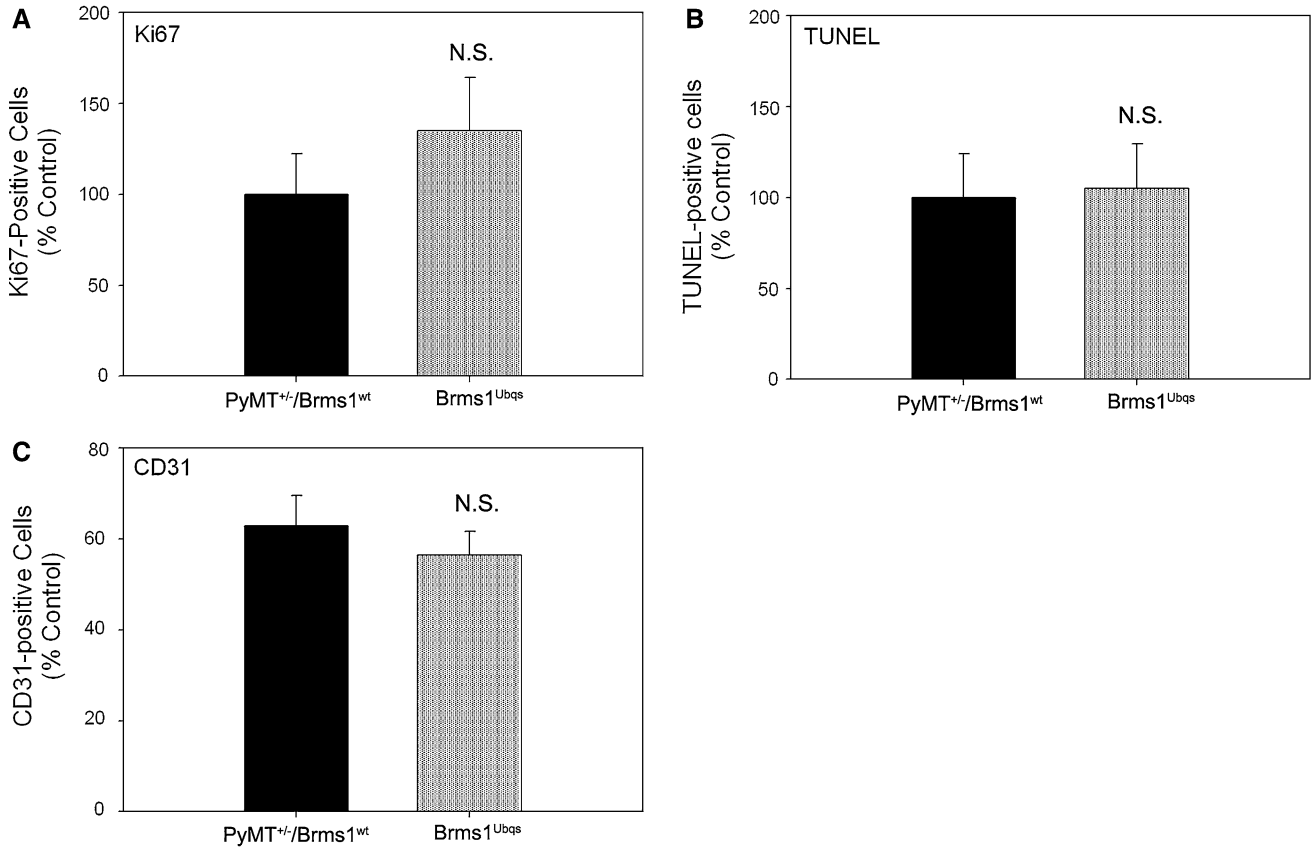

Fig. 4.

Brms1 does not alter primary tumor proliferation, apoptosis, or intratumoral microvessel density (iMVD). Proliferation and iMVD were analyzed by IHC using antibodies to the proliferation marker, Ki67, and CD31, a marker for blood vessels. Apoptosis was analyzed using TUNEL assay. Changes were determined by percentage positive cells (stained brown via DAB peroxidase substrate) per high-powered microscopic field. Analysis was performed via ImageJ software. Brms1^{Ubqs} denotes F_1 crossed mice of PyMT$^{+/-}$ × Brm-s1^{Ubqs}. **a** *Graph* percentage of Ki67-positive cells in primary mammary tumors from transgenic *Brms1* mice, PyMT$^{+/-}$/Brms1^{Ubqs} mice ($n = 8$) normalized to control mice, PyMT$^{+/-}$/Brms1^{wt} ($n = 12$). A minimum of 1,000 cells per sample were counted. Statistical analysis using two-sample student's *t* test revealed no significant change ($P = 0.33$). *Error bars* standard error of the mean for biological replicates. **b** *Graph* percentage of TUNEL-stained cells in primary mammary tumors from PyMT$^{+/-}$/Brms1^{Ubqs} ($n = 6$) normalized to control mice, PyMT$^{+/-}$/Brms1^{wt} ($n = 9$). Statistical analysis using two-sample student's *t* test revealed no significant change ($P = 0.97$). *Error bars* standard error of the mean for biological replicates. **c** *Graph* percentage of CD31-stained cells in primary mammary tumors from PyMT$^{+/-}$/Brms1^{Ubqs} ($n = 7$) normalized to control mice, PyMT$^{+/-}$/Brms1^{wt} ($n = 12$). iMVD was determined by averaging the number of CD31-positive vessels in designated "hot spots". A minimum of three "hot spots" were counted per sample. Statistical analysis using two-sample student's *t* test revealed no significant change ($P = 0.25$). *Error bars* standard error of the mean for biological replicates

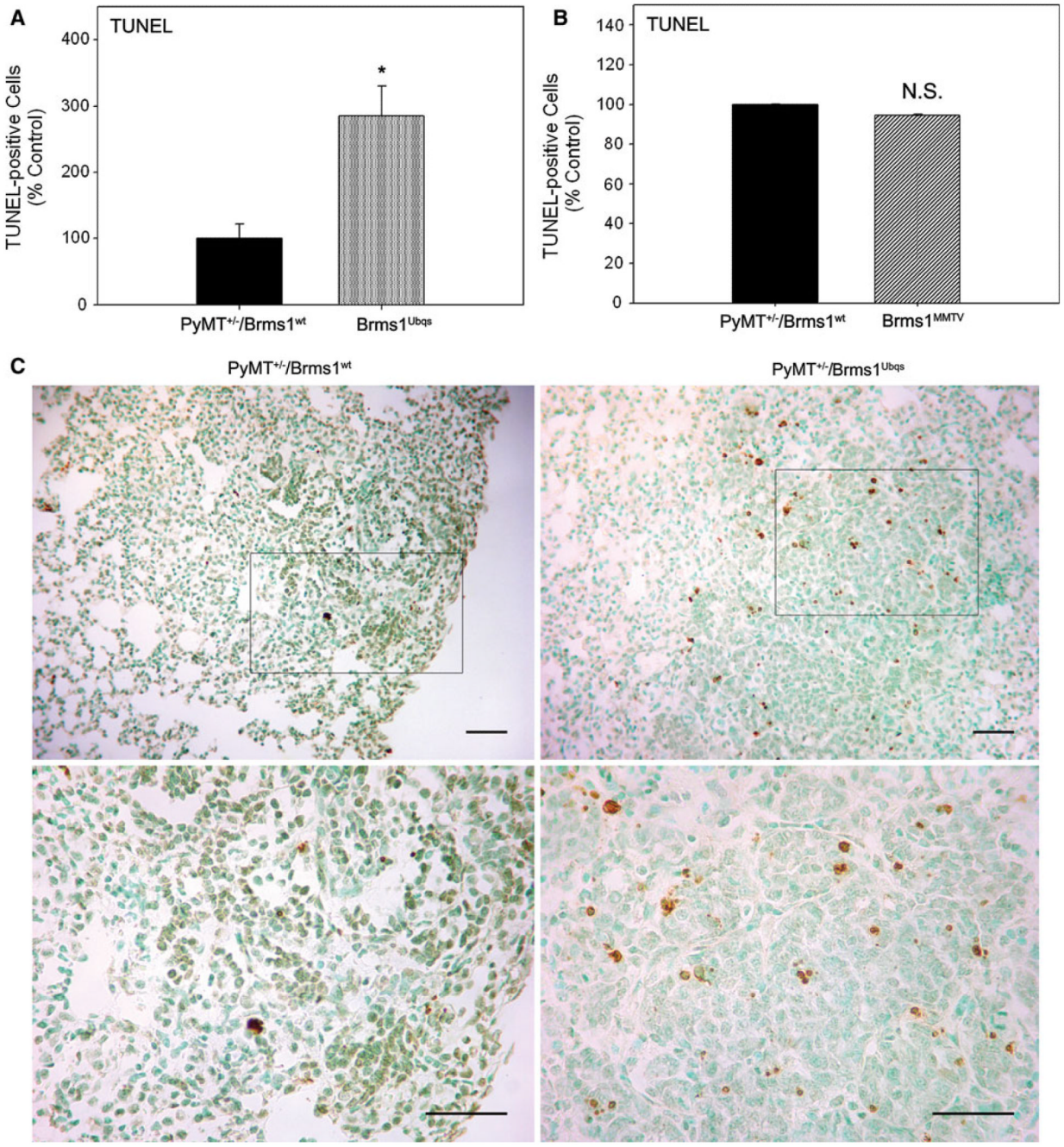

Fig. 5.

Ubiquitous expression of *Brms1* significantly induces apoptosis in lung metastases. Apoptosis in lung metastatic lesions was analyzed using TUNEL assay. Changes were determined by percentage positive cells (stained brown via DAB peroxidase substrate) per lesion. Analysis was performed via ImageJ software. $Brms1^{MMTV}$ and $Brms1^{Ubqs}$ denote F_1 crossed mice of $PyMT^{+/-} \times Brms1^{MMTV}$ and $PyMT^{+/-} \times Brms1^{Ubqs}$, respectively. **a** *Graph* average percentage of TUNEL-stained cells in lung metastases in $PyMT^{+/-}/Brms1^{Ubqs}$ ($n = 5$) mice normalized to control mice, $PyMT^{+/-}/Brms1^{wt}$ ($n = 9$). Statistical analysis using two-sample student's t test revealed a significant increase in apoptosis in lung metastases of $PyMT^{+/-}/Brms1^{Ubqs}$ mice ($P = 0.02$). **b** *Graph* average percentage of TUNEL-stained cells

in lung metastases in $PyMT^{+/-}/Brms1^{MMTV}$ mice ($n = 6$) normalized to control mice, $PyMT^{+/-}/Brms1^{wt}$ ($n = 8$). Statistical analysis using two-sample student's *t* test revealed no significant change in apoptosis ($P = 0.79$). **c** Representative images of apoptotic bodies in metastases of $PyMT^{+/-}/Brms1^{wt}$ and $PyMT^{+/-}/Brms1^{Ubqs}$ mice. The top panels show 200× magnification. *Bottom panels* (400× magnification) inset within top panels, *size bar* 50 μm

Table 1

Descriptive statistics for tumors and metastases in PyMT$^{+/-}$ × transgenic *Brms1* over-expressing mice

Parameter	**PyMT$^{+/-}$/Brms1^{wt} (*n* = 19)**		**PyMT$^{+/-}$/Brms1^{MMTV} (*n* = 47)**	
	Median	**Range**	**Median**	**Range**
Body weight (g)	30.9	21.7–56.0	29.4	20.7–56.0
Age at euthanasia (wk)	17.0	15–19	16	13–19
Tumor burden (g)	5.0	2.3–18.9	6.0	0.0–18.9
No. macroscopic lung metastases	6	0–136	5	0–136
Parameter	PyMT$^{+/-}$/Brms1^{wt} (*n* = 17)		PyMT$^{+/-}$/Brms1^{Ubqs} (*n* = 11)	
	Median	Range	Median	Range
Body weight (g)	29.0	23.0–47.3	31.0	24.8–35.8
Age at euthanasia (wk)	18	12–22	19	14–21
Tumor mass (g)	6.4	2.2–23.9	9.4	5.3–13.1
No. macroscopic lung metastases	31	0–223	8	0–146

There is a known tendency for the mice to reduce aggressiveness if bred for prolonged times. We observed this during the Brms1^{MMTV} crosses. Therefore, MMTV-PyMT mice were re-derived by the supplier and purchased by us for the Brms1^{Ubqs} crosses. This is why there is an apparent increase in basal metastatic potential