βIII-Tubulin Regulates Breast Cancer Metastases to the Brain

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Abstract

Brain metastases occur in about 10% to 30% of breast cancer patients, which culminates in a poor prognosis. It is, therefore, critical to understand the molecular mechanisms underlying brain metastatic processes to identify relevant targets. We hypothesized that breast cancer cells must express brain-associated markers that would enable their invasion and survival in the brain microenvironment. We assessed a panel of brain-predominant markers and found an elevation of several neuronal markers (βIII-tubulin, Nestin, and AchE) in brain metastatic breast cancer cells. Among these neuronal predominant markers, in silico analysis revealed overexpression of βIII-tubulin (TUBB3) in breast cancer brain metastases (BCBM) and its expression was significantly associated with distant metastases. TUBB3 knockdown studies were conducted in breast cancer models (MDA-Br, GLIM2, and MDA-MB-468), which revealed significant reduction in their invasive capabilities. MDA-Br cells with suppressed TUBB3 also demonstrated loss of key signaling molecules such as β3 integrin, pFAK, and pSrc in vitro. Furthermore, TUBB3 knockdown in a brain metastatic breast cancer cell line compromised its metastatic ability in vivo, and significantly improved survival in a brain metastasis model. These results implicate a critical role of TUBB3 in conferring brain metastatic potential to breast cancer cells. Mol Cancer Ther; 14(5): 1–10. ©2015 AACR.

Introduction

Brain metastases occur in about 10% to 30% of breast cancer patients (1, 2). The median survival of patients with brain metastases is extremely poor (< 10 months), and at present there is a lack of targeted therapy. To identify appropriate treatment regimens, it is critical to understand the biology of breast cancer brain metastases (BCBM). To comprehend the brain metastatic process, researchers developed paired models systems of brain metastasis. MDA-MD-231/MDA-MB-231BrM2 and CN34/CN34BrM2 are such paired model systems in which MDA-MB-231BrM2/CN34BrM2 cells exhibit a high propensity to metastasize to the brain after intracardiac administration in athymic nude mice.

Recent literature using this model system combined with BCBM tissues documents that brain metastatic cells overexpress neuronal markers such as ST6GALNAc5 (ST6; ref. 3), Nestin/CD133 (4), GABA receptor (5), and neuroserpin (6). This overexpression of neuronal markers by brain metastatic cells is believed to be an adaptation to survive in the brain microenvironment. However, the active role of these predominant brain markers in conferring BCBM has not been evaluated. Studies on molecular mechanisms of brain metastasis have also led to the identification of hyperactivated signaling pathways, including Src (7), STAT3(8), Notch (9), IGFBP3 (10), and EGFR (11). The inhibition of these signaling mediators either decreased the formation of brain metastases (9–11) or improved survival in murine models of preexisting brain metastases (7).

The unique cytoskeletal characteristics of a cancer cell are also critical mediators of its invasive behavior. Actin microfilaments are essential components for conferring metastatic potential through formation of invasive structures (12), whereas the role of microtubules is still under investigation (12). Microtubules are heterodimers of α and β subunits that are expressed in a tissue-specific manner (13). One such isotype is βIII-tubulin (TUBB3), which is specifically expressed by neurons (14). TUBB3 is absent in normal mammary epithelia (15); however, it is ectopically expressed in breast, lung, ovarian, and colon cancers, and has been found to confer paclitaxel resistance (16–19). Moreover, TUBB3 overexpression is strongly associated with poor prognosis of epithelial malignancies (16–20).

In an attempt to understand the role of neuronal predominant markers in brain metastasis of breast cancer, we screened a panel of neuronal markers and found ectopic overexpression of TUBB3, Nestin, and AchE in breast metastatic cells as compared with parental cells. Among these neuronal predominant markers, in silico analysis revealed overexpression of TUBB3 in BCBM patients, and its expression was significantly associated with distant metastases of breast cancer. Therefore, we focused our study on defining the role of TUBB3 in brain metastases. Through in vitro and in vivo analysis, our results uncover a vital role of the cytoskeletal protein βIII-tubulin in conferring brain metastatic potential to breast cancer cells. Our findings, along with availability of clinically approved antimicrotubule agents, warrants investigation of TUBB3 as a target for prevention of brain metastases in patients with breast cancer.
Materials and Methods

Cell culture and reagents

MDA-MB-231/MDA-MB-231 BrM2 (will be referred to as MDA-231/MDA-Br) cells were obtained from Dr. Joan Massague (MSKCC) and were maintained in DMEM (Corning) with 10% FBS (Hyclone). MDA-MB-468 cells (courtesy of Dr. Suzanne Conzen, University of Chicago) were maintained in RPMI-1640 (Corning) medium with 10% FBS (Hyclone) whereas GJL1M2 cells (obtained from Dr. Vincent Cryns, University of Wisconsin) were maintained in DMEM F/12 medium with 10% FBS and insulin/transferrin/sodium selenite mix (Invitrogen). CN34/34-Br cells were obtained from Dr. Joan Massague (MSKCC) and were maintained in M199 medium supplemented with 2.5% FBS, 10 µg/mL insulin, 0.5 µg/mL hydrocortisone, 20 ng/mL EGF, and 100 ng/mL cholera toxin (Sigma). No cell line authentication was done by the authors. Penicillin-streptomycin (100 µg/mL) was added to all the cell culture media. Cells were routinely screened and found to be free of mycoplasma. For lentiviral transduction, non-target control shRNA and two different shRNA specific to TUBB3 were procured (Sigma). Lentiviral particles were generated by transfection of shRNA and packaging vectors in HEK293 cells (Invitrogen). The primary antibodies used were βIII-tubulin, phosphor-FAK, Src, phospho-Src, STAT3, phosphor-STAT3, and actin (Cell Signaling Technology), βIV-tubulin (Sigma), L1CAM (Thermo Fisher scientific), Integrin β3 (BioLegend), and Nestin (BD Pharmingen). Densitometric analysis was conducted using Image J software. Flow cytometry was conducted as described before (21).

Patient samples and immunohistochemistry

The tissues were collected in accordance with a protocol approved by the Institutional Review Board at the University of Chicago. Sections (5-µm-thick) of breast cancer and BCBM were deparaffinized in xylene and then rehydrated. After deparaffinization and rehydration, tissue sections were treated with antigen retrieval buffer (S1699; DAKO) in a steamer for 20 minutes. βIII-tubulin rabbit monoclonal antibody (Cell Signaling Technology), βIV-tubulin (Sigma), L1CAM (Thermo Fisher scientific), Integrin β3 (BioLegend), and Nestin (BD Pharmingen) was applied on tissue sections at a dilution of 1:20 for 1-hour and incubated at room temperature in a humidity chamber. The antigen–antibody binding was detected by Bond Polymer Refine Detection (DS9800; Leica Microsystems).

Quantitative real-time polymerase chain reaction analysis

RNA (1 µg) was isolated from breast cancer cells using the RNeasy Plus Kit (Qiagen) and was reverse-transcribed using the iScript cDNA Conversion Kit (Bio-Rad) according to the manufacturer's instructions. qRT-PCR was conducted using the SYBR Green qPCR Kit (Bio-Rad) using primers indicated in Supplementary Table S1. Data analysis was performed using the 2-ΔΔCt method for relative quantification, and all sample values were normalized to the GAPDH expression value.

Migration assay

Cells (1 x 10⁶) were seeded in 6-well plates and grown to 95% confluency. A wound was created using a sterile 200 µL pipette tip. The cells were fed with fresh medium with 1% serum and observed under inverted microscope for 28 hours. Images were captured at various time points and migration of cells was measured using Imagem software. The experiments were conducted three times in duplicates.

Invasion assay

Matrigel invasion assay was conducted using BD Biocoat Matrigel invasion chambers according to the manufacturer’s instructions. Briefly, cells were seeded in serum-free medium, in the top inserts and complete medium was used as a chemotactic at the bottom chamber. After 22 hours, the noninvasive cells were removed and the inserts were fixed and stained with crystal violet. Five different fields of each chamber were photographed and cells were counted manually. Graph was plotted as relative invasion to cells to the vector control cells. The experiments were repeated twice in triplicates.

Adhesion assays

Ninety-six well microplate was coated with laminin (5 µg/mL), fibronectin (2.5 µg/mL), and collagen (50 µg/mL). Plates were incubated overnight at 4°C for polymerization of ECM proteins. Unpolymerized substrates were washed with PBS and the plates were blocked with 2% BSA for 2 hours at 37°C. Vector control and TUBB3 knockdown cells were plated (4 x 10⁴) and cell adhesion was monitored for 15 to 60 minutes at 37°C. Following incubation, the nonadherent cells were removed by two washes with PBS. The adherent cells were quantified using MIT assay (Roche) and expressed as a percentage relative of the respective total unwashed cells. The experiments were repeated twice in triplicates.

Animal experiments

All surgical procedures were completed in accordance with NIH guidelines on the care and use of laboratory animals for research purpose. The protocols were approved by the Institutional Committee on Animal Use at the University of Chicago. Six- to 8-week-old athymic nude female mice were obtained from Harlan laboratories and maintained in a specific pathogen-free facility. Mice were anesthetized with an i.p. injection of 0.1 mL of a stock solution containing ketamine HCl (25 mg/mL), xylazine (2.5 mg/mL), and 14.25% ethyl alcohol (diluted 1:3 IN 0.9% NaCl) and inoculated with 250,000 MDA-Br Vector control and TUBB3 knockdown cells in 100 µL PBS via injection into the left ventricle of the heart. After 28 days or appearance of signs of morbidity, mice were sacrificed and whole-brain tissues were excised immediately and embedded in OCT freezing agent. Approximately 100 sections with thickness of 10 µm across the horizontal plane were cut, air dried, and stained with hematoxylin and eosin. For orthotopic mammary gland assay, subconfluent MDA-Br Vector control and TUBB3 knockdown cells were suspended in plain DMEM medium and mixed with Matrigel (Becton Dickinson) at a ratio of 1:1 in a volume of 50 µL. A total of 1 x 10⁶ cells were injected in third or fourth Mammary gland of Hsd: athymic nude female mice, and tumor size was monitored for 15 weeks.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 4 (GraphPad Software Inc.). The sample size for each group was ≥3 and numerical data were reported as mean ± SEM. The RNA levels of brain predominant markers were assessed using the one-sample Student t test, and the differences on protein level were assessed using the two-sample Student t test. Comparisons on cell migration, adhesion, and invasion were conducted using one-way or two-way ANOVA with Bonferroni or Dunnett post hoc test as appropriate. For animal survival experiments, Kaplan-Meier
survival curve was generated and log-rank test was applied to compare survival distributions. Cox proportional-hazard regression was used to investigate the association between TUBB3 and breast cancer metastasis on patient data (22), and distant metastasis-free survival curve was generated for patients grouped as high vs. low expression by one SD from mean. All reported P values were two-sided and were considered to be statistically significant at *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Results

Overexpression of predominant brain markers in BCBM

Given the ectopic overexpression of neuronal markers in BCBM (3–6), we hypothesized that there may be other neuronal markers that are involved in breast cancer metastasis to the brain. To evaluate our hypothesis, we used two different paired model systems, MDA-231/MDA-Br and CN34/CN34-Br. MDA-Br/CN34-Br cells are the brain metastatic derivatives of the parental MDA-231/CN34 cells (respectively), which were isolated after two rounds of intracardiac injection with isolation of transplanted cells from brain tissue (3).

We conducted qRT-PCR to assess the RNA levels of a panel of brain predominant markers (TUBB3, Nestin, AchE, LMO3, Grin1, Grin2b, GFAP, PIN1, NSE2, NFL, NGFR, and NCAM1) and found upregulation of TUBB3, Nestin, and AchE by 1.5-, 2-, and 1.6-fold, respectively, in MDA-Br cells versus their corresponding parental cells (Fig. 1A). These results were further confirmed in CN34-Br cells in which TUBB3, Nestin, and AchE were upregulated by 3.6-, 1.46-, and 2.1-fold, respectively (Fig. 1B). However, there was no significant difference in other brain predominant markers that were evaluated (data not shown). We further confirmed the alterations in RNA levels of TUBB3 and Nestin at the protein level using Western blotting (βIII-tubulin; Fig. 1C) and flow cytometry (Nestin; Fig. 1E). Densitometric analysis revealed a

![Graphs and images](image-url)
A 2.5/4.9-fold increase of TUBB3 protein in MDA-Br/CN34-Br cells, respectively (Fig. 1D), whereas nestin protein levels were elevated by 1.4/2-fold in MDA-Br/CN34-Br cells as compared with parental cells, respectively (Fig. 1E and F).

Furthermore, we conducted gene-expression analysis using gene set enrichment (GSE) datasets, where nestin and AchE overexpression did not correlate with brain metastases (data not shown). However, we found significant upregulation in TUBB3 RNA levels in BCBM samples as compared with the primary breast cancer cells (n = 5); scale bar, 50 μm. There is some weaker staining in the adjacent brain parenchyma and in particular in gemistocytic astrocytes at the edge of the lesion. Arrow indicates BCBM and arrowhead indicates brain tissue; scale bar, 100 μm. **P < 0.01; *P < 0.05.

Knockdown of TUBB3 in breast cancer cells decreases invasion in vitro

To understand the active role of TUBB3 in breast cancer cells, we silenced TUBB3 expression via lentiviral transduction using two different shRNA specific to TUBB3 (shRNA1 and shRNA2) in widely used breast cancer cells, namely MDA-Br, GLIM2, and MDA-MB-468 cells. Densitometric analysis of Western blots revealed 95% and 88% downregulation of βIII-tubulin in shRNA1 and shRNA2, respectively, as compared with scrambled vector control cells (Supplementary Fig. S1A–S1C).

TUBB3 knockdown cells exhibited gross morphologic alterations like elongation of cells and loss of cellular structures (Supplementary Fig. S2A) as compared with the vector control cells. This observation prompted us to evaluate whether there are alterations in migration of TUBB3 suppressed MDA-Br cells by a scratch wound migration assay. MDA-Br cells with downregulated TUBB3 migrated at significantly slower rate (n = 6; P < 0.05) as shown by the images captured after 18 hours (Fig. 3A) in comparison with vector control cells. In addition, when complete closure of...
the wound was noted in vector control cells, the knockdown cells were unable to close the wound even at the end of 28 hours (Fig. 3A). These results indicate reduced migratory potential of TUBB3 knockdown MDA-Br cells.

It is well known that augmented invasion is essential for metastasis of cancer cells. Hence, to evaluate whether TUBB3 knockdown cells possess the ability to invade the basement membrane components, an invasion assay was performed using Matrigel invasion chambers. MDA-Br cells with suppressed TUBB3 demonstrated 2.7- and 2.3-fold reduction in invasion for shRNA1 and shRNA2, respectively (Fig. 3B), as compared with vector control cells. To substantiate the role of TUBB3 in invasion, we conducted invasion assay in GLIM2 cells, which were previously shown to have intrinsic brain metastasis capability (24). We noted 8-(shRNA1) and 2.1 (shRNA2)-fold reduction in invasion of TUBB3-suppressed MDA-MB-468 cells as compared with vector control cells (Fig. 3C). Moreover, TUBB3-suppressed GLIM2 cells demonstrated 9.8-(shRNA1) and 4.5 (shRNA2)-fold decrease in invasion (Fig. 3D). These results demonstrate that ectopic expression of TUBB3 regulates invasive migration of breast cancer and brain metastatic cells.

TUBB3 knockdown in brain metastatic cells decreases cell adhesion to ECM accompanied by decreased levels of β3 integrins

Adhesion to extracellular matrix (ECM) components assists the cancer cells in the metastatic cascade. Laminin, fibronectin, and collagen are abundant ECM proteins in the brain endothelial cells. Hence, we conducted adhesion assays using laminin-, fibronectin-, and collagen-coated dishes. TUBB3 knockdown cells exhibited a significantly decreased ability to attach to laminin and fibronectin (Fig. 4A–C). These results depict that βIII-tubulin modulates the adhesion of cells to the ECM components.

Integrins are cell-surface proteins that mediate the process cell adhesion with ECM proteins. Previous reports documented elevated levels of αβ3, αβ6, and αβ8 integrins in human BCBM (25). Therefore, we evaluated the transcript levels of these integrins in our brain metastasis model. Using the parental and brain

Figure 3.

TUBB3 suppression decreases migration and invasion of breast cancer cells. A, scratch wound assay for migration. Images demonstrating wound closure of vector control MDA-Br and TUBB3 knockdown cells at 0 and 28 hours time points. Right, statistical analysis of migration using ImageJ software. Images showing invasion of vector control and TUBB3 knockdown cells. MDA-Br (B), MDA-MB-468 (C), and GLIM2 (D) cell lines, respectively. Left to right, vector control, shRNA1, and shRNA2. Graph indicates the percentage of invasion as compared with vector control cells. The results represent means ± SEM for triplicate experiments; ***, P < 0.001; **, P < 0.01; *, P < 0.05.
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Figure 4.
Decreased adhesion of MDA-Br TUBB3 knockdown cells to laminin, fibronectin, and collagen. Adhesion assay was performed by seeding equal amount of cells of different groups in 96-well tissue culture plates coated with laminin (A), fibronectin (B), and collagen (C). The results represent means ± SEM for triplicate experiments. D, quantitative RT-PCR analysis of the expression of Integrin genes in MDA-Br cells as compared with parental MDA-MB-231 cells. E, flow cytometric analysis of β3 integrin showing TUBB3 shRNA cells demonstrating reduction in β3 integrin levels; ***, P < 0.01 and ***, P < 0.001.

metastatic derivative of MDA-231 cells, qRT-PCR analysis revealed a 3-fold increase in integrin β3 transcripts in brain metastatic cells (MDA-Br) as compared with parental cells (MDA-231; Fig. 4D). This prompted us to evaluate whether there are any alterations in integrin β3 levels when TUBB3 was suppressed in brain metastatic cells. Flow-cytometry analysis of TUBB3 knockdown cells demonstrated 2.2- and 3.2-fold down-regulation of cell surface β3 integrin levels in shRNA1 and shRNA2, respectively (Fig. 4E), as compared with the vector control cells. These results demonstrate that βIII-tubulin regulates adhesion ability of brain metastatic cells possibly through integrin β3. Integrin downstream signaling is known to promote metastasis in a number of epithelial malignancies; therefore, it was of interest to evaluate whether the alteration in the integrin β3 surface expression in knockdown cells was accompanied by alterations in integrin downstream signaling.

Knockdown of TUBB3 alters integrin–Src and STAT3 signaling

Integrins mediate signaling through phosphorylation of focal adhesion kinase (FAK), which is activated when autophosphorylated at Y397 residues. To ascertain whether decreased integrin β3 in TUBB3 knockdown cells leads to decrease in FAK phosphorylation, we performed Western blotting in vector control and TUBB3 knockdown cells, which revealed reduced FAK phosphorylation (Fig. 5A). Furthermore, reduced FAK activation also resulted in blunted phosphorylation of Src at Y416 residues (Fig. 5A).

Integrin-mediated adhesion and signaling regulates activation of STAT3 (26). To evaluate whether abrogated integrin–FAK–Src signaling leads to alterations in STAT3 signaling, we conducted Western blotting in MDA-Br vector control and TUBB3 knockdown cells. We found attenuated STAT3 phosphorylation at Y705, which is responsible for its transcriptional activation (Fig. 5A). These results demonstrate an important role of TUBB3 in maintaining the integrin–Src–STAT3 axis in the brain metastatic cells (Fig. 5A).

Recently, it was shown that L1 cell adhesion molecule (L1CAM) is important for the colonization of metastatic cells in the brain (6). To address the role of TUBB3 in regulation of L1CAM levels, we conducted Western blotting using MDA-Br vector control and TUBB3 knockdown cells. We found dramatic reduction in L1CAM protein levels in of L1CAM in TUBB3 knockdown cells (Fig. 5B). These results underscore the role of βIII-tubulin in regulating the key signaling molecules that are involved in the process of BCBM.

Knockdown of TUBB3 decreases the brain metastatic capabilities of breast cancer cells and increases survival of mice without altering primary tumor growth

TUBB3 knockdown alters in vitro migratory and invasive properties. However, our in vitro results demonstrated a marginal statistical difference in the proliferative ability of TUBB3 knockdown cells (Supplementary Fig. 2SB–2SD). Therefore, it was of interest to understand whether there are any differences in the ability of vector control MDA-Br and TUBB3 knockdown cells to grow in orthotopic mammary gland. Vector control MDA-Br, TUBB3 shRNA1, and TUBB3 shRNA2 cells were injected in the third and fourth mammary gland of nude mice and tumor volume was monitored for a period of 15 weeks. There were no significant differences in the growth of vector control and TUBB3 knockdown cells (Fig. 6A).

To evaluate the in vivo functional consequences of TUBB3 knockdown on metastatic behavior of cells, a brain metastasis model was used. Vector control and TUBB3 knockdown cells were injected through an intracardiac route, and after 28 days, whole-brain tissues were sectioned to determine the number of metastatic lesions. One hundred sections (10 μm) were isolated from each mouse followed by hematoxylin and eosin staining. Ten
sections of each brain were analyzed for the presence of number of micrometastasis. Within each group an average of 9 mice were considered for calculating the number of micrometastasis. Mice injected with TUBB3 knockdown cells exhibited significant reduction in the formation of micrometastasis (Fig. 6B and C).

To understand the functional consequences of TUBB3 knockdown in a metastatic model on the survival of mice, we conducted survival experiments. Here, the vector control and stable TUBB3 knockdown cells were injected via an intracardiac route in the left ventricle of nude mice, and survival outcomes were determined. The vector control group exhibited a median survival of 34 days whereas shRNA1 and shRNA2 demonstrated median survival of 45 (P = 0.0053) and 50 (P = 0.0011) days, respectively (Fig. 6D). These results demonstrate that TUBB3 knockdown increases survival of mice via decreasing brain metastatic ability of breast cancer cells.

Discussion

There is an urgent need to identify rational molecular targets for the treatment of brain metastases. In this report, we present the first evidence that a microtubule family member, βIII-tubulin, confers brain metastatic potential to breast cancer cells by regulating invasion and Integrin-Src signaling. Our studies support the concept that (i) TUBB3 overexpression is a malignant adaptation of breast cancer cells for invasion in the brain and (ii) suppression of TUBB3 function holds the potential for improving the survival in brain metastasis patients.

Figure 5.
TUBB3 knockdown in brain-seeking MDA-Br cells attenuates FAK, Src, and STAT3 signaling. A, Western blotting demonstrating decrease in phosphorylation of FAK/Src/STAT3 and decreased levels of L1CAM in TUBB3 knockdown cells as compared with vector control MDA-Br cells. B, densitometric analysis of pFAK, pSrc, pSTAT3, and L1CAM. The experiments were repeated three times. ***, P < 0.001; **, P < 0.01; *, P < 0.05.

TUBB3 plays an important role in axonal guidance of neurons and perturbations in TUBB3 function lead to ocular motility disorders (14). Within the brain only a specific subset of tubulins (TUBB3, TUBB2C, TUBB, TUBB2A, and TUBB2B) are expressed (15), whereas in normal breast epithelium (which express TUBB2C, TUBB1, TUBB2A, and TUBB6) TUBB3 is virtually absent (15). Overexpression of TUBB3 is associated with poor prognosis of multiple epithelial malignancies, including lung, colorectal, and ovarian cancers (16–20). Moreover, 85% of small-cell lung cancer patients exhibit TUBB3 overexpression (27), a cohort of which shows the highest incidence of CNS metastasis (28). These reports support the notion that TUBB3 overexpression imparts metastatic potential to cancer cells. However, there are no studies demonstrating a direct correlation of TUBB3 overexpression and brain metastases. We found overexpression of TUBB3 in human BCBM tissues in silico, which is in agreement with the report that TUBB3 overexpression in primary breast cancers is associated with poor prognosis (29). Although a clear relation between overexpression of TUBB3 and prognosis exist in the literature, its role in invasion and metastasis has not been evaluated.

Invasion of cancer cells holds a central role in the process of metastasis. It is a combined interplay of various cancer hallmarks that are governed by both interactions of cancer cell with the ECM components and cell signaling through surface receptors. In this context, we have evaluated the functional role of TUBB3 in invasion by generating TUBB3 knockdown cells in different breast cancer cell lines. In all these models, knockdown of TUBB3 levels significantly decreased the invasion of cells. Moreover, we found
significant reduction in the adhesion ability of $TUBB3$-suppressed brain metastatic cells to ECM components (Laminin, fibronectin and collagen). This decrease in adhesion was also associated with compromised levels of L1CAM, a critical protein involved in the process of BCBM (6).

L1CAM binds to integrin $\beta_1$, $\beta_2$, and $\beta_3$, and heterodimerization of L1CAM to integrins results in recruitment and auto phosphorylation of FAK. Phosphorylation of FAK recruits Src, which is then autophosphorylated, leading to activation of MAPK (30). This adhesion-mediated cascade of signaling then promotes invasion and colonization of cancer cells (6, 30). Integrins are overexpressed in malignant cells, and hence promote invasion and metastasis (31). Integrin $\alpha\beta_3$, $\alpha\beta_6$ and $\alpha\beta_8$ are overexpressed in human BCBM tissues (25). Given the critical role of integrin signaling in invasion and metastasis, we investigated the levels of integrin and found downregulation of integrin $\beta_3$ levels in $TUBB3$-suppressed cells. These results are in concordance with decrease in invasion in MDA-Br $TUBB3$ knockout cells. Taken together, our results suggest that $TUBB3$ regulates L1CAM and Integrin $\beta_3$ levels to regulate the invasive nature of brain metastatic cells.

Moreover, to evaluate the consequences of $TUBB3$ knockdown on metastatic burden, we conducted an in vivo brain metastasis assay, which demonstrated a significant decrease in the number of spontaneous micrometastasis in mice injected with $TUBB3$-suppressed cells. Decreased metastatic load also correlated with improved survival in the preclinical model, which establishes $TUBB3$ as a critical mediator of brain metastasis.

Our results on proliferation of $TUBB3$-suppressed cells in vitro demonstrated marginal difference in proliferation (Supplementary Fig. S2B–S2D). However, tumor volume analysis of $TUBB3$-suppressed metastatic MDA-Br cells showed no difference. These results are in concordance with a very recent report on non–small cell lung cancer model, in which $TUBB3$ regulates lung cancer metastasis without altering growth of tumor cells in vivo in an anchorage-dependent manner (32). Our results are further supported by the fact that parental (MDA-231) and brain metastatic cells (MDA-Br) do not exhibit differences in proliferation (3), although MDA-Br demonstrated a 2.5-fold increase in $\betaIII$-tubulin protein levels. We therefore believe that $TUBB3$ in brain metastatic cells predominantly regulate the molecular players of invasion and metastasis.

Pharmacologic inhibition of invasion is believed to be a plausible approach for successful intervention of cancer...
progression. For example, inhibition of cathepsin B (via VBY-999; ref. 33) and COX2 (via cetuximab; ref. 3) decrease the occurrence of metastasis. These reports provide a clear evidence of preventing brain metastases before they arise. βIII-tubulin protein is known to be sensitive to vinorelbine-mediated drug therapy. In systemic lung cancer patients with TUBB3 overexpression, it has been shown that these patients benefit from vinorelbine-mediated chemotherapy with improvements seen in progression-free survival (34). Hence, it will be of interest to evaluate vinorelbine-mediated prevention studies for inhibiting the occurrence of BCBM. In this context, our analysis of TCGA datasets (22, 23) implicates that, over time, the presence of high levels of TUBB3 in primary breast cancers predicts distant metastasis. Furthermore, our results in a brain metastasis model reveal that the suppression of TUBB3 in these cells decreases the incidence of BCBM. Taken together, TUBB3 in the primary/disseminated breast cancer cells regulates brain metastases of breast cancer and TUBB3 is a potential target for prevention of BCBM. Moreover, given that overexpression of TUBB3 predicts poor prognosis of lung (34, 35) and ovarian (19) cancer, this finding supports a broad role of TUBB3 in metastasis of different malignancies, which are known to metastasize to the brain (36–39). Hence, research on the role of TUBB3 in brain metastasis of other malignancies warrants further investigation.

In conclusion, our work suggests for the first time a novel role of TUBB3/βIII-tubulin in regulation of metastases and integrin-Src signaling. TUBB3/βIII-tubulin is, therefore, a possible marker for high-risk patients for development of brain metastases. Our work also demonstrates that TUBB3/βIII-tubulin regulates key mediators of brain metastases (Src and STAT3), and thus TUBB3/βIII-tubulin should be targeted to improve the prognosis of patients with metastatic brain cancer.

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

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