Published in final edited form as:

Clin Exp Metastasis. 2013 August ; 30(6): . doi:10.1007/s10585-013-9576-7.

Co-evolution of breast-to-brain metastasis and neural progenitor cells

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Abstract

Brain colonization by metastatic tumor cells offers a unique opportunity to investigate microenvironmental influences on the neoplastic process. The bi-directional interplay of breast cancer cells (mesodermal origin) and brain cells (neuroectodermal origin) is poorly understood and rarely investigated. In our patients undergoing neurosurgical resection of breast-to-brain

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Electronic supplementary material The online version of this article (doi:10.1007/s10585-013-9576-7) contains supplementary material, which is available to authorized users.

Conflict of interest The authors declare no competing interests exist.

metastases, specimens from the tumor/brain interface exhibited increased active gliosis as previously described. In addition, our histological characterization revealed infiltration of neural progenitor cells (NPCs) both outside and inside the tumor margin, leading us to investigate the cellular and molecular interactions between NPCs and metastases. Since signaling by the TGF-superfamily is involved in both developmental neurobiology and breast cancer pathogenesis, we examined the role of these proteins in the context of brain metastases. The brain-metastatic breast cancer cell line MDA-MB-231Br (231Br) expressed BMP-2 at significantly higher levels compared to its matched primary breast cancer cell line MDA-MB-231 (231). Co-culturing was used to examine bi-directional cellular effects and the relevance of BMP-2 overexpression. When co-cultured with NPCs, 231 (primary) tumor cells failed to proliferate over 15 days. However, 231Br (brain meta-static) tumor cells co-cultured with NPCs escaped growth inhibition after day 5 and proliferated, occurring in parallel with NPC differentiation into astrocytes. Using shRNA and gene knock-in, we then demonstrated BMP-2 secreted by 231Br cells mediated NPC differentiation into astrocytes and concomitant tumor cell proliferation in vitro. In xenografts, overexpression of BMP-2 in primary breast cancer cells significantly enhanced their ability to engraft and colonize the brain, thereby creating a metastatic phenotype. Conversely, BMP-2 knockdown in metastatic breast cancer cells significantly diminished engraftment and colonization. The results suggest metastatic tumor cells create a permissive neural niche by steering NPC differentiation toward astrocytes through paracrine BMP-2 signaling.

Keywords

Breast cancer; Brain metastasis; Neural progenitor cells; Astrocytes; BMP-2; Tumor microenvironment

Introduction

Metastases are responsible for 90 % of deaths from cancer, and patients diagnosed with brain metastasis have only a 20 % probability of surviving for more than 1 year. There is an increase in the brain as a site of untreatable cancer relapse, and autopsy studies show up to 25 % of patients who have cancer develop brain metastases [1]. Breast cancer is the most common malignancy in women in the United States [2], and breast-to-brain metastases (BBM) now have a 5-year cumulative incidence rate of 7 %. The median survival of untreated patients is 1–2 months [3, 4]. Chemotherapy, in conjunction with surgery and radiation therapy, extends survival by only 4–6 months [4].

Multiple steps are required for cancer cells to form viable brain metastases, the last of which appears to require distinct interactions between the cancer cells (seed) and the brain microenvironment (soil). In breast cancer, tumor cells are thought to be in circulation early in the course of disease, yet diagnosis of brain metastases occurs with a latency of several years to decades. Because circulating tumor cells have been shown to extravasate and either die or become dormant, their colonization of the brain may represent the rate limiting step in the metastatic cascade [5]. Accordingly, the final and potentially most critical step in the metastatic cascade, brain colonization, requires a bi-directional communication between the cancer cells and the brain microenvironment. Alternatively, permissive conditions may develop in the brain microenvironment allowing the cancer cells to colonize and proliferate. However, little progress has been made in understanding the processes facilitating breast cancer colonization of the brain and metastatic outgrowth; processes distinct from the ability of cells to leave the primary tumor, intravasate into the circulation, and then extravasate into the brain.

Astrocytes, the predominant glial cells in the central nervous system (CNS), are responsible for homeostasis of the brain microenvironment. Astrocytes transport nutrients from the circulatory system to neurons and oligodendrocytes, participate in neural signal transduction, and buffer the ionic balance of the extracellular matrix. Astrocyte activation in response to pathological conditions such as trauma, ischemia, and neurodegenerative diseases has been shown to protect neurons from injury-induced apoptosis [6, 7]. Intriguingly, recent evidence demonstrates astrocytes protecting breast cancer cells from chemotherapeutic agents through upregulation of survival genes [8].

The transforming growth factor- β (TGF- β) family of proteins plays an important role in normal development and cancer pathogenesis. Although TGF- β is a key negative regulator of normal breast cell growth, it promotes proliferation in advanced cancers. Furthermore, certain members of the TGF- β superfamily, the bone morphogenic proteins (BMPs), have key functions in breast cancer and distant metastases [9–14]. Like most TGF- β family members, the role of BMP-2 in breast cancer development is dependent on its context. The stage of pathogenesis and the microenvironment can determine if BMP-2 inhibits or enhances the tumorigenic properties of breast cancer. Under the right conditions, BMP-2 can stimulate cell growth, migration, invasion, and differentiation towards a more aggressive phenotype [15–17].

TGF- β family proteins also have a fundamental role in neuro- and gliogenesis. The majority of BMPs have specific functions in neurogenesis, BMP-2 is crucial in gliogenesis [18, 19]. Mainly, when telencephalic neural progenitors are exposed to BMP-2, their developmental fate is switched to the astrocytic cell lineage [20, 21]. Specifically, BMP-2 treatment significantly reduces the number of cells expressing markers of undifferentiated neural progenitor cells (NPCs) and neurons while simultaneously increasing the number of astrocytes [20, 22]. Therefore, BMP-2 has distinct effects on both breast cancer cells and NPCs.

In the process of brain metastasis, the interactions between cancer and host cells are key if colonization is to occur, yet the molecular mechanisms underlying this bi-directional communication remain poorly understood. Determining how metastatic breast cancer cells and the resident CNS cells interact and co-evolve could reveal the underlying molecular mechanisms in BBM. In this study, we analyzed the unique contribution of both NPCs and astrocytes to BBM formation. We show NPCs, astrocyte progenitors (AP), and reactive astrocytes were present at the border between the brain and tumor in surgical sections of patient metastatic breast-to-brain tissues. Furthermore, BBM cells overexpressing BMP-2 promote the differentiation of NPCs into astrocytes and created a permissive niche favoring tumor growth.

Materials and methods

BC cultures

MDA-MB-231 (231) and MDA-MB-231Br (231Br) cell lines (purchased from the Characterized Cell Line Core Facility at the University of Texas MD Anderson Cancer Center) were grown in DMEM/F12 supplemented with 10 % FBS, Glutamax, Pen/Strep, and maintained at 37 °C and 5 % CO₂. For visualization purposes 231 and 231Br cell lines were transduced in the presence of 4 µg/ml Polybrene with lentivirus containing ZsGreen1 and firefly luciferase at a MOI of one. ZsGreen1+ cells were determined and sorted by flow cytometry after 72 h.

NPC cultures

Human Rencell CX NPCs (Millipore) were cultured in NPC defined media (DMEM/F12 supplemented with B27, Glutamax, Pen/Strep, EGF (20 ng/ml) and FGF-2 (20 ng/ml), on laminin-coated T-75 flasks, and maintained at 37 °C and 5 % CO₂. To differentiate NPCs into astrocytes for control experiments, exogenous recombinant human BMP-2 (50 ng/ml) was added to cultures for 15 days.

Co-cultures

Multi-well thincert (0.4 µm) plates were used for transwell co-cultures. Laminin coated glass coverslips with 10⁴ human Rencell CX NPCs were seeded in NPC defined media for 24 h. 10⁴ breast cancer cells were then seeded on the thincert in equal volumes of NPC defined media. Half the NPC defined media was changed every 2–3 days. For BMP-2 antagonist experiments, Noggin was added to the bottom wells of control and co-culture experiments every 2 days at a final concentration of 250 ng/ml.

pLV-BMP2-Puro plasmid construction

To construct a lentiviral-based BMP2 expression plasmid, we isolated RNA from MDA-MD-231Br cells using the RNeasy 96 kit (Qiagen) and prepared cDNA using the Transcriptor First Strand cDNA synthesis kit (Roche). The cDNA was amplified using the Phusion High Fidelity PCR master mix (NEB) according to the manufacturer's instructions. Primer sequences were: 5'-TGAGTGAATTC AAGGTCGACCATGGTGGC and 5'-TATATGGATCCT AGCGACACCCACAACCC (*Eco*RI and *Bam*HI recognition sites, underlined, respectively). The 1222-bp PCR product was excised with *Eco*RI and *Bam*HI and cloned into pLVX-EF1a-IRES-Puro (Clontech).

BMP-2 knock-down and overexpression

Plasmids were prepared using the Qiagen Plasmid Maxi kit. 293T cells were transfected by calcium phosphate co-precipitation with 10 µg of pPACK HIV packaging plasmid mix (SBI) and 20 µg of the appropriate plasmid [pLV-BMP2-Puro, pLKO.1-BMP2 shRNA (TRCN0000 058194, Sigma) or pLKO.1-non targeting shRNA (Sigma)]. The culture medium was replaced with fresh medium after 6 h, and supernatants were collected 24 and 48 h after transfection. To determine viral titers, 10⁵ HT1080 cells were seeded in six-well plates and transduced with various dilutions of vector in the presence of 4 µg/ml Polybrene (Sigma). The culture medium was replaced 24 h later with fresh medium containing 1.5 µg/ml puromycin (Sigma), and puromycin-resistant colonies were counted 10 days later. 231Br and 231 cells were transduced with the appropriate vectors at a MOI of 0.5.

ELISA

BMP-2 and BMP-4 ELISA Kits (R&D Systems) were used according to manufacturer's protocol.

qRT-PCR arrays

Total RNA was isolated using the RNeasy 96 Kit (Qiagen). 500 ng of each RNA sample were converted to cDNA with the RT² First Strand Kit (Qiagen) and then combined with the RT² SYBR Green qPCR Mastermix (Qiagen), according to the manufacturer's instructions. Equal volumes (25 µl) of this mixture were loaded into each well of TGF- RT² Profiler PCR array plates (Qiagen) then qPCR performed using an iQ5 Real-time PCR Detection System (Bio-Rad, Hercules, CA).

qRT-PCR

Total RNA was extracted using TRIzol (Invitrogen), following the manufacturer's protocol. Samples were further purified by treatment with TURBO DNA-free™ (Ambion) followed by a second extraction with phenol/chloroform. Total RNA was converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time RT-PCR was performed using an IQ Real-time RT-PCR Detection System for *Sox2*, *Sox9*, *Nestin*, *glial fibrillary acidic protein (GFAP)*, *Vimentin*, *Map2*, and *PDGFR* (Supplemental Table 1). PCR efficiency for each primer was determined using a standard curve generated from cDNA serial dilutions. For each sample, expression levels were normalized to *GAPDH* (Applied Biosystems).

Immunofluorescence labeling of formalin-fixed paraffin-embedded (FFPE) tissue

Patient breast to brain metastases tissue samples (HER2⁺ = BBM1–2, triple negative = BBM3) were collected in accordance to an approved City of Hope Institutional Review Board ($n = 3$). Tissues were then formalin-fixed, embedded, and stained as follows: Paraffin sections were first dewaxed with xylene and then hydrated through alcohol gradient (100, 95, 70 %). Antigen recovery was performed on sections in Na-Citrate buffer (10 mM, pH 6.0) in a 95 °C water bath for 30 min. Sections were then permeabilized in 0.3 % tween-20 for 30 min at 37 °C. Finally, sections were blocked for non-specific sites by incubating in PBS + 1 % bovine serum albumin (BSA) + 10 % fetal bovine serum (FBS). For detection, sections were incubated in appropriate primary antibody (Supplemental Table 1) over night at 4 °C, in PBS + 1.5 % FBS + 1 % BSA. The next day, sections were incubated with the appropriate secondary antibody in PBS for 1 h at room temperature protected from light and then mounted with Immunogold + DAPI (Fisher). Samples were analyzed on a Carl Zeiss LSM Confocal Microscope. NIH Image Software (Image J, <http://rsb.info.nih.gov/ij/>) was used for quantification of infiltrated and non-infiltrated proliferative astrocytes by calculating the number of cells in every fifth 10 µm sections over range of 250 µm.

Immunocytochemistry

Cultures were stained as previously described [23]. Briefly, cells were fixed with 4 % paraformaldehyde at 4 °C. Samples were permeabilized in 0.3 % Triton X-100 for 30 min. Samples were then blocked for 1 h in 10 % normal goat serum. Primary antibodies (Supplemental Table 1) were diluted in 1 % normal goat serum and incubated overnight at 4 °C. The next day, samples were incubated with the appropriate secondary antibody in PBS for 1 h at room temperature protected from light, mounted with Immunogold plus DAPI (Fisher), and analyzed on a Carl Zeiss Axio LSM Confocal Microscope.

Western analysis

Cells were homogenized in Pierce IP Lysis Buffer (Thermo Scientific) containing Halt Protease Inhibitor Cocktail (Thermo Scientific), Halt Phosphatase Inhibitor Cocktail (Thermo Scientific), and 0.5 M EDTA solution (Thermo Scientific). 40 µg of total proteins were loaded onto Mini-PROTEAN TGX Gels (Bio-Rad). Protein bands were incubated with appropriate primary antibodies and then detected by horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling) and super-signal west pico chemiluminescent substrate (Thermo Scientific). Each extract was also stained for α -actin to calculate relative levels. Relative intensities of protein bands were quantified by scanning densitometry using NIH Image Software (Image J, <http://rsb.info.nih.gov/ij/>) and by calculating the average of three assays.

Xenografts

Breast cancer cells expressing ZsGreen-firefly luciferase were suspended in PBS, and then 10^5 cells/ $2\mu\text{L}$ were injected in the brains (2 mm right and 1 mm anterior to the bregma suture) of female NOD/SCID mice (6 mice/group, total = 24) in accordance with approved City of Hope IACUC protocol. In vivo bioluminescence imaging (BLI) was performed with the Xenogen System and Living Image software for data acquisition (Xenogen Corp.).

Statistical analyses

Data are represented as mean values $\pm$ standard error of the mean. Statistical significance was assessed using Student's *t* test and 1-way ANOVA with Bonferroni's multiple comparison test (*p* value: **p* < 0.05, ***p* < 0.01, and ****p* < 0.001; 95 % confidence interval of difference).

Results

Neural progenitor-like cells and astrocyte-like cells appear adjacent to metastatic breast cancer cells

Brain metastases from breast cancer patients contain astrocytic cells positive for GFAP [24]. However, the role of astrocytes and NPCs in the peritumoral brain region remains unclear. We used immunohistochemistry to analyze patient tissue from BBM. First, we show T1-gadolinium enhanced axial brain MRIs from a patient who had a right occipital periventricular ring-enhancing breast metastasis surrounded by hypo-intense vasogenic edema before and after surgical resection (Fig. 1a). H&E staining of sections of post-operative, resected tissue revealed the peritumoral brain, tumor edge, and breast tumor periphery (Fig. 1b). We observed breast metastatic cells accompanied by an increasing gradient of GFAP+ astrocytes in both the peritumoral brain and tumor regions, with the highest accumulation having occurred at the tumor edge (Fig. 1c). These GFAP+ astrocytes exhibited a hypertrophic morphology comparable to glial scars that form subsequent to traumatic brain injury. Because glial scars are normally associated with neural trauma [25], we asked if the GFAP+ cells that accumulated in and around tumor tissues were also scar-like and contained proliferative astrocytes. We found 20 % of the GFAP+ cell population in the peritumoral brain region co-express the proliferative marker Ki67; with 30 % of these Ki67+/GFAP+ proliferative astrocytes appearing to have infiltrated at the tumor edge (Fig. 1d, e).

Since progenitor cells have been shown to generate new cells under pathological conditions in the CNS, we asked if cells surrounding the brain metastasis exhibited NPC features [26, 27]. To determine this we stained sections for Nestin and the multipotency and self-renewal marker Sox2, both of which are expressed in majority of NPCs [28]. We found Sox2+/Nestin+ cells were present were located at the peritumoral brain-tumor edge, adjacent to metastatic tumor cells (Fig. 1f). Our examination of adjacent tissue suggested Sox2+/Nestin+ cells in the peritumoral region (described above) had infiltrated the tumor (Fig. 1f, i–iii). Breast metastasis cells at the tumor periphery also expressed Sox2, as previously reported [29]. However, the positivity of Nestin combined with Sox2 confirmed the cells, which had infiltrated the tumor originated from the neuroepithelium.

The Sox9 transcription factor is upregulated when CNS NPCs differentiate into glial rather than neuronal lineages [30]. We observed Nestin+ NPCs in the peritumoral brain co-expressed Sox9, suggesting they had differentiated toward glial fates (Fig. 1g). Metastatic tumor cells in the tumor periphery were Sox9+/Nestin-. In addition, Sox9 localized to both the nuclear and cytoplasmic compartments of the majority of the tumor cells, as previously reported [31]. Moreover, breast cancer patients whose tumor cells contain cytoplasmic Sox9

have worse clinical outcomes; the phenotype is associated with distant metastasis [32], as was the case for these cancers.

Because the NPCs observed adjacent to the tumor were committed toward a glial fate, we looked at astrocyte differentiation by staining tissue sections for the glutamate transporter (GLAST), an astrocyte-specific marker is also expressed in AP cells [33]. Recently, it was reported AP cells in the developing brain also express CD44+, a marker associated with breast cancer and metastatic breast cancer cell invasion [34]. Immunofluorescence staining revealed GLAST+/CD44+ AP cells in peritumoral brain (Fig. 2a) and inner tumor regions (Fig. 2b). Moreover, breast cancer cells had differential expression for CD44, with inner tumor regions having stronger expression (Fig. 2b) than peritumoral brain region (Fig. 2a), suggesting a heterogeneous population.

Because BMPs play important roles in both AP cell development and maturation in the CNS [20], and the pathogenesis of metastatic breast cancers [35, 36], we examined metastatic breast-brain sections for BMP-2 expression. We found metastatic breast cancer cells express BMP-2 at the tumor periphery and edge of tissue sections (Fig. 2c). Moreover, the metastatic cells showed robust BMP-2 expression at tumor and Vimentin+ astrocyte interfaces (Fig. 2c, d, arrows). Although Vimentin has been shown to be expressed in breast cancer cells in the epithelial-to-mesenchymal transition (EMT) [37], the vast majority of breast metastatic cells in patient BBMs did not stain for Vimentin in the tumor edge or periphery.

BMP-2 is overexpressed in BBM versus primary breast cancer cell lines

Since TGF- β signaling normally has tumor-suppressive effects [38], we investigated the role of TGF- β proteins and their effectors on metastatic breast-to-brain cancer and the tumor-host cell microenvironment. We selected the primary breast carcinoma cell line 231 and its matched brain-trophic metastatic subline 231Br to study the expression of components of the TGF- β signaling pathway. Gene expression analysis of *BMP-1*, *BMP-3-7*, *TGF- β 1-3* and their downstream effectors *SMADs 1-5*, revealed less than twofold difference in the mRNA levels of these genes in metastatic 231Br cells compared to 231 cells. However, 231Br cells expressed *BMP-2* mRNA at significantly higher levels (27.2-fold; $p < 0.001$) compared to 231 cells (Fig. 3a), suggesting BMP-2 signaling could be associated with metastatic activity.

Western blot analysis of TGF- β signaling components (Fig. 3b) revealed elevation of intracellular BMP-2 protein levels (5.2 fold; $p < 0.001$) and its transcription effectors SMADs 1/5/8 (2.2 fold; $p < 0.001$) in the metastatic 231Br cells compared to 231 cells. In contrast, BMP-1, BMP-7, and TGF- β 1-3 protein levels were not significantly different between the two cell types. Conversely, BMP-4 protein levels were higher in 231 cells (2.2 fold; $p < 0.001$) compared to 231Br cells.

Because BMP-2 and BMP-4 play roles in astrocyte differentiation of brain NPCs [39], we further quantified the secreted levels of these proteins by 231Br and 231 cells for possible paracrine signaling. Analysis of cell culture media revealed BMP-2 concentrations were significantly higher in 231Br media compared to 231 media (Fig. 3e). However, there was no significant difference in the concentration of BMP-4 between the 231Br metastatic and 231 primary breast cancer cell lines (Fig. 3e) and the levels of BMP-4 were lower overall compared to BMP-2. Immunocytochemistry was used to qualitatively validate the Western results for differential expression of BMP-2/4 proteins in 231Br and 231 cells (Fig. 3c, d). One possible reason for the differential expression of BMP-4 mRNA and protein could be due to the variance in mRNA silencing process in 231 versus 231Br thus affecting protein translation [40–42].

NPCs co-cultured with MD-MBA 231Br cells differentiate into astrocytes via a BMP-2 dependent mechanism

Our examination of the surgical sections of BBM indicated the NPCs were present in the peritumoral region and some NPCs had infiltrated the tumor. Because *BMP-2* mRNA is up-regulated in the metastatic breast cancer cells with a corresponding increase in the secretion of BMP-2 protein, we investigated the effects of paracrine BMP-2 signaling from breast cancer cells on NPCs.

We first verified human NPCs expressed the BMP-2 receptor BMPRI1A/B and were competent to respond to exogenous BMP-2 (Supplemental Fig. 1). We found exogenous BMP-2 treatment differentiated Sox2⁺ NPCs into astrocytes within 15 days. During this period, the cells lost their bipolar processes, began co-expressing GFAP/Vimentin and developed a flat star-shaped morphology, all phenotypes are associated with astrocyte differentiation (Supplemental Fig. 1a). We also observed an overall decrease in *SOX2* mRNA expression, which indicates the cells lost NPC multipotency (Supplemental Fig. 1b). The neuro- to glial switch during BMP-2 treatment was observed early (1–5 days), as evidenced by increased *SOX9* mRNA expression 1–5 days in vitro (Supplemental Fig. 1b). There was also an overall increase in Nestin expression, which is a marker for both NPCs and astrocytes. BMP-2 treatment did not promote neuronal or oligodendrocyte differentiation, which was evidenced by decreased *MAP-2* and *PDGFR* mRNA expression over the 15-day period (Supplemental Fig. 1b). Immunocytochemistry confirmed the expression of the Sox2, Sox9, Nestin, GFAP, and Vimentin proteins correlated with the changes in mRNA expression observed during BMP-2 treatment.

To mimic paracrine interactions between tumor cells and the host microenvironment, we established transwell co-cultures of the 231 primary and 231Br metastatic breast cancer cells with NPCs. When co-cultured with 231 primary cancer cells, the NPCs did not undergo any type of differentiation (Fig. 4a, b). Closer analysis revealed the NPC *SOX2* mRNA expression was unchanged (Fig. 4a), and cells stained positive for Nestin but negative for Vimentin at 1 and 15 days in vitro, indicating the NPCs were not affected by the presence of 231 cells (Fig. 4b). Moreover, co-culture of NPCs with 231 cells did not induce the expression of any lineage-specific mRNA markers for astrocytes (*SOX9*, *GFAP*, *VIMENTIN*), neurons (*MAP-2*), or oligodendrocytes (*PDGFR*) (Fig. 4a). Consistent with the effects of exogenous BMP-2 treatment (Supplemental Fig. 1), when NPCs were co-cultured with metastatic 231Br cells for 15 days they differentiated into astrocytes (Fig. 4c). We observed a decrease in the levels of *SOX2* mRNA expression in NPCs over 15 days in vitro (Fig. 4c). Commitment of the NPCs to a glial lineage was indicated by increased mRNA expression for *SOX9*, as well as the astrocyte specific *GFAP* and *VIMENTIN* mRNA, and cells appeared flat with star-shaped processes (Fig. 4d). Similar to the 231 cells, the 231Br tumor cells did not induce any changes in the neuronal or oligodendroglial markers.

To determine if the differentiation of NPCs into astrocytes in metastatic 231Br co-cultures was dependent on paracrine BMP, we added the BMP antagonist Noggin, which sequesters BMPs and blocks their binding to surface receptors [43]. By using relative *GFAP* mRNA expression as an indicator of astrocyte differentiation, we found Noggin treatment significantly decreased *GFAP* expression when NPCs were cultured alone (Fig. 5a, b, dashed line). Similar to previous experiment, NPCs co-cultured with metastatic 231Br cells showed significant increase in *GFAP* expression over 15 days, indicating astrocyte differentiation (Fig. 5a, green line). However, when NPCs were co-cultured with 231Br cells and Noggin, there was minimal upregulation of *GFAP* expression over the 15-day period, suggesting astrocyte differentiation was blocked (Fig. 5a, red line). As expected, exogenous Noggin had no effect on NPCs co-cultured with primary 231 cells (Fig. 5b, red line).

Overall, our data suggest BMP-2 signaling from metastatic breast cancer cells promotes the differentiation of NPCs into astrocytes.

Astrocyte differentiation of NPCs enhances the proliferation of co-cultured tumor cells

We next asked if the proliferation of 231Br cells was affected by the NPCs differentiating into astrocytes. When 231Br cells were cultured alone, they had an overall doubling time of 3.94 days. However, when co-cultured with NPCs, the overall doubling time 231Br tumor cells increased to 5.06 days, suggesting slower growth (Fig. 5c). However, closer analysis revealed during 1–5 days in vitro, just prior to NPC differentiation into astrocytes, the 231Br cell doubling time was 11.4 days. In contrast, 5–15 days in vitro, after astrocyte differentiation occurred, the doubling time of 231Br accelerated to 2.42 days, and was more rapid than observed when 231Br cells were cultured alone (3.90 days). Furthermore, when astrocyte differentiation was blocked by addition of Noggin, the 231Br cells had a doubling time of 27.73 days (Fig. 5c). The growth curve analysis of the 231 cells cultured alone revealed they had a doubling time of 5.10 days (Fig. 5d). When 231 cells were co-cultured with NPCs with or without Noggin they exhibited a similar and slower doubling time of 13.59 days (Fig. 5d). These data suggest increased in breast cancer cell proliferation in vitro correlated positively with BMP differentiation of NPCs into astrocytes (Table 1).

Targeting of BMP-2 alters primary and metastatic tumor phenotypes

To determine if the tumor-secreted BMP-2 specifically caused NPCs to differentiate into astrocytes, we used shRNA to knockdown BMP-2 in the 231Br metastatic cells (231BrKD) and lentivirus to overexpress BMP-2 in the 231 non-metastatic primary cells (231KI). We first confirmed the knockdown and induction of BMP-2 expression by these cells. Real-time RT-PCR analysis showed 231BrKD and 231BrNT (the scrambled shRNA control) had a 0.19 fold decrease and a 1.1 fold change, respectively, in the amount of *BMP-2* mRNA compared to the non-transfected 231Br cells (Supplementary Fig. 2a). In contrast, 231KI cells had a 10.1 fold increase in the amount of *BMP-2* mRNA compared to 231 cells (Supplementary Fig. 2a). ELISA data showed the extracellular concentration of BMP-2 was significantly higher in 231KI cultures compared to 231BrKD cultures. Western blot analysis also confirmed 231KI cells had significantly higher levels of intracellular BMP-2 compared to 231BrKD cells (Supplementary Fig. 2b, c).

We also analyzed BMP-4, BMP-7, TGF- β 1, TGF- β 3 and their effector SMADs 1/5/8 in the 231BrKD and 231KI cells to determine if dramatically altering BMP-2 levels in tumor cells affected these other molecules. Protein analysis showed higher amounts of intracellular SMAD1/5/8 in 231KI cells compared to 231BrKD cells. In addition, the intracellular levels of BMP-4 and TGF- β 3 were significantly higher in 231BrKD cells versus 231KI cells (Supplementary Fig. 2b). ELISA data showed the concentration of extracellular BMP-4 was significantly higher in the 231KI compared to 231BrKD and 231BrNT cells (Supplementary Fig. 2c). Immunocytochemistry was used to qualitatively verify the protein analysis results for BMP-2 and BMP-4 in the 231KI, 23BrKD, and 231BrNT cells (Supplementary Fig. 2d).

We then determined if altered BMP-2 in the tumor cells affected the ability of NPCs to undergo astrocytic differentiation. We found NPCs did not differentiate into astrocytes when cultured with the 231BrKD cells (Fig. 6a). The NPCs showed no change in *SOX2* or *NESTIN* mRNA expression relative to NPCs cultured alone. The expression of lineage-specific genes for astrocytes (*GFAP*, *VIMENTIN*), neurons (*MAP-2*), or oligodendrocytes (*PDGFR*) was also unaffected by co-culture with 231BrKD (Fig. 6a, i). The state of the NPCs as undifferentiated at 15 days was further confirmed qualitatively by staining for Nestin and Vimentin (Fig. 6a, ii). Thus overall, 231BrKD behaved similarly to 231 cells in co-culture. In contrast, when co-cultured for 15 days with primary 231KI cells over-

expressing BMP-2, NPCs differentiated into astrocytes (Fig. 6b). The differentiation of NPCs into astrocytes in 231KI co-cultures was accompanied by the suppression of *SOX2* and the increased expression of *SOX9*, *GFAP*, and *VIMENTIN* mRNA (Fig. 6b, i). Astrocyte differentiation was further verified qualitatively by co-staining for GFAP and Vimentin (Fig. 6b, ii). Similar to co-culture of 231Br with NPCs, the 231KI cells did not induce changes in the expression of neuronal or oligodendroglial mRNAs. Overall, the 231KI behaved similarly to 231Br metastatic cells in co-culture.

Next we determined how altered BMP-2 expression in 231BrKD and 231KI cells affected their proliferation when co-cultured with NPCs over time. We found differentiation of NPCs into astrocytes in co-cultures enhanced 231KI proliferation. Growth curves of 231KI cells cultured alone showed an overall doubling time of 3.71 days (Fig. 6b, iii). When co-cultured with NPCs, 231KI exhibited an overall doubling time of 4.06 days (Fig. 6b, iii). From 1 to 5 days when cells are more like NPCs than astrocytes, the 231KI doubling time was 9.32 days. However, from 5 to 15 days after astrocytes has differentiated, the doubling time of 231KI accelerated to 3.09 days. In contrast, the failure of NPCs to differentiate into astrocytes had a dramatic effect on 231BrKD tumor cell proliferation (Fig. 6a, iii). Growth curve analysis of 231BrKD cultured alone revealed a doubling time of 4.73 days (Fig. 6a, iii). When co-cultured with NPCs that fail to differentiate into astrocytes, 231Br KD cells exhibited an overall doubling time of 14.40 days (Fig. 6a, iii). These effects were not observed with scrambled mRNA control 231BrNT cells; when co-cultured with NPCs their growth curves mimicked those of the non-transfected 231Br cells (Supplementary Fig. 2e, f). These data suggest increased in breast cancer cell proliferation in vitro correlated positively with BMP-2 differentiation of NPCs into astrocytes (Table 2).

We then determined if the knock-in of BMP-2 in 231 cells and knockdown of BMP-2 in 231Br cells affected their ability to colonize mouse brain and develop into tumors in vivo. When breast cancer cells were xenografted into the brains of female NOD/SCID mice, serial BLI acquisition over 21 days revealed 231BrKD had a delayed time for tumor cell engraftment and colonization (10 vs. 14 days, post injection) and a significant overall slower tumor growth compared to 231Br (Fig. 7a). In contrast, xenografted 231KI cells had a shorter time for tumor cell engraftment and colonization (3 vs. 14 days, post injection) and a significant overall more aggressive tumor growth compared to 231 (Fig. 7b).

Discussion

The tumor microenvironment is a highly specialized niche determined by its tissue-specific location and cell-derived contents, including the extracellular matrix and soluble factors. In this study we investigated the role of peritumoral NPCs and astrocytes in BBM within the context of molecular mechanisms involved in brain trauma.

Because of the metastatic site, the surgical resection of these three tissues included a rare small cuff of brain adjacent to the tumor. These extremely scarce BBM specimens allowed us a unique opportunity to investigate the bi-directional interplay of brain and neoplastic cells at the tumor boundary and edge. We determine that these BBMs display active gliosis accompanied by NPCs and glial progenitor cells in the peritumoral region and tumor periphery. Furthermore, these astrocytes resemble those observed in the glial scars forming at the sites of traumatic brain injury [44], and the highest accumulation of these cells was at the tumor edges in the patient samples. Recent studies of pediatric and adult post-natal brain germinal niches (subventricular and subgranular zones) for progenitor cells have revealed there is a dynamic CNS environment with a cellular plasticity previously thought not to exist. Depending on specific cues, NPCs can retain multi-potency or differentiate into astrocytes, oligodendrocytes, or neurons [6, 45]. These physiological processes are

accentuated near sites of traumatic brain injury, and ischemia, suggesting a role for NPCs and their differentiated descendants in pathological responses. [25, 46]. Thus, although migratory NPCs are known to exist in the post-natal mammalian brain, little is known about any molecular cues mediating their activation, migration, and differentiation in during brain metastasis [47]. Here we confirm cross-talk between NPCs and metastatic tumor cells creates an optimal niche for tumor cell colonization and proliferation in the brain. Our studies demonstrate that metastatic growth is inhibited by NPCs, until they differentiate into reactive astrocytes in response to metastatic breast cancer cells overexpressing and secreting BMP-2. This step promotes a favorable environment in which the cancer cells can proliferate. Our data suggest a role for BMP-2 in promoting the divergent evolution of resident neural progenitor and metastatic breast cancer cells in the brain microenvironment. In breast cancer cells, BMP-2 expression during specific pathogenic stages can be oncogenic but in NPCs its expression promotes differentiation.

Since brain metastases are increasingly the first site of relapse after treatment for metastatic disease in patients with HER2⁺ breast cancer, a link between HER2 and BMP-2 may exist. Studies have shown the close interaction of HER2 and TGF- β in controlling the pathogenesis of breast cancer from mammary epithelial cells through the MAPK/Ras pathways [48–52]. In addition, the cooperation between the TGF- β and HER2/Ras/MAPK pathways leads to the secretion of other cytokines supporting EMT, migration, and invasion [53–55]. In addition, higher levels of BMP-2 may promote the growth, invasion and metastases of breast cancer cells having undergone EMT [12, 56, 57]. This hypothesis is supported by the triple negative, basal-like status of 231Br cells confirmed by the PAM50 intrinsic molecular phenotype classifier, strongly correlating with the “EMT-like” status of breast cancer cells [58]. Indeed, our experiments show metastatic patient lesions were CD44⁺ and Sox9⁺/Nestin[–], which also correlates with the “EMT-like” status. We propose these breast cancer cells may behave similarly and are undergoing EMT through autocrine BMP-2 expression.

Signaling from the brain niche can potentially affect tumor cell growth in a negative or positive manner [59–63]. The specific dynamic processes resulting in the interaction of BMP-2 with both the breast cancer and NPCs being favorable to breast cancer proliferation, are unclear. However, recently published studies of the effect of BMPs on the neural crest progenitor cell (NCPC) population in the early vertebrate embryo may yield some clues. In zebra fish and *Xenopus* embryos, a BMP gradient establishes the future NCPC domain during gastrulation. BMPs are believed to pattern the ectoderm of zebrafish and *Xenopus* in a gradient fashion, such that high levels of activity induce epidermis, intermediate levels induce neural crest, and the absence of BMPs results in neurectoderm formation [64, 65]. Therefore, the effect of BMP-2 on NPCs may be directly tied to a gradient, with intermediate BMP-2 levels driving NPC differentiation towards the astrocytic lineage.

We propose NPCs initially migrate towards a brain lesion to impose a protective effect. However, after they are juxtaposed to the metastatic breast tumor cells expressing BMP-2, they differentiate into astrocytes resulting in the promotion of tumor cell proliferation (Fig. 7c). This BMP-2 NPC-to-astrocyte differentiation may be regulated by downstream expression of either SMAD 1/5/8, RE1 silencer of transcription (REST), or neuron-restrictive silencer factor (NRSF) [66, 67]. Furthermore, astrocytes cause an up-regulation of the survival genes, GSTA5, BCL2L1, and TWIST1 in breast cancer cells resulting in an increased resistance to chemotherapeutic agents [8]. Overall, our results highlight the need for complementary treatment strategies for brain metastasis patients targeting both the cancer cells and the changes in the brain niche supporting them. We intend to investigate the penetrance of these phenomena in breast-to-brain cancer metastases, and evaluate therapeutic strategies targeting this intercellular communication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Margaret Morgan for her comments and careful editing of this manuscript. Rahul Jandial is supported by National Institutes of Health (NIH) Grant 2K12CA001727-16A1. Josh Neman is supported by California Institute for Regenerative Medicine (CIRM) Grant TG2-01150. Additional project support was provided by National Cancer Institute Grant (NCI) P30 CA033572.

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Fig. 1.

Patient BBM reveal active gliosis and infiltrated NPCs. **a** Representative pre- (*top*) and post- (*bottom*) operative T1 gadolinium-enhanced axial brain MRIs from patient BBM1. *Red circle* indicates part of surgical specimen containing brain and tumor analyzed. **b** Representative H&E section of patient BBM showing peritumoral brain, tumor edge (*dash line*), and tumor periphery. **c** Immunofluorescence of BBM1 showing HER2+ tumor cells and GFAP+ astrocytes in peritumoral brain and tumor edge. **d, e** Immunofluorescence from BBM3 showing GFAP+/Ki67+ proliferative astrocytes (*solid arrows*) adjacent to Ki67+/GFAP- BBM cells (*open arrows*) in peritumoral brain and tumor edge. **e** High power magnification from BBM3 showing infiltrated and non-infiltrated proliferative astrocytes (*solid arrows*) adjacent to BBM cell (*open arrow*). **f** Quantification of infiltrated and non-infiltrated proliferative astrocytes across all patient BBM tissue specimens collected. **g** Immunofluorescence from BBM2 showing infiltrated Sox2+/Nestin+ NPCs (*arrows, insert*) adjacent to Sox2+/Nestin- BBM cells in peritumoral brain and tumor periphery. **h** Immunofluorescence from BBM2 of Sox9+/Nestin+ NPCs (*arrow*) adjacent to Sox9+/Nestin- breast cancer cells in peritumoral brain and tumor periphery

Fig. 2.

Patient BBM reveal active gliogenesis and expression of BMP-2. **a** Immunofluorescence from BBM1 showing infiltrating AP cells in peritumoral brain (*solid arrows*, GLAST+/CD44+) and BBM cells at the tumor edge (*dash arrows*, GLAST-/CD44+). **b** Immunofluorescence from inner tumor regions of BBM1 showing GLAST+/CD44+ AP cells (*arrows*, *inserts i-ii*) and GLAST-/CD44+ BBM cells. **c** Immunofluorescence from BBM1 showing BMP-2+ tumor cells and Vimentin+ astrocytes at tumor edge. **d** Immunofluorescence from BBM3 showing BMP-2+ tumor cells and Vimentin+ astrocytes in tumor periphery. BBM cells with stronger expression of BMP-2 (*solid arrows*) can be seen along the tumor edge (**c**) and periphery (**d**). *BV* blood vessels

Fig. 3.

Analysis of TGF- superfamily members reveals BMP-2 overexpression in brain trophic 231Br versus primary 231 breast cancer cells. **a** Real-time qPCR of TGF- superfamily expression in 231Br relative to 231. **b** Western blot analysis of BMP-1, BMP-2, BMP-4, BMP-7, TGF- 1, TGF- 2, TGF- 3, and SMAD1/5/8 proteins. Immunofluorescence of ZsGreen+ 231Br (**c**) and 231 (**d**) stained for BMP-2 (*red*), BMP-4 (*white*), nuclear DAPI (*blue*). **e** ELISA for BMP-2 and BMP-4 release in 231 and 231Br. Data are represented as mean values $\pm$ standard error of the mean ($n = 3$). Statistical significance was assessed using Student's *t* test or 1-way ANOVA with Bonferroni's multiple comparison test (p value: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; 95 % confidence interval of difference)

Fig. 4.

Human NPCs co-cultured with metastatic 231Br cells differentiate into astrocytes. **a** mRNA analysis and **b** protein expression reveal NPCs remain in a progenitor-like state after co-culturing with 231 for 15 days in vitro. **c** mRNA analysis and **d** protein expression reveal NPCs differentiate into astrocytes after co-culture with 231Br for 15 days in vitro. Grey zone denotes NPC to astrocyte differentiation. Data are represented as mean values $\pm$ standard error of the mean ($n = 3$)

Fig. 5.

Breast metastasis cell proliferation is associated with the differentiation of NPCs to astrocytes. Noggin treatment only affects the differentiation of NPCs into astrocytes (grey zone) in 231Br co-culture (a) and not 231 (b) over 15 days in vitro. Growth curve analysis of metastatic 231Br (c) and 231 cell (d) co-cultured with NPCs for 15 days in vitro along with quantification of tumor growth at day 15. The differentiation of NPCs into astrocytes was accompanied by increased tumor cell proliferation. Data are represented as mean values $\pm$ standard error of the mean ($n = 3$). Statistical significance was assessed using Student's t test or 1-way ANOVA with Bonferroni's multiple comparison test (p value: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; 95 % confidence interval of difference)

Fig. 6.

Targeting BMP-2 altered primary and metastatic tumor phenotypes. **a** 231BrKD cells lacking expression of BMP-2 fail to differentiate NPCs into astrocytes in co-culture (*i*). Immunocytochemistry of NPCs in 231BrKD co-cultures after 15 days in vitro (*ii*). 231BrKD proliferation is abolished because NPCs fail to differentiate into astrocytes (*iii*). **b** 231KI cells overexpressing BMP-2 differentiated NPCs into astrocytes when co-cultured (*i*). Immunocytochemistry of NPC derived astrocytes in 231KI co-cultures after 15 days in vitro (*ii*). 231KI proliferation increase as NPCs differentiated into astrocytes (*iii*). Data are represented as mean values $\pm$ standard error of the mean ($n = 3$). Statistical significance was assessed using Student's *t* test (p value: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$; 95 % confidence interval of difference)

Fig. 7. Serial BLI monitoring of naïve and BMP-2 altered primary and metastatic tumor cells. In vivo monitoring and time series of average BLI (count/s) of luciferase positive in **a** 231Br and 231BrKD cells, and **b** 231 and 231KI cells, over a 21-day interval. Representative in vivo brain BLI images were acquired on day 21-post injection. **c** Model for the co-evolution of breast metastasis cells and NPCs. Data are represented as mean values $\pm$ standard error of the mean ($n = 3$). Statistical significance was assessed using Student's t test (p value: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$; 95 % confidence interval of difference)

Table 1

231Br and 231 cell doubling breakdown

	Doubling (days)		
	Days 1–5	Days 5–15	Overall
231Br	4.30	3.90	3.94
231Br + NPC	11.4	2.42	5.06
231Br + Noggin	5.3	3.63	4.1
231Br + NPC + Noggin	15.3	46	27.73
231	5.31	5.07	5.07
231 + NPC	6.63	28.75	13.53
231 + Noggin	2.34	4.6	5.31
231 + NPC + Noggin	6.27	32.86	13.53

Table 2

231KI and 231BrKD cell doubling breakdown

	Doubling (days)		
	Days 1–5	Days 5–15	Overall
231KI	3.59	3.75	3.71
231KI + NPC	9.32	3.09	4.06
231BrKD	4.89	4.66	4.73
231BrKD + NPC	7.04	30.53	14.4