Molecular Pathways Mediating Metastases to the Brain *via* Epithelial-to-Mesenchymal Transition: Genes, Proteins, and Functional Analysis

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Abstract. *Background: Brain metastases are the leading cause of morbidity and mortality among patients with disseminated cancer. The development of metastatic disease involves an orderly sequence of steps enabling tumor cells to migrate from the primary tumor and colonize at secondary locations. In order to achieve this complex metastatic potential, a cancer cell is believed to undergo a cellular reprogramming process involving the development of a degree of stemness, via a proposed process termed epithelial-to-mesenchymal transition (EMT). Upon reaching its secondary site, these reprogrammed cancer stem cells submit to a reversal process designated mesenchymal-to-epithelial transition (MET), enabling establishment of metastases. Here, we examined the expression of markers of EMT, MET, and stem cells in metastatic brain tumor samples. Materials and Methods: Immunohistochemical analyses were performed to establish the markers of EMT and MET. Co-expression of these markers was determined by immunofluorescence analysis. Gene-expression analysis was conducted using tissues from brain metastases of primary adenocarcinoma of the lung compared to non-metastatic tissue. Cell proliferation was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide S-phase entry analysis, by determining the 5-ethynyl-2'-deoxyuridine incorporation. Scratch wound and chemotactic migration assays were performed in an astrocytic setting. Results: Metastatic brain tumor samples displayed expression of epithelial markers (zinc finger protein SNAI1 and Twist-related protein-1), as well as the mesenchymal marker vimentin. The stem cell marker CD44 was also highly expressed. Moreover, co-expression of the epithelial marker E-cadherin with the mesenchymal marker vimentin was evident, suggesting a state of transition. Expression analysis of transcription factor genes in metastatic brain tumor samples demonstrated an alteration in genes associated with neurogenesis, differentiation, and reprogramming. Furthermore, tumor cells grown in astrocytic medium displayed increased cell proliferation and enhanced S-phase cell-cycle entry. Additionally, chemotactic signaling from the astrocytic environment promoted tumor cell migration. Primary tumor cells and astrocytes were also shown to grow amicably together, forming cell-to-cell interactions. Conclusion: These findings suggest that cellular reprogramming via EMT/MET plays a critical step in the formation of brain metastases, where the cerebral milieu provides a microenvironment suitable for the development of metastatic disease.*

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Brain metastases occur in about 40% of patients with cancer in the United States, and are the major cause of morbidity and mortality in these individuals (1). The incidence is estimated to be about 170,000/year in the USA, approximately 10-times higher than that of primary malignant brain tumors. The most common types of cancer, which have a propensity for metastasizing to the brain, include lung (20%), breast (5%), melanoma (7%), renal (7%), and colorectal cancer (2%), with patients aged 20 to 39 years with breast cancer having the highest proportional risk of cerebral metastases (2). Following the development of brain metastases, there is a median survival of approximately 2-25 months depending on the origin of the primary tumor (3, 4).

Dissemination to distant organs from the primary site is a complex process that involves multiple steps. The primary tumor cells must detach from the tumor site, migrate into the bloodstream or lymphatic system, and then invade and proliferate at the secondary site (5-7). The formation of brain metastases, in particular, requires tumor cells to travel and attach to the cerebral microvasculature, penetrate the brain parenchyma through the blood–brain barrier, induce

angiogenesis, and proliferate in response to local growth factors (8, 9). Those that survive, form micrometastases that may, in turn, establish clinically significant lesions after a fairly variable period of dormancy, in which requirements for cell division are acquired (10, 11). Metastatic deposits in the brain are generally seen forming at the grey-white matter junction, with the largest percentage of these lesions involving the cerebrum (80%), while cerebellum and brainstem lesions collectively account for only 20% of all lesions (12, 13).

Classic views on the formation of metastases argue that during tumor progression, cancer cells acquire multiple alterations that render them increasingly competent to establish metastatic lesions in specific organs (6, 14). How different genes may cooperate in fulfilling various requirements for the establishment of tissue-specific metastases remains to be elucidated. The fact that gene-expression signatures indicative of poor prognosis present in the primary tumor can predict the clinical development of metastases, has challenged this hypothesis (15, 16). Recent studies have been able to isolate specific genes from primary cancer cell lines that have a role in determining organ-specific metastases (17-20) and also show that expression of these genes are regulated by specific factors such as transforming growth factor beta (TGFβ) (18). Taken together, these studies imply that the gene-expression signature already present in the parental tumor cell population promotes organ-specific metastasis, and that the process of metastasis requires a set of functions beyond those underlying the emergence of the primary tumor (18).

While the genetic basis of tumorigenesis varies extensively between different tumor types, the cellular and molecular steps required for metastasis remain similar for all solid tumors (21, 22). The embryologically conserved mechanism of epithelial-to-mesechymal transition (EMT), which allows cells to break cell–cell adhesions and migrate to a secondary site, followed by the reverse process of mesenchymal-to-epithelial transition (MET), is an attractive mechanism for explaining the development of metastases. EMT programming is associated with complex genetic alterations allowing epithelial and mesenchymal cells to be distinguished by the expression of a number of classical markers (23). For example, epithelial markers include adherins and tight junction proteins such as E-cadherin, whereas mesenchymal markers include the extracellular matrix component fibronectin and the intermediate filament protein vimentin (24).

Parallels between EMT in development and tumor progression have already been suggested (25). For instance, the intratumoral and intertumoral heterogeneity of human breast cancer are considered as differentiation repertoires available to the neoplastic cells in response to the tumor microenvironment, including reversion to a 'normal' phenotype (26), rather than a consequence of phenotypic drifting due to genetic instability. While studies have charted the phenotypic switch between EMT and MET in malignant progression of colorectal (27), bladder (28), and ovarian (29) cancer, the process is yet to be demonstrated in the formation of brain metastases.

The finding that metastatic sites have a heterogeneous characteristic suggests that the cells establishing metastases have the ability to survive, self-renew, differentiate and modify. These properties are often associated with stemness, similar to cancer stem cells (CSC). This process could mirror developmentally-regulated EMT signaling pathways, such as wingless-type MMTV integration site family (WNT), Notch and Hedgehog, which drive both normal and stem-cell renewal, as well as maintenance (30-32). Empirical evidence connecting EMT to the emergence of stem cells has also been reported. Differentiated mammary epithelial cells that have undergone EMT, either on TGFβ treatment or by forced suppression of E-cadherin expression, give rise to $CD44^+/CD24^-$ CSCs (33). Furthermore, disseminated breast cancer cells found in pleural effusions are enriched for a $CD44^+/CD24^-$ CSC-like population (34).

While the process of EMT and MET has been well-described in numerous types of metastatic cancer, its role in cerebral metastases has yet to be established. In this study, we identified the markers of EMT and MET as they are expressed in metastatic brain tumors. Furthermore, we studied the transcription factors associated with EMT/MET using gene-expression profiling in order to identify genes that are altered through these processes. Lastly, we established the relationship between the astrocytic environment and primary tumor cells in promoting and maintaining cerebral metastases. We observed that the process of EMT exists in metastatic brain lesions and that local environmental factors interact with the primary tumor cells to promote growth, proliferation and migration in the cerebral microenvironment.

Materials and Methods

Tumor samples and cell lines. Twenty-six samples of confirmed metastatic brain tumors were obtained from the Department of Pathology at Westchester Medical Center, Valhalla, NY, USA, according to Institutional Review Board guidelines and approval. Human breast cancer cell line MDA-MB-231 [American Tissue Culture Collection (ATCC), Manassas, VA, USA] was maintained in RPMI medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin/amphotericin. The human astrocytic (glioblastoma) cell lines LN18 and U87 (hereafter designated as Astro 1 and Astro 2, respectively) (ATCC) were used to attain astrocytic cells, and were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin. All cells were maintained at 37˚C with 5% CO_2 in a humidified incubator at 3-5 passages.

Immunohistochemistry and fluorescence immunohistochemistry. The standard immunohistochemical technique was utilized to determine the expression of zinc finger protein SNAI1 (SNAIL), Twist-related protein-1 (TWIST), and vimentin, as well as the stem cell marker CD44. Briefly, metastatic brain tumor samples were de-paraffinized, underwent antigen retrieval in citrate solution and were incubated with antibodies against TWIST, SNAIL (Abcam, Cambridge, MA, USA), vimentin and CD44 (Cell Signaling Technology, Inc., Danvers, MA, USA). After incubation overnight at 4˚C, antibody detection was performed with Super Picture Poly-HRP conjugate (Invitrogen, Camarillo, CA, USA) for 15 minutes, color developed with DAB substrate solution (Invitrogen, Camarillo, CA, USA), and washed in running tap water. Counterstaining with hematoxylin (Mayer hematoxylin S3309; DakoCytomation, Carpinteria, CA, USA) was used to maximize signal contrast. Secondary antibody alone served as negative control. Images of three randomly selected microscope fields for each sample were taken. Staining of grade three or greater was considered positive for a particular protein.

For immunofluorescence analysis, antigen retrieval was carried out as described above, then the tumor sections were incubated with blocking buffer for 2 h, treated with mouse primary antibody to E-cadherin (Abcam) and incubated overnight at 4˚C. The sections were then exposed to rhodamine anti-goat fluorochrome-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and incubated for 2 h in the dark. Sections were then re-blocked in buffer for 2 h and subjected to rabbit primary antibody to vimentin (Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4˚C. Sections were then exposed to fluorescein isothiocynate anti-rabbit fluorochrome-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Tumor sections were washed three times in phosphate-buffered saline (PBS). Mounting was accomplished using medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA), placement of a cover slip and storage of samples at −20˚C under light protection. Expression of E-cadherin and vimentin was visualized using the imaging Zeiss Axiovert 100M imaging system (Carl Zeiss, Thornwood, NY, USA).

Gene-expression profiling analysis. RNA extracted from brain metastases of primary adenocarcinoma of the lung (two patients: following Health Insurance Portability and Accountability Act regulation) was compared with non-metastatic lung tissue. Total RNA was extracted using the TRIzol reagent (Life Technologies, Inc.). Isolated RNA was electrophoresed through 1.0% agarose formaldehyde gels to verify the quality. The concentration and purity of RNA were determined from absorbance measurements at 260 and 280 nm. Total cellular RNA (7 μg) was annealed to oligo(dT) and reverse-transcribed in the presence of Cy3-labeled or of CY5-labeled dUTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA), using 10,000 units/ml of Superscript II reverse transcriptase (LifeTechnologies, Inc.). The resulting Cy3- and Cy5-labeled cDNAs were treated with RNase One (Promega Corporation, Madison, WI, USA) for 10 min at 37˚C, combined, and purified by passing through a Centricon-50 filtration spin column (Millipore, Bedford, MA, USA). Hybridizations were performed on cDNA microarray glass overnight at 50˚C in a humidified chamber. Each slide contained 17,000 unique human cDNA clones. Slides were scanned using a microarray scanner Scan Array Lite (Perkin Elmer, Waltham, MA, USA). The scanner output .tif images were gridded, and the fluorescence intensities of microarrays were calculated using the Iobion Genetraffic software (Iobion Informatics, La Jolla, CA, USA). Final intensities of green and red channels were filtered and the ratios of the red intensity to the green intensity were determined. The average expression level of genes, as presented in Table I, in the two groups was calculated, and the cutoff value was set to 2-fold up- or down-regulation after normalization of data with respect to that for non-malignant tissue.

Cell proliferation assays. Cell proliferation was assessed using the MTT Cell Growth Assay Kit (Chemicon International, CA, USA). Approximately 15,000 MDA-MB-231 breast tumor cells/well were seeded in a 96-well flat-bottomed microtiter culture plate (Falcon Labs, Colorado Springs, CO, USA) and incubated for 24 h at 37˚C. Cells were made quiescent by incubating in serum-free medium for 24 h and then exposed to astrocytic conditioned medium (Astro 2) for 24 h. At the termination of the experiment, the medium was removed and 1% 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) in cell medium was added to each well. Ten microliters of AB solution (50 mg MTT), was added to each well. Cells were incubated in a CO_2 incubator at 37˚C for 4 h in order for cleavage of MTT to occur. The reaction was terminated by removal of the MTT and addition of dimethyl sulfoxide. Absorbance (595 nm and 630 nm) was measured using a Multiscan FC Microplate Reader (Fisher Scientific, Waltham, MA, USA).

Cell-cycle entry analysis. Proliferating cells were visualized by utilizing the Click-iT EdU Imaging Kit (Invitrogen). MDA-MB-231 cells were exposed to astrocytic conditioned medium (Astro 1 or Astro 2) for 4 h and were subsequently incubated for 4 h in 10 μM 5-ethynyl-2-deoxyuridine (EdU). Cells were fixed in 4% paraformaldehyde for 15 min at room temperature and permeabilized for 15 min in 0.1% Triton X-100 in PBS. EdU incorporation was detected by incubation in the Click-iT reaction cocktail (as prescribed by the kit) at room temperature. The samples were then washed three times for 5 min in PBS. Frequency maps of the cell proliferation were constructed from fluorescence images using a Zeiss microscope (Axiovision; Carl Zeiss, Thornwood, NY, USA) and quantification analysis was performed using Image J (National Institutes of Health, Bethesda, MD, USA).

Migration assays. Cell migration was performed using chemotactic (Boyden chamber) and scratch wound-healing assays. Quiescent MDA-MB-231 cells in the upper compartment of a Boyden chamber were allowed to migrate through an 8-μm pore filter in response to chemotactant signaling from astrocytic medium (Astro 2) in the lower compartment. Starvation medium (without FBS) and complete medium (with FBS) were used as controls. After 24 h, cells attached to the upper side of the filter were wiped off and cells migrated through the filters were fixed, stained with hematoxylin and eosin, mounted onto a glass slide and visualized using an Axiovert Zeiss 200 microscope (Carl Zeiss).

For the scratch wound migration assay, a wound was introduced into a confluent monolayer of MDA-MB-231 breast cancer cells. The initial wounding and the movement of the cells in the scratched area were photographically monitored using an Axiovert Zeiss 200 microscope (Carl Zeiss) at ×10 magnification (NA 0.25) for 24 h. The migration rate was expressed as a percentage that of the control, and was calculated as the ratio of the mean distance between both borderlines caused by scratching to the distance that remained cell-free after migration. Two independent series of experiments were performed in quadruplicate.

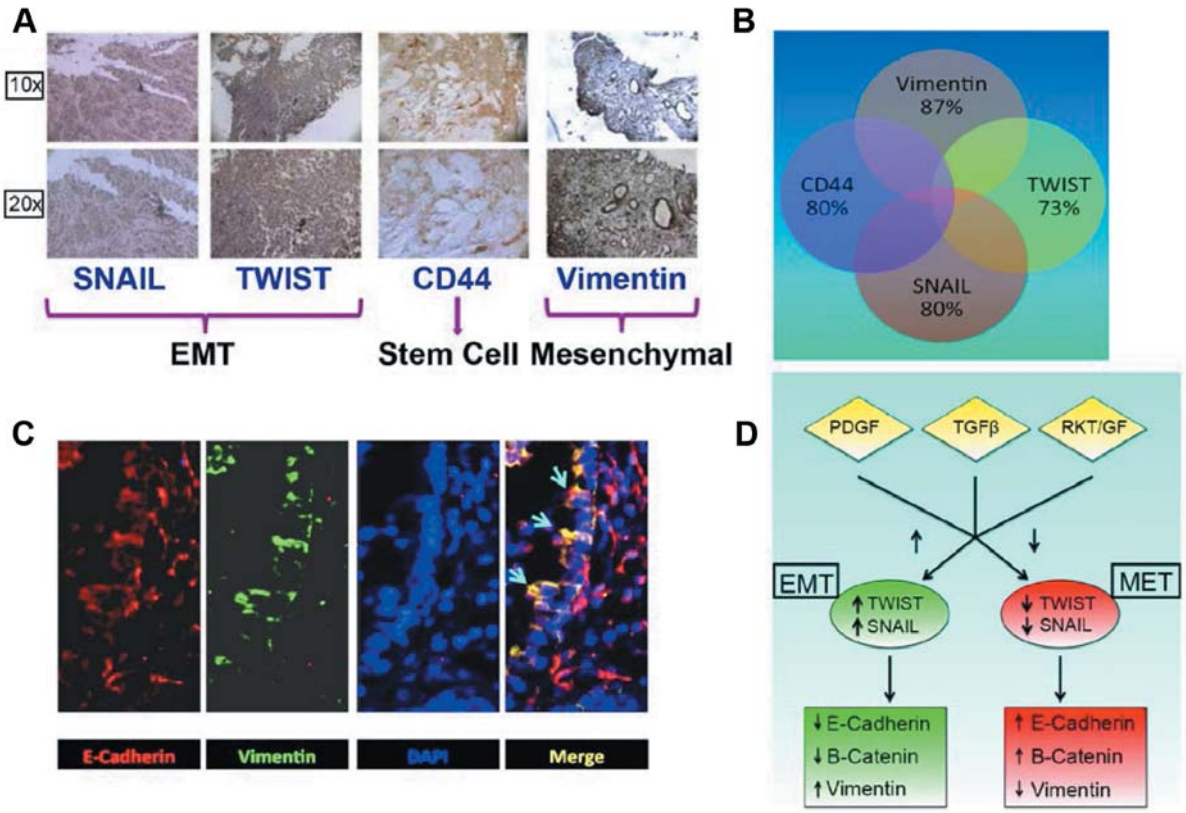

Figure 1. *Markers of epithelial-to-mesenchymal transition (EMT), mesenchymal-to-epithelial transition (MET) and stem cells in metastatic brain tumors. Immunohistochemical analysis demonstrating the expression of EMT, MET and stem cell markers. A: Expression of EMT markers zinc finger protein SNAI1 (SNAIL) and twist-related protein 1 (TWIST), mesenchymal marker vimentin and stem cell marker CD44 in metastatic tumor samples. Images are shown at ×10 and ×20 magnifications. B: Venn diagram displaying the percentage of SNAIL, TWIST, vimentin and CD44 expression in metastatic brain tumor samples (n=26), with varying degree of overlap amongst the markers within samples. C: Fluorescent immunohistochemical analysis of antibody-tagged E-cadherin (red) and vimentin (green) revealing areas of expression and colocalization in metastatic brain tumor samples (arrows). D: Cartoon diagram depicting the signaling pathway of platelet-derived growth factor (PDGF), tumor growth factor beta (TGFβ), receptor tyrosine kinase (RTK) and other growth factors (GF) promoting EMT and MET. Stem cells may lie at the interface of this transition.*

Co-culture. A total of 1000 MDA-MB-231 cells were plated onto 4-well slides, and after 24 h, 1000 Astro 2 cells were added. The cell–cell interaction between MDA-MB-231 and Astro 2 cell lines was monitored for 48 h using an Axiovert Zeiss microscope (Carl Zeiss) every 6 h.

Statistical analysis. Values are presented as the mean±SEM. One-way ANOVA was carried out for multiple comparisons and two-tailed *t*-tests were performed for single comparisons. A *p*-value of less than 0.05 was considered significant.

Results

Markers of EMT, MET and stem cells are expressed in metastatic brain tumors. We analyzed specimens of resected brain metastases from primary sites, including the lungs, breast, colon and renal system. These metastatic samples were immunostained for markers of EMT and MET, namely vimentin, SNAIL and TWIST, alongside stem cell marker CD44. A significant number of tumor specimens expressed markers of EMT: 80% SNAIL, and 73% TWIST (Figure 1A). Mesenchymal marker vimentin was also expressed in a high proportion of samples (87%) (Figure 1A). CD44 was expressed in about 80% of metastatic brain tumors. Among those samples with CD44-expression, coexpression was greatest with vimentin (60%) and SNAIL (73%) (Figure 1B). The expression of these markers was often heterogeneous (Figure 1B), implying that the process of cerebral metastasis consists of both EMT and MET.

Coexpression of vimentin and E-cadherin as markers for EMT and MET was confirmed by immunofluorescent analysis of metastatic brain tumors. Expression of E-cadherin was often seen to be colocalized with vimentin, suggesting the possible state of reversibility that exists in these tumors (Figure 1C).

Table I. *Categorization of genes differentially expressed in metastases of primary adenocarcinoma of the lung.*

Gene symbol	Gene ID	Chromosomal location	Gene description
Up-regulated genes			
IFIH1	NM_022168	2q24	Interferon induced with helicase C domain 1
MMP12	NM_002426	11q22.3	Matrix metallopeptidase 12 (macrophage elastase)
MESDC2	D42039	15q13	Mesoderm development candidate 2
NDUFS8	AK002110	11q13	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23 kDa (NADH-coenzyme Q reductase)
NMI	NM_004688	2q23	N-myc (and STAT) interactor
INO80B	NM_031288	2p13.1	INO80 complex subunit B
SAFB	NM_002967	19p13.3-p13.2	Scaffold attachment factor B
SFRP1	NM_003012	8p11.21	Secreted frizzled-related protein 1
BTF3	NM_001207	5q13.2	Basic transcription factor 3
BTF3P12	M90355	8q13.3	Basic transcription factor 3 pseudogene 12
TWIST1	NM_004374	12q24.2	Twist-related protein 1 basic helix-loop-helix transcription factor 1
UTF1	NM_003577	10q26	Undifferentiated embryonic cell transcription fator 1
TFAP2C	U85658	20q13.2	Transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma)
GATA1	NM_002049	Xp11.23	GATA binding protein 1 (globin transcription factor 1)
CREB3L3	NM_032607	19p13.3	cAMP responsive element binding protein 3-like 3
Down-regulated genes			
SOX7	NM_031439	8p22	Sex determining region Y-box 7
NFYC	NM_014223	1p32	Nuclear transcription factor Y, gamma
DCP1A	NM_018403	3p21.1	mRNA-decapping enzyme 1A (S. cerevisiae)
ATF5	NM_012068	19q13.3	Activating transcription factor 5
USF1	NM_007122	19q13.3	Upstream transcription factor 1
ATF6	NM_007348	1q22-q23	Activating transcription factor 6
DMTF1	NM_021145	7q21	Cyclin D-binding myb-like transcription factor 1

Gene-expression profiling suggests alterations in genes associated with processes leading to EMT and MET. Gene-expression profiling of metastatic adenocarcinoma of the lung was performed against that of non-malignant lung microRNA, utilizing cDNA microarrays. Varied expression of several transcription factor genes associated with neurogenesis, differentiation and reprogramming was evident in metastatic tumor samples (Table I). Genes involved in neurogenesis, growth and differentiation, including matrix metallopeptidase-12 (*MMP12*), n-myc interactor (*NMI*), secreted frizzled-related protein 1 (*SFRP1*), undifferentiated embryonic cell transcription factor 1 (*UTF1*), transcription factor AP-2 gamma (*TFAP2C*) and GATA binding protein 1 (*GATA1*) were up-regulated. Furthermore, *TWIST1* and *GATA1*, with well-established roles in EMT, cancer initiation, progression and metastasis, were also shown to be up-regulated. Among the down-regulated genes were activating transcription factor 5 (*ATF5*), thought to have a role in neurogenesis and differentiation, and SRY box 7 (*SOX7*), involved in stem cell proliferation and dissemination. The alterations in these genes suggest that primary cells undergo processing that allows for metastasis and assimilation into the brain.

An astrocytic environment enhances cell proliferation, S-phase entry and migration of primary tumor MDA-MB-231 cells. In order to examine the contribution of an astrocytic milieu to cerebral metastasis, we assessed proliferation, cell-cycle entry and migration of MDA-MB 231 cells following exposure to astrocytic media.

MTT analysis was performed to assess the ability of metastatic tumor cells to grow in an astrocytic environment (Figure 2A). The MDA-MB 231 mesenchymal breast cancer cells proliferated abundantly in the astrocytic medium compared to control medium ($p<0.05$).

Using the Click-It EdU technique, cell-cycle S-phase-entry analysis was performed. Quantification of cells undergoing S-phase entry was accomplished by dividing the number of EdU-stained cells, representing cells that had entered S-phase, by the number of DAPI-stained cells. A significantly greater percentage of tumor cells entered S-phase when exposed to astrocytic media (Astro 1 or Astro 2), relative to control medium ($p<0.05$) (Figure 2B).

Tumor-cell migration towards an astrocytic environment was assessed using both scratch wound migration and chemotactic analysis. Quiescent cells were allowed to migrate across a scratch wound in control and two astrocytic

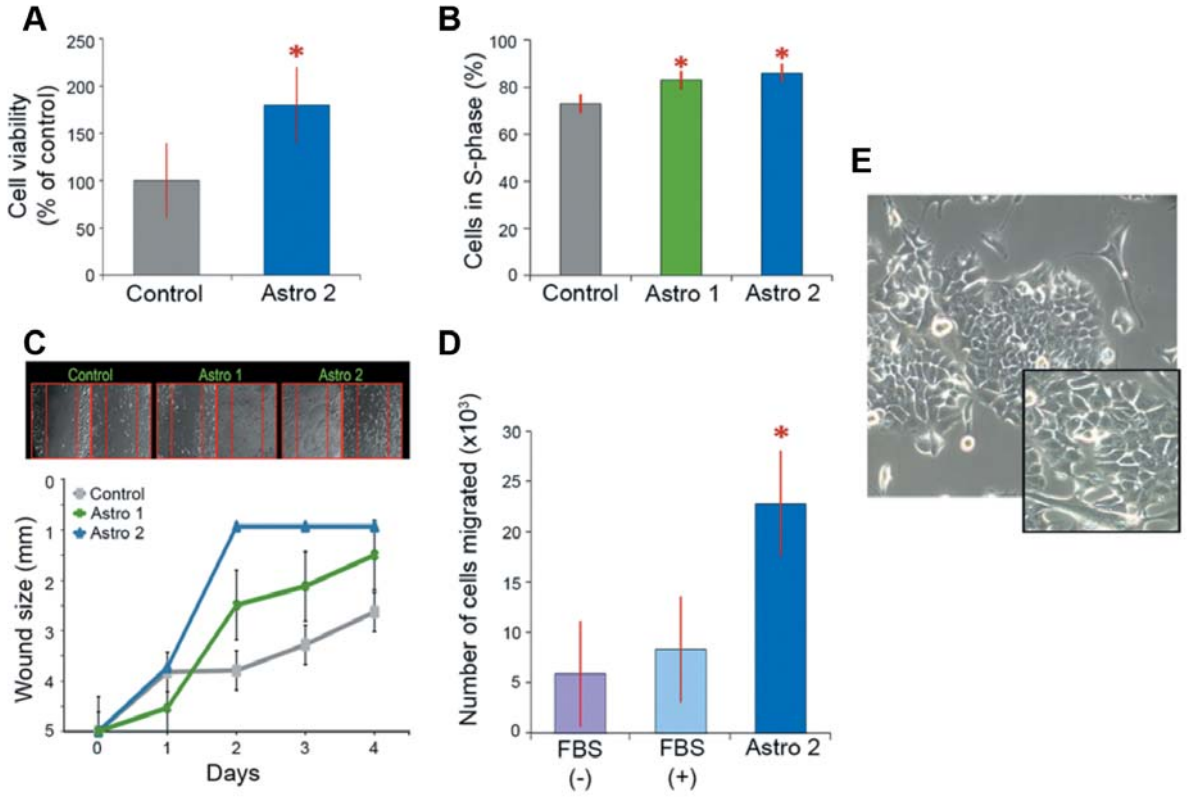

Figure 2. *Influence of the astrocytic milieu on primary tumor cell proliferation, S-phase entry and migration. A: Cell proliferation was significantly enhanced in the presence of astrocytic medium (Astro 1) (*p<0.05) compared to control medium (set to a value of 1). B: Cell-cycle entry analysis revealing significantly increased primary tumor cell entry into S-phase, as quantified by 5-ethynyl-2'-deoxyuridine positivity, when exposed to an astrocytic environment (Astro 1 and 2) (*p<0.05). C: Scratch wound-migration analysis demonstrating enhanced migration of primary tumor cells in astrocytic media (Astro 1 and 2), relative to control medium (*p<0.05). D: Chemotactic migration of cells which were allowed to migrate towards astrocytic medium revealed extensively increased migration, as compared to cells exposed to starvation or FBS- enriched media (*p<0.05). E: Phase-contrast image displaying coculture of astrocytic cells and metastatic breast cancer cells growing amicably after plating for 48 h.*

media and the migration period was recorded daily for four days. We observed that a significantly greater proportion of primary tumor cells (MD-MB-231) migrated when exposed to astrocytic media compared to those exposed to control medium ($p<0.05$) (Figure 2C). Furthermore, we assessed the affinity of tumor cells towards astrocytic medium by chemotactic migration using a Boyden Chamber. The number of migrating tumor cells was dramatically greater towards astrocytic medium (Astro 2), relative to starvation or control media ($p<0.05$) (Figure 2D).

Co-culture of breast cancer cell lines was carried out using an astrocytic cell line (Astro 2) to determine the relationship between these two cell types. Primary cells (MD-MB-231) were plated beside astrocytic cells and the pattern of growth was observed for 48 h. No signs of cell death were detected; rather both cell types were discovered to grow amicably in this environment (Figure 2E).

Discussion

The results of this study demonstrated that the markers of EMT (TWIST and SNAIL) and the mesenchymal marker vimentin were overexpressed in metastatic brain tumors. Expression of the stem cell marker CD44 was also observed. In addition to the EMT markers, transcription factor genes implicated in neurogenesis, differentiation and reprogramming were also differentially expressed. Interaction of primary tumor cells with the astrocytic milieu revealed that these cells had higher proliferation and migration with a strong affinity for astrocytic cerebral components.

While the process of EMT is complex, the molecular mechanisms that control the steps are regulated by multigenic phenomena as well as alterations in metastatic suppressor genes, growth factors and their receptors (17, 18, 20). EMT requires convergence of signaling from various

pathways including TGFβ, hepatocyte growth factor, epidermal growth factor, insulin-like growth factor, fibroblast growth factor, and members of the Notch signaling family (13). In order to complete metastasis, the processes of EMT and MET must be carefully coordinated. In our study, we analyzed various metastatic brain tumors with diverse primary tumor origins; a majority of these samples were derived from the lung (53%) and breast (27%), with the remaining samples from the renal system, colon and gallbladder. Expression of EMT markers TWIST and SNAIL were represented in a majority of these tumors (73% and 80%, respectively). Furthermore, the mesenchymal marker vimentin was recorded in 87% of samples. Interestingly, we found a significant overlap in expression of these EMT and MET markers across tumor samples (Figure 1A and B). Moreover, certain regions of tumor sections were found to coexpress markers of EMT and MET as evidenced by visualization of fluorescence-derived probes for E-cadherin and vimentin (Figure 1B and C). These observations suggest that a conversion may exist between these processes upon reaching the secondary site. These findings also help us to understand the mechanism of EMT and MET, that may be uniform to the metastatic process from all unique primary sites (Figure 1D). Despite this, however, the established metastatic lesion may or may not be distinguishable depending on the site of origin.

As previously described in other metastatic cancer types, high expression of $CD44^+$ in our metastatic brain tumor samples represents the role of stem cells in this process as well as the process of adhesion, homing and migration (Figure 1A and B) (35). Of note, in a model of lung cancer, higher levels of $CD44^+$ expression were observed in specific differentiation phenotypes, suggesting its role in progression of disease, as well as its potential utility as a prognostic indicator (36). In addition, recent studies have demonstrated that increased expression of the $CD44^+$ marker correlates with development of cerebral metastases in breast cancer, and that up-regulation of Notch pathway activity may contribute to this process (37). The notion that stem cells emerge through a process of EMT is supported by the fact that differentiated mammary epithelial cells which have undergone EMT, either by TGFβ treatment or forced suppression of E-cadherin, give rise to $CD44^+/CD24^-$ CSCs (33). In fact, the process of EMT may be responsible for triggering differentiated cells to acquire a multipotent stem cell-like phenotype. This process could mirror developmentally regulated EMT signaling pathways, such as WNT, Notch and Hedgehog, which drive both normal and stem cell renewal as well as maintenance (30-32). Such metastatic cells displaying $CD44^+$ expression, possessing stem cell-like properties are termed CSCs and are considered a major contributor to the relative resistance of metastatic lesions to standard chemotherapeutics. For instance, the $CD44^+$ non-small cell lung cancer cell sub-population, expressing markers of pluripotency (OCT4, NANOG and SOX2), displays resistance to chemotherapy with capability for *in vivo* serial transplantability (38).

The local milieu contributes enormously to the process allowing primary cells to metastasize to a distant organ. The activation of EMT and MET appears to occur as a result of signaling interactions between stromal and tumor cells. Studies have shown that these complex processes can be achieved by inducing certain discrete transcription factors and signals from within the adjacent stroma (33, 39). *In vitro* studies have shown that astrocytes play an important role in the development of cerebral metastases, where they promote cell growth and survival of human lung adenocarcinoma as shown in co-culture assays (40). Furthermore, cells isolated from breast cancer-derived brain metastases display an increased adhesion to astrocytes and enhanced growth in the presence of media from stimulated astrocytes, in comparison to parental cells (41). Consistent with these observations, we found vigorous proliferation and higher S-phase entry of primary breast cancer cells exposed to an astrocytic environment (Figure 2A and B). Furthermore, scratch wound migration and chemotactic migration assays revealed an increased migratory potential of breast cancer cells exposed to the astrocytic media (Figure 2C and D). Taken together, our results, along with the findings of others, establish that factors present in the cerebral environment provide support for growth and proliferation of primary tumor cells to metastasize to the brain.

Attempts have been made in recent years to further decipher the genes associated with cerebral metastasis. Through gene profiling and protein expression analysis, we found TWIST1 to be highly up-regulated (Figure 1A and B; Table I). EMT regulator TWIST1 has long been known to have a pivotal role in embryogenesis through its interaction with multiple proteins involved in this process (42). Since TWIST has a well-defined role in EMT and stem cell regulation, its expression in our samples strengthens the notion that metastatic brain tumors undergo such processes. Moreover, a correlation has been shown between expression of TWIST, CXC chemokine receptor type 4 (CXCR4) and CC chemokine receptor type 7 (CCR7), markers of metastasis, suggesting that TWIST may actually have multiple roles in tumor dissemination (42). Increased expression of GATA1, as identified by gene profiling analysis, provides additional support for the role of EMT in metastatic dissemination to the brain (Table I). Transcription factor GATA1 serves a critical function in hematopoiesis with roles in the differentiation, proliferation and apoptosis of erythroid cells (43). Recent findings suggest that GATA1 acts as an E-cadherin repressor *via* recruitment of histone deacetylase 3/4 (HDAC3/4) to the E-cadherin promoter (43). Of the genes up-regulated in our metastatic brain tumor samples, *MMP12* has been shown to take part in

oligodendrocyte maturation and development of oligodendrocyte processes. MMP12 also has a role in lung inflammation and increased expression is associated with disease progression (44). In addition, *NMI* expression was up-regulated in metastatic tumor samples (Table I). Recent studies have shown that *NMI* expression is up-regulated in response to various cytokines, and interacts with several proteins involved in oncogenesis and tumor progression including MYC, signal transducer and activator of transcription (STAT), breast cancer gene 1 (BRCA1), SOX10 and TAT interactive protein 60 (TIP60) (45). More importantly, interaction between NMI and the TGFβ signaling pathway helps solidify its role in EMT (46). Interestingly, NMI has also been implicated in gliomagenesis, where it interacts with STAT3 protein to stimulate tumor growth (47). Additional genes found to be up-regulated in gene-expression profiling analysis included *SFRP1*, *TFAP2C*, and *UTF1*, shown to be associated with differentiation of pluripotent stem cells (48-50), as well as scaffold attachment factor B (*SAFB*) and basic transcription factor 3 (*BTF3*), which have roles in transcriptional control, cell-cycle regulation and apoptosis (51).

Among the down-regulated genes, *SOX7* belongs to a family of SRY-related HMG box transcription factors with established regulatory roles in hematopoiesis, vasculogenesis and cardiogenesis (52). In particular, SOX7 activates GATA4 and GATA6 in stimulation of extraembryonic endodermal differentiation (53). Differential expression of SOX7 has a fundamental position in the development of hematogeneous malignancies; however, in recent years it has been made clear that SOX7 also plays an important role in solid tumors. Consistent with recent findings, our studies revealed a significant decrease in *SOX7* gene expression (Table I). SOX7 might be considered a negative regulator of the WNT/β-catenin pathway and in this capacity it serves as a tumor suppressor (54). Moreover, SOX7 levels may also be regulated by vascular endothelial growth factor (VEGF), as it has been shown that VEGF down-regulates SOX7, thereby reducing the expression of vascular endothelial-cadherin (VE-cadherin) and providing an avenue for the metastatic process by loosening endothelial barriers (52). ATF5 was also found to be down-regulated in metastatic tumor samples. ATF5 has been shown to have a critical role in development, differentiation, cellular proliferation and inhibition of apoptosis, through interactions with cAMP-response elements (55). ATF5 is often found highly expressed in a variety of cancer types, including glioblastoma, with evidence of a possible inverse correlation to prognosis (55). While increased levels of ATF5 have been correlated with disease progression, it is the down-regulation of ATF5 by various neurotrophic factors that allows neuroprogenitor cells to exit the cell cycle and progress toward differentiation (56). This role is particularly pronounced in cells destined to become astrocytes and oligodendrocytes (55). Our observation of down-regulation of ATF5 expression suggests that alterations in the local environment may also play a critical role in supporting the primary tumor cells within the brain. Several additional genes were also shown to be down-regulated in metastatic tumor samples, which are involved in chromatin remodeling, cell-cycle regulation, inflammation and apoptosis (Table I).

In conclusion, the results of this study demonstrate that EMT and MET are involved in the process of metastatic dissemination to the brain. More importantly, we provided evidence that metastatic cells exist in a state of perpetual EMT/MET stemness when establishing brain metastases. Gene-expression profiling analysis provided additional evidence of differentially expressed genes involved in the regulation and maintenance of EMT, as well as differentiation of stem cells. We believe that the identification and characterization of factors involved in maintaining this equilibrium may lead to the elucidation of useful prognostic indicators suggesting the impending development of metastatic brain disease, and provide a means for discovery of novel targets for therapeutic intervention. Lastly, targeting key regulators of this pathway may play a crucial role in altering the progression of metastasis as inhibition of this process has been demonstrated to disrupt cell proliferation and migration in *in vitro* studies (57).

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