Background  
Brain metastases are most common in adults with lung cancer, predicting uniformly poor patient outcome, with a median survival of only months. Despite their frequency and severity, very little is known about tumorigenesis in brain metastases.

Methods  
We applied previously developed primary solid tumor-initiating cell models to the study of brain metastases from the lung to evaluate the presence of a cancer stem cell population. Patient-derived brain metastases (n = 20) and the NCI-H1915 cell line were cultured as stem-enriching tumorspheres. We used in vitro limiting-dilution and sphere-forming assays, as well as intracranial human–mouse xenograft models. To determine genes over-expressed in brain metastasis tumorspheres, we performed comparative transcriptome analysis. All statistical analyses were two-sided.

Results  
Patient-derived brain metastasis tumorspheres had a mean sphere-forming capacity of 33 spheres/2000 cells (SD = 33.40) and median stem-cell frequency of 1/60 (range = 0–1/141), comparable to that of primary brain tumorspheres (P = .53 and P = .20, respectively). Brain metastases also expressed CD15 and CD133, markers suggestive of a stemlike population. Through intracranial xenotransplantation, brain metastasis tumorspheres were found to recapitulate the original patient tumor heterogeneity. We also identified several genes overexpressed in brain metastasis tumorspheres as statistically significant predictors of poor survival in primary lung cancer.

Conclusions  
For the first time, we demonstrate the presence of a stemlike population in brain metastases from the lung. We also show that NCI-H1915 tumorspheres could be useful in studying self-renewal and tumor initiation in brain metastases. Our candidate genes may be essential to metastatic stem cell populations, where pathway interference may be able to transform a uniformly fatal disease into a more localized and treatable one.

A Cancer Stem Cell Model for Studying Brain Metastases From Primary Lung Cancer


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Manuscript received May 30, 2012; revised October 1, 2012; accepted January 4, 2013.

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Brain metastases are common in adults suffering from a variety of primary cancers, including those of the lung, breast, and colon (1). Up to 50% of lung cancer patients develop brain metastases, the majority within a few years of diagnosis (2–5). Due to their subcortical location, lung-derived brain metastases are less easily surgically excised than the dural-based metastases, typical of breast cancer (6). Their invasive nature and ability to escape current treatments predicts uniformly poor patient outcome, with a median survival time of only months (7, 8). Despite the frequency and severity, very little research exists on brain metastases from lung cancer, nor is there an appropriate experimental model with which to study them. The idea of a subpopulation of cells initiating tumor growth is quite similar to the metastatic process (26). Although many cells may be shed from the primary tumor, only a small number of cells are able to survive in the circulation and seed a secondary site (27, 28). An even...
smaller number are able to propagate in a new niche into a clinically apparent tumor (27, 28). In fact, many studies have reported increased adhesion and invasiveness in CSCs of various primary tumor models, implicating the involvement of CSCs in the development of metastases (29–33). These observations, the metastatic process, and existence of CSCs in lung cancer led us to hypothesize that brain metastases from the lung may also arise from a TIC population.

A stem cell population can be identified through assessment of self-renewal in vitro, and through serial passage and tumor recapitulation in vivo (34). In the current study, patient tumor samples and cell lines were cultured as tumorspheres in neural stem cell (NSC) conditions to enrich for stemlike populations. Using sphere-forming assays to assess self-renewal, we show that brain metastases from the lung possess sphere-forming capacities similar to those of primary brain tumors. Intracranial injection of minimally cultured, patient-derived, and NCI-H1915 tumorspheres into nonobese diabetic severe combined immunodeficient (NOD-SCID) mice demonstrated similar multifocal tumor formation, and recapitulated tumor cytoarchitecture and heterogeneity, indicating that NCI-H1915 cells can be used as a surrogate for patient samples in vivo. We also used serial intracranial injection of NCI-H1915 tumorspheres into NOD-SCID mice to show that the putative TIC population can be serially passaged.

We performed RNA-sequencing (RNA-Seq) and subsequent comparative analysis of brain metastasis tumorspheres, primary lung and brain tumors, to identify genes overexpressed in stem cell–enriched brain metastases. These candidate genes predicted decreased survival in primary lung cancer patients, and are known to be involved in cell adhesion, cytoskeleton rearrangements, proliferation/tumorigenesis, and formation/disruption of cell–cell junctions. Further characterization of these genes in pathways essential for the metastasis of lung cancer to the brain may prove to be useful and effective as therapeutic targets.

Methods
Patient Sample Processing and Cell Culture
Consecutive brain tumor samples (1–2 cm³) were collected from consenting patients undergoing surgical tumor resection (Hamilton Health Sciences Research Ethics Board, #07-366). Specimens pathologically diagnosed as lung-derived brain metastases from patients with a history of lung cancer were used in the study (n = 20), alongside primary brain tumor controls (n = 3). Nonviable samples were excluded from the study, as defined by failure to propagate as tumorspheres, ≥80% cell death, and pathological diagnosis of high necrotic tissue content (approximately 20% of all samples collected). Upon collection, samples were mechanically dissociated and subjected to enzymatic digestion with 200 ìL Liberase Blendzyme (0.2 Wunisch units/mL, Roche) for 15 minutes at 37°C on an incubator rocker (VWR). Undigested tissue was removed, and red blood cells were lysed (RBC Lysis Buffer, Stem Cell Technologies). Cells were washed with phosphate-buffered saline (Hyclone), subsequently resuspended in complete NSC (cNSC) media, and plated in an ultra-low attachment plate (Corning). Complete NSC media is comprised of NSC basal media (1% N2 supplement [Gibco], 0.2% 60 µg/mL N-acetylcystine, 2% neural survival factor-1 [Lonza], 1% HEPES, and 6 mg/mL glucose in 1:1 Dulbecco's Modified Eagle Medium and F12 media [Gibco], supplemented with 1x antibiotic–antimycotic (Wisent), 20 ng/mL human epidermal growth factor (Sigma), 20 ng/mL basic fibroblast growth factor (Invitrogen), and 10 ng/mL leukemia inhibitory factor (Chemicon). We also used two cell lines cultured in cNSC media: NCI-H1915 (American Type Culture Collection) cells were isolated from a brain metastasis of a 61-year-old woman with a large cell, poorly differentiated lung carcinoma; BT241 is a lab-derived glioblastoma (GBM) cell line from a 68-year-old woman. Cultures were maintained at 37°C, 5% CO₂, and media was changed every other day, or as needed.

Sphere Formation Assay and Limiting Dilution Analysis
Tumorspheres were dissociated using 5–10 ìL Liberase Blendzyme in 1 mL of phosphate-buffered saline for 5 minutes at 37°C. Cells were plated at limiting dilution (200 to 2 cells per well) in 100 μL of cNSC media in quadruplicate in a 96-well plate. After seven days, the number of spheres per well was counted for each dilution, and was used to estimate the mean number of spheres per 2000 cells. For patient samples, this assay estimated secondary sphere formation, whereas cell lines were passage three or higher. The fraction of negative wells vs cell dilution was graphed and fitted with a linear regression to estimate stem cell frequency, as in Tropepe et al (35). Following the assumption that a single stem cell gives rise to one sphere (36), the proportion of negative wells can be defined by the zero point (F₀) of the Poisson distribution: F₀ = e⁻x, where x is the mean number of cells per well. The dilution at which it is expected to have one stem cell (one sphere) per well can be identified by the point at which the line-of-best-fit crosses 0.37 (when x = 1, F₀ = e⁻¹ = 0.37) (35).

Flow Cytometry and Cell Sorting
The Aldefluor assay ( Stem Cell Technologies) was used as a nonimmunological method to analyze the ALDH activity of samples, where high enzyme activity is an indication of stem cell populations. Cells were also stained and assessed for surface expression of CD15 and CD133 by flow cytometry (Supplementary Materials, available online). Cells were sorted (purify settings) into tubes of NSC basal media and allowed to equilibrate at 37°C for 1–2 hours after sorting, prior to their use in experiments. Aliquots of sorted populations were taken for purity assessment immediately after sorting. For clonal analysis of single cells, CD15⁻ or CD15⁺ cells were sorted one cell per well of a 96-well plate into 100 μL of 25% NCI-H1915 conditioned cNSC media. Cultures were left undisturbed for 7 days after sorting, and received an additional 50 μL of cNSC media after 7 days.

Statistical Analyses
Gene Signature Survival Analyses. Genes found to be overexpressed in brain metastases (Supplementary Materials, available online) were ranked according to their ability to predict patient survival in a dataset of 226 primary lung tumor samples (GSE31210) (37) using a median cut-point to calculate hazard ratios and perform log-rank tests. In brief, raw Affymetrix.CEL files (Affymetrix Human Genome U133A Array) and associated clinical data were downloaded from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; GSE31210), and normalized using robust multiarray analysis (38). Probe expression levels were transformed into...
standard normal deviates; patient gene scores were calculated as the mean of the standardized scores from the probe sets undergoing analysis (39–40). Genes producing a log-rank test of \( P \) less than or equal to .05 (two-sided) were considered to be statistically significantly associated with length of survival. We used Cox proportional hazards regression to complete forward selected stepwise regression to identify an optimal model for predicting patient outcome. Kaplan–Meier survival analysis was performed, where the patient cohort was divided into tertiles to discriminate patients into high, medium, or low expression groups.

Other Experiments. Statistical analyses were performed using GraphPad Prism 5. Two-sided \( t \) tests were used, and data are presented as mean (SD), unless otherwise stated. Survival curve analysis for in vivo experiments was performed using the log-rank (Mantel–Cox) test. Statistical significance was set at \( P \) less than .05 (two-sided).

Results

Patient-Derived Brain Metastases and NCI-H1915 Cells Form Stem-Enriched Tumorspheres and Exhibit Self-Renewal Capacity

It has previously been shown that several cancers, such as breast (17–19), colon (16, 18, 20), lung (15–22), and brain (10–14) tumors possess TIC subpopulations. The presence of these TIC populations has a strong correlation with the development of metastases (29–33). On the basis of these observations, and the fact that brain metastases derive from a limited number of primary tumor cells (27, 28), we wondered whether brain metastases also possessed a self-renewing stemlike population.

Patient samples of primary brain tumors and lung-derived brain metastases were cultured as tumorspheres in cNSC media (Supplementary Table 1, available online). We observed similar secondary sphere-forming capacities between primary brain tumors (19 spheres/2000 cells [SD = 12.55]) and brain metastases (33 spheres/2000 cells [SD = 33.40]; \( P = .53 \); Figure 1A), where secondary sphere formation is a hallmark of the stem cell property of self-renewal (34). As the brain metastases derived from lung adenocarcinomas, individual cells are much larger and glandular in appearance, resulting in slightly less homogenously spherical and compact tumorspheres, compared to those of primary brain tumors (Figure 1A, lower panels). As a sphere is thought to represent all progeny from a single stem cell, sphere formation reflects the frequency of a stem cell population (35); thus, the presence of tumorspheres is indicative of a culture enriched for stem cells. Stem cell frequency was estimated through limiting-dilution analysis. Median frequencies for patient samples were 1/117 cells (range 1/90–1/160 cells) for primary brain tumors, and 1/60 cells (range 0–1/41 cells) for brain metastases, and were not statistically significantly different (\( P = .20 \); Figure 1C).

Due to limitations surrounding the use of patient samples (eg, low cell number, low viability, unpredictable acquisition), we supplemented our work with representative cell lines. The NCI-H1915 cell line is derived from a brain metastasis originating from a lung tumor, whereas BT241 is a lab-derived GBM cell line. NCI-H1915 cells, originally grown adherently in RPMI (+10% fetal bovine serum) conditions, were characterized in both RPMI and in cNSC conditions (Supplementary Figure 1, available online). Like their patient-derived counterparts, when in cNSC conditions, both cell lines formed spheres—BT241, 57 spheres/2000 cells (SD = 42.30); NCI-H1915, 217 spheres/2000 cells (SD = 143.50)—and were not statistically significantly different (\( P = .14 \); Figure 1B); however, their sphere-forming capacity appears to be much higher than that of minimally cultured patient samples. The increased ability to form secondary or higher spheres is likely due to prolonged culture, which can increase stem cell frequency (41, 42), as evidenced by frequencies of 1/78 cells and 1/38 cells for BT241 and NCI-H1915 cultures, respectively (Figure 1D). Despite this increase in sphere-forming capacity, NCI-H1915 and patient-derived brain metastasis tumorspheres were of similar morphology and size (99.07 \( \mu m \) [SD = 16.80] and 163.2 \( \mu m \) [SD = 54.42], respectively; Figure 1E). Together, these data suggest that despite a potential enhanced stem cell compartment in the cell line, NCI-H1915 cells may be appropriate for studying self-renewal in brain metastases, as validated by patient samples.

Known TIC Markers Are Variably Expressed by Patient-Derived and NCI-H1915 Tumorspheres

Primary brain tumor CSC (in vitro) and BTIC (in vivo) populations were previously identified using CD133, CD15, and Aldefluor, where positive populations possess increased self-renewal, hierarchical differentiation, and tumor-initiating capacities (10–14). Both CD133 (15, 16, 18) and Aldefluor (20, 21, 29, 43, 44) have been used to identify TICs in various epithelial cancers, including lung cancer, suggesting that these may be universal markers of stemlike populations in solid tumors. We wondered if these TIC markers were expressed by brain metastases, correlating with the presence of a stemlike population.

We analysed patient-derived tumorspheres for their expression of known brain and lung TIC markers using flow cytometry. All markers selected for subpopulations within the samples, but to varying degrees. CD133+ and Aldefluor+ cells were relatively small populations (9.7% [SD = 11.8] and 10.8% [SD = 15.8], respectively); conversely, CD15 selected for a larger population of cells (39.2% [SD = 33.3]), but with much more variability (Figure 2, A and C).

When looking at the same markers in NCI-H1915 tumorspheres, we found that very few cells were positive for CD133 (0.05% [SD = 0.08]); whereas Aldefluor (36.6% [SD = 20.2]) and CD15 (14.8% [SD = 8.1]) demonstrated much more positivity (Figure 2, B and D). Only CD15 selected for a distinct population (Figure 2D, left panel), in contrast to the absence of CD133+ cells, or whole population shift seen when using Aldefluor (Figure 2D, right panel). CD15 expression was comparable in both NCI-H1915 and patient-derived tumorspheres (Figure 2, A and B); any discrepancies can be attributed to the variability among individual patient tumors. Interestingly, unlike CD133 (15, 16, 18) and Aldefluor (20, 21, 29, 43, 44), CD15 has yet to be shown to select for epithelial TICs, and may be suggestive of a population within the primary tumor primed for growth in the neural microenvironment, as CD15 has previously identified NSC (45) and BTIC (10, 13, 14) populations. Sphere-formation assessment of flow-sorted CD15+ and CD15− populations from NCI-H1915 tumorspheres indicated no functional difference between the two populations (Supplementary Figure 2, A–E, available online). Flow sorting of patient samples precluded any subsequent experiments.
Figure 1. Patient-derived samples and cell lines of primary brain tumors (BTs) and brain metastases (BMs) have similar sphere-forming capacities. Tumorsphere-derived cells were plated at limiting dilution to estimate secondary sphere formation capacity per 2000 cells, averaged over dilutions of 1–200 cells. Corresponding bright-field images of representative spheres are shown below each graph; scale bar is 100 µm.

A) Sphere formation of patient-derived primary brain tumors (glioblastoma (GBM); n = 3: BT41, BT47, BT82) and patient-derived brain metastasis samples (from lung; n = 4: BT199, BT219, BT222, BT256), was not statistically significantly different (P = .53). Each dot is representative of one patient sample; bar represents mean of biological replicates.

B) Sphere formation of cell lines BT241 (GBM cell line) and NCI-H1915 (lung-to-brain metastasis cell line) was not statistically significantly different (P = .14). Results of three independent experiments are shown.

C) and D) Limiting dilution analysis with the zero point of the Poisson distribution F0 = e−x was used to determine the dilution at which a single stem cell (x = 1, F0 = 0.37) would give rise to a single tumorsphere, thus estimating stem cell frequency.

C) Analysis of patient-derived primary brain tumors (left panel) and brain metastases (right panel).

D) Analysis of cell lines. A single data point represents the mean sphere size for a single patient sample; bar represents mean of biological replicates (n = 3: BT390, BT391, BT397). Right panel: results of three independent experiments are shown (n = 3).
Figure 2. Brain tumor-initiating cell (BTIC) markers CD133 and aldehyde dehydrogenase (ALDH) are differentially expressed in patient-derived brain metastasis and NCI-H1915 tumorspheres. Tumorsphere cells were stained with CD133-APC, CD15-PE, and/or Aldefluor, and assessed by flow cytometry. Results are presented as mean ± SD. A) Patient-derived brain metastases (from lung) cultured in complete neural stem cell (cNSC) media. Each dot represents the percentage positive for a single patient sample; bar represents the mean of biological replicates: CD133, n = 5 (BT219, BT222, BT291, BT296, BT381); CD15, n = 7 (BT219, BT222, BT291, BT296, BT381, BT382, BT390); Aldefluor, n = 5 (BT296, BT298, BT382, BT390, BT397). B) The NCI-H1915 brain metastasis (from lung) cell line was cultured in cNSC media. The results of three independent experiments are shown as mean ± SD. C-D) Representative flow plots of patient samples (left to right) BT219 and BT381 (CD15 and CD133), and BT296 and BT390 (Aldefluor), (C) and the NCI-H1915 cell line (D) are shown. Plots from left to right are CD15-PE/CD133-APC, and side-scatter/Aldefluor, respective isotype, and DEAB (diethylaminobenzaldehyde) controls are shown below the corresponding flow plot.
Patient-Derived and NCI-H1915 Tumorspheres Form Multifocal Tumors in Mice After Intracranial Injection, Recapitulating Patient Brain Metastases

The gold standard for identification of a TIC population is serial tumor formation in immunocompromised mice (34). As such, we assessed tumor formation of patient-derived and NCI-H1915 tumorspheres with intracranial injections into NOD-SCID mice (n = 16 and n = 4, respectively). The cell line and patient samples behaved similarly in vivo, forming multifocal masses seeded throughout the ventricles and cerebellum (Figure 3A), despite being injected into the right frontal lobe. This is consistent with presentation of brain metastases in patients with metastatic lung cancer, in whom brain metastases are often multifocal, border the ventricles, and often develop in the cerebellum. The majority of injected patient samples formed tumors (Table 1), where xenografts recapitulated the patient tumor histology and cytokeratocuture (Figure 3B).

To further assess the degree to which the xenograft model recapitulates the original patient tumor, a subset of xenografts (n = 3) were stained with the same marker profile used to clinically diagnose patient brain metastases. Astonishingly, the staining profiles and patterns in the xenografts were identical to that of the original patient brain metastasis (Figure 3C, Table 1). This indicates that not only does injection of brain metastasis tumorspheres lead to the recapitulation of the patient tumor, but it also demonstrates that tumorsphere cells possess the TIC capacity to differentiate into the various tumor cell types in vivo.

NCI-H1915 Tumorsphere-Derived Tumors Are Serially Transplantable in NOD-SCID Mice

We next performed serial intracranial injections using NCI-H1915 tumorspheres to assess their ability to be serially passaged in vivo. Two parental (P1) tumors were processed and cultured to select for human stemlike tumor cells, as confirmed by flow cytometry analysis of EpCAM expression (data not shown). Three mice each were injected with 100,000 P1 tumor cells (n = 6 total). One of each P1 derivative from the subsequent (F1) tumors was similarly cultured and injected to give rise to F2 tumors (n = 6 total). F1 and F2 tumors resembled the histology and cytokeratoculture of the P1 tumors (Figure 4A and B). Percentage of survival statistically significantly decreased with passaging (P = .02; Figure 4C), reflecting increased tumor growth rates. This may be a result of TIC outgrowth, or an artefact of in vivo selection for cells more adaptable to survival in a mouse neural microenvironment.

To address the issue of in vivo selection, we assessed sphere formation and expression of CD15 and CD133 on P1, F1, and F2 tumors. Outgrowth of a TIC population should be accompanied by increased sphere formation, and possibly increased expression of CD15 and/or CD133. Contrary to our prediction, experimental efforts to determine an increased TIC population were inconclusive (data not shown). This indicates that CD15 or CD133-expressing cells are not selected for during serial in vivo passing, suggesting these may not be TIC markers of brain metastases. This is further supported by the absence of a difference in tumor formation, survival, and tumor phenotype of mice injected with CD15+ or CD15+ flow-sorted NCI-H1915 tumorsphere cells (Supplementary Figure 2, F–H, available online).

Tumor formation suggests that there is likely a TIC population within brain metastases; however, in the case of NCI-H1915 tumorspheres, this population is not identifiable by BTIC marker expression.

Identification of Candidate Genes Overexpressed in Brain Metastasis Tumorspheres

After unsuccessful attempts to identify the TIC population in brain metastases using primary TIC markers, we wished to identify novel genes and/or pathways specifically overexpressed in brain metastasis tumorspheres. Two patient samples of brain metastasis (lung-derived) and early-passage BT241 tumorspheres were subjected to RNA-Seq (which is less noisy and requires fewer replicates than microarrays [46]) and subsequent analysis. Additional RNA-Seq data from two oligodendrogliomas (grade III primary brain tumors) and two primary lung tumors in the GSC Human Variation Database (47) were also used for comparative analysis. Comparisons between brain metastasis tumorspheres, primary lung and brain tumors were done to determine genes overexpressed exclusively in the brain metastasis stem cell population (Benjamini–Hochberg–adjusted q < .05). Thirty candidate genes were identified (Figure 5A), including genes involved in cell adhesion, cytoskeleton rearrangements, proliferation/tumorigenesis, and formation/disruption of cell–cell junctions.

To confirm their relevance to brain metastasis, we tested the predictive power of the candidate genes for overall patient survival, as poor survival of lung cancer patients strongly correlates with brain metastasis (2–5, 48). Because of our limited sample size, we examined patient survival in a larger homogenous dataset of 226 primary lung tumors (GSE31210). Of the 30 candidate genes, 25 were represented by the probeset used to acquire gene expression data in the dataset, and, as a whole, were associated with length of patient survival (P = .002, log-rank, Figure 5B). Due to its clinical implications, EIF4EBP1 (Figure 5C), a repressor of mammalian target of rapamycin (mTOR)–mediated proliferation, where its repressive effects are relieved by phosphorylation by mTOR complex 1, was of particular interest (49). High levels of phosphorylated EIF4EBP1 are associated with poor clinical prognosis and increased proliferation in breast (50, 51) and ovarian (52) cancers.

We performed survival analysis with 25 candidate genes to identify which specific genes were related to patient survival, and found that elevated expression of 11 of 25 genes was statistically significantly associated with poor patient outcome (log-rank, P ≤ .05; Figure 5D; Table 2). We also constructed a forward selection proportional hazard model, and identified four genes (DSP, DSG2, NOCAL, and KRT7) that optimally predicted patient outcome (log-rank, P = .001; Figure 5E). Although these genes may be useful for prognostic or diagnostic purposes, their functional involvement in disease has yet to be identified, warranting further study.

Discussion

Primary solid tumors have been shown to follow a CSC model of tumorigenesis, where TIC populations are exclusively responsible for tumor generation (9–22). In vitro, these CSC populations...
demonstrate increased self-renewal and differentiation into all tumor cell types; in vivo, TIC populations have the ability to serially recapitulate the original tumor. In this report, we demonstrate that brain metastases also possess such stemlike populations, as they have comparable sphere-forming capacity and stem cell frequency to primary brain tumor controls (Figure 1), are able to initiate tumor growth, reproduce original tumor heterogeneity through in vivo differentiation, and can be serially passaged in vivo (Figures 3 and 4).

NCI-H1915 cells grown in stem-enriching cNSC conditions appear to be an appropriate model for the study of stem cell populations in brain metastases. Particularly valid assays include sphere
Figure 4. Serially transplanted tumorsphere-derived NCI-H1915 tumors resemble the parental tumor and lead to decreased survival. A) Serial injection of NCI-H1915 tumorsphere-derived parental tumors demonstrated growth patterns similar to the original tumor, F1 (i) and F2 (ii). Representative hematoxylin-and-eosin (H&E) sections are shown; scale = 1 mm. B) NCI-H1915 tumors maintain the histology and cytoarchitecture seen in the P1 tumor (i) through in vivo passaging, F2 tumor (ii). Representative H&E sections are shown; scale = 50 µm. C) Serially injected NCI-H1915 tumorsphere cells resulted in a statistically significant ($P = .02$) decrease in overall survival with subsequent injections (F1, F2; n = 6), compared to the initial injection (P1, n = 4). *$P < .05$.

Table 1. Tumor formation of tumorsphere cells from patient-derived brain metastases*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cell No.</th>
<th>Tumor formation</th>
<th>Xenograft profile</th>
<th>Patient profile</th>
</tr>
</thead>
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<tr>
<td>BT16</td>
<td>100,000</td>
<td>1 / 2</td>
<td>CK7− CK20− AE.1− TTF1−</td>
<td>CK7− CK20− AE.1− TTF1−</td>
</tr>
<tr>
<td></td>
<td>50,000</td>
<td>2 / 2</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>BT69</td>
<td>34,000</td>
<td>2 / 4</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>BT250S</td>
<td>100,000</td>
<td>1 / 1</td>
<td>CK7− CK20− AE.1− TTF1−</td>
<td>CK7− CK20− AE.1− TTF1−</td>
</tr>
<tr>
<td></td>
<td>50,000</td>
<td>0 / 1</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>BT296</td>
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<td>1 / 2†</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>BT367</td>
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<td>1 / 1</td>
<td>CK7− CK20− AE.1− TTF1−</td>
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</tr>
<tr>
<td>BT370</td>
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<td>1 / 1†</td>
<td>n.d.</td>
<td>n.a.</td>
</tr>
<tr>
<td>BT381</td>
<td>200,000</td>
<td>1 / 1‡</td>
<td>n.d.</td>
<td>n.a.</td>
</tr>
<tr>
<td>BT382</td>
<td>275,000</td>
<td>1 / 1‡</td>
<td>n.d.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

* NOD-SCID mice were subjected to intracranial injection of the indicated numbers of tumor cells from patient samples. Tumor formation was based on hematoxylin-and-eosin staining of mouse brains upon reaching endpoint, the first of death or 6 months postinjection. n.d. = not determined; n.a. = not applicable; CK = cytokeratin; A.E.1 = cytokeratin cocktail (CKs 1–8, 10, 14–16, 19); TTF1 = thyroid transcription factor 1.
† Tumor cell infiltration into the cerebellum in the absence of a tumor mass.
‡ Cells injected into 4-week-old NOD-SCID gamma mice.
and tumor formation. Our system provides a much needed method to study brain metastases from the lung, and is unique in its application of the CSC model to in vitro and in vivo assay development.

The minimally cultured tumorsphere-derived xenografts accurately recapitulate the patient tumor and may also provide a novel method with which to study the nascent metastatic cells. This cell
Table 2. Ranking of individual genes associated with lung cancer patient survival*

<table>
<thead>
<tr>
<th>Gene</th>
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<th>P</th>
<th>Gene</th>
<th>HR</th>
<th>P</th>
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<td>.005</td>
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<td>---</td>
<td>n.s.</td>
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<tr>
<td>LAMC2</td>
<td>2.4</td>
<td>.01</td>
<td>RASAL1</td>
<td>---</td>
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<tr>
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<td>.01</td>
<td>IRF6</td>
<td>---</td>
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<td>.01</td>
<td>TCP1</td>
<td>---</td>
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<tr>
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<td>.01</td>
<td>LPGAT1</td>
<td>---</td>
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<tr>
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<td>.02</td>
<td>NARS</td>
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<td>CYC1</td>
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<td>.03</td>
<td>SRC</td>
<td>---</td>
<td>n.s.</td>
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<td>CTNN1D1</td>
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<tr>
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<td>---</td>
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<td>PROM1</td>
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<tr>
<td>SLCA9A3R2</td>
<td>---</td>
<td>n.s.</td>
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</table>

* Our 25 candidate genes were independently subjected to log-rank testing to determine their association with length of patient survival. Genes with P values ≤0.05 were considered to be statistically significantly associated with length of patient survival; the HR for each of these genes is presented. HR = hazard ratio; n.s. = not statistically significant.

line may also be a suitable supply of cells when assessing the effects of new therapeutics on self-renewal or tumor growth.

Primary BTIC populations have been identified using cell surface markers CD133 and CD15, as well as the Aldefluor assay for ALDH activity. Our patient-derived brain metastasis and NCI-H1915 tumorspheres expressed these BTIC markers within or close to established ranges (CD15, 2.4%–70.5% [10, 13]; CD133, 19%–29% [12]; and Aldefluor, 4.8%–12.3% [14]); however, unlike in their primary brain tumor counterparts, none were useful in population segregation.

Recent studies have demonstrated equilibrium between stem and nonstem populations, where isolated nonstem populations can spontaneously convert to stemlike cells, reestablishing equilibrium (53–55). This dynamic relationship, along with other disadvantages of surface markers, such as glycosylation-dependent detection (56, 57), discrepancies in long- vs short-term culture (11, 12, 58), and lack of functionality, are likely reasons why they have yet to be explicitly applied to the study of metastasis.

Several reports suggest that stemlike cells of primary breast tumors may be intrinsically invasive in vitro, and metastatic in vivo (29, 30, 59–61), while others suggest that migratory cells can acquire self-renewal and tumorigenic capabilities through an epithelial–mesenchymal transition (62–66). Again, such seemingly dynamic relationships between cell populations indicate that targeting marker-specific populations in metastasis may not be ideal. In the case of brain metastasis, perhaps the best approach is to explore genes and signaling pathways required for the metastasis of lung cancer to the brain. In fact, using a breast-derived brain metastasis model, McGowan et al. demonstrate that depletion of CD44hi/CD24lo CSCs, by inhibiting Notch signaling, led to less invasive behavior and fewer brain metastases (31). This suggests that not only are CSC populations relevant in brain metastases from other primary tumors, but also that these putative brain metastasis-initiating populations can be affected by disruption of important pathways.

Although some studies have identified organ-specific metastasis gene signatures from breast tumors, they neglect to study the candidate genes in the context of the CSC model, and depend on the potentially biased process of in vivo selection (67–71). Similar disadvantages surround gene signatures for lung metastasis to the brain (72). For the first time, we have identified prognostic candidate genes, based on their expression in stem cell-enriched brain metastasis patient samples. Similar patient-derived tumorspheres in the same stem-enriching conditions demonstrate stem cell properties of self-renewal and tumor formation, implicating the expression of these candidate genes in a CSC or TIC population. Survival analyses highlighted the importance of the 11 genes in the outcome of lung cancer patients, further implicating the importance and novelty of our brain metastasis tumorsphere-based signature.

It is important to note that only the NCI-H1915 cell line was used to establish our in vitro and in vivo CSC models of brain metastases. The only alternate commercial cell line was neither biologically relevant (small cell carcinoma), nor viable in cNSC conditions. We emphasize that a single cell line is not necessarily representative of the intertumor variation seen in patients; therefore, findings of any work performed using NCI-H1915 cells should be confirmed through validation in a subset of patient samples or patient-derived cell lines. Other limitations of this study include the fact that identification of our candidate genes was limited by our small sample size. Despite validation using survival analyses in much larger datasets, we were unable to determine their direct prognostic value for the occurrence of brain metastases. Additional functional studies are required to make definitive conclusions for the prediction of brain metastasis.

Through characterization of genes and pathways essential for the metastasis of lung cancer to the brain, not only in the context of invasion and metastasis, but also in that of the CSC model, more effective targets may be identified. Not only would such genes and pathways be useful therapeutic targets, but it is also possible that they may be expressed in the rare subset of cells that successfully form brain metastases from the bulk primary tumor. Identification of a putative brain metastasis-initiating population would not only provide an alternative therapeutic target to genes or pathways, but would also help elucidate the relationship between TIC and metastatic potential, as it has been suggested that these are divergent characteristics (73). By blocking the metastatic process, formation of brain and other metastases could be prevented, transforming a systemic and fatal disease into a locally controlled and much more treatable one.

References


Funding
This work was supported by the McMaster University Department of Surgery (not applicable), the Canadian Cancer Society Research Institute (2012–701052), and the Ontario Institute for Cancer Research (CSC-PRAR-MAC).

Note
We thank Bernardo Trigatti and Katrin Scheinemann for their excellent discussion and experimental recommendations for this manuscript, and the Hamilton Regional Lab Medicine Program for performing the immunohistochemistry on our samples.

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