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PLEKHA5 as a Biomarker and Potential Mediator of Melanoma Brain Metastasis

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Translational Relevance

In this study we used an integrated clinical and bench-based approach to identify genes aberrantly expressed in metastatic melanoma samples that might be associated with a greater propensity for brain metastasis. Our clinical and mechanistic findings suggest that PLEKHA5 might be a mediator of the brain homing phenotype. PLEKHA5 expression was higher in patients with early brain metastasis compared to patients without, in both cerebral- and extra-cerebral metastases and expression was associated with a shorter time to development of brain metastases. PLEKHA5 appears to be a regulator of survival and proliferation of cerebrotropic melanoma cells and a mediator of their passage through the blood brain barrier. PLEKHA5 could therefore be a novel and biologically important target for pharmacological inhibition in melanoma patients with brain metastases.

Abstract

Purpose: Approximately 40% of metastatic melanoma patients develop brain metastases. Our purpose was to identify genes aberrantly expressed in melanoma that might be associated with propensity for brain homing.

Experimental Design: We studied gene expression profiles in a cell line model of brain metastasis (cerebrotropic A375Br cells versus parental A375P cells) and compared them to profiles of patients who developed early brain metastases and who did not. A tissue microarray containing 169 metastatic melanoma cases with variable time to brain metastasis was constructed to further study marker expression by quantitative immunofluorescence. An *in vitro* model of the blood brain barrier (BBB) was generated to evaluate potential mediators of brain metastases.

Results: PLEKHA5 was differentially expressed in both the A375 cell line model and patient samples subjected to gene expression profiling. At the protein level, by quantitative immunofluorescence, PLEKHA5 was associated with decreased brain metastasis free survival. PLEKHA5 over-expression was not associated with other metastatic sites. Knock-down of PLEKHA5 decreases viability of A375Br cells, inhibits BBB transmigration and invasion *in vitro*. Similar results were found with YUMUL cells, cultured from a patient with overwhelming brain metastases. PLEKHA5 knock-down did not affect the viability of A375P cells.

Conclusions: PLEKHA5 expression in melanoma tumors was associated with early development of brain metastases. Inhibition of PLEKHA5 might decrease passage across the BBB and decrease proliferation and survival of melanoma cells both in the brain and in extra-cerebral sites.

Introduction:

The incidence of melanoma is constantly increasing (1), and the prognosis can be poor once the disease metastasizes. Melanoma is relatively resistant to classic chemotherapy and radiotherapy, but in recent years a number of promising systemic therapies have been approved for this disease (2). These include ipilimumab (anti-CTLA4 antibodies), vemurafenib and dabrafenib (selective inhibitors of mutant BRAF), trametinib (MEK inhibitor), or nivolumab and pembrolizumab, (PD-1 inhibitors) and with these newer drugs the median survival for metastatic melanoma patients has started to improve, providing new hope of prolonged disease response in subsets of patients with metastatic disease (2). These drugs, however, have been less extensively studied in patients with brain metastases.

Of all solid tumors, melanoma metastasizes to the brain with the highest frequency; approximately 40% of metastatic melanoma patients develop brain metastases during the course of their illness, and brain metastases are found in up to 75% at autopsy (3). Brain metastases have historically been associated with a median overall survival of only 2.5-4 months in older series, but recent series report a median survival 8-10.6 months, likely due to the impact of improved brain imaging modalities, frequent surveillance and improved local central nervous system (CNS) therapies, particularly stereotactic radiosurgery (4). The only standard melanoma drug known to penetrate the BBB is temozolamide. Dabrafenib also has intracranial activity; response rates in brain metastases to these drugs are: <10% and 31-39%, respectively, with a median progression free survival of 1.2 and 4 months, respectively (5-6). Ipilimumab induces response in untreated brain metastases at a frequency similar to extra-cerebral metastases (7). Most drugs are only studied in the brain metastasis population after they have been fully studied in patients with extracerebral metastases in phase III trials, delaying access to newer therapies for

the brain metastasis population. This is due to historical poor survival in this population, limited studies of drug blood brain barrier penetration in tumors, and limited understanding of the biology of brain metastases.

The present dearth of knowledge regarding the biology and molecular basis of melanoma brain metastasis is a major obstacle to progress against this disease. Identifying new biomarkers is necessary to enable future development of targeted therapies for treatment of melanoma brain metastases.

Distant metastasis is a multiple-step process which includes local invasion from the primary site, intravasation into blood or lymphatic flow, survival in the circulation, extravasation into a target organ, and re-growth. The natural barriers to disseminated cancer cells are unique to each organ and the ability of metastatic cells to adapt to different environments is distinct (8). This has been demonstrated in studies of metastatic subpopulations of cells with different organotropisms, which have uncovered unique gene signatures associated with breast cancer metastasis to specific organs, such as bones, lungs or brain (9-11).

In melanoma, a few studies have identified molecules involved in the pathogenesis of the disease and mediators of invasion or angiogenesis. For example, in preclinical models, elevated levels of VEGF-A (Vascular Endothelial Growth Factor-A) were found to cause deterioration of the blood-brain barrier (BBB) function, vascular hyperpermeability and rapid growth of melanoma brain metastasis, while increased activity of HSPE (heparanase), an enzyme that degrades polymeric heparan sulfate molecules which are major constituents of the endothelial cell layer, enhances the invasiveness of melanoma cells to the brain (12-13). The JAK-STAT (Janus Kinase – Signal Transducer and Activator of Transcription) signaling pathway has been shown to have an important role in melanoma brain metastasis specifically through STAT3

activation and/or SOCS-1 (Suppressor of Cytokine Signaling-1) downregulation (14-15). In an analysis of melanoma patient specimens PI3K-AKT pathway activation was found in brain metastases compared to extracerebral sites (16-17). More recently, Gap junction proteins (Connexins), were shown to mediate early events in brain metastasis, such as tumor cell extravasation and blood vessel co-option (18). EDNRB (Endothelin Receptor B) was found to be a mediator of spontaneous brain metastasis, while overexpression of this protein also exacerbated the extracerebral metastasis (19). Profiling of primary melanoma specimens revealed a seven miRNA signature that can predict brain metastasis (20).

We hypothesized that melanoma cells with the ability to cross the BBB, and grow and survive in the central nervous system (CNS) microenvironment, are molecularly distinct from those that cannot. Genes associated with CNS tropism might be harnessed for drug targeting in patients with cerebrotropic melanoma. In this study we analyzed cell line models and patient specimens to identify additional biomarkers of cerebral metastasis in melanoma and conducted functional studies for further characterization.

Materials and Methods:

Cell lines

YUGANK, YUMUL, YUGEN, YUSIK, YUKOLI, YUMUT, YUSIV, YUKRIN, YUSIT, YUMAC, YURIF, YUKADI, YUCOT, YURDE, YUSIPU, YUGOE, YUHUY and YUHEF are early passage (<20) cell cultures derived from tumors of patients treated at Yale University. Cells in culture were tested for mutations in BRAF and NRAS by Sanger sequencing. PTEN loss was also assessed by Western blot. Mutation patterns were compared to the tumors

from which they were derived for authentication. Cells underwent less than 20 passages prior to use in these studies. After 20 passages, cells were discarded and a vial from an early passage from the cell bank was used. A375P and A375Br were kindly provided by Dr. S. Huang from M.D. Anderson Cancer Center in Houston and profiled within 6 months of receipt. A375P is a human melanoma cell line derived from a lymph node metastasis, which fails to produce brain metastases in mice. A375Br is a highly cerebrotropic derivative, described previously, derived by repeat injection of parental cells in the carotid artery of mice and re-passaging of cells recovered from the brain (14-15). In contrast to A375P, A375Br cells yield brain metastasis in 100% of mice. Additional information is provided in the supplementary materials.

Patient samples for gene expression studies

With Institutional Review Board approval, we reviewed charts of metastatic melanoma patients treated at our institution between 2005 and 2011. For our discovery studies we identified patients with available extra-cerebral metastatic tumors for expression profiling who had been closely followed with serial surveillance MRI imaging every 3 months or less, and selected patients who developed CNS metastases within 6 months of diagnosis of metastatic disease and who did not develop brain metastases for over 18 months. Age at diagnosis ranged from 20 to 80 (mean - 55.4 years). The cohort included 65% males and 35% females. Specimens included metastases from several sites: lymph nodes, skin, soft tissue and visceral metastases. The two groups were balanced for gender, age, site of primary lesion and presence of lung metastases.

Gene expression profiling and data analysis

RNA extraction, quality control measures and data pre-processing are described in the supplementary materials. Data have been deposited in NCBI's Gene Expression Omnibus (GEO), (accession number: GSE60464).

Since gene expression can be influenced by the microenvironment, for our initial analysis we compared samples from similar anatomical sites representing metastases from lymph nodes, soft tissue and skin for both cerebrotropic and non-cerebrotropic cases (17 and 25 respectively). Both groups were represented by 30% and 36% lymph nodes, by 47% and 40% soft tissue and 23% and 20% skin, respectively. To test the association between gene expression and cerebrotropism we used the Wilcoxon rank-sum test. We set the significance cutoff at 0.05 for q-values that were adjusted following the Benjamini-Hochberg correction for multiple testing.

Tissue microarray construction

We constructed a metastatic melanoma tissue microarray (TMA) from paraffin-embedded, formalin-fixed tissue blocks from 169 patients.

Clinical characteristics: The cohort included 63% males and 37% females. Age at diagnosis ranged from 20 to 82 (mean - 55 years). 89% had primary cutaneous (non-acral) melanomas, 8% had mucosal or acral-lentiginous melanoma and 3% had uveal melanoma. Of the non-acral cutaneous melanomas, 43% originated in the trunk, 29% in the extremities and 28% in the head and neck area. At initial diagnosis of stage IV disease, 16% had M1a disease, 30% M1b disease and 55% M1c disease, 31% had elevated LDH. Median survival from diagnosis of stage IV disease was 55 months in patients with M1a disease compared to 24 and 22 months in M1b and M1c disease patients, respectively. BRAF mutations were found in 43% and NRAS in 19%. Brain metastasis free survival (BMFS) follow up time was up to 187.65 months, with a mean

follow-up time of 19.34 months. Other non-cerebral metastases were present in 50% of patients prior to or at the time of sample acquisition. In 70% of cases first cerebral metastasis was diagnosed at or before specimen collection. Prior to sample acquisition, systemic therapy had been administered to 55% of patients.

Specimens included metastases from several sites: lymph nodes, skin, soft tissue and visceral metastases. Information regarding sites of metastatic disease was only collected from patients who had imaging to capture involvement of those sites. A pathologist (DLR) re-examined each case and selected a representative region of invasive tumor for coring two cores for each block.

Immunofluorescent staining, automated image acquisition and analysis

Staining is detailed in supplementary material. Automated image acquisition and analysis have been previously described (21). JMP version 5.0 software was used (SAS Institute). Organ specific metastasis free survival analyses were depicted using the Kaplan-Meier method. Analyses were based on sites of metastases at any time during the course of the illness. The association between continuous AQUA scores and other clinical/pathological parameters was assessed by analysis of variance.

BBB transmigration assay

For transmigration assays we adapted a co-culture model of the BBB which was previously described (11). The *in vitro* BBB model is described in supplementary material. PLEKHA5 expression was silenced with 40nM siRNA, and 24 hours prior to performing transmigration assays, cells were labeled with CFMDA cell tracker green and recovered in

culture. Approximately 50,000 CFMDA-labeled cells were seeded in the upper well, on top of the endothelial layer and incubated for various times. Transmigration was quantified at 8 and 16 hours. Depletion of medium of fetal bovine serum in the upper well to inhibit proliferation did not affect the results. Each experiment was run in triplicate. Inserts were examined microscopically for holes in the insert. Inserts with holes were discarded. Inserts were washed with PBS and membranes containing BBB cells as well as melanoma cells in the process of transmigration were fixed with 4% paraformaldehyde, mounted on slides and analyzed microscopically. An average of 10 pictures was taken from each insert and the number of transmigrated cells were counted for each insert and averaged for each experiment.

Matrigel basement membrane invasion assay

For invasion assays we used the BioCoat tumor invasion 24-multiwell transwell system (Corning). PLEKHA5 expression was silenced with 40nM siRNA, and 100,000 CFMDA-labeled cells were seeded in the upper well in serum free medium. The lower well was filled with medium containing 10% fetal bovine serum. After 18 hours, inserts were washed with PBS and membranes containing invading melanoma cells were analyzed microscopically as described in the BBB transmigration assay.

Results:

Gene expression studies of cerebrotropic and non-cerebrotropic cell lines and specimens from patients with and without early brain metastases

To identify genes specifically associated with brain homing and less likely to be associated with metastases in general or aggressive metastatic melanoma, we studied gene

expression profiles of the A375P and A375Br cell line model. These are isogenic cell lines and provide an excellent system to study molecular determinants of brain metastases. Comparison of transcript profiles between the parental cell line and its cerebrotropic derivative yielded a relatively small set of differentially expressed genes, similar to results of studies done in other disease models (11, 22). Supplementary Table S1 shows genes that had a three-fold or higher change (over- or under-expressed) in expression level between A375Br and A375P. We also analyzed transcript profiles of extra-cerebral metastases of patients with cerebrotropism (defined here as development of brain metastasis within < 6 months of stage IV disease), compared to patients who did not develop brain metastases for >18 months, using the Wilcoxon rank-sum test (Supplementary Table S2). These samples were balanced for age, gender, site of primary lesion, presence of lung metastases and metastatic site of lesion (using metastases from soft tissue, skin and lymph nodes, but not from visceral organs). To identify clinically significant candidate genes to study in the A375P/Br model, we restricted the analysis of patient data to those genes that had a 3 fold difference in expression levels in the cell line model. Following the Benjamini-Hochberg correction for multiple hypotheses testing of the two-sided Wilcoxon sign-rank test, we found that PLEKHA5 was the only gene that was up-regulated in cerebrotropic patients (adjusted P value = 0.02) and over-expressed in the A375Br cells.

In Figure 1A we show a box plot demonstrating the distribution of expression of PLEKHA5 in extra-cerebral specimens from patients with cerebrotropic melanoma and without. PLEKHA5 expression was higher in the group of patients with early brain metastases. 14 specimens taken from the brain were also available for analysis. Expression of PLEKHA5 in cerebral and non-cerebral metastases in patients with early brain metastases demonstrated similar distribution (data not shown).

PLEKHA5 protein expression and correlation with brain metastasis free survival (BMFS)

Correlation between mRNA and protein levels cannot be assumed and previous studies have shown that they correlate about half of the time (23). Furthermore, in order for a gene to be considered a true mediator of brain homing, we need to confirm the association between its protein expression and brain propensity, since most of the biological functions are carried out by proteins and not mRNA. PLEKHA5 was first studied by Western blot in a panel of melanoma cell lines which included the A375P/A375Br cells and a second isogenic cell line model of cerebrotropism – 113/6-4L and 131/4-5B1 cells (24). Differential expression at the mRNA level translated into significant alteration at the protein level; PLEKHA5 expression was found to be globally higher in the cerebrotropic and brain metastasis derived cells when compared to non-cerebrotropic cells. PLEKHA5 was not detected or was weak in A375P and 113/6-4L parental cells but expressed at high levels in the cerebrotropic derivatives, A375Br and 131/4-5B1, respectively (Figure 1B). PLEKHA5 expression was elevated in YUGEN8 and YUKRIN cells, derived from brain metastases, and YUMUL, YUKOLI, YUMUT cultures, all derived from patients with early melanoma brain metastases. However, PLEKHA5 was low or minimal in two cerebrotropic cell lines while expression was high in another two non-cerebrotropic cell lines.

We then tested the association between PLEKHA5 protein expression level and brain propensity using a method of quantitative immunofluorescence (AQUA). Staining of our TMA of 169 metastatic melanoma patients with variable patterns of disease dissemination was done for AQUA, as depicted in Figure 1C. Staining within a histospot was fairly homogenous and predominantly membranous and cytoplasmic. At least two cores per patient were included in

the TMA and AQUA scores were averaged to give one AQUA score per patient. AQUA scores ranged from 2.73 to 101.99 (median: 12.8). Using the Pearson correlation test we compared scores from matching cores included on the array for each patient and found a high degree of correlation; $R = 0.88$ ($P < 0.0001$). Unpaired t-tests showed that expression of PLEKHA5 was higher in patients who developed brain metastases at some point during their illness ($P = 0.015$) (Table 1). PLEKHA5 expression did not correlate with an increased incidence of bone, liver, lung, soft tissue and skin or lymph node metastases, nor was it associated with the site of the biopsy, demonstrating that it is elevated in all sites of metastases of patients with brain involvement (Table 1).

Our TMA included 11 cases with matched tissue cores from a cerebral and extracerebral site or two different extracerebral sites. Comparison of PLEKHA5 AQUA scores by the Pearson correlation test showed a high degree of correlation of PLEKHA5 expression in these paired samples; $R = 0.86$ ($P < 0.0001$), suggesting once again that molecular aberrations associated with the brain homing phenotype can also be found in extra-cerebral samples.

ANOVA analysis or t-test was used to test the association between PLEKHA5 expression and other clinical variables. No correlation was found between PLEKHA5 expression and gender or age or BRAF/NRAS mutational status ($P > 0.05$). By Cox univariate survival analysis we found no association between PLEKHA5 expression and overall survival, once again suggesting that PLEKHA5 expression is associated specifically with the brain homing phenotype and not with aggressive disease in general.

We next assessed the association between PLEKHA5 levels and brain metastasis free survival (BMFS) by Cox univariate analysis. For each patient, BMFS is defined as the time interval between diagnosis of first distant metastasis and imaging diagnosis of brain metastasis.

We note that patients in this cohort were under active surveillance for development of brain metastasis by frequent brain imaging. Patients without brain metastasis were censored at the time of their last MRI or CT scan. High PLEKHA5 expression was associated with decreased BMFS ($P = 0.007$, HR=1.3). To visually assess the association between PLEKHA5 and BMFS we dichotomized scores by the median value and performed Kaplan-Meier analysis using log rank statistics. High PLEKHA5 expression was associated with decreased BMFS (log-rank $P = 0.02$) (Figure 1D). Using the Cox proportional hazards model, we did multivariable analysis, and found that high PLEKHA5 expression remained an independent predictor of short BMFS ($P = 0.015$). The only other variable associated with BMFS by multivariate analysis was American Joint Committee on Cancer (AJCC) M stage ($P = 0.04$). All other variables included in the model (age, gender, and LDH) were not associated with BMFS (Supplementary Table S3). No association was found between PLEKHA5 expression and time to development of lung, liver or bone metastasis (Supplementary Figure S1).

PLEKHA5 knock-down in cerebrotropic melanoma cell lines

To address the role of PLEKHA5 in proliferation of cerebrotropic melanoma cell lines, we used siRNAs to inhibit expression of PLEKHA5 and studied their effects on cell viability. At 96 hours, three of the four different siRNA clones tested completely abrogated expression of PLEKHA5 in A375Br cells at a concentration of 40nM (Supplementary Figure S2). Target knock-down was furthermore confirmed in YUMUL cells, which similarly to A375Br cells express high levels of PLEKHA5 (Supplementary Figure S3). YUMUL was established in culture from a metastasis of a patient with early and overwhelming brain metastases. PLEKHA5 knockdown inhibited viability of A375Br and YUMUL cells in culture at each of

the three siRNA concentrations (Figure 2). The degree of knockdown for each of the three siRNA concentrations is shown in Supplementary Figure S3. In contrast, knock-down of PLEKHA5 expression did not affect viability of A375P cells (Figure 2). A scrambled siRNA was utilized as a negative control and did not show an effect on proliferation in any of the three cell lines. Assessment of PARP cleavage and annexin V/propidium iodide staining showed that PLEKHA5 knockdown does not induce apoptosis (data not shown). This suggests that the decrease in cell number (total viable cells) following PLEKHA5 silencing is mediated by a reduction in cell growth.

Previous studies by Lin et al showed that astrocytes can have a protective effect on melanoma cells and prevent them from undergoing apoptosis (25). In addition, astrocytes have been shown to enhance proliferation of cancer cells through paracrine signaling (26). To test whether astrocytes could potentiate a protective effect and reduce the cytotoxicity of PLEKHA5 knockdown, we cultured A375Br cells with conditioned medium and in close proximity to astrocytes using a transwell system. PLEKHA5 silencing inhibited viability of A375Br cells, both in the absence and in the presence of astrocytes and the effect was comparable (Supplementary Figure S4).

PLEKHA5 and melanoma transmigration through an *in vitro* model of the BBB and invasion through matrigel matrix.

Given the association between PLEKHA5 and decreased BMFS, we sought to determine if this molecule was a mediator of BBB passage. We utilized siRNAs to inhibit expression of PLEKHA5 in the highly cerebrotropic A375Br and YUMUL cells and then tested the ability of these cells to transmigrate through an *in vitro* model of the BBB (Figure 3).

A375Br cells were more able to transmigrate through the *in vitro* BBB than their parental A375P cells (Figure 4). Knockdown of PLEKHA5 expression inhibited transmigration of A375Br through the *in vitro* BBB more than four-fold (Figure 4). Silencing of PLEKHA5 expression also inhibited transmigration of YUMUL cells, although the effect was less pronounced (two fold). Finally to investigate the role of PLEKHA5 in invasion of cerebrotropic cells through reconstituted basement membranes, we utilized a matrigel matrix transwell system. We found that PLEKHA5 knockdown also inhibited matrigel invasion of A375Br and YUMUL cells (Figure 5). The effects of PLEKHA5 silencing on motility utilizing a wound healing assay could not be assessed due to the low baseline migration of the two cell lines tested (data not shown).

Discussion:

Our purpose was to identify candidate genes that might mediate melanoma cerebrotropism. We compared genome-wide expression analysis of a melanoma cell line derived from a lymph node metastasis (A375P) and compared it to a cerebrotropic derivative (A375Br) generated by repeated carotid artery injection in mice. We also profiled tissues from patients who developed early brain metastases with patients who did not. PLEKHA5 was significantly up-regulated in cerebrotropic patients and over-expressed in A375Br cells. Using a method of quantitative immunofluorescence on a larger sample set, we confirmed the association between high PLEKHA5 expression and brain propensity (using BMFS as the endpoint). PLEKHA5 expression was not associated with development of metastases at other sites and expression was high in samples from patients with brain metastases, regardless of the

anatomical location of the specimen assayed. To determine whether PLEKHA5 is mechanistically associated with CNS dissemination and homing, we knocked down PLEKHA5 expression by siRNA and found that this inhibited proliferation of cerebrotropic cells, both alone and in the presence of astrocytes. Furthermore, we found that transient suppression of PLEKHA5 diminished transmigration of cerebrotropic cells across an *in vitro* BBB model and inhibited their invasion through reconstituted basement membranes.

PLEKHA5 belongs to the PLEKHA family (PLEKHA1 -7) and contains both the PH (pleckstrin homology) and Trp-Trp WW putative domains. The PH domain is shared by proteins with phosphoinositide-binding specificities that are key mediators of cellular events such as signaling, cytoskeleton rearrangement, membrane protein targeting and vesicular trafficking. PLEKHA proteins are thought to function mostly as adaptor proteins, since they lack catalytic subunits (27). PLEKHA5 expression was found to be elevated in melanoma by Dowler et al (27). More recently multi-splicing PLEKHA5 variants have been identified in mice and humans, but their functions are poorly characterized (28). The L-PLEKHA5 form (1282 amino acids) is the dominant variant in adulthood and is expressed predominantly in the brain, while a shorter form, S-PLEKHA5 (1116 amino acids) is ubiquitous (28). Both variants play a role in normal brain development (28). Full length PLEKHA5 is expressed at the plasma membrane and is associated with microtubules (29). Increased expression of membrane-associated PLEKHA5 was found at cell-cell contacts and also at the actin-polymerization site at the leading edge of motile cells in a wound healing assay, suggesting an important role for PLEKHA5 in cell migration (29). It is unclear whether PLEKHA5 function is exerted primarily by its PH domain-specific lipid binding affinity or by other yet uncharacterized protein-protein interactions similar to other PH-domain containing proteins, which usually

engage in a variety of protein-protein and protein-lipid interactions and formation of membrane associated signaling complexes. Interestingly, previous studies have shown a higher degree of PI3K-AKT pathway activation in melanoma brain metastases when compared to extra-cranial sites (16-17). In addition, many lines of evidence suggest possible links between PI3K pathway activation and expression or activity of a number of known melanoma brain metastasis mediators and their respective pathways, such as VEGF-A, HSPE, STAT3 and Connexins. For example, HSPE stimulates PI3K and AKT and enhances VEGF expression (30). Connexin43 co-localizes with AKT and its cellular trafficking seems to be dependent on AKT, while PI3K and STAT3 signaling seems to be interdependent (31-32). PLEKHA5 clearly has phosphoinositide-binding specificity and studies are currently underway in our laboratory to assess the role of PI3K in its membrane recruitment and to investigate the functional crosstalk between PI3K-AKT signaling and PLEKHA5 in melanoma brain metastasis.

Both, transmigration across the BBB and proliferation and survival within the CNS microenvironment are likely to be necessary for the formation of brain metastases. The role of PLEKHA5 in mediating CNS penetration of melanoma cells and/or facilitating proliferation and survival in the CNS has not been characterized. Its established role in cell migration might facilitate BBB transmigration, while binding to its downstream messengers might result in increased cell survival. Down-regulation of PLEKHA5 in A375P cells had little effect on proliferation, as baseline levels were low, while down-regulation in A375Br and YUMUL cells (cerebrotropic with high levels at baseline) inhibited proliferation. The inhibitory effect of PLEKHA5 knockdown was also seen in the presence of astrocytes, though this effect was not studied during direct physical contact between astrocytes and tumor cells. Our results suggest that PLEKHA5 might be an important mediator of melanoma brain metastasis and therefore an

attractive target for pharmacological inhibition in both cerebral and extra-cerebral metastases in patients with metastatic melanoma.

PLEKHA5 can be inhibited by emerging gene silencing technologies, such as synthetic U1 adaptors (33). Given that PLEKHA5 does not have a known critical kinase domain, its function cannot be inhibited by small molecule kinase inhibitors. An alternative approach for inhibition is specifically targeting the PH domain of PLEKHA5 protein. This class of inhibitors has been used to selectively bind to the PH domain and inhibit the function of proteins such as Akt and PDPK1, resulting in anti tumor activity (34). This approach has also been proposed for inhibiting PLEKHA7, another PLEKHA family member, and novel target for inhibition in KRAS mutated colon cancer (35).

Our gene expression profiles identified a large number of potential drug targets phenomenologically associated with early development of brain metastases. We note that we focused our studies on PLEKHA5 as it was differentially expressed in the cell line model, although other genes of potential importance were differentially expressed in the human tumors and require functional validation in different models. For example, BCHE (Butyrylcholinesterase) was represented on the Illumina platform by two probes, both of which were the most differentially expressed in the human samples, but not in the A375 cell line model. BCHE would be an excellent candidate for future studies using other models. We also found up-regulation of additional genes with known roles in neural cell development, such as ECE2, NRCAM, SOX13 and STMN1, or genes expressed in the brain and associated with normal neural function, including RHEB, SNCA, STXBP6 and CC2D1A (36-44). High expression of these genes in extra-cerebral sites further suggests that they might facilitate the brain homing process. Some of these genes have also been found to have a role in the

development, progression and dissemination of melanoma or other malignant processes. For example, increased expression of NRCAM and STMN1, potentiates proliferation and migration of melanoma cells, while SNCA, a gene primarily expressed in the brain, but also in various tumors including ovarian, colorectal and melanoma, is involved in tumorigenesis (45-48).

One of the limitations of this study is that the timing of resection or biopsy of metastatic sites was not uniform, and not done in all cases at initial diagnosis of metastatic disease. It is unknown whether expression of PLEKHA5 changes over time, and this needs to be evaluated in future studies.

In previous studies, mRNA profiling of matched melanoma cranial and extra-cranial metastases did not identify PLEKHA5 as differentially expressed between these two anatomical locations (17, 49). Our paired cerebral and extra-cerebral samples indicate that PLEKHA5 expression at the two sites is similar, and PLEKHA5 expression is high in both sites. Our study suggests that some of the molecular aberrations associated with brain metastasis can also be evident in other metastatic locations, and that in melanoma, genes mediating the metastatic spread to the brain and brain homing can also mediate their metastasis and growth at other visceral sites. This is supported by studies by Bos et al, who identified biomarkers of brain dissemination in extra-cerebral tumors, and showed that these can mediate the metastatic spread of breast cancer cells to other sites as well (11). This paradigm was also more recently reported by Cruz-Munoz et al. who utilized a spontaneous brain metastasis mouse model and showed that overexpression of Endothelin receptor B (EDNRB), a mediator of spontaneous melanoma brain metastases, results in enhanced overall metastatic disease and increased incidence of spontaneous brain metastases, while inhibition significantly suppresses both extra-cerebral and cerebral metastatic burden (19).

Our RNA profiling studies were limited by the small cohort size and the fact that patients had metastases at other sites as well. Our gene selection was based on differential expression in the A375Br cells, a BRAF mutated cell line and expression patterns might be driven by BRAF status. However, there was no association between BRAF or NRAS mutational status and PLEKHA5 expression in human tumors. A375Br cells are not uniquely brain metastatic since they form extra-cranial metastases as well. Differentially expressed genes might therefore be associated with metastases and aggressive disease in general, and might not be specific for development of brain metastases. However, our validation studies in a larger cohort of patients using brain metastasis free survival as endpoint, coupled with our functional analyses, provides further confidence that PLEKHA5 is associated with a brain metastatic phenotype. The majority of melanoma patients with brain involvement have extra-cerebral metastases as well, and an ideal drug for the brain metastatic population would target melanomas at all sites. Moreover, we note that the expression studies were primarily conducted on extra-cerebral metastases, both for practical reasons (availability of a large number of specimens for study) and due to the need to co-targeting drivers of both proliferation and survival in CNS lesions and extra-cerebral sites. Prospective validation of the association between PLEKHA5 and development of brain metastases is warranted and clinically relevant, as this might be useful for selection of patients for more frequent brain imaging. In addition efforts are ongoing in our laboratory to develop clinically relevant xenograft models to further study the role of PLEKHA5 in the development of brain metastases *in vivo*.

In conclusion, our studies suggest that melanomas that tend to home to the brain might be pre-programmed to passage through the BBB and then grow and survive in the CNS microenvironment. Mediators of the brain CNS penetration and proliferation can be identified

and potentially inhibited in both cerebral- and extra-cerebral metastases. PLEKHA5 is possibly one such mediator. Although PLEKHA5 likely cooperates with additional molecules in facilitating brain metastasis, our studies indicate that systemic pharmacological targeting of PLEKHA5 might affect the brain homing process and inhibit growth in both cerebral and extra-cerebral metastatic sites. Further studies are warranted to elucidate the specific role of PLEKHA5 in brain homing and to identify up-stream and down-stream regulators of PLEKHA5 function, which might be easily druggable.

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Table 1. Association between PLEKHA5 and clinical or tumor characteristics

		t-statistic	<i>P</i> value
Clinical Characteristics	Soft tissue and Skin metastasis	-1.59	0.117
	Lymph node metastasis	-1.182	0.241
	Lung metastasis	-0.12	0.905
	Liver metastasis	-1.449	0.152
	Bone metastasis	0.096	0.924
	CNS metastasis	2.491	0.015
	Site of Biopsy	Soft tissue and Skin	-1.34
Lymph node		-1.517	0.132
Viscera		1.122	0.274
Brain		1.44	0.155

Note: Two-independent sample t test comparing PLEKHA5 expression (continuous AQUA scores) between patients who developed metastases at specific sites at any time during the course of illness and those who didn't, and between specimens of a specific origin and all others. Significant correlations ($P < 0.05$) are marked in bold.

Figure Legends

Figure 1. Association of PLEKHA5 expression and development of brain metastases. **A)** Box plot showing that PLEKHA5 expression by DASL assay is higher in patients with cerebrotropic disease. **B)** Analysis of PLEKHA5 expression by Western blot. Equal protein gel loading was assessed by probing for expression of β -actin. YUGANK, YUMUL, YUGEN, YUSIK, YUKOLI, YUMUT, YUSIV and YUKRIN were all metastatic melanoma patients with early melanoma brain metastases, whereas YURIF, YUKADI, YUCOT, YURDE, YUSIPU, YUGOE, YUHUY and YUHEF did not develop brain metastases. The course of YUSIT and YUMAC is unknown. **C)** Analysis of PLEKHA5 expression by AQUA. S100 protein is used to locate the tumor. Holes are filled to create a tumor mask and DAPI is used to locate nuclei. Signal from nuclei is subtracted from that of the mask to create a cytoplasmic compartment. Target was measured on a scale of 0-255 within each compartment **D)** Kaplan-Meier curves for PLEKHA5 demonstrating the correlation between marker expression (dichotomized scores) and BMFS. High PLEKHA5 expression was significantly associated with decreased BMFS.

Figure 2. Effect of PLEKHA5 silencing on cell viability. A375Br, A37P and YUMUL cells were transfected with 10, 20 or 40 nmol/L of either PLEKHA5 siRNA or a scrambled, control siRNA for 96 h. PLEKHA5 siRNA inhibited cell viability of A375Br and YUMUL cell lines by 43% to 69% and 49% to 59%, respectively. Viability of the A375P cell line was less affected at all siRNA concentrations. A control scrambled siRNA did not affect viability in any of the cell lines. Significant *P* values from the Student's t-test comparing the viability between treated vs. untreated cells are indicated by * for *P* < 0.05 or ** for *P* < 0.001.

Figure 3. Establishment of the *in vitro* BBB model **A)** Schematic of the model. Arrows mark a cross-section of the porous PET membrane at 60X magnification showing contact between an astrocytic cell and an endothelial cell through the membrane pore (8 μ m) **B)** Western blot analysis of the endothelial (anti-vWF) (TOP) and astrocytic (anti-GFAP) (BOTTOM) layers of the BBB membrane. **C)** Western blot analysis of TOP and BOTTOM layers of the BBB showing increasing expression of GGT1 and GLUT1 **D)** Immunofluorescence of the top endothelial layer showing the establishment of tight junctions (anti-ZO-1) and astrocytic (anti-GFAP) layers of a BBB insert.

Figure 4. Effect of PLEKHA5 silencing on BBB transmigration of cerebrotropic cells. *In vitro* BBB transmigration activity of A375Br and YUMUL (+/- anti-*PLEKHA5* siRNA) was compared to A375P cells. The number of transmigrated cells was summed for ten different fields, averaged for triplicate determinations and plotted as shown. Knockdown of *PLEKHA5* expression inhibited transmigration of A375Br and YUMUL cells through the *in vitro* BBB approximately four and two fold, respectively. Significant *P* values from the Student's t-test comparing levels of transmigration between treated vs. untreated cell lines are indicated by * for *P* < 0.05.

Figure 5. Effect of PLEKHA5 silencing on invasion of cerebrotropic cells. Reconstituted basement membrane invasion of A375Br and YUMUL (+/- anti-*PLEKHA5* siRNA) was compared to A375P cells. The number of invading cells was summed for ten different fields, averaged for triplicate determinations and plotted as shown. Knockdown of *PLEKHA5* expression inhibited matrigel invasion of A375Br and YUMUL cells. Significant *P* values from the Student's *t*-test comparing levels of invasion between treated vs. untreated cells are indicated by * for *P* < 0.05.

Figure 1

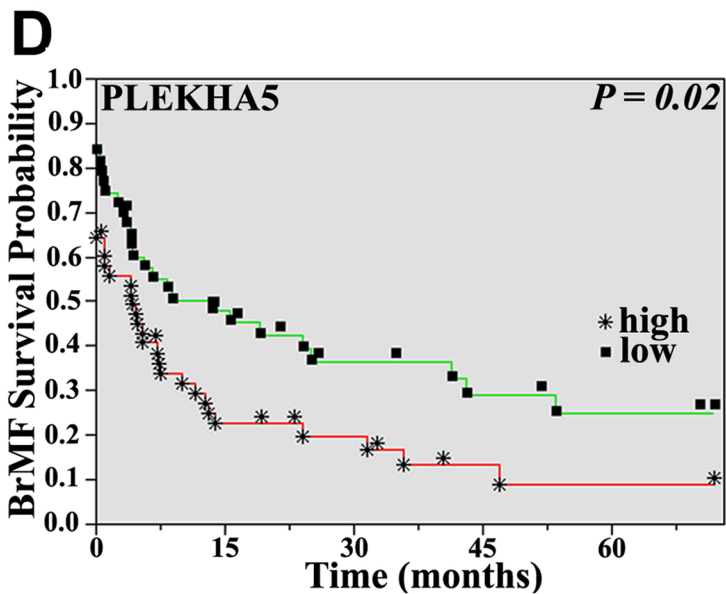
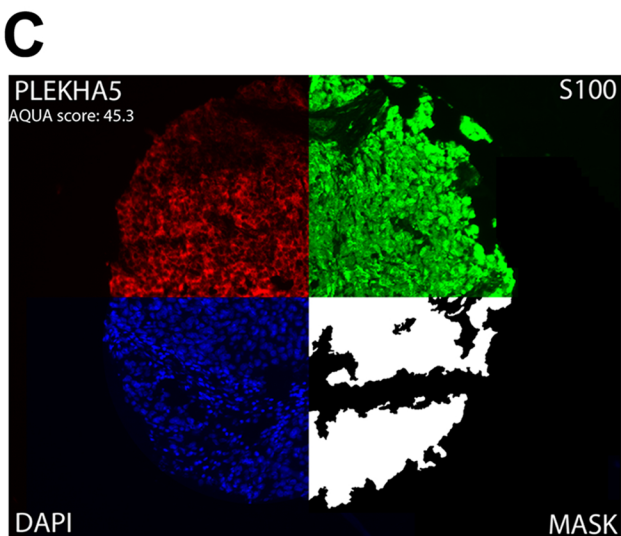
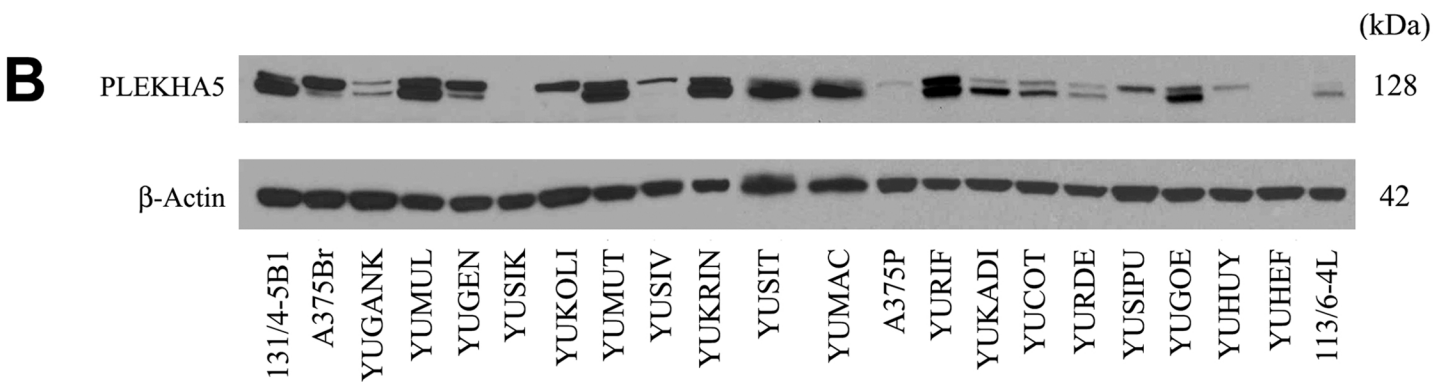
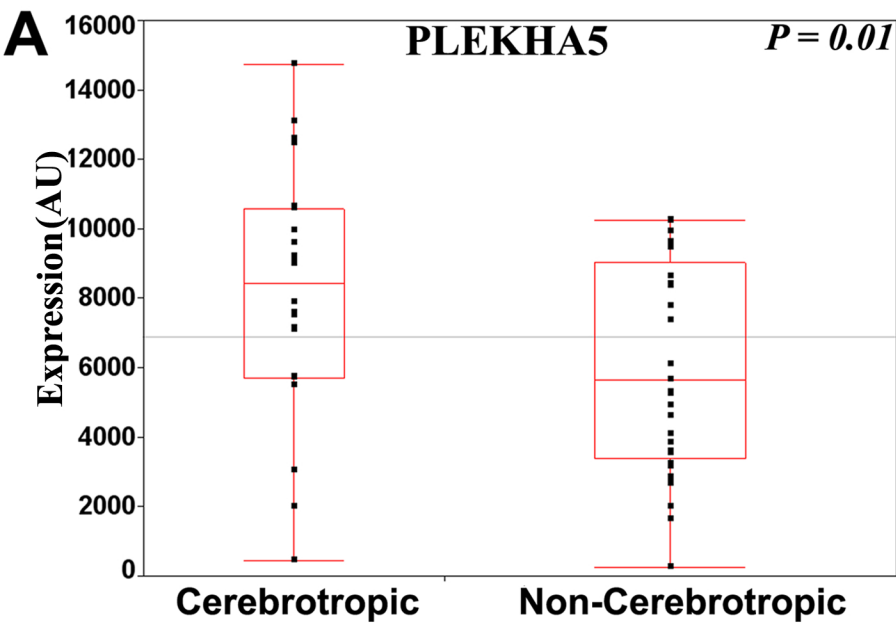


Figure 2

PLEKHA5 siRNA

Scrambled siRNA

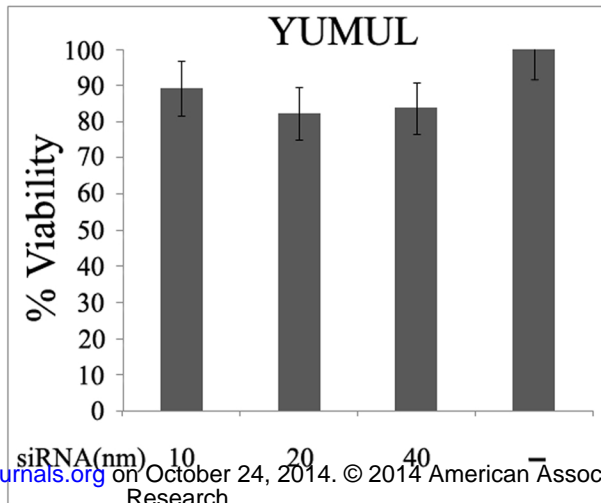
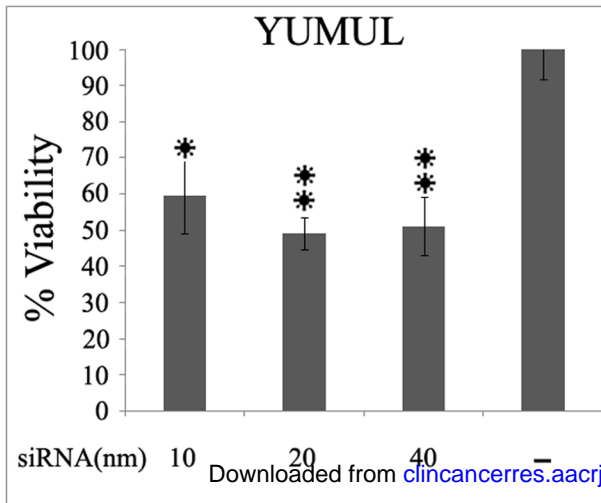
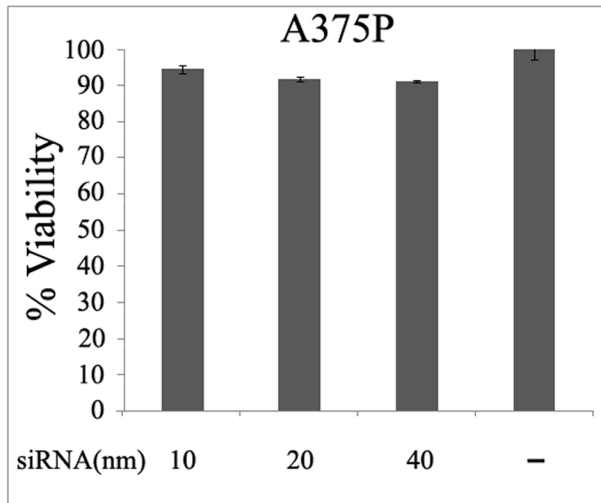
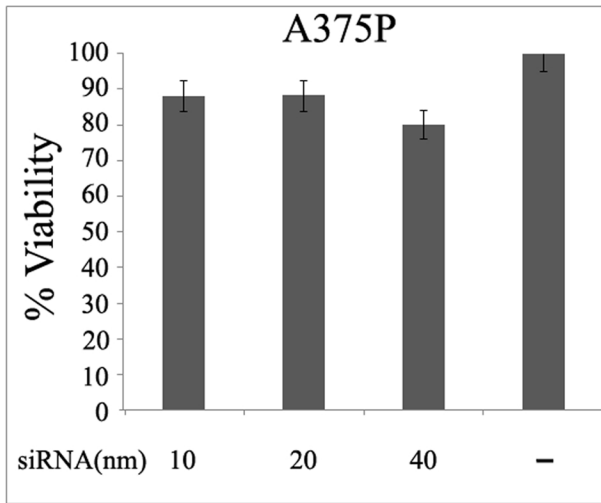
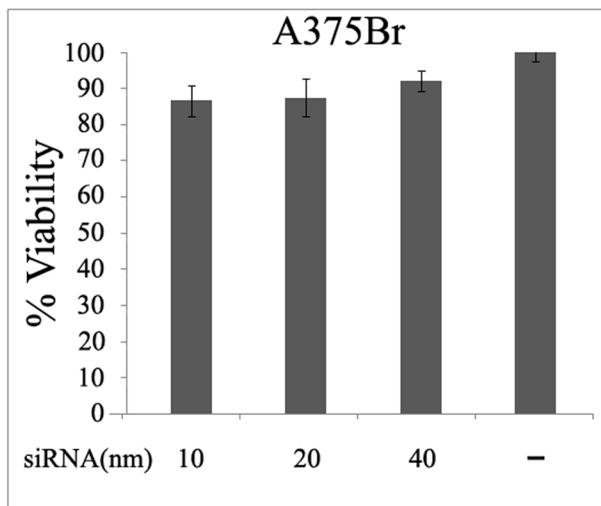
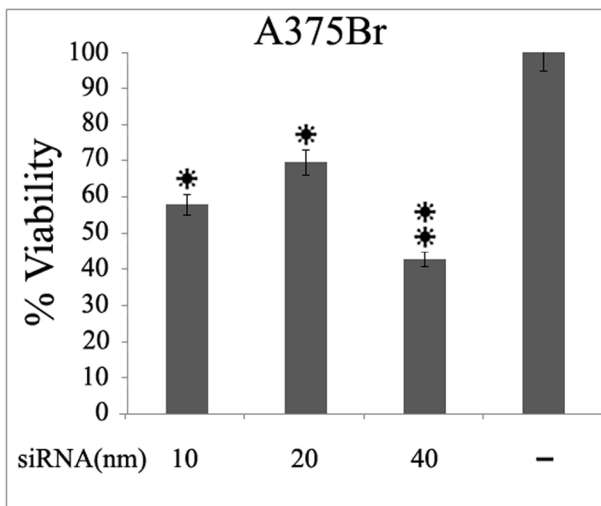
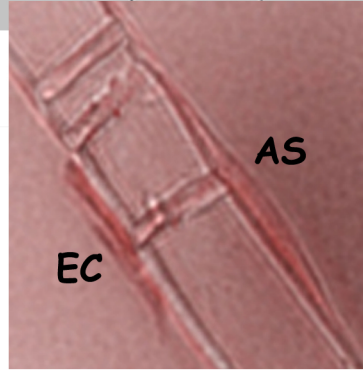
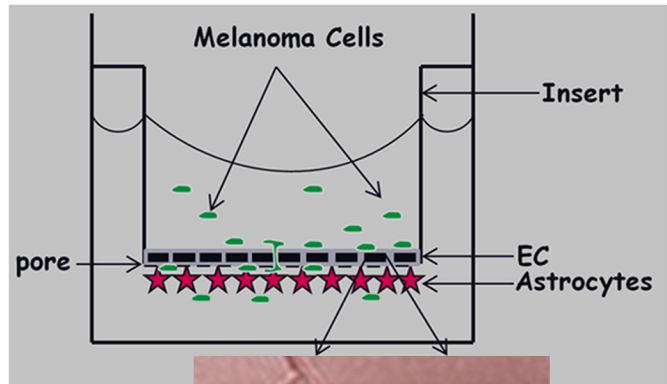
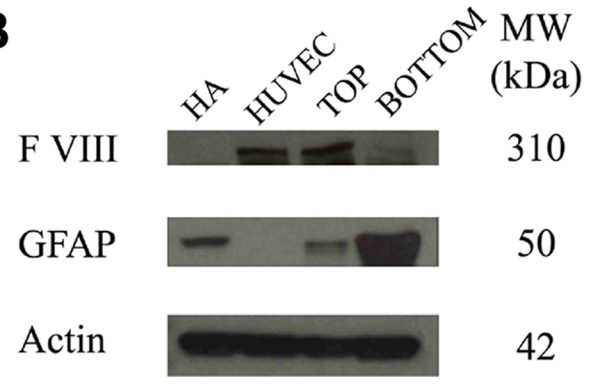


Figure 3

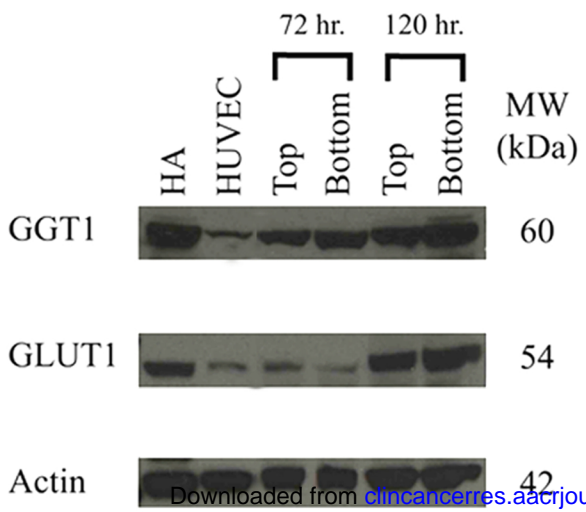
A



B



C



D

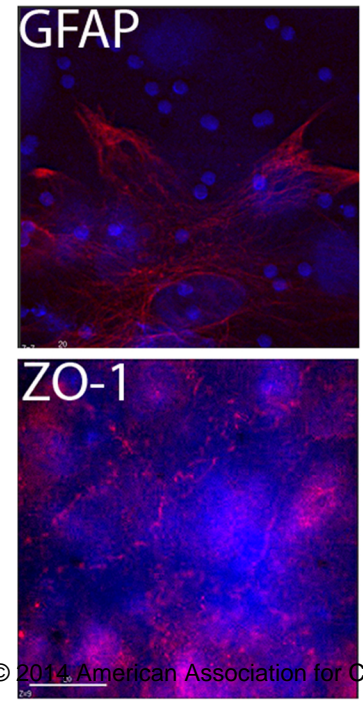
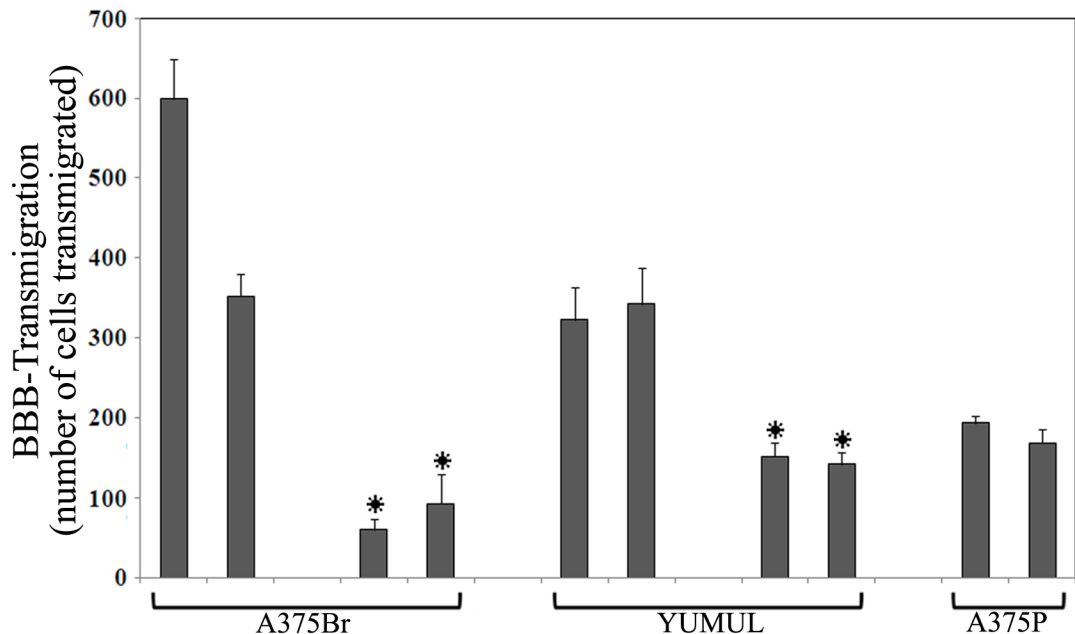


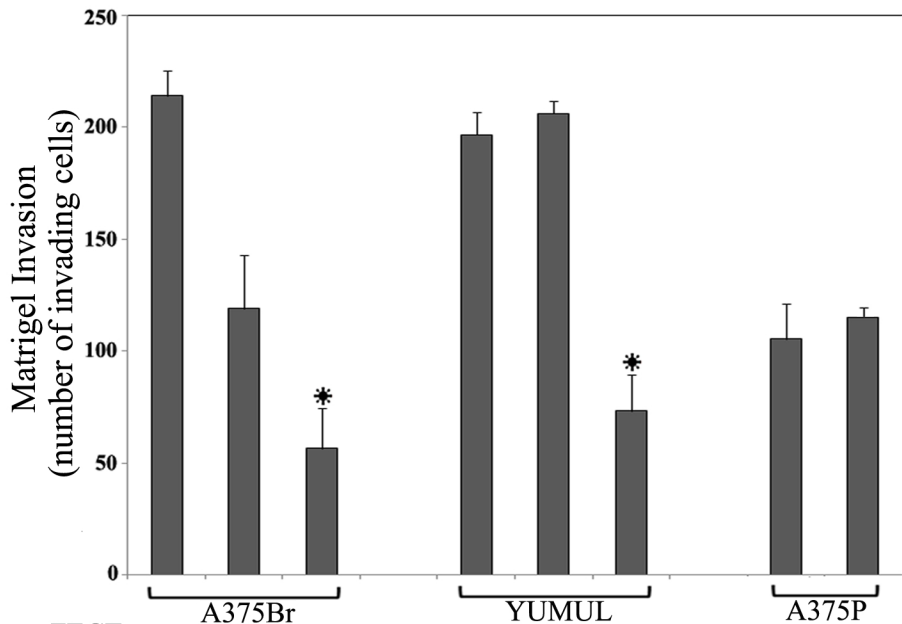
Figure 4



DharmaFECT
40nM siRNA C11
40nM siRNA C12

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Figure 5



DharmaFECT
40nM siRNA

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Research.