HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2016 March 10.

Published in final edited form as:

Oncogene. 2015 September 10; 34(37): 4890–4900. doi:10.1038/onc.2014.412.

miR-509 suppresses brain metastasis of breast cancer cells by modulating RhoC and TNF α

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Abstract

The median survival time of breast cancer patients with brain metastasis is less than 6 months, and even a small metastatic lesion often causes severe neurological disabilities. Because of the location of metastatic lesions, a surgical approach is limited and most chemotherapeutic drugs are ineffective due to the blood brain barrier (BBB). Despite this clinical importance, the molecular basis of the brain metastasis is poorly understood. In this study, we have isolated RNA from samples obtained from primary breast tumors and also from brain metastatic lesions followed by microRNA profiling analysis. Our results revealed that the miR-509 is highly expressed in the primary tumors, while the expression of this microRNA is significantly decreased in the brain metastatic lesions. MicroRNA target prediction and the analysis of cytokine array for the cells ectopically expressed with miR-509 demonstrated that this microRNA was capable of modulating two genes essential for brain invasion, RhoC and TNFα that affect the invasion of cancer cells and permeability of BBB, respectively. Importantly, high levels of TNFα and RhoC-induced MMP9 were significantly correlated with brain metastasis-free survival of breast cancer patients. Furthermore, the results of our *in vivo* experiments indicate that miR-509 significantly suppressed the ability of cancer cells to metastasize to the brain. These findings suggest that miR-509 plays a critical role in brain metastasis of breast cancer by modulating the RhoC-TNFα network and that this miR-509 axis may represent a potential therapeutic target or serve as a prognostic tool for brain metastasis.

Keywords

miR-509; RhoC; TNFα; breast cancer; brain metastasis

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Conflict of interest

The authors declare no conflict of interest.

Introduction

Due to the recent intense effort in public education and mammogram screening followed by early treatments, breast cancer is becoming a curable disease. However, more than 90% of the cancer deaths are still attributed to metastatic disease, and brain is one of the most common metastatic sites of breast tumor[1, 2]. Approximately 25% of patients with advanced stages of breast cancer eventually develop brain metastasis, and their chance of one year survival is less than 20%[3]. Despite this clinical importance, the exact molecular mechanisms and pathological process of brain metastasis are yet poorly understood. Because of the delicate structure of the brain, surgery is limited only to tumors that are not localized in the vital areas[4, 5]. Most chemotherapeutic drugs that are effective in treating primary tumors fail to suppress the growth of brain metastatic tumors because of the blood brain barrier (BBB) and chemo-resistant property of the metastatic cells due to the genetic and epigenetic alternations acquired during the chemotherapy[6, 7].

MicroRNAs (miRNA) are non-coding RNAs that regulate gene expression by targeting the sequences on their untranslated region (UTR)[8]. More than one thousand human miRNAs have been identified, and many of them are found to be involved in tumorigenesis of breast cancer[9]. Let-7 is the most well studied miRNA and it functions as a tumor suppressor by targeting multiple oncogenes including cyclin D, MYC and H-RAS that enhance the proliferation and stemness of breast cancer cells[10, 11]. In the case of ER-positive breast cancer, miR-191 was found to be regulated by estrogen through a positive feedback loop and to promote cancer cell proliferation and migration by targeting SATB1 which plays a key role in chromatin remolding[12]. In another study, miR-30c which is transcriptionally regulated by GATA3 was shown to attenuate the chemoresistance property of breast cancer cells by targeting TWF1 and IL-11[13]. Moreover, some microRNAs are known to be highly expressed only in certain subtypes of breast cancers such as triple negative or Her2-positive, both of which are known to preferentially metastasize to the brain[14]. In case of metastasis, Volinia *et al*. performed a survival analysis in 466 patients with invasive ductal carcinoma (IDC) based on their microRNA expression in primary tumors and found that seven miRNAs (miR-103, miR-1307, miR-148b, miR328, miR-484, miR-874 and miR-93) were significantly correlated with distant relapse-free survival, which was further validated in other patient cohorts[15]. Moreover, Rosenfeld and colleagues identified a group of microRNAs that are exclusively expressed in metastatic tumors based on the analysis of 336 breast cancer patients, suggesting the existence of metastasis specific microRNAs[16]. These results strongly suggest that microRNAs may serve as attractive biomarkers and therapeutic targets for metastatic breast cancer. However, this requires more systematic approaches using tumor tissues derived from both primary and metastatic lesions and further understanding of the mechanistic roles of microRNA in tumor progression.

In this study, we isolated RNA from tumor samples obtained from primary breast tumors and also from brain metastatic lesions followed by microRNA profiling. The results of our analysis revealed that primary tumors expressed high level of the miR-509 while the expression levels of these microRNAs were significantly reduced in brain metastatic lesions. We demonstrated that the down-regulation of miR-509 induces the expression of two essential genes for brain metastasis, RhoC and TNFα, followed by up-regulating the MMP9

expression, which together facilitates increase in the permeability of BBB and penetration of tumor cells in the brain.

Results

The expression of miR-509 is attenuated in brain metastatic lesions of breast cancer patients

To identify microRNAs that are specifically involved in the progression of brain metastasis, we obtained clinical specimens of primary tumors and metastatic lesions in the brain. We isolated total RNA only from the tumor areas to avoid the potential contamination by stromal cells or normal tissues. Total RNA was subjected to the Affymatrix GeneChip miRNA 1.0 Array followed by RMA normalization, and a heatmap of the most up and down regulated microRNAs between two groups was generated (Figures 1a and b). The results of our analysis revealed that the expression of 25 microRNAs were significantly up-regulated while 14 microRNAs were down-regulated in brain metastatic lesions (more than 2 fold change, $p<0.01$). Of particular interest are the two forms of miR-509, miR-509-3-5p and miR-509-5p that were both significantly enriched in the primary tumors compared to metastatic brain tumors. We further verified the microarray result by performing Taqman qPCR for the primary tumors from patients with or without brain metastasis and also tumors from brain metastatic lesions. As shown in Figure 1c, the expression levels of both microRNAs were indeed significantly decreased in the primary tumors derived from patients with brain metastasis compared to that from patients without brain metastasis. Importantly, the level of the microRNAs was significantly further down-regulated in the brain metastatic lesions, suggesting a potential role of miR-509 in brain metastasis. To examine whether downstream target genes of the miR-509 which are correlated with the status of brain metastasis in clinical settings, we analyzed a series of clinical microarray cohort data (GSE12276, GSE2034, GSE2603, GSE5327, and GSE14020) that contain the brain relapse information of a total of 710 patients. We also performed GSEA (Gene-set enrichment analysis) for the patients with brain metastasis (n=47) and patients without any distant metastasis (n=315) by using top 60 most predicted miR-509 target genes based on TargetScan analysis. Notably, the expressions of miR-509 target genes were significantly enriched in the patients with brain metastasis, which further support our notion that the miR-509 is involved in the pathogenesis of brain metastasis by modulating groups of target genes (Figure 1d and Supplementary Figure 1a). In addition, we analyzed another microRNA array cohort data (GSE31309) which contains comprehensive microRNA profiling from the blood of healthy donors and early stage breast cancer patients. Notably, a significantly higher level of miR-509 was detected in the circulation of healthy donors (n=57) compared to the patients with early stage of breast cancers (n=48) (Supplementary Figure 1b). Furthermore, we examined the expression of miR-509 in MDA-MB-231 (MDA231), MDA-MB-231BoM-1833 (231BoM) and MDA-MB-231BrM2a (231BrM) cells. The latter two cell lines were generated from MDA231 through multiple rounds of *in vivo* selections of clones that preferentially metastasized to bone and brain, respectively. We also examined MDA-MB-231-HM (231-HM) cells that were derived from a spontaneous brain metastasis of MDA231 cells in NOD/SCID/IL2R$_\gamma$ (NSG) mice[17]. We found that the endogenous expressions of miR-509 were significantly reduced in 231BrM and 231-HM

cells compared to the parental cells and bone metastatic cells, suggesting that miR-509 is involved in the pathogenesis of brain metastasis (Figure 1e).

miR-509 suppresses the expression of RhoC gene

To study the function of miR-509 in brain metastasis, we searched potential target genes using four database search engines (TragetScan, miRanda, miRDB and miRWalk) and found that 9 genes were commonly identified by all four databases with a cutoff p-value of 0.0005 (Supplementary Figure 2a). Among these genes, we focused on RhoC gene which ranked the highest and has also been known to play a critical role in metastasis[18, 19]. The complementary binding sequences of RhoC 3'UTR and miR-509 are shown in Figure 2a. It should be noted that miR-509 was also ranked highest when we conversely searched potential RhoC-targeting microRNA in the same databases. To examine the effect of miR-509 on the expression of RhoC, we ectopically expressed pre-miR-509 in 231BrM and CN34BrM, another cell line that preferentially metastasizes to the brain by lentiviral infection followed by Western blot analysis. As shown in Figure 2b, we found that RhoC was highly expressed in 231BrM and CN34BrM cells compared to their parental cells and ectopic expression of pre- miR-509 significantly suppressed the RhoC protein expression. On the other hand, transfection of miR-509 LNA significantly enhanced RhoC expression in MDA231, MCF7 and MCF10A cells (Figures 2c). To further validate our results, we co-transfected miR-509 expression plasmid and a 3'-UTR reporter plasmid of the RhoC gene into 293TN cells followed by reporter assay. As shown in Figure 2d, the reporter activity of 3'UTR of RhoC was significantly suppressed by miR-509; however, deletion of miR-509 binding site attenuated the suppressive effect. These results strongly suggest that miR-509 is capable of suppressing the expression of RhoC by directly targeting its 3'-UTR. Furthermore, we examined the relationship of miR-509 and RhoC in the clinical samples derived from brain metastatic samples by Taqman PCR, and we indeed found a significant negative correlation between the expression of RhoC and miR-509 in the brain metastatic lesions (Figure. 2e). We also did meta-analysis of the RhoC expression for the patients with or without brain metastasis using a combined existing database (GSE12276, GSE2034, GSE2603, GSE5327, and GSE14020) and found that RhoC was indeed significantly up-regulated in the patient with brain metastasis (Figure 2f).

miR-509 suppresses trans-endothelial cell migration by blocking RhoC-induced MMP9

To further investigate the effect of miR-509 and RhoC on brain metastasis, we examined the trans-endothelial cell migration ability of cancer cells using a trans-well culture insert coated with mouse brain endothelial cells (mBrEC) and primary human astrocytes, a system which mimics BBB (Figure 3a). As shown in Figure 3b, we found that miR-509 significantly suppressed transmigration of 231BrM cells, while ectopic expression of RhoC blocked the suppressive effect of miR-509. We also tested the effect of RhoC on the trans-endothelial cell migration of MDA231 cells using the same assay system and found that ectopic expression of RhoC indeed significantly enhanced the transmigratory ability of MDA231 cells (Figure 3c). Furthermore, to test whether miR-509 affects the basic migratory ability, we performed the wound healing assays in the same set of cells shown in Figure 3b and found that the ectopic expression of miR-509 significantly reduced the mobility of 231BrM cells. Similarly, the ectopic expression of RhoC in MDA231 cell was able to restore the

original invasive phenotype (Figure 3d). In addition, we transfected miR-509 LNA or scramble LNA into MCF7 and MDA231 cells followed by examining their migratory abilities. As shown in Figure 3e, inhibition of miR-509 significantly enhanced the migratory abilities of both MCF7and MDA231 cells. However, miR-509 did not affect the growth ability of 231BrM cells, suggesting that miR-509 suppresses brain metastasis by attenuating its transmigration and migration ability without affecting the proliferation of cell (Supplementary Figure 3a). Our previous study showed that MMP9 which plays a key role in tumor invasion was regulated by RhoC through the activation of Pyk2[20]. Therefore, we examined a possibility that RhoC promotes brain metastasis by inducing MMP9. We found that miR-509 indeed significantly decreased the expression and secretion of MMP9 in 231BrM cells (Figure 3f), while transfection of LNA that was targeted to miR-509 in 231BrM cells or ectopic expression of RhoC in MDA231 cells significantly increased the MMP9 expression (Figures 3g and h). Furthermore, to examine the clinical relevance of MMP9 in brain metastasis, we analyzed the relationship between the MMP9 expression and metastatic status of breast cancer patients using the existing GEO data base that was used in Fig 1d. We found that the expression of MMP9 was indeed significantly correlated with brain and overall metastasis-free survival but not with bone metastasis-free survival, suggesting that MMP9 promotes metastasis in an organ preferential manner (Figure 3i and Supplementary Figure 3b).

Suppressing miR-509 increases BBB permeability by promoting secretion of TNF α

Our data in Figure 3b indicates that miR-509 is capable of blocking trans-endothelial migration, but this effect is only partially restored by ectopic expression of RhoC. This result strongly suggests that there are other downstream effectors of miR-509 which affect the BBB permeability. BBB serves as a strong barrier against cancer cell invasion to the brain; however, the permeability of BBB is known to be increased by various tumor-induced inflammatory cytokines[21, 22]. Therefore, we sought a possibility that miR-509 indirectly modulates such cytokine expression by examining the cytokine profile in the medium of 231BrM with or without expression of pre- miR-509 using the Cytokine Expression Array (RayBio) which includes antibodies for 120 cytokines and growth factors. As shown in Figure 4a, we found that TNFα, which is known to enhance the permeability of BBB[23] was the most significantly suppressed cytokine in the conditioned medium of 231BrM cells that ectopically expressed miR-509. We further validated this suppressive effect of miR-509 on TNFα in 231BrM and CN34BrM cells by RT-PCR and immunocytochemistry (Figure 4b and c). We also transfected MDA231 cells with LNA which was targeted to miR-509 and found that knock-down of miR-509 significantly increased the TNFα expression (Figure 4d). To test whether miR-509 suppresses TNFα by directly binding to its 3'UTR, we performed the TNFα 3'UTR reporter assay with or without miR-509 expression and found that there was no significant difference between two groups, indicating that miR-509 regulates TNFα in an indirect manner (Supplementary Figure 4a). Furthermore, to examine the effect of TNFα in promoting brain metastasis by increasing the permeability of BBB, we performed trans-endothelial cell migration assays by treating MDA231cells with different doses of TNFα or ectopically expressing TNF±. As shown in Figure 4e and f, recombinant TNFα and ectopic expression of TNFα indeed significantly enhanced the transmigratory ability of MDA231 cells. To further investigate whether TNFα enhances the permeability of

BBB, we performed the Trans Epithelial Electric Resistance (TEER) assay by treating brain endothelial cells with recombinant TNFα or conditioned medium derived from 231BrM cells with or without expressing miR-509. We found that conditioned medium from 231BrM and recombinant TNFα indeed significantly decreased the TEER value of brain endothelial cells compared to the conditioned medium from 231BrM-miR-509 and the control medium (Figure 4g). These results strongly suggest that miR-509 suppresses brain metastasis in part by blocking TNFα–induced BBB penetration.

TNFα is highly expressed in brain metastatic tumors and brain metastatic lesions

To further investigate the relationship between the expression of TNFα and brain metastasis in clinical settings, we performed immunohistochemical analysis of TNFα for breast cancer specimens. As shown in Figures 5a and b, brain metastatic lesions and primary tumors from patients who eventually developed brain metastasis expressed significantly higher levels of TNFα compared to the tumors from overall metastasis-free patients with the similar clinical grades. The results of correlation analysis between miR-509 and TNFα indicate that there was a significant inverse correlation between these two genes in brain metastatic samples (Figure 5c). We also examined the clinical relevance between TNFα and the status of brain metastasis in the same cohort as mentioned in Figure 1d. We found that a higher level of TNFα was significantly correlated with a poorer brain metastasis-free survival of breast cancer patients, while it was not related with lung or bone metastasis (Figure 5d). Our results strongly indicate that the secreted TNFα from brain metastatic cells plays critical roles in metastatic growth and that the expression of TNF± can be used as a biomarker for predicting risk of brain metastasis in breast cancer patients.

miR-509 suppresses brain metastasis in vivo

The results of our *in vitro* experiments and clinical analysis strongly suggest that miR-509 suppresses brain metastasis by modulating the expression of RhoC and TNF α. To further validate these observations, we examined the effect of miR-509, RhoC and TNF α on the metastatic growth of 231BrM and MDA231cells *in vivo*. Cells were prepared from 231BrM that were infected with lentivirus carrying with or without the expression of pre-miR-509, and they were transplanted into nude mice through intracardiac injection followed by monitoring metastatic tumor growth in the brain. Metastatic tumor growth in the brain was observed in all mice after 4 weeks that received 231BrM cells infected with vector control. On the other hand, animals that received 231BrM cells carrying miR-509 showed significantly less metastatic lesions in the brain, suggesting that miR-509 was indeed capable of suppressing brain metastasis (Figures 6a and b). On the other hand, the knockdown of miR-509 in MDA231 cells by LNA significantly enhanced their brain metastatic ability after implantation of these cells via intracardiac injection (Figure 6c). Next, we ectopically expressed RhoC or TNF α in MDA231 cells, and they were transplanted into nude mice through intracardiac injection. As shown in Figures 6d and e, both RhoC and TNF α significantly enhanced the brain metastatic ability of MDA231 cells. We then performed a similar experiment using the bone metastatic cell line, 231BoM, which carried lentivirus with or without the expression of pre-miR-509. Interestingly, we found that miR-509 did not affect the incidence or growth of bone metastasis (Supplementary

Figure 5a and b). These results further support our notion that miR-509 specifically suppresses brain metastasis by down-regulating RhoC and TNF α.

Discussion

In this study, we performed microRNA profiling for the primary and brain metastatic tumor samples of breast cancer patients and found that the miR-509 is capable of suppressing the brain metastasis by targeting TNFα and RhoC-induced MMP9 secretion which affect the permeability of BBB (Figure 6f). Our findings match with the notion that brain metastasis is a highly dynamic multi-step process which requires the contribution of several key factors, and most importantly, they are regulated by at least one or few microRNAs. It is well recognized that tumor cells often metastasize to preferred organs due to their requirement of particular microenvironment which is also known as the seed-soil theory, and it is plausible that this organ preference is partly controlled by microRNAs[24]. Previously, Korpal *et al*. found that miR-200s facilitated breast cancer lung colonization and promoted an epithelial phenotype by targeting Zeb1/2, and on the other hand, inhibiting Sec23a- induced Ifgbp4 and Tinagl1 secretion[25]. In the case of brain metastasis, we have previously demonstrated that miR-7 was significantly decreased in the breast cancer stem cell population that were isolated from brain metastatic cells and it suppressed the self-renewal and invasion of cancer stem cells by targeting KLF4[26]. Arora and colleagues have recently shown that miR-328 promotes brain metastasis of non-small cell lung cancer (NSCLC) by enhancing cell migration through activating PRKCA expression[27]. It is worth noting that the expression of miR-328 is also highly elevated in brain metastatic lesions of breast cancer patients in our cohort data obtained in this study, suggesting that some miroRNAs promote organ preferential metastasis in multiple types of cancers. More recently, Zhou *et al*. demonstrated that miR-105 which is secreted from highly metastatic breast cancer cells promotes distant metastasis by affecting the integrity of vascular endothelial barriers and that the serum levels of miR-105 is associated with the level of tumor progression in breast cancer patients[17]. These results indicate that an activation of a microRNA network plays a critical role in metastatic process of breast cancer.

Our *in vitro* and *in vivo* data strongly indicates that RhoC is a key mediator in brain metastasis and its expression is regulated by miR-509. RhoC is a well-known oncogene which enhances the migration and invasive ability of cancer cells by activating various pathways[18]. Rosenthal *et al* found that the level of RhoC expression was positively correlated with that of ALDH1, a cancer stem cell marker, in the breast cancer specimens and that overexpression of RhoC enhanced metastasis in a xenograft model[28]. Although we found no significant correlation between the mRNA levels of RhoC and brain metastasis-free survival of patients by analyzing GEO database (data not shown), ectopic expression of RhoC significantly enhanced the brain metastatic ability in weakly brain metastatic cell line, suggesting that post-transcriptional regulation is also likely to be involved in the RhoC-mediated brain metastasis.

Metastasis is a multi-step cascade which includes intravasation, survival in the circulation, extravasation and colonization[29]. The rate limiting step of brain metastasis is the extravasation when circulating tumor cells seed in the brain by penetrating BBB which

requires the collaboration of multiple key oncogenes[30]. Therefore, to establish colonization in the brain, a cancer cell has to acquire the properties which enable them to extravasate the brain blood vessel[31]. In this study, we have shown that miR-509 suppressed the secretion of TNFα which in turn decreased the BBB permeability and the penetration of cancer cells into the brain. Several studies have previously shown that pro-inflammatory cytokines such as TNFα and IL1β were able to alter the permeability of BBB by affecting the tight junction proteins[32, 33,34]. Indeed, Yang *et al*. treated mice with anti-TNF α antibody and found that blocking TNFα significantly decreased the disruption of BBB in a reperfusion model[35]. These results are in good agreement with our results that miR-509 mediated TNFα plays a critical role in brain metastasis of breast cancer cells. How miR-509 modulates TNFα expression is an intriguing question. When we tested the effect of miR-509 on TNFα using a 3'UTR reporter plasmid, we did not see any effect of miR-509 (data not shown), suggesting that the effect is indirect. We then searched transcription factors that potentially control TNFα expression using the human transcription factor ChIP sequence data which was released by the ENCODE project[36]. We found 19 such factors; however, none of them were predicted targets of miR-509 based on our Targetscan analysis. Indeed, our expression analysis for cell lines that preferentially metastasize to brain (MDA231BrM and CN34BrM) indicated that these transcription factors were not significantly altered in these cell lines. We also considered the possibility of link between RhoC/MMP9 and TNFα, by examining the TNFα expression in MDA231 with or without the ectopic expression of RhoC and we found that the TNFα expression was not controlled by RhoC (data not shown). Therefore, miR-509 modulates TNFα expression through an unknown pathway which is likely to be preferentially involved in the regulatory network of brain metastasis.

Recently, Bos *et al*. have established variant cell lines that preferentially metastasize to the brain from two triple negative breast cancer cell lines, MDA-MB231 and CN34. By performing the global expression analysis of these cells, 17 genes that were found to be significantly up- or down-regulated in the established brain metastatic cell lines as well as in the primary tumors of breast cancer patients with brain metastasis and these genes were designated as a brain metastasis signature which may serve as a prognostic tool[37]. In this study, we explored a possibility that microRNAs can be used as a potential prognostic marker for breast cancer patients with brain metastasis, and we performed microRNA array for the samples obtained directly from metastatic tumors and found that the expression of several microRNAs including the miR-509 are significantly altered in metastasized tumors in the brain. Our GSEA data demonstrated that miR-509 suppresses brain metastasis in clinical settings by regulating a group of genes that were predicted by Target scan-based analysis. This is, to our knowledge, the first cohort based study showing the clinical relevance between microRNA target genes and the brain metastasis status. To further explore the potential clinical utility of miR-509 as a biomarker for assessing the brain metastasis risk in breast cancer patients, we analyzed the existing microRNA cohorts database and found that miR-509 expression was not only correlated with the relapse-free survival but also highly enriched in the serum of healthy donor while it is significantly reduced in the circulation of patients with early stage breast cancer. These results strongly suggest that the level of miR-509 in serum and the expression profile of the downstream

target genes may be used as biomarkers for assessing the risk of distant relapse of breast cancer even at an early stage and that these genes may also serve as therapeutic targets.

Materials and Methods

Cells and cell culture

We purchased MDA-MB-231 and MCF7 breast cancer cell lines from ATCC. The MDA-MB231BrM-2a, CN34 and CN34-BrM2c cells were a kind gift from Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center)[37]. The MDA-MB-231-HM cells were a kind gift from Shizhen Emily Wang (City of hope)[17]. Firefly luciferase-labeled cells were generated by lentivirus infection. 293TN cells were obtained from System Bioscience. MDA231, MDA231 variant cells and 293TN cells were cultured in DMEM medium supplemented with 10% FBS and antibiotics. CN34 and its variant cells were cultured in Medium199 supplemented with 2.5% FBS, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 20 ng/ml EGF, 100 ng/ml cholera toxin and antibiotics. The immortalized mouse brain microvascular endothelial cells (mBrEC) were supplied by Dr. Isaiah J. Fidler (M.D. Anderson Cancer Center)[38]. mBrEC was maintained at 8% CO_2 at 33 °C in DMEM with 10% FBS, 2 mM of L-glutamine, 1 mM of sodium pyruvate, 1% of non-essential amino acids and 1% of vitamin mixture. Primary astrocytes were purchased from clonexpress.

Plasmids

The plasmids expressing hsa-mir-509 precursor was cloned into lentiviral pCDH-CMV-MCS-EF1-copGFP vector (System Bioscience). pSin-TNFα-puro lentiviral plasmid was constructed by sub cloning from PCMV6-TNFα (Origene). RhoC reporter plasmid, pMir-Report-RhoC-3'-UTR was constructed by cloning RhoC 3'UTR sequence into pMir-Report plasmid by PCR amplifying the regions using the primer set for RhoC 3'UTR F : GGGAAGCTTGTTAACAAACAATTCTTTGTACAGTG and RhoC 3'UTR R: CGAGCTCGATCCCCAAGGCCTTTCCTAC. Deletion of the miR-509 binding core sequence on 3'-UTR region of RhoC gene was achieved by the overlap extension PCR methods[39] using the following primers for RhoC 3'UTR F : GGGAAGCTTGTTAACAAACAATTCTTTGTACAGTG and RhoC 3'UTR R: CGAGCTCGATCCCCAAGGCCTTTCCTAC, RhoC mut F : TGGCTCACAGGAAAGGGGGACCCAGAGTC and RhoC mut R: GACTCTGGGTCCCCCTTTCCTGTGAGCCA. TNFα reporter plasmid, pMir-Report-TNFα- 3'-UTR was constructed by cloning TNFα 3'UTR sequence into pMir-Report plasmid by PCR amplifying the regions using the primer set for TNFα 3'UTR F: CCCAAGCTTGGAGGACGAACATCCAACC and TNFα 3'UTR R: CGAGCTCTTC TTT TCTAAGCAAACTTTATTTCTCGCC.

RNA isolation from FFPE samples and microRNA microarray profiling

The primary tumor tissues and brain metastatic lesions of breast cancer patients were formalin fixed and paraffin embedded and sliced to10 μm thickness. Tumor lesions were microdissected and total RNAs were isolated from samples using miRNeasy FFPE kit (Qiagen). Expression profiling of microRNA was performed by using the human miRNA

chip (GeneChip miRNA 1.0 Array). Clustering and its visualization were performed using Cluster3.0 and TreeView.

Real-time PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) and also from paraffin-embedded human tissue samples using miRNeasy FFPE RNA purification kit (Qiagen), respectively. TaqMan MicroRNA Reverse Transcription kit, High Capacity RNA-to-cDNA kit were used for microRNA and mRNA reverse-transcription (Applied Biosystems and BioRad). qPCR was performed by using TaqMan Universal Master Mix II and TaqMan microRNA assays or TaqMan gene expression assays (Applied Biosystems). A human RNU48 and actin TaqMan probes were used as endogenous controls for micro RNA and regular gene expression analysis, respectively.

Western blot

Western blot analysis was performed as described previously using antibodies against RhoC (1/500; Cell Signaling Technology), TNFα (1/1000; Cell Signaling Technology) and α-tubulin (1/1,000; Cell Signaling Technology)[40].

Immunohistochemistry

Human primary breast cancer samples and brain metastatic samples were obtained from CHTN (Cooperative Human Tissue Network). All of the tissue sections were obtained by surgical resection. Sections of 4 μm thickness were cut out from the formaldehyde-fixed and paraffin-embedded tissue specimens. The sections were deparaffinized and antigens were retrieved by heating the slides in 10 mM sodium citrate (pH 6.0) at 85°C for 30min. The slides were treated with 3% H_2O_2 and then incubated overnight at 4°C with anti-TNFα antibody (1/500; Cell Signaling Technology). The sections were then incubated with secondary antibodies and visualized using the Envision-plus kit (Dako Corp).

Immunocytochemistry

Cells were fixed with 70% ethanol, washed with PBS and blocked by 2%BSA for 1hr. After blocking, cells were washed again with PBS and incubated with anti-TNFα rabbit polyclonal antibody (1/500; Cell signaling Technology) overnight at 4°C. Cells were then incubated with anti-rabbit IgG Alexa Fluor (R) 555 molecular probe (1/500; Cell Signaling Technology) for 1hr at room temperature. Fluorescence images were taken by a fluorescent microscope.

3'UTR Reporter assay

Ten ng of reporter plasmid and 200ng of miR-509 expression plasmid were co-transfected with 1 ng of phRG-TK Renilla luciferase internal control plasmid (Promega) into 293TN cells using Lipofectamine 2000 reagent (Invitrogen). After 24 hours, luciferase activities were measured by using dual-luciferase reporter assay system (Promega).

Transfection

For reporter assay, cells were transfected with Lipofectamine 2000. For knock down of miR-509, cells were transfected with Locked nucleic acid (LNA) targeting miR-509 (Exiqon) using RNAifectin reagent (Applied Biological Materials) at the final concentration of 5nM.

Transmigration assays

For transmigration assay, 5×10^5 immortalized mBrEC and 5×10^5 human astrocytes were seeded on the both side of a trans-well insert (Corning, pore size 3 μm) and allowed to grow to confluence. Cancer cells were labeled with Cell tracker green (Life Technologies) and 5×10^4 cells were seeded into trans-well inserts supplemented with culture medium with 10% serum in triplicate. The bottom part of trans-wells was filled with same culture medium with 20% serum. After 24 hours, the total number of labeled cells that were invaded through the endothelial cells was counted under the fluorescent microscope.

Transendothelial electrical resistance (TEER) Assay

TEER was assessed in confluent mBMECs monolayers using an EVOM™ Epithelial Voltammeter (World Precision Instruments, Sarasota, FL). 5×10^5 Mouse brain endothelial cells and 5×10^5 human astrocytes were cultured in the both sides of the upper chamber of trans-well until they reach confluency. Conditioned medium from 231BrM, 231 BrM-miR-509 and recombinant TNFα were added into the upper chamber and TEER was measured every 24hrs.

Animal experiments

For experimental metastasis assay, nude mice (7–8 weeks) were injected with 10^5 or 5×10^5 luciferase-labeled cancer cells in PBS into left cardiac ventricle in a total volume of 100 μl. To confirm a successful injection, the photon flux from whole body of the mice was immediately measured using IVIS Xenogen bioimager (Caliper). The data was normalized by the brain photon measured at day 0, and the brain metastasis progression was monitored by quantifying the luminescence in the brain. For the brain metastasis-free survival analysis, we set the threshold as 2.5×10^5 (photon/s). At the endpoint of this study, whole brain was removed, incubated in RPMI-1640 medium with 0.6 mg/ml luciferin for 5 min and photon flux was measured.

MTS assay

Five thousand cancer cells were seeded in a 96-well plate for 48 hours. Cell proliferation was measured by the MTS assay every 24 hrs (Promega).

Wound healing assay

For the wound healing assay, confluent cells were scratched by a 1ml pipette tip, and migration of cells was monitored under microscopy after 24hrs. The % of wound healing was measured in three different fields of each cell line.

Statistical analysis

For *in vitro* experiments, T-test or one-way ANOVA was used to calculate the p-values. The Kaplan-Meier method was used to calculate the survival rates and was evaluated by the log-rank test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant support

This work was supported by the National Institutes of Health (grants R01CA124650, R01CA129000 and R01CA173499 to K. Watabe) and the US Department of Defense (BC096982 to K. Watabe).

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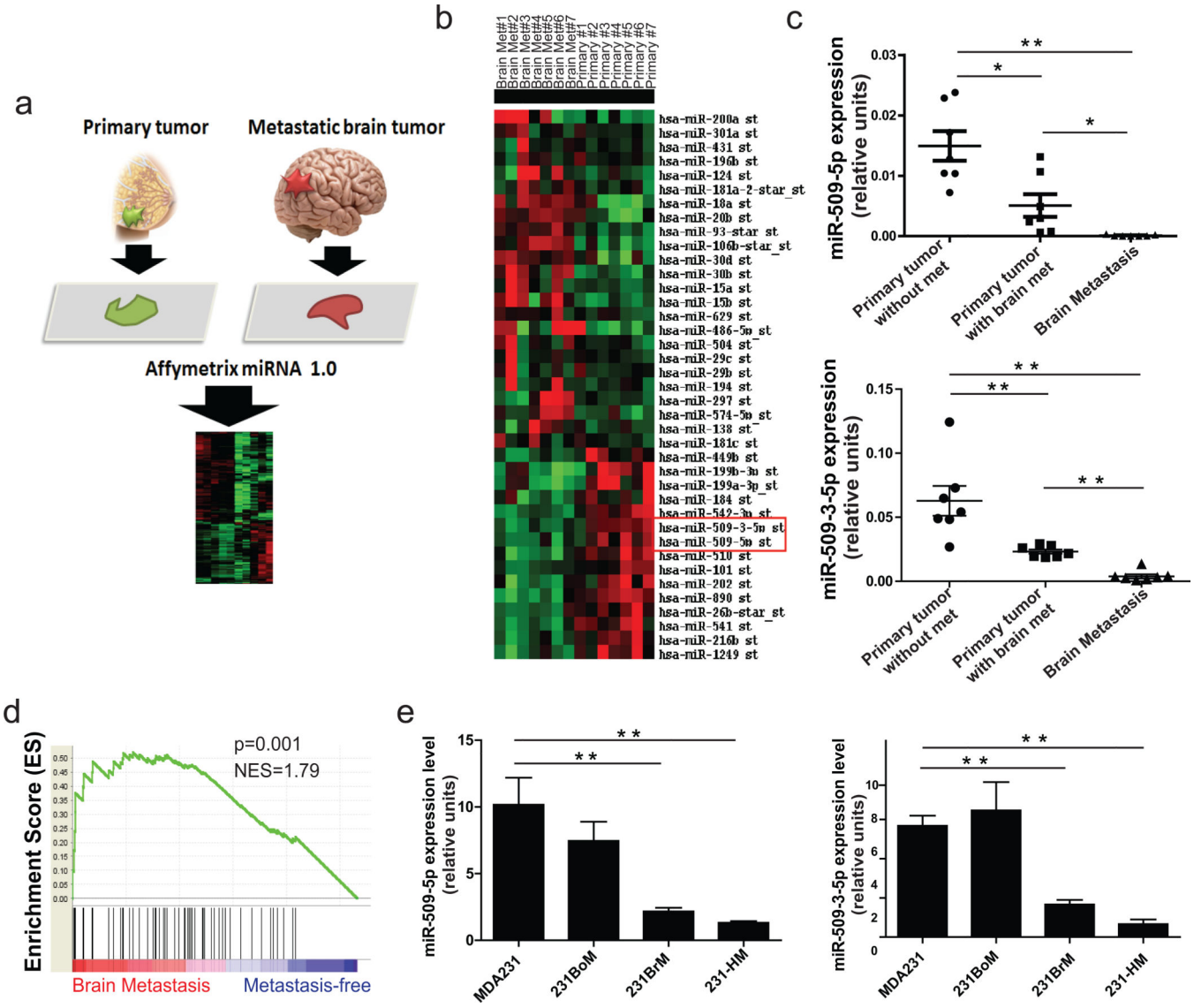

Figure 1. miR-509 is down-regulated in breast cancer patients with brain metastasis

(a) Schematic cartoon of miroRNA array analysis of primary and metastatic brain tumors (b) mRNAs were isolated from primary (n=7) and brain metastatic lesions (n=7), and they were subjected to microRNA 1.0 array analysis. A heatmap was generated for the microRNAs that were significantly up- or down- regulated in brain metastatic lesions compared to the primary tumors (cut off is 2-fold change and $p<0.05$). (c) The expression of miR-509-5p and miR-509-3-5p were examined by TaqMan qRT-PCR for primary breast tumors with or without brain metastasis (n=7) and brain metastatic lesions (n=7). (d) The target genes of miR-509 were chosen from the top 60 genes predicted by Target scan followed by GSEA (Gene Set Enrichment Analysis) between patients with or without brain metastasis. NES, normalized enrichment score. (e) The expression of miR-509-5p and miR-509-3-5p was examined by TaqMan qRT-PCR for, MDA231, 231BoM, 231BrM and 231-HM cells.* indicates $p<0.05$, ** indicates $p<0.001$

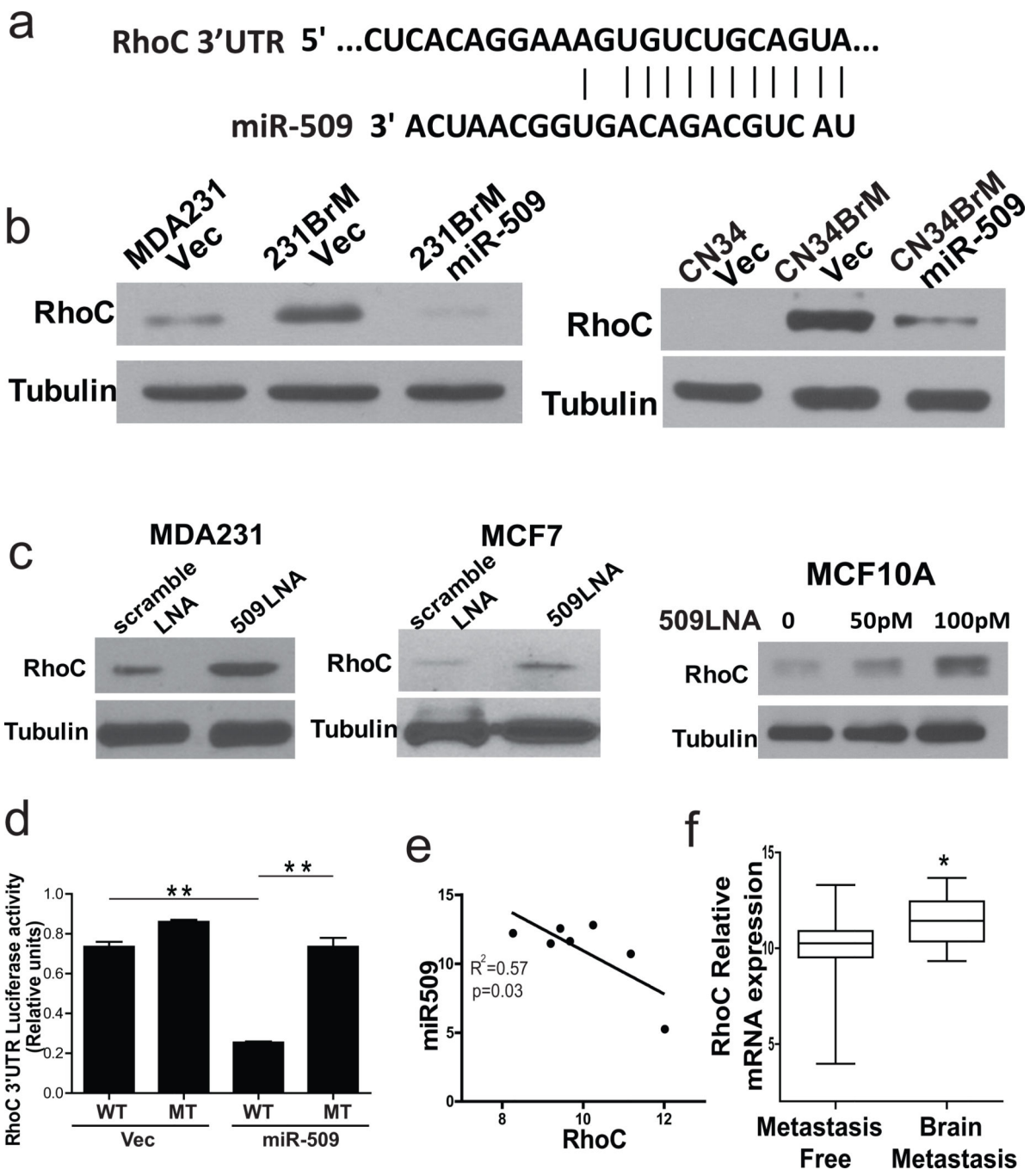

Figure 2. miR-509 directly targets RhoC

(a) Schematic representation of the RhoC 3'-UTR with a miR-509 binding site. (b) 231BrM (left panel) and CN34BrM (right panel) were infected with lentivirus expressing pre-miR-509 or vector only, and the expression of RhoC was examined by Western blot. (c) MDA231 (left panel) and MCF7 (middle panel) were transfected with miR-509 LNA (50pM) or scramble LNA, and the expression of RhoC was examined by Western blot. MCF10A (right panel) was transfected with various doses of miR-509 LNA, and the expression of RhoC was examined by Western blot. (d) Constructs carrying RhoC 3' UTR

luciferase reporter (WT) or deletion mutant of miR-509 binding site (MT) were transfected to 293TN cells with the miR-509 expression plasmid or vector plasmid. Cells were harvested and luciferase activities were measured after 24hrs transfection. (e) Expression of miR-509-5p and TNFα were measured by TaqMan PCR in brain metastasis samples after micro-dissection. The relative expression level of miR-509-5p to RNU48 and the expression level of RhoC to ACTB were plotted (n=7). (f) RhoC mRNA expression was measured between the primary tumors with or without brain metastasis in a combined GEO data bases (GSE12276, GSE2034, GSE2603, GSE5327, and GSE14020). * indicates $p<0.05$, ** indicates $p<0.001$

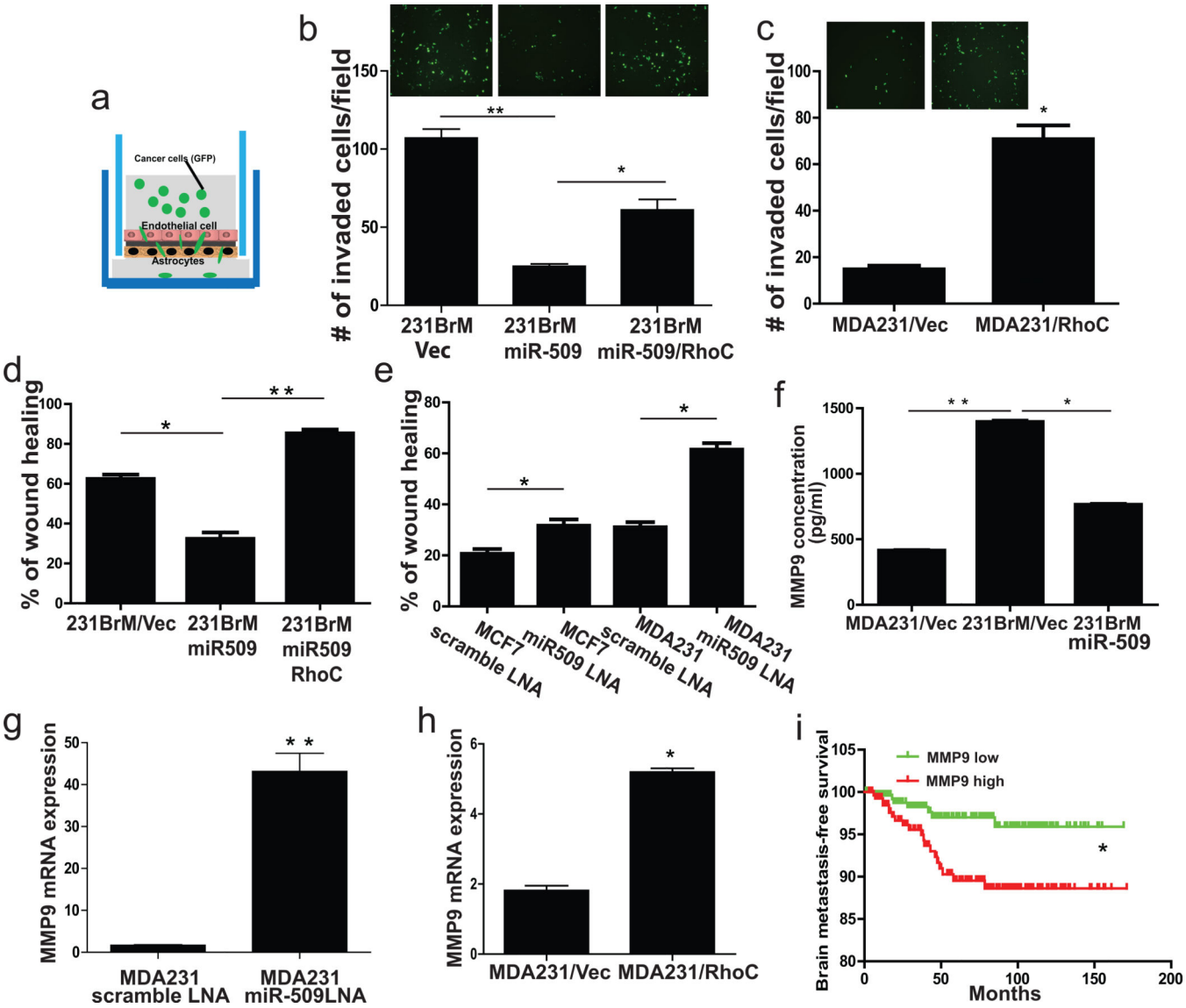

Figure 3. miR-509 suppresses the metastatic ability of 231BrM by inhibiting RhoC-mediated MMP9 expression

(a) A schematic diagram of BBB-mimic transwell assay. (b) 231BrM cells with the ectopic expression of pre-miR-509, or both pre-miR-509 and RhoC were seeded on top of a transwell insert coated with astrocytes and mouse brain endothelial cells on both sides, and the number of invaded cells was counted after 24hrs. (c) MDA231 cells with the ectopic expression of RhoC were seeded on top of a transwell and the number of invaded cells was counted after 24hrs. (d) Wound healing assays for 231BrM cells with the ectopic expression of pre-miR-509, or both pre-miR-509 and RhoC. (e) Wound healing assays for MCF7 and MDA231with or without miR-509 LNA transfection. (f) MMP9 expression was measured in 231BrM cells and MDA231 cells that were infected with lentivirus expressing miR-509 by ELISA. (g) MMP9 expression was measured in MDA231 cells that were transfected with miR-509-LNA or scramble LNA by qRT-PCR. (h) MMP9 expression was measured for MDA231 infected with lentivirus expressing RhoC by qRT-PCR. (i) Kaplan-Meier analysis

for brain metastasis-free survival of 710 breast cancer patients using a combined GEO data bases (GSE12276, GSE2034, GSE2603, GSE5327, and GSE14020). Patients were divided into two groups based on the expression status of MMP9 in their primary tumors. * indicates $p<0.05$, ** indicates $p<0.001$

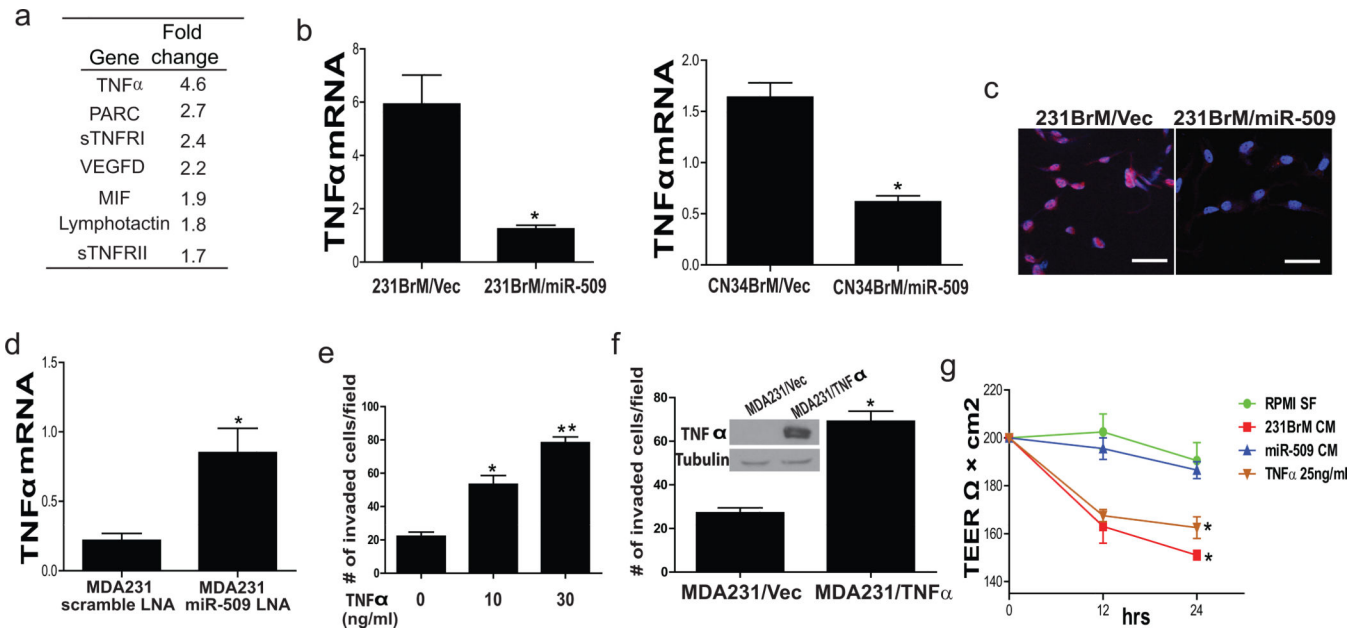

Figure 4. miR-509 suppresses brain metastasis by targeting TNFα

(a) Conditioned medium from 231BrM that were infected with lentivirus carrying vector or pre-miR-509 were subjected to cytokine array (RayBiotech) analysis. Fold change was measured by Image J software. (b) 231BrM (left panel) and CN34BrM cells (right panel) were infected with lentivirus expressing pre-miR-509, and the mRNA level of TNFα was measured by qRT-PCR. (c) 231BrM was infected with lentivirus expressing pre-miR-509, and the expression of TNFα was examined by immunocytochemical staining. (d) MDA231 was transfected with miR-509 LNA or scramble LNA, and the mRNA of TNFα was measured by qRT-PCR. (e) MDA231 cells were seeded on top of a transwell insert in the presence of various doses of TNFα, and the number of invaded cells was counted after 24hrs. (f) MDA231 cells with the ectopic expression of TNFα were seeded on top of a transwell insert, and the number of invaded cells was counted after 24hrs. Expression of TNFα was examined by Western blot (inserted fig). (g) Mouse brain endothelial cells were treated with conditioned medium of 231BrM, 231BrM-miR-509 and recombinant TNFα, the trans-endothelial electrical resistance was measured by EVOM^2 epithelial voltohmmeter. Bar, 100μm * indicates $p<0.05$, ** indicates $p<0.001$

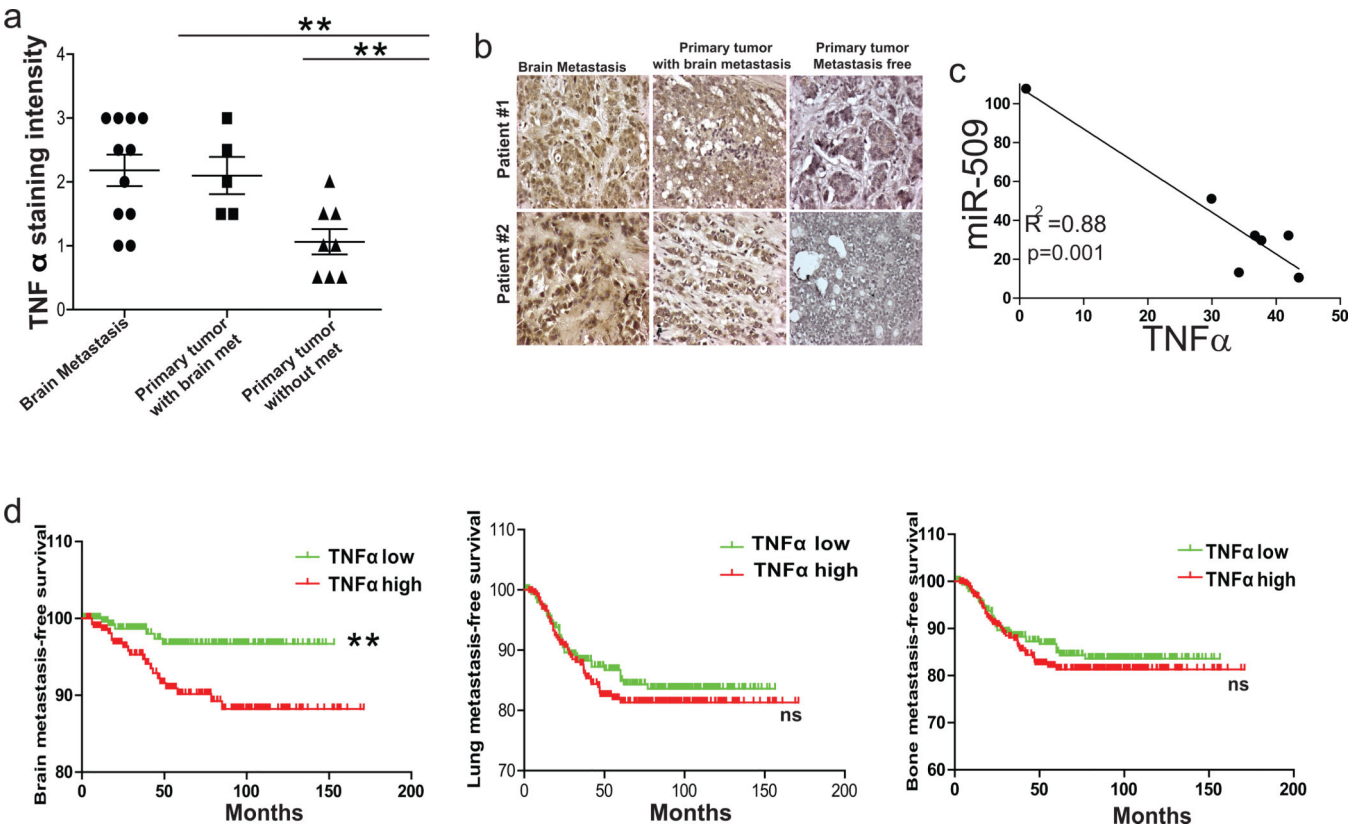

Figure 5. TNFα is up-regulated in brain metastatic lesion of breast cancer patients

(a)The expression of TNFα was measured by immunohistochemistry, and the staining intensity of TNFα in brain metastatic lesions, primary tumor with brain metastasis and primary tumors without distant metastasis were assessed (n=5–11). (b) Representative pictures of (a) were shown. (c) Expression of miR-509-5p and TNFα were measured by TaqMan PCR in brain metastasis samples after micro-dissection. The relative expression level of miR-509-5p to RNU48 and the expression level of TNFα to ACTB were plotted (n=7) (d) Kaplan-Meier analysis for brain and bone metastasis-free survival of 710 breast cancer patients using the combined GEO data bases (GSE12276, GSE2034, GSE2603, GSE5327, and GSE14020). Patients were divided into two groups based on the expression status of TNFα in their primary tumors. * indicates $p<0.05$, ** indicates $p<0.001$

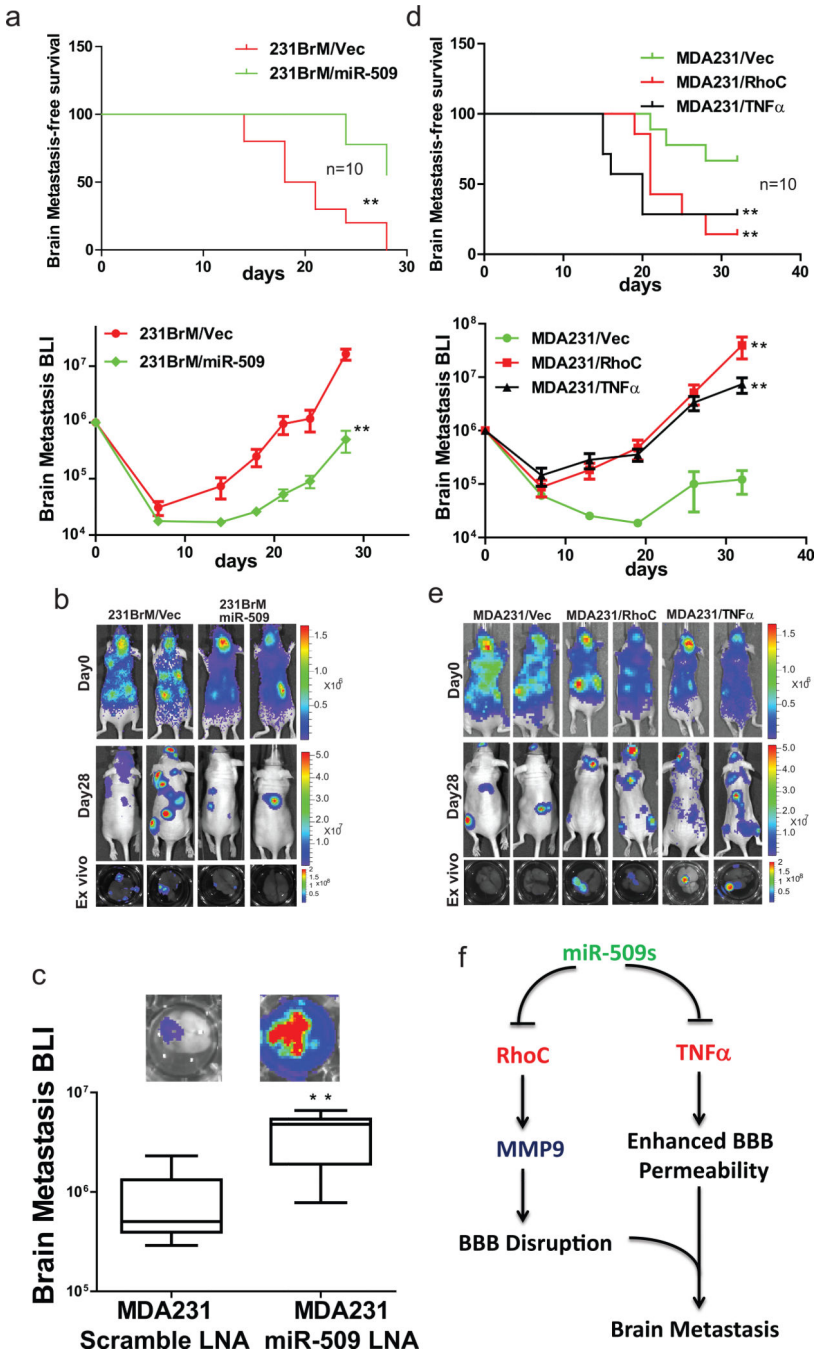

Figure 6. miR-509 suppresses brain metastasis *in vivo*

(a) Cancer cells were prepared from 231BrM/Vec and 231BrM/ miR-509 and 10^5 cells were intracardially injected into nude mice (n=10) followed by monitoring tumor growth by measuring the total photon flux in the brain. Upper panel; Kaplan-Meier analysis for brain metastasis-free survival of mice that were inoculated with 231BrM/Vec or 231BrM/ miR-509. Lower panel; total photon flux of brain metastatic lesions was measured by BLI at the time indicated. (b) BLI images of brain metastatic lesions of two representative mice from each experimental group. (c) Cancer cells were prepared from MDA231transfected

with scrambled LNA or miR-509 LNA, and 5×10^5 cells were intracardially injected into nude mice (n=10). At the end point, mice were sacrificed and the total photon of the brain was measured. (d) Cancer cells were prepared from MDA231/Vec, MDA231/RhoC and MDA231/TNFα and 5×10^5 cells were intracardially injected into nude mice (n=10) followed by monitoring tumor growth by measuring the total photon flux in the brain. Upper panel; Kaplan-Meier analysis for brain metastasis-free survival of mice that were inoculated with MDA231/Vec, MDA231/RhoC or MDA231/TNFα. Lower panel; total photon flux of brain metastatic lesions was measured by BLI at the time indicated. (e) BLI images of brain metastatic lesions of two representative mice from each experimental group. (f) Schematic model of miR-509 function in brain metastasis. * indicates $p<0.05$, ** indicates $p<0.001$