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MICROENVIRONMENT-INDUCED PTEN LOSS BY EXOSOMAL MICRORNA PRIMES BRAIN METASTASIS OUTGROWTH

Lin Zhang

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by

Lin Zhang, BS

APPROVED:

_____
Dihua Yu, M.D., Ph.D.
Supervisory Professor

_____
Mien-Chie Hung, Ph.D.

_____
Menashe Bar-Eli, Ph.D.

_____
Zhimin Lu, M.D., Ph.D.

_____
Xiaobing Shi, Ph.D.

APPROVED:

_____
Dean, Graduate School of Biomedical Sciences

The University of Texas Health Science Center at Houston

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A

DISSERTATION

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DOCTOR OF PHILOSOPHY

By

Lin Zhang, B.S.
Houston, Texas
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Dedication

The dissertation is dedicated to my dearest husband for his loving support!

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I would like to thank to my family. My parents and my sister gave me all their love and support. They respected all of my personal decisions. My husband is extremely supportive for my work. He has taken care of me very well over the past five years, especially while revising my paper. Without his understanding and support, I could not achieve my Ph.D. and stand here today. Finally, I would like to acknowledge my grandfather. He was my first mentor, and taught me how to write my first word and solve my first math problem. He showed me how to be successful in the life when I was a child. Thanks to my grandparents for their endless love.

ABSTRACT

MICROENVIRONMENT-INDUCED PTEN LOSS BY EXOSOMAL MICRORNA PRIMES BRAIN METASTASIS OUTGROWTH

Lin Zhang, B.S.

Supervisory professor: Dr. Dihua Yu, Ph.D., M.D.

Development of life-threatening cancer metastases at distant organs requires disseminated tumor cells' adaptation to and co-evolution with the drastically different microenvironments of metastatic sites. Cancer cells of common origin manifest distinct gene expression patterns after metastasizing to different organs. Clearly, the dynamic interplay between metastatic tumor cells and extrinsic signals at individual metastatic organ sites critically impacts the subsequent metastatic outgrowth. Yet, it is unclear when and how disseminated tumor cells acquire the essential traits from the microenvironment of metastatic organs that prime their subsequent outgrowth. Here we show that primary tumor cells with normal expression of PTEN, an important tumor suppressor, lose PTEN expression after dissemination to the brain, but not to other organs. PTEN level in PTEN-loss brain metastatic tumor cells is restored after leaving brain microenvironment. This brain microenvironment-dependent, reversible PTEN mRNA and protein down-regulation is epigenetically regulated by microRNAs (miRNAs) from astrocytes. Mechanistically, astrocyte-derived exosomes mediate an intercellular transfer of PTEN-targeting miRNAs to metastatic tumor cells, while astrocyte-specific depletion of PTEN-targeting miRNAs or blockade of astrocyte exosome secretion rescues the PTEN loss and suppresses brain metastasis *in vivo*. Furthermore, this adaptive PTEN loss in brain metastatic tumor cells leads to an increased secretion of cytokine chemokine (C-C motif) ligand 2 (CCL2), which recruits Iba1+ myeloid cells that reciprocally enhance outgrowth of brain metastatic tumor cells via enhanced

proliferation and reduced apoptosis. Our findings demonstrate a remarkable plasticity of PTEN expression in metastatic tumor cells in response to different organ microenvironments, underpinning an essential role of co-evolution between the metastatic cells and their microenvironment during the adaptive metastatic outgrowth. Our findings signify the dynamic and reciprocal cross-talk between tumor cells and the metastatic niche; importantly, they provide new opportunities for effective anti-metastasis therapies, especially of consequence for those brain metastasis patients who are in dire need.

Table of Contents

ABBREVIATIONS

TWIST1	Twist family BHLH transcription factor 1
SNAI1	Snail Family Zinc Finger 1
SNAI2	Snail Family Zinc Finger 2
EMT	epithelial-mesenchymal transition
PTGS2	prostaglandin G/H synthase 2
EGER	epiregulin
MMP1	matrix metalloproteinase 1.
PTHRP	parathyroid hormone-related protein
IL-11	interleukin-11
IL-6	interleukin-6
TNF-α	tumor necrosis factor-α
TGF- β	transforming growth factor-β
YAP	yes-associated protein
CUP	cancer of unknown primary
TME	tumor micro-environment
CAF	cancer associated fibroblast
αSMA	α-smooth muscle actin
PDAC	pancreatic ductal adenocarcinoma
CTC	circulating tumor cells
DTC	disseminated tumor cells
NK	natural killer
TH1	type 1 T helper cell
BMPs	bone morphogenetic proteins
HSC	hematopoietic stem cells
CXCL12	chemokine (C-X-C Motif) ligand 12
RANKL	receptor activator of nuclear factor kappa-B ligand
OPN	osteopontin
CLDN2	claudin-2
PyMT	polyoma virus middle T antigen

MMTV	mouse mammary tumor virus
MHC I	major histocompatibility complex class I
LMP	latent membrane protein
TAMs	tumor associated macrophage
CSF-1	colony stimulating factor 1
CSF-1R	CSF-1 receptor
CCL2	chemokine (C-C motif) ligand 2
CCR2	CCL2 receptor
miR, miRNA	microRNA
SRS	stereotactic radiosurgery
WBRT	whole-brain radiotherapy
BBB	blood-brain barrier
CTSS	cathepsin S
COX2	cyclooxygenase-2
ST6GaLNac5ST6	N-acetylgalactosaminide alpha-2,6-Sialyltransferase 5
HBEGF	heparin-binding EGF-like growth factor
MMP2	matrix metalloproteinase-2
MMP9	matrix metalloproteinase-9
LICAM	L1 cell adhesion molecule (L1CAM)
VEGF	vascular endothelial growth factor
FasL/FASLG	cytokine Fas ligand
PA	plasminogen activator
MIF	macrophage inhibitory factor
IL-8	interleukin-8
PAI-1	plasminogen activator inhibitor 1
PDGFRβ	beta-type platelet-derived growth factor receptor
IL-1β	interleukin-1 beta
EGFR	epidermal growth factor receptor
S100A	S100 calcium-binding protein A1
cGAMP	2′3′-cyclic GMP-AMP

IL-1	interleukin-1
NO	nitric oxide
EGF	epidermal growth factor
GABA	γ-aminobutyric acid
GABAAR	type A GABA receptor
LCC	latency competent cancer cells
CNS	central nervous system
MVB	multi-vesicular body
Alix	ALG-2-Interacting Protein X
TSG101	tumor susceptibility gene 101
EVs	extracellular vehicles
PM	plasma membrane
ESCRT	endosomal sorting complex required for transport
SNARE	NSF-attachment protein receptor complex
Tspan8	tetrapanin 8
CML	chronic myeloid leukemia
Dll4	Notch ligand Delta-like 4
EBV	B-Epstein–Barr virus
DCs	dendritic cells
Tregs	regulatory T cell
MDSC	myeloid-derived suppressor cell
FasL	FAS ligand (FasL)
TRAIL	TNF-related apoptosis-inducing ligand (TRAIL)
PCP	planar cell polarity
HIFα	hypoxia-inducible factor alpha
MMP 13	matrix metalloproteinases 13
LMP1	latent membrane protein 1
Lamp2b	lysosome-associated membrane protein 2b
GPC1	glypican-1
PTEN	phosphatase and tensin homolog deleted on chromosome 10

PHTS	PTEN hamartoma tumor syndromes
PTP	protein tyrosine phosphatase
TCGA	the cancer genome atlas
LOH	loss of heterozygosity
EGR-1	early growth response protein 1
PPARγ	peroxisome proliferator-activated receptor γ
NF-κβ	nuclear factor-κB
lncRNAs	long non-coding RNAs
NHERF1	sodium-hydrogen exchanger regulatory factor 1
NHERF2	sodium-hydrogen exchanger regulatory factor 2
PHLPP1	PH domain leucine-rich repeat protein phosphatase 1
PI3K	phosphatidylinositol-3-kinase
SHARPIN	shank-interacting protein-like 1
MAN2C1	mannosidase Alpha Class 2C Member 1
PI (3,4,5)P3	phosphatidylinositol (3,4,5)-trisphosphate
PI (4, 5) P2	phosphatidylinositol 4,5-bisphosphate
PI3K	phosphatidylinositol-3-kinase
Bim	Bcl-2 interacting mediator of cell death
TSC2	tuberous sclerosis complex 2
CREB	cAMP response element binding protein
SHC	Src Homology 2 Domain Containing
APC/C	the anaphase-promoting complex
CENP-C	centromeric protein-C
PTEN-L	PTEN-long
FAK	focal adhesion kinase
CRC	colorectal cancer cell
CAF	cancer associated fibroblasts
MFP	mammary fad pad
ICA	intracarotid artery
RPPA	reverse phase protein array

FPPE	formalin fixed and paraffin-embedded
TMA	tissue microarray
IHC	immunohistochemistry
IF	immunofluorescence
IRS	immunoreactive score
TNBC	triple negative breast cancers
HR	hormone receptor
UTR	untranslated region
EpCAM	epithelial cell adhesion molecule
DMA	dimethyl amiloride
shRNA	short hairpin RNA
Dox	doxycycline
RFP	red fluorescent protein
GFP	green fluorescent protein
PDTC	pyrrolidine dithiocarbamate
IBA1	ionized calcium-binding adapter molecule 1
CSF	cerebrospinal fluid

LIST OF FIGURES

LIST OF TABLES

Chapter 1: Introduction

1.1 Metastasis

Metastasis, the dissemination of cancer cells to second organs, is an important hallmark of cancer. About 90% of cancer patient death are caused by metastasis [1]. While therapeutic efficacy in the control of primary tumors has improved, effective methods to prevent or treat metastatic disease remain rare. Metastasis is an extremely complex process including multiple steps: local invasion, escape to the circulation (intravasation), survival and transport in the circulation, exit from the circulation to the parenchyma of organ (extravasation), and colonization and outgrowth in the distant organs [2, 3] (Fig.1). Understanding the mechanism of metastasis will serve as the base of effective prevention and treatment for metastasis. Dr. Joan Massague and colleagues sorted the metastatic related genes into three groups: metastasis "initiation genes", "progression genes" and "virulence genes" [3]. Metastasis initiation genes regulate cancer cell intravasation. These genes, such as twist family BHLH transcription factor 1 (TWIST1) and snail family zinc finger 1 and 2 (SNAI1 and SNAI2), promote cell mobility or epithelial-mesenchymal transition (EMT). Progression genes control the extravasation of disseminated cells, and include prostaglandin G/H synthase 2 (PTGS2), epiregulin (EREG), and matrix metalloproteinase 1 (MMP1). Meanwhile, virulence genes modulate the metastatic cells' colonization and growth in the distant organs, and include parathyroid hormone-related protein (PTHRP), interleukin-11 (IL-11), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). However, these gene categories are not absolutely exclusive. Some genes are involved in different steps of metastasis. For example, transforming growth factor-β (TGF- β) is well known to promote cell intravasation by EMT [4, 5]. However, TGF- β also contributes to the tumor growth in the metastatic organs by inducing angiogenesis and inhibiting immune reactions [6-9]. Furthermore, whether a gene has metastasis promoting properties or suppressing properties is

largely cancer type-dependent. Nuclear yes-associated protein (YAP) promotes tumor progression and metastasis in breast cancer [10, 11], whereas cytosolic YAP restricts the growth of colon cancer [12].

Figure 1

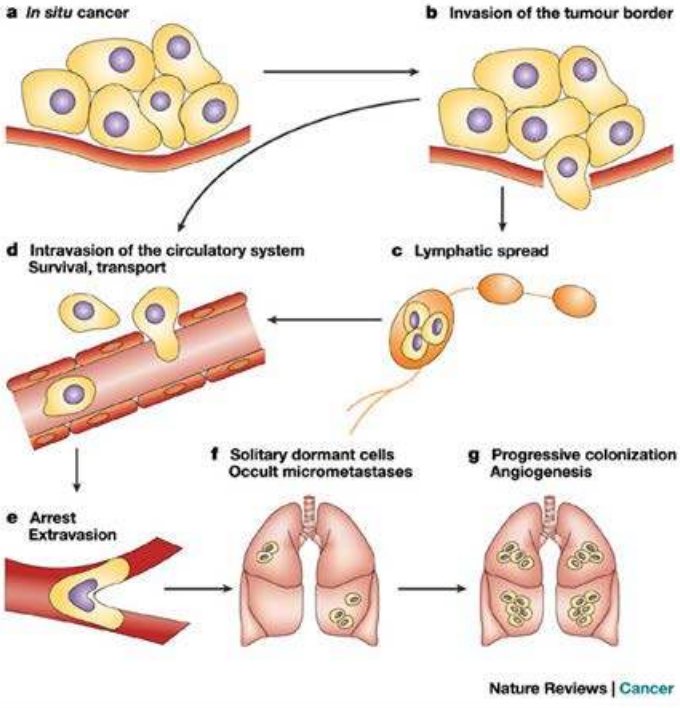


Figure 1. **| The multistep process of metastasis.** A schematic of the metastatic process includes several challenging steps. It begins with local invasion, then tumor cells enter the circulation (intravasation) via lymphatics or directly enter the blood vessel, survive and transport in the circulation, exit from the circulation to the parenchyma of organ (extravasation), followed by colonization and outgrowth in the distant organs. Metastatic cells in a distant site progresses might remain dormant for years, and then progressively grow to generate macrometastases. *Reprinted with permission from Patricia S. Steeg "Metastasis suppressors alter the signal transduction of cancer cells."* Nature Reviews Cancer *2003. **3**: 55-63.*

1.1.1 The characteristics of metastasis

Metastasis has several striking features. First, the pattern of metastasis in distant organs is cancer type-specific [3, 13]. For example, prostate cancer predominantly metastasizes to bone, whereas pancreatic cancer normally metastasizes to liver and lungs. This phenomenon can be partly explained by "seed and soil" hypothesis [14], which suggests that disseminated cancer cells can colonize in selective distant organs which have similar growth environment as primary organs. The detail of this hypothesis will be discussed later. However, "seed and soil" hypothesis does not include the exact mechanism for this organ tropism. The possible mechanism is that a subgroup of primary tumor cells might contain genes with tissue-specific metastatic tendency [15, 16] or stromal cells educate tumor cells for tissue-specific gene expression [17].

The second feature for metastasis is that the duration of metastatic dormancy in distant organs differs among tumor types. Brain metastasis originating from lung cancer can be detected as the same time as the diagnosis of the primary tumor. However, distant metastases of breast cancer are often detected several years after the removal of primary tumor. Short latency of metastatic relapse might indicate that metastatic cells hold strong competency to colonize, and overcome obstacles for growth in the distant organs; long latency of metastatic relapse indicates that the distant microenvironment inhibits tumor growth or cell death counterbalance the cell proliferation[18]. Although latent metastatic cells do not grow fast for long periods after extravasation, they reside there and wait for the "kindling" to generate macro-metastasis. Metastasis to different organs requires distinct abilities to infiltrate and colonize. Meanwhile, the relatively short latency of metastatic relapse also raises the question about the metastasis model: is it linear or parallel evolution? In 2-10% of clinical cases, one or more metastatic sites are detected without a clear determined primary site, which is termed as "cancer of unknown

primary" (CUP) [19]. Obviously, CUP and the short latency of metastatic relapse oppose the traditional model of metastasis, which considers metastasis as a late-arising phenotype in the linear progression model. However, metastasis might occur in the early stage of tumorigenesis in parallel with the primary tumor regardless of the size of primary tumors [20]. So far, there is not a clear picture of the metastasis model, but it seems most likely that linear and parallel models co-exist.

Recently, two other general concepts of metastasis have been challenged. The first question is whether EMT is required for metastasis. Generally, EMT was considered to be crucial for intravasation of carcinoma, because EMT can reduce the intracellular adhesion of epithelial cells, and therefore enhance cancer cells' motility and invasiveness [21]. However, recent studies of breast cancer [22] and pancreatic cancer [23] showed that although EMT contributed to drug resistance, it was not necessary for metastasis progression. It was found that the majority of metastatic tumors was derived from epithelial-like cells, rather than cells with mesenchymal phenotypes. Moreover, inhibition of the EMT-inducing genes did not change metastasis rate. As a result the role of EMT in metastasis thereby became controversial and should be investigated further.

The second evolving concept regards the status of cancer cells migrating in the blood vessels. The conventional view is that cancer cells migrate clonally or with platelets while travelling to the distant organs. However, there is growing evidence supporting that metastatic cells migrate collectively as a cluster and the clusters are more efficient than single cell to produce metastasis. The different clonal combination in the cluster can promote mutual survival and the success of metastasis [24-26].

In addition, the influence of tumor micro-environment (TME) on tumor progression and metastasis has gained more attention. Cancer associated fibroblasts (CAFs), normal fibroblasts, immune cells, and the vascular network all play roles in tumor growth, invasion and metastasis. However, their effects can be both pro-tumorigenic and anti-tumorigenic. For example, classically activated M1 macrophages exert anti-tumorigenic effect, whereas alternatively activated M2 macrophages exert opposite effect to promote tumorigenesis [27, 28]. Generally CAF is considered to promote tumor growth and metastasis [29, 30]. However, depletion of α-smooth muscle actin (αSMA)-positive CAFs in pancreatic ductal adenocarcinoma (PDAC) enhanced EMT and tumor progression [31]. Therefore, the individual and integral functions of these stromal cells in tumor progression are dynamic and context-dependent. It is a challenge to target or re-educate the heterogeneity of TME for tumor treatment, but new avenues for cancer therapy could emerge in light of the growing availability of TME-targeting drugs in clinical phase [32, 33].

In the process of metastasis, organ colonization is the rate-limiting and the least efficient step. Most disseminated cells die very soon after they enter the new organs. In my thesis, I focus on how the interaction of TME with disseminated tumor cells promotes tumor growth in second organs.

1.1.2 The interaction of TME with disseminated tumor cells

The "seed and soil" hypothesis might be the first theory to explain the relationship between tumor cells and tumor microenvironment. Stephen Paget, a surgeon and pathologist, first claimed that metastasis did not occur randomly in 1889. According to his observations, one type of cancer prefers to seed at specific secondary distant organs [14, 34]. This hypothesis was validated and advanced by Dr. Isaiah Fidler. The "seed" refers to the disseminated cancer cells or

cancer stem cells, and the "soil" to the specific TME. Successful metastasis depends not only on the intrinsic factors of cancer cells, but also on the "nourishment" that cancer cells can get from the microenvironment [14]. Most circulating tumor cells (CTC) and disseminated tumor cells (DTC) can invade into secondary organs, but cannot survive nor generate macro-metastasis. This indicates that the "soil" is not very welcoming to most of the "seeds," or most seeds do not contain the intrinsic traits to overcome the obstacles for outgrowth in the new "soil" [35, 36].

How does the microenvironment protect from the invasion of tumor cells? The first defense system is immune cells, including cytotoxic T cells, natural killer (NK) cells and Type 1 T helper cells' (TH1) cytokines (interferon-γ or interleukin-12) [37]. Depletion of NK cells promotes breast cancer liver metastasis [38, 39], breast cancer brain metastasis, and lung cancer bone metastasis[40]. Microglia are the most important immune cells in the central nervous system. Activated microglia induces cytotoxicity to cancer cells [41]. Second, stromal cells in the microenvironment can also defend the disseminate cancer cells. The stromal cells release growth inhibitory factors, such TGF-β and bone morphogenetic proteins (BMPs), to hinder the tumor growth in lung metastasis or bone metastasis [42-44].

However, the microenvironment also facilitates the growth of disseminated tumor cells under certain circumstances. One good example is in the bone marrow. Bone marrow stromal cells prepare a niche for hematopoietic stem cells (HSC)' seeding through protein interactions and secreted cytokines, such as chemokine (C-X-C Motif) ligand 12 (CXCL12), receptor activator of nuclear factor kappa-B ligand (RANKL), osteopontin (OPN) or BMPs [45, 46]. Likewise, metastatic cells can appropriate the same mechanism to seed into bone marrow [45, 46].

The TME controls the fate of metastatic cancer cells. Reciprocally, cancer cells also actively modulate or educate the microenvironment. Breast cancer cells interact with liver stromal cells by claudin-2 (CLDN2) to educate hepatocytes to support tumor growth [47]. In the MMTV-PyMT mouse model, which expresses the oncogene of polyoma virus middle T antigen (PyMT) driven by the mouse mammary tumor virus (MMTV) promoter/enhancer, breast cancer lung metastatic cells stimulate the extracellular-matrix protein periostin in lung fibroblast by TGF-β. Periostin, together with tenascin C, stimulates the WNT pathway in tumor cells [48, 49], thereby promoting tumor colonization in the lung.

Dormant cancer cells can escape immunosurveillance by the loss of major histocompatibility complex class I (MHC I) or latent membrane protein (LMP) expression [50]. Cancer cells recruit tumor-associated macrophages (TAMs) or tumor-associated lymphomonocytes to evade immunity and enhance tumor growth. TAM or inflammatory monocytes mediate breast cancer cell extravasation and outgrowth in the lung [51-53]. Prevention of the recruitment of TAM or inflammatory monocytes by the inhibition of colony stimulating factor 1 (CSF-1) and its receptor (CSF-1R) [54], or chemokine (C-C motif) ligand 2 (CCL2) and its receptor (CCR2) [51], can repress tumor growth or metastasis.

Primary tumors can release systemic signals, such as cytokines or exosomes, and therefore prepare metastatic sites, which are called pre-metastatic niches. Recently published works endorse the importance of exosome in metastasis. The type of integrins embedded in exosomes pre-determine the breast cancer metastatic organ pattern [55]. Melanoma exosomes can induce inflammation in the distant organs to build pre-metastatic niches [56]. On the other hand, exosomes containing microRNAs (miR-23b, miR-192, or miR-143) can inhibit metastasis [57-59].

Therefore, the dynamic and bidirectional interplay between disseminate cancer cells and metastatic tumor microenvironment is critical to subsequent cancer colonization and outgrowth. I will discuss brain microenvironment and brain metastasis later in detail.

1.1.3 Brain metastasis

In the United States, brain metastasis affects millions of cancer patients each year, at numbers ten times more than primary brain tumors [60]. The incidence of brain metastases is the highest in patients with lung cancer, with nearly 40-50% of patients with lung cancer developing brain metastasis during the course of disease. Breast cancer (25%) and melanoma (20%) have the second- and third-highest rates of brain metastases [61, 62]. Other cancers, such as renal cell carcinoma, gastrointestinal carcinoma and uterine/vulvar carcinoma, also have the possibility to develop brain metastasis [63]. The median survival of all types of brain metastasis is 1 to 2 months. Even after conventional radiotherapy and chemotherapy, the median survival can only be extended to six months [64]. Current treatment for brain metastases are surgery and radiation therapy. Surgery is only feasible for very small lesions in accessible regions. Stereotactic radiosurgery (SRS) and whole-brain radiotherapy (WBRT) are the common alternative treatments for brain metastasis. Unfortunately, both treatments are palliative with severe cognitive complications and associated with poor quality of life. Therefore how to diagnose brain metastasis early and treat it effectively are two most imposing challenges in the clinic [65].

Except for lung cancer brain metastasis, brain metastasis normally happens at the advanced stage with a long latency before detection. The brain is a special organ with unique immune characteristics. One of its important defense systems is the blood-brain barrier (BBB) (Fig. 2). The BBB is composed of endothelial cells surrounded by pericytes, a basement membrane, and astrocytes. The endothelial cells tightly connect to each other by tight junctions

and express active efflux pumps, both of which limit the entrance of large molecules, including chemical drugs, and microorganisms, into the brain parenchyma. However, the BBB can be compromised by dissemination of cancer cells into the brain. To cross the BBB, cancer cells secrete miR-105, cathepsin S (CTSS), cyclooxygenase-2 (COX2), ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 5 (ST6GaLNac5), heparin-binding EGF-like growth factor (HBEGF), matrix metalloproteinase-2 (MMP2), and matrix metalloproteinase-9 (MMP9), which help break the tight joints of the BBB [66-68]. The BBB seems to be intact when tumor is smaller, but the dissociation of astrocytes and pericytes with blood vessel happens and the BBB becomes leaky when a tumor is larger than 0.25 mm^2 [69, 70].

After extravasation, brain metastatic cells express L1 cell adhesion molecule (L1CAM) [71] or integrin β1 [72] for adhesion to brain capillaries. This adhesion seems the key for the initiation of tumor growth in the brain. Brain metastasis generally displays neovascularization or vascular remodeling [69]. Vascular endothelial growth factor (VEGF) has a vital role in the angiogenesis of some brain metastasis [69]. Anti-VEGF treatment inhibits the progressive growth of brain metastasis of lung carcinoma, but not those of melanoma, in experimental models [69, 73].

Figure 2

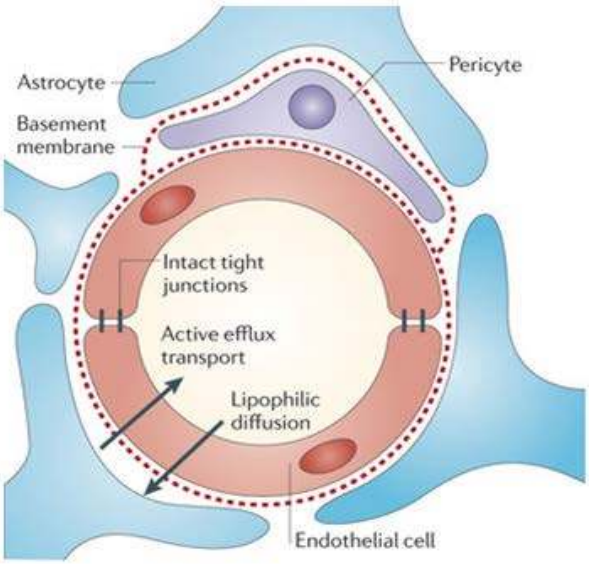


Figure 2. | The composition of BBB. The blood-brain barrier (BBB) is a selective permitting access to only certain substances in order to protect the normal brain from pathogens. It is formed by brain endothelial cells, tightly connected by continuous tight junctions. Pericytes and the astrocyte foot cover the surface of endothelial cells by the basement membrane. Endothelial cells can actively pumps drugs, microorganisms, or other species back into the blood, but lipophilic substance can pass through BBB and diffuse into brain. *Reprinted with permission from Patricia S. Steeg, Kevin A. Camphausen & Quentin R."Brain Metastases as preventive and therapeutic targets."* Nature Reviews Cancer *2011.5: 352-363.*

Indeed, the bidirectional interaction of cancer cells and brain TME is poorly understood and understudied (Fig. 3). There are mainly three types of brain stromal cells in the unique brain environment: astrocytes, microglia, and neurons. They have both positive and negative effects on brain metastasis.

Figure 3

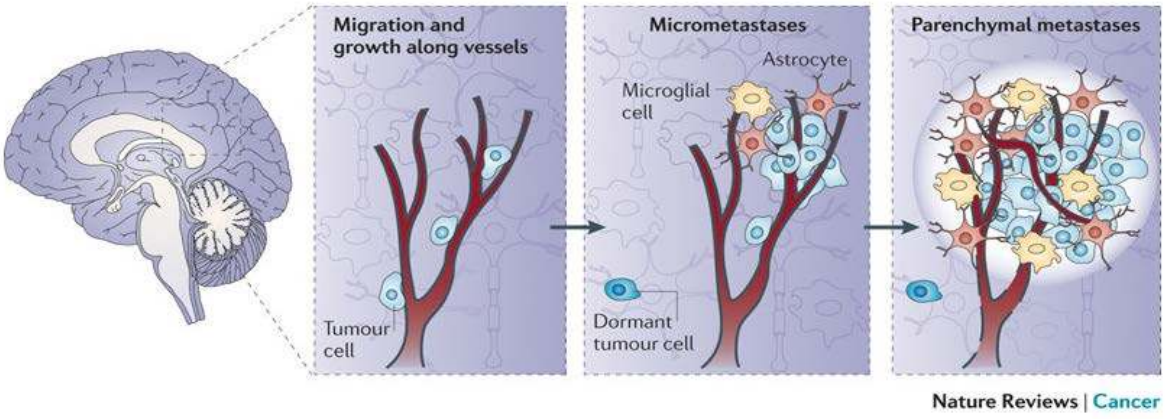


Figure 3. | Process of brain metastases and the interaction between brain TME and tumor cells. After brain metastatic cancer cells traverse from blood vessel, they attach to the outside of vessels. Tumor cells' extravasation induces gliosis. Activated microglia or macrophage-like cells (shown in yellow) and astrocytes (shown in orange) provide resistance or support for tumor cells. As the metastasis expands, the local brain function is damaged, including neuronal damage, edema (white halo) and the disruption of the blood–brain barrier (shown in dashed black lines). Dormant solitary tumor cells can also reside in the brain, and they have the potential to develop additional metastases. *Reprinted with permission from Patricia S. Steeg, Kevin A. Camphausen & Quentin R."Brain Metastases as preventive and therapeutic targets."* Nature Reviews Cancer *2011.5: 352-363.*

Astrocytes originally were considered as a line of defense against tumor cells. Brain metastasis causes reactive astrogliosis. Activated astrocytes kill the disseminated cancer cells by releasing the proapoptotic cytokine Fas ligand (FasL/FASLG), which is activated by plasminogen activator (PA). However, brain metastatic cells express anti-PA serpins that protect cancer cells from the threat of astrocytes [71]. On the other hand, astrocytes can also play an active supporting role to tumor cells. Activated astrocytes are recruited by tumor-derived cytokines, such as macrophage inhibitory factor (MIF), interleukin-8 (IL-8), and plasminogen activator inhibitor 1 (PAI-1), to congregate around the developing metastasis [74, 75]. These recruited astrocytes secrete beta-type platelet-derived growth factor receptor (PDGFRβ) [76], IL-6 [75, 77], interleukin-1 beta (IL-1β), and TNF-α [75], or upregulate JAG-1, the ligand of NOTCH 1 receptor, to activate the NOTCH pathway in cancer cells [74], subsequently promoting colonization and tumor growth. Astrocytes can be further activated by estrogen to promote the proliferation, migration, and invasion of cancer cells by epidermal growth factor receptor (EGFR)-S100 calcium-binding protein A1 (S100A) pathway [78]. Recently it was reported that tumor cells transferred 2′3′-cyclic GMP-AMP (cGAMP) to astrocytes by gap junctions, activating the STING pathway in astrocytes, which in turn released inflammatory cytokines (interferon α 1, TNF) to promote brain metastasis growth[79]. Importantly, the application of gap junction inhibitor dramatically decreased brain metastasis. Astrocytes also can protect tumor cells from chemotherapy [80]. Under paclitaxel treatment, astrocytes induced elevated levels of p-AKT and p-MAPK in tumor cells, thereby inhibiting tumor cells' apoptosis and prolong their survival [80].

Microglia are considered the immune effector cells in the brain, but the role of microglia in brain tumor or brain metastasis is not clear. Activated microglia can induce antigen-mediated

lymphocyte reaction [81] and produce a variety of chemokines and cytokines, such as interleukin-1 (IL-1), IL-6, TNF-α, TGF-β), nitric oxide (NO), and superoxide. NO was revealed to be able to kill tumor cells [82-84]. However, it was also reported that activated microglia promoted metastasis outgrowth by WNT pathway [85], lead to angiogenesis by VEGF and promoted tumor proliferation by epidermal growth factor (EGF) [86, 87]. In addition to resident microglia, infiltrating macrophages or monocytes can also have positive roles for tumor growth in the brain. M2-polarized TAMs (including microglia) supported the growth of high-grade gliomas as inhibiting CSF-1R resulted in drastic tumor regression in one study [88]. Although it remains elusive whether this treatment has similar effect on brain metastasis, this study indicates microglia have positive impact on the growth of tumor cells. On the contrary, another study showed direct depletion of TAM (including microglia) in the tumor promoted glioma tumor growth [89]. In this study ganciclovir was injected to deplete macrophages in transgenic mice that expressed thymidine kinase under the macrophage/microglia specific CD11b promoter. Thus, it seems microglia/macrophages have opposite functions in these two studies, but the different consequences in these two studies might have resulted from the different methods and time points of depletion of macrophages.

The relationship between neuron and brain metastasis is rarely studied. One study showed that brain metastatic cells may upregulate γ-aminobutyric acid (GABA) transporters, type A GABA receptor ($GABA_AR$), and GABA transaminase to take up and catabolize neuron-released GABA neurotransmitter as a metabolite, supporting tumor outgrowth in the brain [90].

Although the mechanisms of brain metastasis development are being revealed gradually, the whole landscape is still under extensive investigation. Several questions still need to be addressed: 1) it is not clear that how the quiescent tumor cells in the brain acquire the essential

traits from the brain microenvironment that prime their subsequent outgrowth, and what the exact function of brain stromal cells is for brain metastasis. Dr. Joan Massague's group showed that latency-competent cancer cells (LCC) maintained their slow-cycling by secretion of WNT inhibitor and evading NK-cell-mediated clearance [40], but it is not clear how single or small cluster of LCCs generate macro-metastases. The continuous crosstalk between the brain microenvironment and LCC at the late stage of latency is essential for tumor growth in the brain. 2) It is unknown whether brain metastases hijack metabolic mechanism to utilize nutrients from the unique brain microenvironment. It was reported that brain tumors had the special ability to oxidize acetate in the citric acid cycle to generate the fuel for growth [91]. Breast cancer brain metastatic cells can survive and proliferate by gluconeogenesis, or the oxidation of glutamine and branched chain amino acids, independent of glucose [92]. However, these reports only uncover the tip of the iceberg of the metabolic changes that may benefit brain metastasis. We are curious to explore a more complete picture of metabolism utilized by brain metastasis in the future. 3) The discovery of lymph vessels in the outer layers of brain [93] provides a new insight to study immune responses in the central nervous system (CNS) and the potential to explore how brain metastatic cells evade the immune surveillance in the brain. Several groups detected bone marrow-derived macrophages in the brain and studied their function as well [88]; however, the functions of other immune cells, such as neutrophils, monocytes, T-cells, and NK-cells in brain metastasis, remain to be elucidated.

My project focuses on the crosstalk between the brain TME and tumor cells and attempts to answer the question of how the quiescent tumor cells in the brain acquire the essential traits from the brain microenvironment to promote their growth.

1.2. Exosomes and cancer

1.2.1 Exosome composition and regulation

Exosomes, one specific subtype of membrane vesicles, arise from the membranes of multi-vesicular body (MVB) [94, 95]. Exosomes are 50-150 nm in diameter. Exosomes can be isolated by high speed centrifugation (100,000g) and then floated at sucrose density from 1.13 to 1.19g/ml. CD63, ALG-2-interacting protein X (Alix), tumor susceptibility gene 101 (Tsg101), and proteasome component Hsc10 are highly enriched in the exosomes [96]. Another type of membrane-enclosed vesicle is extracellular vesicles (EV), which are also called microvesicles, microparticles, ectosomes, or oncosomes. They germinate from plasma membrane (PM), and the sizes of these EVs are 100-1000nm in diameter [97]. Although exosomes and EVs come from different origins, they have similar abilities to communicate with other cells and modulate local/distant microenvironment.

Exosome biogenesis and formation is generally controlled by the endosomal sorting complex required for transport (ESCRT) machinery [98-100]. ESCRT-0, -I, -II, and –III and accessory proteins regulate cargo clustering, bud formation, and vesicle scission. Exosome secretion is regulated by RAB family, especially RAB27a, RAB27b, RAB11, RAB35 and RAB7 [101-103]. The RAB proteins in control of exosome secretion can differ in various cell lines. For example, RAB27a and RAB27b control the exosome release in Hela cells [101], while RAB7 regulates the exosome release in MCF-7 breast cancer cells [103]. Soluble NSF-attachment protein receptor complex (SNARE) is also involved in exosome release [104, 105] (Fig. 4).

Figure 4

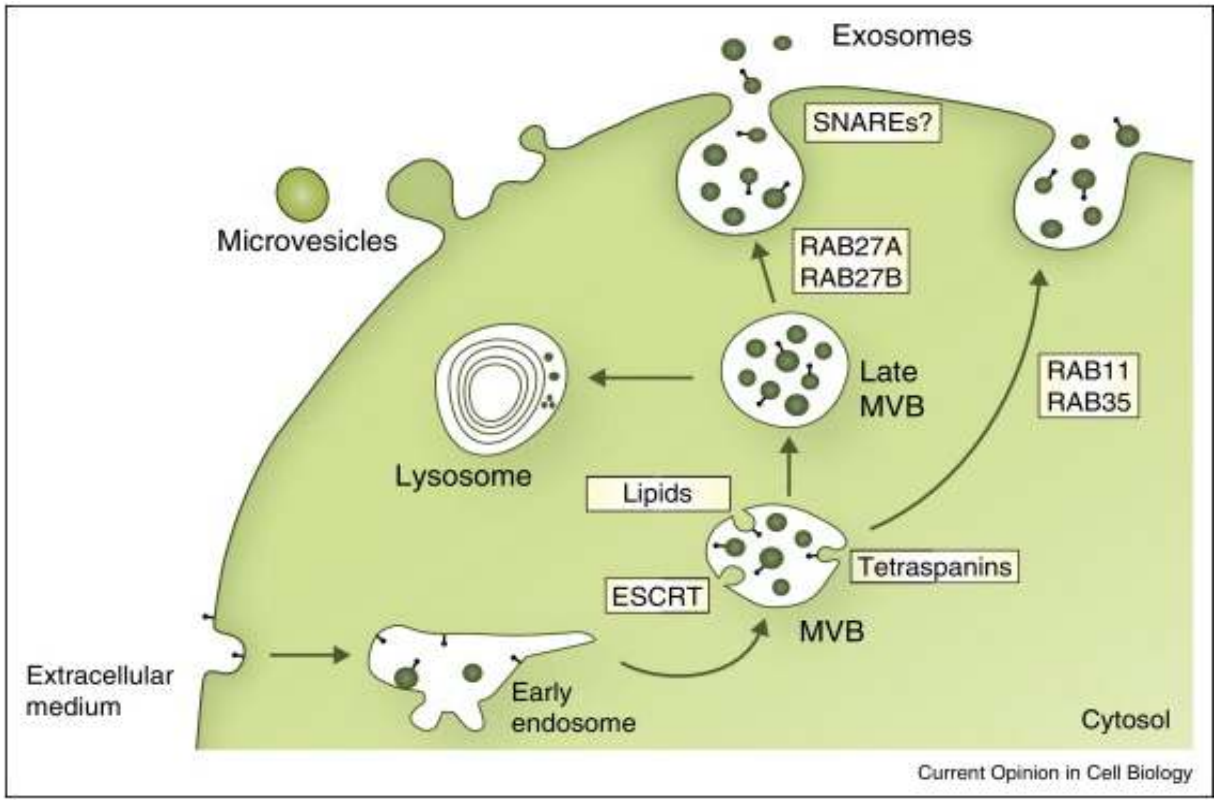


Figure 4. | Exosome biogenesis and secretion in eukaryotic cells. First exosomes fuse into early endosomes and MVBs. ESCRT machinery, lipids, and tetraspanins help exosome budding. Late MVBs fuse with plasma membrane to release exosomes or they fuse with lysosomes for degradation. RAB proteins (RAB11, RAB27 and RAB35) and SNARE are involved in the exosome secretion. On the contrary, EVs (microvesicles) germinate from plasma membrane, not from MVBs. *Reprinted with permission from Joanna Kowal, Mercedes Tkach and Clotilde Thery. "Biogenesis and secretion of exosomes."* Current Opinion in Cell Biology. *2014. 29: 116-125.*

Exosomes are secreted by nearly all mammalian cells, and taken up by many cells. For example, cancer, fibroblast, and immune cells all have been reported to release or take up

exosomes [106, 107]. Exosomes carry protein, lipids, and nucleic acid (mRNA, microRNA, and DNA) [108-110], as messengers for cell communication (Fig. 5). The protein, lipid, or nucleic acid cargo in exosomes is cell-specific [111]. The mechanism of how exosomes selectively package their cargo remains largely unknown and is worthy of further exploration. It has only been reported that mRNA secreted in exosomes have special sequences for export [112], but it is unclear what the criteria for exosomal packaging of protein, lipid, and microRNAs is. In order to demonstrate that mRNA loading in EVs were exchanged between tumor cells *in vivo*, Zomer and the colleagues transfected tumor cells with Cre recombinase (Cre^+ cells). After the reporter-expressing cells took up EVs, their color was changed from pre-labeled red to green by the Cre-LoxP system [113]. This directly showed that EVs containing mRNA can be transferred to other tumor cells. Although the same experiment was not performed with exosomes, it is very likely that exosomes have a similar ability to transfer mRNAs between cells.

Of note, the uptake of exosomes by recipient cells is not random. The interaction involves specific receptors on the surface of both exosomes and recipient cells. Several reports indicate that selective exosome uptake might be determined by adhesion-associated molecules on the surface of exosomes, such as tetrapanins, glycoproteins, integrins and SNAREs [114, 115]. For example, $CD54^+$ pancreas cells prefer to take up exosomes expressing tetrapanin 8 (Tspan8) and integrin α4 [115]. In light of exosomes being targeted to specific cells, exosomes have become a potential mediator to deliver microRNA, siRNAs, proteins, and others for tumor therapy.

Figure 5

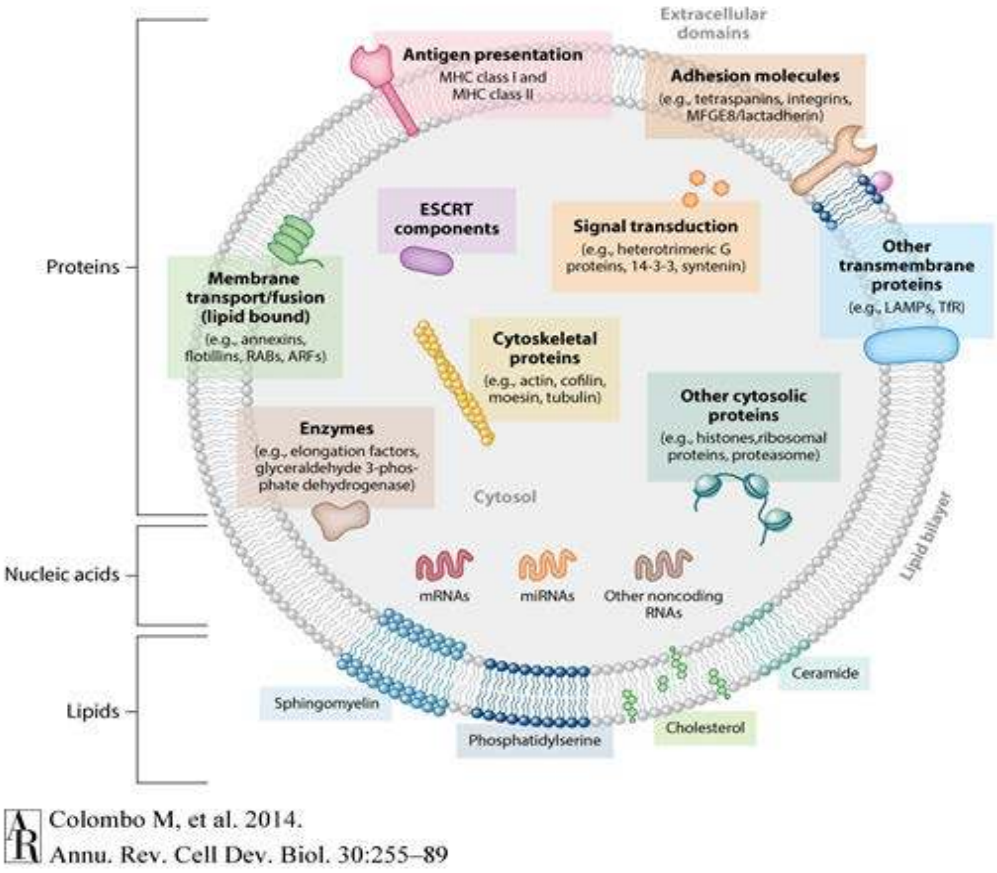


Figure 5. | The composition of exosome and EVs. Exosomes or EVs contain components, such as proteins, lipids, and nucleic acids. The cargos of exosomes or EVs are cell-specific. Some subtypes of EVs, but not others, take up all the listed components. Big Evs can carry histones, proteasome, and ribosome proteins. *Reprinted with permission from Marina Colombo, Graca Raposo and Clotilde Thery. "Biogenesis, Secretion and Intercellular Interactions of Exosomes and Other Extracellular Vesicles."* Annu. Rev. Cell. Dev. Biol*. 2014. 30: 255-89.*

1.2.2 Exosomal function in tumor

Recently, exosomes have been widely studied for their roles in cell communication, especially in tumor progression and metastasis. Exosomes can change cell fate by autocrine signaling. Exosomes produced by chronic myeloid leukemia (CML) have an autocrine effect to activate anti-apoptotic pathways by TGF-β [116]. More importantly, exosomes mediate intercellular interactions and modulate the microenvironment by paracrine mechanisms. Exosomes from host cells serve as external stimuli for recipient cells and modulate their signaling pathways. Cancer cell-released exosomes are taken up by stromal cells to build tumor-prone microenvironment; reciprocally, stromal cells in the tumor microenvironment release exosomes, which are taken up by cancer cells to facilitate their own growth. Due to the heterogeneous nature of cancer cells, exosomes or EVs secreted by tumor cells can change the protein expression in their neighboring tumor cells to alter their biological phenotypes. For example, glioma cells transferred the oncogenic receptor EGFRvIII by EVs to other glioma cells lacking this receptor [117], thereby activating the AKT pathway and promoting anchorage-independent growth.

Exosomes can induce drug resistance and modulate angiogenesis. Drug sensitive cells become drug resistant because they take up exosomes containing P-glycoprotein from drug resistant cells [118]. Exosomes from melanoma and ovarian cancer help tumor cells expel cytotoxic drugs [119-122]. Exosomes presenting tetraspanins also promote tumor growth by increasing angiogenesis [123]. Tspan8 combined with CD49d enhances endothelial cell proliferation and angiogenesis [124]. Notch ligand Delta-like 4 (Dll4) presented by tumor cells' exosomes increases vessel density and branching *in vivo* [125].

Additionally, another important character of exosomes is modulating tumor immune response. The evidence of exosome-mediated intercellular antigen transfer was first reported by Dr. Clotilde Thery's group [126, 127]. They showed that Epstein–Barr virus (EBV)-transformed B cells released exosomes containing MHC-II and specifically activated CD4+ T cells, while dendritic cells (DCs) secreted exosomes containing MHC-I and promoted CD8+ cellular immune response *in vitro*. Such functions expanded to tumor immunity. Tumor-derived exosomes or EVs were reported to activate immune response. Exosomes derived from tumor cells present neoantigens that are transferred by DC cells to activate the function of T cells, NK cells, or macrophages [128-132]. However, the opposite function, that tumor exosomes inhibited anti-tumor immune response, was also reported [133]. Exosomes derived from tumors can contain proteins, such as TGF-β, or C-type lectin-like receptor NKG2D, which inhibit T cell and NK cell activation, and promote regulatory T cell (Treg) function or myeloid-derived suppressor cell (MDSC) development [132, 134]. Exosomes presenting FAS ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) induce the apoptosis of activated T cells [135, 136]. In glioma and lung carcinoma tumors, the majority of exosome-recipient cells are MDSCs. After taking the exosomes, MDSCs display an enhanced immunosuppressive phenotype [137]. Therefore, the exosomes exert both immune active and suppressive functions. Dissecting the mechanism of both functions should be helpful for targeting exosomes in cancer treatment.

1.2.3 Exosomes and cancer metastasis

As mentioned before, exosomes have become a hot topic in cancer biology, partly because of their extensive impact on cancer metastasis.

1.2.3.a Cell migration and invasion

Exosomes control directional cell movement. Sung and colleagues showed exosomes were required for the persistent and efficient movement of cancer cells. The mechanism is that fibronectin in autocrine-secreted exosomes bound with integrin, and then was converted into an adhesive form to promote cell migration [138]. Tumor associated fibroblasts secrete CD81-containing exosomes to promote breast cancer cell migration and invasion by activating WNT-planar cell polarity (PCP) signaling in tumor cells [139]. In order to promote cell invasion, exosomes derived from different cancer cells modulate the extracellular matrix. For example, exosomes derived from gynecological tumors induce extracellular matrix degradation by metalloproteinase [140]. Exosomes containing tetraspanins CD151 and Tspan8 derived from the rat pancreatic adenocarcinoma line ASML are essential for matrix degradation and tumor microenvironment reprogramming [141]. Exosomes also target the tight and adherent junctions. Exosomes from breast cancer can open the tight junctions of endothelial cells by transferring miR-105 to endothelial cells and therefore inhibiting the expression of ZO-1, a tight junction protein. [68]. Exosomes derived from cancer cells activate the EMT program in recipient cells. Exosomes contain EMT drivers, such as hypoxia-inducible factor alpha (HIFα), matrix metalloproteinase 13 (MMP 13), casein kinase Iiα, annexin A2, and latent membrane protein 1 (LMP1), and lead to increased migration and invasion of recipient cells [142-145]. Zomer and colleagues found that less malignant tumor cells that took up EVs from malignant tumor cells displayed enhanced migratory behavior and metastatic capacity by *in vivo* imaging analysis [113].

1.2.3.b Pre-metastatic niches

Exosomes are capable of building pre-metastatic niches for disseminated cancer cells. This novel function of exosome has mainly been advanced by Dr. David Lyden and his colleagues. Exosomes from melanoma cells educate bone marrow progenitor cells through the receptor tyrosine kinase MET, supporting tumor growth [146]. The exosomes from pancreatic cancer cells contribute to the formation of pre-metastatic niches in the liver. PDAC exosomes are taken up by Kupffer cells. Highly expressed MIF in PDAC exosomes activates TGF-β release from Kupffer cells, which in turn increase fibronectin production and recruit bone marrow-derived macrophages and neutrophils to form a fibrotic microenvironment for tumor growth [147]. Breast cancer exosomes that contain different integrins pre-determine the metastatic organ pattern. In particular, exosomal integrins $\alpha_6\beta_4$ and $\alpha_6\beta_1$ prime the lungs as the metastatic site, while exosomal integrin $\alpha_v\beta_5$ primes the liver [55]. The effect of exosomes on the pre-metastatic niches complements the "seed and soil" hypothesis, where the cancer cells modify the distant organs before they arrive by releasing the exosomes.

1.2.4 Exosomes as therapeutic tools and biomarkers

Exosomes represent a type of bio-vehicles that can be considered natural liposomes. They are membrane-permeable, well-tolerated, and can cross the blood-brain barrier. Therefore, exosomes have emerged as favorable candidate carriers for therapeutics of cancer and other diseases. The first appealing application of exosome is the delivery of drugs, microRNAs, siRNAs, and other therapeutic compounds, which stay stable in exosomes [148]. For example, exosomes could be used to deliver tumor suppressor microRNAs or siRNAs that knock down oncogenes to inhibit tumor growth. As exosomes target specific cell types or tissues, delivery by exosomes may also improve efficacy and reduce off-target effects. Synthetic target ligand expression on the surface of exosomes can further improve their cell or tissue targeting

specificity. For example, exosomes were engineered to express lysosome-associated membrane protein 2b (Lamp2b) with a tumor-targeting integrin to enhance tumor-specific uptake [149]. Second, exosomes can be applied for cancer vaccination. Exosomes can deliver tumor-derived antigens to induce anti-tumor immune responses. Dendritic cell-derived exosomes induced anti-tumor immunity and have been used in clinical trials [150-152]. Third, given that cancer-cell derived exosomes can modify pre-metastatic niches [55], depleting these exosomes from the circulatory system is a possible option for blocking cancer metastasis. Inhibiting exosome assembly and release from tumor cells by inhibitors, or eliminating exosomes from cancer patients' circulation by extracorporeal purification, leads to the reduction of exosomes in the circulation. In summary, using exosomes as a new venue for cancer therapy is promising despite the technical challenges.

Many investigators have shown evidence supporting the application of exosomes for cancer treatment *in vitro* and *in vivo* [106]. However, currently only a few clinical trials using exosomes in cancer therapy have been implemented. The main challenges of using exosomes in cancer therapy include, but are not limited to, 1) how to load exogenous microRNAs, siRNAs, or drugs into exosomes and increase cell-specific transfer; 2) how to prevent inflammation and immune reactions when developing non-autologous exosomes or bioengineered exosomes; 3) how to increase the half-life of exosomes in the body and avoid rapid clearance by liver and kidney after injection; 4) when depleting exosomes in the blood of patients, how to prevent the loss of normal physiological functions of non-tumor-promoting exosomes; and 5) the cost concerns and technological challenges related to isolating large amounts of exosomes required for the treatment of patients. Exosomes can partake in both pro- and anti-tumor functions.

Therefore, we should thoroughly understand the complexity of exosome biology before widely implementing exosomes in clinical trials.

Significant applications of exosomes include using exosomes as biomarkers for disease diagnosis, prognosis, and therapeutics. Exosome biomarkers for diagnosis or prognosis seem to be on the fast track, so cancer diagnosis based on exosomes has a bright future. Exosomes can be detected in bodily fluid, such as blood, urine, saliva, and cerebrospinal fluid. Therefore, it is an ideal non-invasive biomarker for tumor diagnosis. A great example is that exosomes from pancreatic cancers were found to be enriched with a cell surface proteoglycan, glypican-1 (GPC1), which can be used as a highly sensitive and specific biomarker of pancreatic cancer [153]. Although GPC1 as a biomarker of pancreatic cancer needs to be validated in larger patient cohorts, this finding exemplifies the power of utilizing exosomes for cancer diagnosis. The main challenges of using exosomes in cancer diagnosis are sensitivity and specificity. Not every exosomal protein serving as a biomarker is as sensitive and specific as GPC1. Many biomarker candidates' sensitivity and specificity are no better than current cancer biomarkers and most of them have not shown a clear promise for clinical application. The technical aspects for isolating and enriching exosomes from the blood or other bodily fluids also need to be further improved.

In my project, I explore the function of exosomes in the bio-information communication between the brain microenvironment and tumor cells.

1.3 PTEN

PTEN (Phosphatase and tensin homolog deleted on chromosome 10) is considered as one of the most important tumor suppressors [154, 155] for several reasons: 1) Germline mutation of *PTEN* exists in the *PTEN* hamartoma tumor syndromes (PHTS), including Lhermitte-Duclos disease, Bannayan-Riley-Ruvalcaba syndrome, Cowden disease, Cowden-like syndrome, and Proteus/Proteus-like syndrome [156-158]. 2) Heterozygous $Pten^{+/-}$ mice develop breast cancer, endometrial carcinoma, T-cell lymphoma/leukemia, and neoplasms of liver, prostate, gastrointestinal tract, thyroid, and thymus [159-161]. 3) PTEN loss is found in many advanced cancers, such as prostate cancer, breast cancer, melanoma, glioblastoma, and others [154, 155]. While PTEN protein structure, regulation, and function have been studied for twenty years, the complexity of PTEN in normal cells or cancers remains to be elucidated.

1.3.1 PTEN structure

Human PTEN contains 403 amino acids and is composed of three key functional domains: a protein tyrosine phosphatase (PTP) domain, a C2-membrane binding domain, and a C-terminal tail. The PTP domain is close to the N-terminal. The amino acids 1-185 at N-terminal are homologous to tensin and auxilin [154]. The PTP domain of PTEN contains motif $C(x)_5R$, the specific feature of the protein tyrosine phosphatase family. The cysteine (C residue) in this motif controls the catalytic activity, while the arginine (R residue) controls the substrate binding [162]. The C2 domain regulates the binding of PTEN to phospholipid bilayers, thereby promoting the binding of PTP domain to its substrates [163]. The C-terminal tail contains PDZ (PSD-95, Discs-large, ZO-1)-binding motif, but the exact role of PDZ in PTEN regulation is still

elusive. Phosphorylation of C-terminal tail can regulate PTEN function [164], which will be addressed later.

1.3.2 PTEN regulation

PTEN is inactivated in many cancers, but the nature of PTEN loss is dependent on tumor type. Within the genetic change, PTEN functional loss is through gene deletion or mutation. Germ-line mutations of *PTEN* frequently occur in the PHTSs, while somatic mutations exist in many other cancers [165, 166]. The cancer genome atlas (TCGA) database analysis showed that most prostate and ovarian cancers presented *PTEN* gene deletion, while endometrial carcinomas or glioblastomas frequently contained *PTEN* mutation [167]. Other groups reported loss of heterozygosity (LOH) of *PTEN* also frequently happened in glioma, melanoma, and breast cancer [168], while somatic mutations of *PTEN* were identified in several other types of tumors, such as advanced prostate cancer [169] and late stage of bladder cancer, in addition to endometrial carcinomas and glioblastomas [168] (Table 1). The outcome of loss-of-function *PTEN* mutation is similar as that of loss of whole *PTEN* gene. *PTEN* mutation frequently lies in the PTP domain or C2 domain. Most of mutations in PTP domain are missense mutations, while those in C2 domain are frameshift mutations [167]. PTEN loss can be due to epigenetic mechanisms too. DNA methylation and histone modification at *PTEN* gene promoter are observed in many types of cancer [168, 170, 171].

Table 1. Summary of *PTEN* and *Pten* alteration in specific cancers.

Tissue	*PTEN* alteration in human cancer	Neoplasms and tumours in PHTS	Tumours in *Pten*$^{+/-}$ mice	Mice: tissue-specific deletion outcome	Mice: enhanced tumours in the presence of additional alterations*	Refs
Breast	Mutation <5%, LOH 40%, promoter methylation 50% and loss of expression ~40%	25–50% lifetime risk for women	Yes	Tumours	*Wnt* or *Erbb2* transgenes	39,44, 57,58
Endometrium	Mutation 35–50%	Yes	Yes	NR	*Mlh1*$^{-/-}$ accelerated *Pten* LOH in *Pten*$^{+/-}$ mice	39,60–63, 68
Thyroid	Homozygous deletion <10%, promoter methylation >50%, and rearrangement in most papillary thyroid carcinomas	Yes	Late onset and low frequency	Goiter and benign follicular adenomas in females	Thyroid hormone receptor-β (*Thrb*) transgene, with metastasis	24,70–76
Prostate	Frequent LOH and miR-22 and miR-106b–25 cluster overexpression	NR	Late onset	Early onset of invasive, metastatic prostate tumours	*Cdkn1b*$^{+/-}$, *Nkx31*$^{-/-}$, *Tmprss2–Erg* fusion protein and SV40 Tag	42,74,89, 91–94
Leukaemia or lymphoma	Deletion 10% of T-ALL and 27% mutation in T-ALL	NR	Lymphoma and radiation decreases latency	Early onset lymphoma and autoimmunity (T cell deletion)	NR	40,124, 126–128, 212
Glioma	LOH >70%, mutation 44% (coincident with LOH) and miR-26a amplification	Dysplastic gangliocytoma of the cerebellum in LD	NR	Macrocephaly, seizures and benign cerebellar abnormalities	Mutant *Hras*, SV40 Tag, *Trp53*$^{-/-}$, *Trp53*$^{+/-}$ and *Nf1*$^{+/-}$	31,77–81, 83–87
Melanoma	LOH 30–60%, mutation 10–20% (metastases) and >50% frequent promoter methylation in patients with XP	NR	NR	No spontaneous melanoma but melanoma induced by carcinogen in 50%	*Braf*$^{-/-}$	97, 99–103, 213,214
Lung cancer	Mutation infrequent, promoter methylation frequent, miR-21 upregulation 74% and loss of PTEN 74%	Occasional	NR	Late-onset lung adenocarcinoma 87% and increased carcinogen-induced lung tumours	Mutant *Kras*	30,32,49, 105–109, 112
Liver	Mutation <5%, PTEN expression lost in 12% and PTEN expression lost in HepC HCC	NR	Infrequent	Fatty liver and insulin hypersensitivity	*Vhl*$^{-/-}$	116–118, 215,216
Bladder	LOH 23%, homozygous deletion 6%, mutation 23% (late stage) and decreased or absent PTEN expression 53%	NR	NR	Late-onset transitional cell carcinomas in 10%	*Trp53*$^{-/-}$	48, 120–122
Kidney	LOH 25%	NR	NR	NR	NR	120
Pancreas	Altered localization common	NR	NR	Metaplasia and carcinoma 20%	*Smad4*$^{-/-}$	113–115
Adrenal pheochromocytoma	LOH more common in malignant than in benign tumours	NR	Yes	NR	*Cdkn2a*$^{-/-}$	39,103, 123
Colon and intestine	Up to 18% mutated and up to 19% LOH depending on tumour type	Yes and benign polyps in >90%	Hyperplastic changes	NR	*Apc*$^{+/-}$	37, 217–219

HepC HCC, hepatitis C-positive hepatocellular carcinoma; LD, Lhermitte–Duclos syndrome; LOH, loss of heterozygosity; miRNA, microRNA; NR, not reported; PHTS, PTEN hamartoma tumour syndromes; T-ALL, T cell acute lymphocytic leukaemia; XP, xeroderma pigmentosum. **Pten* alteration led to decreased tumour latency or increased tumour stage in the presence of these additional alterations.

Reprinted with permission from M. Christine Hollander, Gideon M. Blumenthal & Phillip A. Dennis "PTEN loss in the continuum of common cancers, rare syndromes and mouse models." Nature Reviews. *2011. 11: 289-301.*

PTEN mRNA level is positively controlled by a few transcription factors, such as p53, early growth response protein 1 (EGR-1), and peroxisome proliferator-activated receptor γ (PPARγ) [172-174]. p53 is a *PTEN* transcription factor; on the other hand, PTEN stabilizes p53 by inhibiting p53 degradation [172]. Therefore these two important tumor suppressors have positive reciprocity to each other. However, several other transcription factors SNAIL, CBF-1, c-Jun, and nuclear factor-κB (NF-kβ) negatively regulate *PTEN* transcription [175-178]. PTEN is also regulated by microRNAs, which reduce *PTEN* mRNA stability and also inhibit the translation of PTEN protein. PTEN is targeted by many microRNAs, such as miR-21, miR-25, and miR 17-92 cluster [179-182]. In addition, long non-coding RNAs (lncRNAs) control *PTEN* mRNA expression as well. The *PTEN* pseudogene transcript *PTENP1* was reported to sequester PTEN-targeting miRNAs, thereby increasing *PTEN* mRNA expression and protein level [183].

Post-translational modifications can regulate PTEN expression too. PTEN is frequently phosphorylated within C2 domain and C-tails by protein kinases GSK3β, CK2α, ATM, ROCK, or Rak [184-190]. The phosphorylated residues in C2 domain activate PTEN function or stabilize PTEN protein. Phosphorylation of C-tail interacts with C2 domain to keep C2 domain from cell membrane, thereby inhibiting PTEN function. However, C tail can be auto-dephosphorylated and allow the functional competence of PTEN [191]. In the PTP domain, oxidation of catalytic site Cys124 forms a disulfide bond with Cys71 to inhibit PTEN phosphatase function [192, 193]. Catalytic sites Lys125 and Lys128 can be acetylated by acetyltransferase PCAF, thereby decreasing PTEN activity [194]. Another important modification is ubiquitination. PTEN is ubiquitinated by E3 ubiquitin-protein ligases NEDD4-1, XIAP, and WWP2 for degradation [189, 195-197]. PTEN is mono-ubiquitinated by NEDD4-1 which is reported to control PTEN nuclear

localization [195]. SUMOlyation at Lys254 is also important in determining PTEN nuclear localization [198, 199] (Fig. 6).

Figure 6

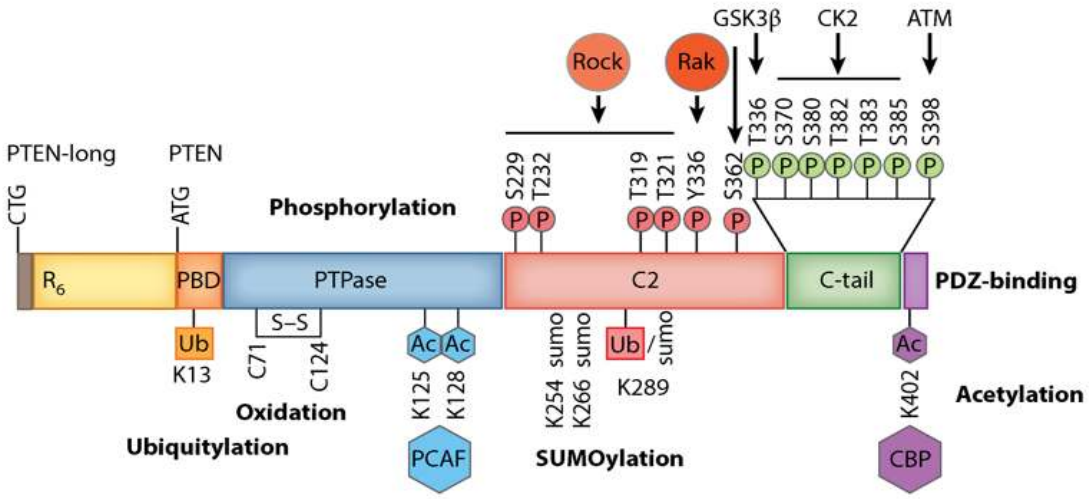


Figure 6. | Posttranslational modifications of PTEN. Most of PTEN phosphorylation sites are in its C2 and C-tail. PTEN also can be regulated by ubiquitination, oxidation, SUMOylation, and acetylation. PTEN-long initiates translation at N-terminal upstream at a CTG codon, containing extra 173 amino acids. *Reprinted with permission from Carolyn A. Wordy and Jack E. Dixon. "PTEN" Annu. Rev.Biochem. 2014. 83:641-69.*

Furthermore, PTEN function is modulated by its binding partners. For example, PTEN binds to the sodium-hydrogen exchanger regulatory factor 1 and 2 (NHERF1 and 2) and PH domain leucine-rich repeat protein phosphatase 1 (PHLPP1) to form an inhibitory complex for phosphatidylinositol-3-kinase (PI3K)/the serine-threonine kinase AKT pathway [200, 201]. p85 alpha, one subunit of PI3K, also binds with PTEN to stimulate PTEN lipid phosphatase activity. It seems paradoxical because p85 alpha generally stimulates the PI3K kinase activity which in

turn inhibits PTEN function, but it shows the complex role of p85 alpha in PI3K-AKT pathway [202]. Meanwhile, several proteins that bind with PTEN inhibit its activity. For example, shank-interacting protein-like 1 (SHARPIN) and mannosidase alpha class 2C member 1 (MAN2C1) interact with PTEN to attenuate PTEN lipid phosphatase function [203, 204].

1.3.3 PTEN function

PTEN was identified as a tumor suppressor for about twenty years and its lipid phosphatase function has been extensively studied. PTEN dephosphorylates phosphatidylinositol (3,*4*,*5*)-trisphosphate (PI(3,4,5)P3) to phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), which reverses the effect of PI3K, therefore antagonizing PI3K-AKT-mTOR pathway. AKT is one of prominent downstream effector of PI3K. AKT activates cell cycle by inhibiting FOXO, p27 and p130 [205, 206], prevents apoptosis by inhibiting pro-apoptotic genes, such as Fas-L and Bcl-2 interacting mediator of cell death (Bim) [207, 208]. AKT also promotes cell growth by negatively regulating tuberous sclerosis complex 2 (TSC2), thereby inducing mTOR pathway [209, 210]. Through antagonizing the PI3K/AKT pathway, PTEN regulates cell cycle, cell growth and metabolism, cell motility, and angiogenesis. PTEN also dephosphorylates β-Catenin, cAMP response element binding protein (CREB), Src Homology 2 Domain Containing (SHC), and SRC [211-214]. Therefore PTEN plays an important role in tumorigenesis, as well in the normal cellular function.

The canonical function of PTEN located at the plasma membrane has been most widely studied. Recently, however, the function of nuclear PTEN and long secreted PTEN have emerged as new focuses as well. PTEN entering the nucleus depends on cell cycle. High nuclear PTEN expression is observed in G0-G1 phase, and low expression is in S phase [215]. Nuclear

PTEN is critical for cell cycle arrest by several mechanisms. First, nuclear PTEN binds p53 and increases p53 level thereby inducing p53-mediated G1 arrest [216]. Second, nuclear PTEN tightens the association of the anaphase-promoting complex (APC/C) with CDH1, and decreases cyclin D1, which leads to cell arrest [217]. Nuclear PTEN also maintains centrosome stability by centromeric protein-C (CENP-C) [218] and controls chromosome integrity by binding with H1 [219]. PTEN upregulates Rad51 expression, which controls DNA double-stranded break repair [220]. In summary, nuclear PTEN regulates DNA stability and cell cycle, independent of PI3K-AKT pathway.

PTEN-long (PTEN-L) contains an extra 173-amino acid domain compared with the classical PTEN. PTEN-L contains a secretion signal sequence so that it can be secreted from cell and received by the neighbor cells where it blocks PI3K-AKT pathway [221]. Detectable PTEN-L in serum or plasm [221] might be a good biomarker for diabetes or other glucose metabolism disorders.

PTEN is a haploinsufficient tumor suppressor, the loss of which induces tumor initiation, combined with other genetic deletion or mutation. PTEN loss promotes tumor growth generally by the canonical AKT-mTOR pathway. But what is the function of PTEN in tumor metastasis? PTEN promoting cell migration *in vitro* has been suggested for a decade. PTEN controls cell motility partly because PIP3 is assembled at the cell's leading edge while PTEN localization is at the opposite side [222]. Another mechanism is that PTEN dephosphorylates focal adhesion kinase (FAK) or SHC to modulate cell motility [223]. PTEN overexpression inhibits the migration of glioblastoma and fibroblasts *in vitro* [224, 225]. However, the role of PTEN in tumor metastasis, at whole organism level, remains controversial and poorly-defined. In a PDAC mouse model, loss of PTEN combined with Kras mutation promoted local, liver, and lung

metastasis by activating NF-kB pathway[226]. In a colorectal cancer cell (CRC) mouse model, restoring PTEN reduced its metastatic capability [227]. However, in breast cancer, one group reported that PTEN loss occurred more often in brain metastasis, especially in HER2-negative brain metastases, compared with primary tumors [228], while another group reported there was no difference in PTEN level between primary breast cancer and metastasis (distant nodes, skin, liver, lung, and bone) [229]. Therefore I decided to explore the exact function of PTEN in brain metastasis as my thesis project.

Chapter 2: Material and Methods

2.1 Reagents and cell culture

All common chemicals were from Sigma (St. Louis, MO). Pyrrolidinedithiocarbamic acid was from Santa Cruz Biotechnology (Santa Cruz, CA). Exo-FBS Exosome-depleted FBS was purchased from System Biosciences (SBI) (Mountain View, CA). PTEN (# 9188), p-Akt (T308) (#9275), p-Akt (S473) (#4060), Pan Akt (#4691), and Bim (#2933) antibodies were from Cell Signaling (Danvers, MA). CD9 (ab92726), Rab27a (ab55667), AMPK (ab3759), CCL2 (ab9899), MAP2 (ab11267), and p-P70S6K (ab60948) antibodies were from Abcam (Cambridge, MA). Tsg101 (14497-1-AP) and Rab27b (13412-1-AP) antibodies were from Proteintech (Chicago, IL). CD81 (104901) antibody was from BioLegend (San Diego, CA). E2F1 (NB600-210) and CCR2 (NBP1-48338) antibodies were from Novus (Littleton, CO). GFAP (Z0334) antibody was from DAKO (Carpinteria, CA). IBA-1 antibody was from WAKO (Richmond, VA). Cre (969050) antibody was from Novagen (Madison, WI). NF-kB p65 (SC-109), CD63 (SC-15363) antibody was from Santa Cruz (Santa Cruz, CA). DMA (sc-202459) and CCR2 antagonist (sc-202525) were from Santa Cruz (Santa Cruz, CA). MK2206 (S1078) was from Selleckchem (Houston, TX). PDTC (P8765) was from Sigma-Aldrich (St. Louis, MO). Human breast cancer cell lines (MDA-MB-231, HCC1954, BT474, and MDA-MB-435) and mouse cell lines (B16BL6 mouse melanoma and 4T1 mouse breast cancer) were purchased from ATCC and verified by MD Anderson Cancer Center (MDACC) Cell Line Characterization Core Facility. Primary glia was isolated as described[13]. Briefly, after homogenization of dissected brain from P0-P2 neonatal mouse pups, all cells were seeded on Poly-D-Lysine coated flasks. 7 days later, flasks with primary culture were placed on an orbital shaker and shaken at 230 rpm for 3 hours. Warm DMEM 10:10:1 (10% of fetal bovine serum, 10% of horse serum, 1% Penicillin/streptomycin) were added and flasks were shaken again at 260 rpm overnight. After

shaking, fresh trypsin was added into the flask and leftover cells were plated with warm DMEM 5:5:1 (5% of fetal bovine serum, 5% of horse serum, 1% Penicillin/streptomycin) to establish primary astrocyte culture. >90% of isolated primary glial cells were GFAP+ astrocytes. Primary cancer associated fibroblasts (CAFs) were isolated by digesting the mammary tumors from MMTV-neu transgenic mouse. 231.xenograft CAFs were isolated by digesting the mammary tumors from MDA.MB.231 xenograft. For the mixed co-culture experiments, tumor cells were mixed with equal number of freshly isolated primary glia or CAFs or NIH3T3 fibroblast cells in six-well plate (1:3 ratio). Co-cultures were maintained for 2-5 days before magnetic bead-based separation. For the trans-well co-culture experiments, tumor cells were seeded in the bottom well and freshly isolated primary glia, CAFs, or NIH3T3 cells were seeded on the upper insert (1:3 ratio). Co-cultures were maintained for 2-5 days for the further experiments. Lentiviral-based packaging vectors (Addgene, Cambridge, MA), pLKO.1 PTEN-targeting shRNAs and all siRNAs (Sigma), Human Cytokine Antibody Array 3 (Ray biotech), and lentiviral-based vector pTRIPZ-PTEN and pTRIPZ-CCL2 shRNAs (MDACC shRNA and ORFome Core, from Open Biosystems) were purchased. The human PTEN-targeting shRNA sequences in the lentiviral constructs were: 5'-CCGGAGGCGCTATGTGTATTATTATCTCGAGATAATAATACACATAGCGCCTTTTTT-3' (targeting coding sequence); 5'-CCGGCCACAAATGAAGGGATATAAACTCGAGTTTATATCCCTTCATTTGTGGTTTTT-3' (targeting 3'-UTR). The human PTEN-targeting siRNA sequences used were: GGUGUAAU GAUAUGUGCAU and GUUAAAGAAUCAUCUGGAU. The human CCL2-targeting siRNA sequences used were: CAGCAAGUGUCCCAAAGAA and CCGAAGACUUGAACACUCA. The mouse Rab27a-targeting siRNA sequences used were: CGAUUGAGAUGCUCCUGGA

and GUCAUUUAGGGAUCCAAGA. Mouse pLKO shRNA (shRab27a: TRCN0000381753; shRab27b: TRCN0000100429) were purchased from Sigma (St. Louis, MO). For lentiviral production, lentiviral expression vector was co-transfected with the third-generation lentivirus packing vectors into 293T cells using Lipo293 DNA *in vitro* Transfection Reagent (SignaGen). 48 to 72 hours after transfection, cancer cell lines were stably infected with viral particles. Transient transfection with siRNA was performed using pepMute siRNA transfection reagent (SignaGen). For *in vivo* intracranial virus injection, lentivirus was harvested from 15cm plates 48 hours after transfection of packaging vectors. After passing a 0.45μm filter, all viruses were centrifuged at 25,000 rpm for 90 min at 4 ^{o}C. Viral pellet was suspended in PBS (~200 fold concentrated). The final virus titer (~1×10^9 UT/ml) was confirmed by limiting dilution.

2.2 Isolation of tumor cells from co-culture

Cell isolation was performed based on the magnetic bead-based cell sorting protocol according to manufacturer's recommendation (Miltenyi Biotec Inc., CA). After preparation of single cell suspension, tumor cells (HCC1954 or BT474) were stained with primary EpCAM-FITC antibody (Catalog #130-098-113) (50 μl per 10^7 total cells) and incubated for 30 min in the dark at 4^{o}C. After washing, the cell pellet was re-suspended and Anti-FITC microbeads (50 μl per 10^7 total cells) were added before loading onto the magnetic column of a MACS separator. Column was washed twice and removed from the separator. The magnetically captured cells were flushed out immediately by firmly applying the plunger. The isolated and labeled cells were analyzed on a Gallios flow cytometer (Beckman Coulter). For EpCAM-negative MDA-MB-231 tumor cells, FACS sorting (ARIAII, Becton Dickinson) was used to isolate GFP+ tumor cells from glia or CAFs.

2.3 Isolation of CD11b+ cells from mouse primary glia

Isolation of primary glia was achieved by homogenization of dissected brain from postnatal days 0-2 mouse pups. After 7 days, trypsin was added onto plate and cells were collected. After centrifugation and re-suspension of cell pellet to single –cell suspension, cells were incubated with CD11b+ microbeads (Miltenyl Biotec) (50 µl per 10^7 total cells) for 30 min at 4 ºC. The cells were washed with buffer and CD11b+ cells were isolated by MACS Column. CD11b+ cells were analyzed by flow cytometry and immunofluorescence staining.

2.4 Western blotting

Western blotting was done as previously described. Briefly, cells were lysed in lysis buffer (20 mM Tris at pH 7.0, 1% Triton-X 100, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, and protease inhibitor cocktail). Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. After membranes were blocked with 5% milk for 30 min, they were probed with various primary antibodies overnight at 4°C, followed by incubation with secondary antibodies for 1 hour at room temperature, and visualized with enhanced chemiluminescence reagent (Thermo Scientific).

2.5 qRT-PCR

Briefly, total RNA was isolated using miRNeasy Mini Kit (Qiagen) and then reverse transcribed using reverse transcriptase kits (iScript cDNA synthesis Kit, Bio-rad). SYBR-based qRT-PCR was performed using pre-designed primers (Life Technologies). miRNA assay was conducted using Taqman miRNA assay kit (Life Technologies). For quantification of gene expression, real-time PCR was conducted using Kapa Probe Fast Universal qPCR, and SYBR

Fast Universal qPCR Master Mix (Kapa Biosystems) on a StepOnePlus real-time PCR system (Applied Biosystems). The relative expression of mRNAs was quantified by $2^{-\Delta\Delta Ct}$ with logarithm transformation. Primers used in qRT-PCR analyses are: Mouse CCL2: forward, 5'-GTTGGCTCAGCCAGATGCA-3'; reverse: 5'-AGCCTACTCATTGGGATCATCTTG-3'. Mouse ACTB: forward: 5'-AGTGTGACGTTGACATCCGT3'; reverse: 5'-TGCTAGGAGCCAGAGCAGTA-3'. Mouse PTEN: forward: 5'-AACTTGCAATCCTCAGTTTG-3'; reverse: 5'-CTACTTTGATATCACCACACAC-3'. Mouse CCR2 primer: Cat: 4351372 ID: Mm04207877_m1 (Life technologies, Grand Island, NY)

2.6 miRNA labeling and transfection

Synthetic miRNAs were purchased from Sigma and labeled with Cy3 by Silencer siRNA labeling kit (Life Technologies). Briefly, miRNAs were incubated with labeling reagent for one hour at 37^{o}C in the dark, and then labeled miRNAs were precipitated by ethanol. Labeled miRNAs (100 pmoles) were transfected into astrocytes or CAFs in a 10 cm-plate. After 48 hours, astrocytes and CAFs containing Cy3-miRNAs were co-cultured with tumor cells (at 5:1 ratio).

2.7 PTEN promoter methylation analysis and luciferase reporter assay of PTEN promoter activity

Genomic DNA was isolated by PreLink genomic DNA mini Kit (Invitrogen), bisulfite conversion was performed by EpiTect Bisulfite Kit and followed by EpiTect methylation-specific PCR (Qiagen). Primers for PTEN CpG island are 5'-TGTAAAACGACGGCCAGTTTGTTATTATTTTTAGGGTTGGGAA-3'and 5'-

CAGGAAACAGCTATGACCCTAAACCTACTTCTCCTCAACAACC-3'. Luciferase reporter assays were done as previously described[27]. The wild-type PTEN promoter driven pGL3-luciferase reporter was a gift from Dr. A. Yung (MD Anderson Cancer Center). The pGL3-PTEN reporter and a control *Renilla* luciferase vector were co-transfected into tumor cells by Lipofectamine 2000 (Life Technologies). After 48-hours, tumor cells were co-cultured with astrocytes or CAFs. Another 48 hours later, luciferase activities were measured by Dual-Luciferase Report Assay Kit (Promega) on Luminometer 20/20 (Turner Biosystems). The PTEN-3'UTRs with various miRNA-binding site mutations were generated by standard PCR-mediated mutagenesis method and inserted downstream of luciferase reporter gene in pGL3 vector. The activities of the luciferase reporter with the wild-type and mutated PTEN-3'UTRs were assayed as described above.

2.8 Exosome isolation and purification

Astrocytes or CAFs were cultured in the media with Exosome-depleted FBS for 48-72 hours and exosomes were collected from their culture media after sequential ultracentrifugation as described previously. Briefly, cells were harvested, centrifuged at 300×g for 10 minutes and the supernatants were collected for centrifugation at 2000×g for 10 minutes, 10,000×g for 30 minutes and 100,000×g for 70 minutes. The pellet was washed once with PBS and purified by centrifugation at 100,000×g for 70 minutes again. The final pellet containing exosomes was re-suspended in PBS and used for *1)* TEM by fixing exosomes with 2% glutaraldehyde in 0.1 M phosophate buffer, pH 7.4; *2)* measure of total exosome protein content using BCA Protein Assay normalized by equal number of primary astrocytes and CAF cells; *3)* Western blotting of

exosome marker protein CD63, CD81 and Tsg101; and *4)* real-time qRT-PCR by extracting miRNAs with miRNeasy Mini Kit (Qiagen).

2.9 Transmission electron microscopy

Fixed samples were placed on 100 mesh carbon coated, formvar coated nickel grids treated with poly-L-lysine for about 30 minutes. After washing the samples on several drops of PBS buffer, samples were incubated on drops of buffered 1% gluteraldehyde for 5 minutes, and then washed several times on drops of distilled water. Afterwards, samples were negatively stained on drops of millipore-filtered aqueous 4% uranyl acetate for 5 minutes. Stain was blotted dry from the grids with filter paper and samples were allowed to dry. Samples were then examined in a JEM 1010 transmission electron microscope (JEOL, USA, Inc., Peabody, MA) at an accelerating voltage of 80 Kv. Digital images were obtained using the AMT Imaging System (Advanced Microscopy Techniques Corp., Danvers, MA).

2.10 Flow cytometry analysis of exosome marker proteins, Annexin V and CCR2

For exosome detection, 100 µl exosome isolated from 10 ml conditioned media of astrocytes or CAFs were incubated with 10 µl of aldehyde/sulfate latex beads (4 µm-diameter, Life Technologies) for 15 min at 4ºC. After 15 min, PBS was added to make sample volume up to 400 µl which was incubated overnight at 4ºC under gentle agitation. Exosome-coated beads were washed twice in FACS washing buffer (1% BSA and 0.1% NaN_3 in PBS), and re-suspended in 400 µl FACS washing buffer, stained with 4 µg of PE-conjugated anti-mouse CD63 antibody (BioLegend) or mouse IgG (Santa Cruz Biotechnology) for 3 hours at 4ºC under gentle agitation and analyzed on a FACS Canto II flow cytometer. Samples were gated on bead singlet

based on FCS and SSC characteristics (4 μm-diameter). For Annexin V apoptosis assay, after 24 hrs doxorubicin (2uM) treatment, the cells were collected, labeled by APC-Annexin V antibody (Biolegend) and analyzed on a FACS Canto II flow cytometer. CD11b+ and BV2 cells were stained with CCR2 antibody (Novus) at 4 ^{o}C overnight; they were then washed and stained with Alexa Fluro 488 anti-rabbit IgG (Life Technologies) at room temperature for 1 hour. Then the cells were analyzed on a FACS Canto II flow cytometer.

2.11 *In vivo* experiments

All animal experiments and terminal endpoints were carried out in accordance with approved protocols from the Institutional Animal Care and Use Committee of MDACC. Animal numbers of each group were calculated by power analysis and animals are grouped randomly for each experiment. No blinding of experiment groups was conducted. Mammary fad pad (MFP) tumors were established by injection of 5×10^6 tumor cells in 100 μl of PBS:Matrigel mixture (1:1 ratio) orthotopically into the MFP of 8-weeks old swiss nude mice as done previously[28]. Brain metastasis tumors were established by intracarotid artery (ICA) injection of tumor cells (250,000 cells in 0.1ml HBSS for MDA-MB-231, HCC1954, MDA-MB-435, 4T1, B16BL6 and 500,000 cells in 0.1ml HBSS for BT474.m1 into the right common carotid artery as done previously[29]. Mice (6-8 weeks) were randomly grouped into designated groups. Female mice are used for breast cancer experiments, both female and male are used for melanoma experiments. Since brain metastasis model does not result in visible tumor burdens in living animal, the endpoints of *in vivo* metastasis experiments are based on the presence of clinical signs of brain metastasis, including but not limited to, primary CNS disturbances, weight loss, and behavioral abnormalities. Animals are sacrificed upon showing above signs or one to two weeks post-

surgery based on specific experimental designs. Brain metastasis lesions are enumerated as experimental readout. Brain metastases were counted as micromets and macromets. The definition of micromets and macromets are based on a comprehensive mouse and human comparison study previously published[30]. Briefly, ten HE-stained serial sagittal sections (300 μm/section) through the left hemisphere of the brain were analyzed for the presence of metastatic lesions. We counted micrometastases (i.e., those ≤50 μm^2) to a maximum of 300 of micrometastases per section and every large metastasis (i.e., those >50 μm^2) in each section. Brain-seeking cells from overt metastases and whole brains were dissected and disaggregated in DMEM/F-12 medium using Tenbroeck homogenizer briefly. Dissociated cell mixtures were plated on tissue culture dish. Two weeks later, tumors cells recovered from brain tissue were collected and expanded as brain-seeking sublines (Br.1). For the astrocyte miR-19 knockout mouse model, $Mirc1^{tm1.1Tyj}$/J mice (Jax lab) (6-8 weeks) were intracranially injected with Ad5.GFAP.Cre virus (Iowa University, Gene Transfer Vector Core) 2 μl (MOI ~10^8 units/μl) per point, total 4 points at the right hemisphere (n=9). Control group (n=7) was injected with the same dose Ad5.RSVβ-Gluc (Ad.GLuc) at the right hemisphere. All intracranial injections were performed by an implantable guide-screw system. One week after virus injection, mice were intracarotidlly injected with 2×10^5 B16BL6 tumor cells. After two weeks, whole brains were dissected and fixed in 4% formaldehyde, and embedded in paraffin. Tumor formation, histological phenotypes of H&E-stained sections, and immunohistochemistry staining were evaluated. Only parenchymal lesions, which are in close proximity of adenovirus injection, were included in our evaluation. Tumor size was calculated as (longest diameter) × (shortest diameter) ^ 2 × 0.5. For intracranial tumor model, $Mirc1^{tm1.1Tyj}$/J mice (Jax lab) (6-8 weeks) were intracranially injected as described above. Seven mice were used in the experiment. One week

later, these mice were intracranially injected with 2.5×10^5 B16BL6 tumor cells at both sides where adenoviruses were injected. After another week, whole brains were dissected and fixed in 4% formaldehyde, and embedded in paraffin. Tumor formation and phenotype were analyzed as above.

For the Rab27a/b knockdown mouse model, seven C57BL6 mice (Jax lab) (6-8 weeks) were intracranially injected with concentrated lentivirus containing shRab27a and shRab27b (ratio 1:2) 2 μl per point, total 3 points at the right hemisphere; concentrated control lentivirus containing pLKO.1 scramble were injected at the left hemisphere. All intracranial injections were performed by an implantable guide-screw system. One week later, mice were intracranially injected with 5×10^4 B16BL6 tumor cells at both sides where they had been infected. After one week, whole brains were dissected and fixed in 4% formaldehyde, and embedded in paraffin. Tumor formation, histological phenotypes of H&E-stained sections, immunohistochemistry staining were evaluated. When perform metastases size quantification, only parenchymal lesions that in close proximity to adenovirus injection sites were included into analyses. Tumor size was calculated as (longest diameter) × (shortest diameter) ^ 2 × 0.5. For exosome rescue experiments, eight C57BL6 mice (Jax lab) (6-8 weeks) were intracranially injected with concentrated lentivirus containing shRab27a and shRab27b (ratio 1:2) 2 μl per point, total 3 points at both hemispheres. One week later, these mice were intracranially injected with 5×10^4 B16BL6 tumor cells with 10 μg exosome isolated from astrocyte media at right sides where they had been injected with lentivirus; 5×10^4 B16BL6 tumor cells with vehicle were injected at left sides where lentivirus had been injected. After another week, whole brains were dissected and fixed in 4% formaldehyde, and embedded in paraffin. Tumor formation and phenotype were analyzed as above.

For *in vivo* extravasation assay, equal number of cells labeled with GFP-control shRNA and RFP-PTEN shRNA (Open Biosystems) were mixed and ICA injected. After cardiac perfusion, brains were collected and sectioned through coronal plan on a vibrotome (Leica) into 50 μm slice. Fluorescent cells were then counted. For inducible PTEN expression *in vivo*, mice were given doxycycline (10 μg/kg) every other day. To quantify brain metastasis incidence and tumor size, brains were excised for imaging and histological examination at the end of experiments. Ten serial sagittal sections every 300 microns throughout the brain were analyzed by at least two pathologists who are blinded to animal groups in all above analyses.

2.12 Reverse Phase Protein Array

Reverse phase protein array (RPPA) of PTEN-overexpressing cells was performed in MDACC Functional Proteomics core facility. Briefly, cellular proteins were denatured by 1% SDS, serial diluted and spotted on nitrocellulose-coated slides. Each slide was probed with a validated primary antibody plus a biotin-conjugated secondary antibody. The signal obtained was amplified using a Dako Cytomation–catalyzed system and visualized by DAB colorimetric reaction. The slides were analyzed using customized Microvigene software (VigeneTech Inc., Carlisle, MA). Each dilution curve was fitted with a logistic model ("Super curve Fitting" developed at MDACC) and normalized by median polish. Differential intensity of normalized log values of each antibody between RFP (control) and PTEN overexpressed cells were compared in GenePattern (http://genepattern.broadinstitute.org). Antibodies with differential expression ($p<0.2$) were selected for clustering and heatmap. The data clustering was performed using GenePattern.

2.13 Patient samples

Two studies in separate cohorts were conducted. The first one was a retrospective evaluation of PTEN in two cohorts. 1) Archived formalin fixed and paraffin-embedded (FFPE) brain metastasis specimens (n=131) from patients with a history of breast cancer who presented with metastasis to the brain parenchyma and had surgery at MDACC (Supplementary information). Tissues were collected under a protocol (LAB 02-486) approved by the institutional review board (IRB) at MDACC. 2) Archived unpaired primary breast cancer FFPE specimens (n=139) collected under an IRB protocol (Lab 02-312) at MDACC (Supplementary information). Formal consent was obtained from all patients. The second study was a retrospective evaluation of PTEN, CCL2 and IBA1 in the matched primary breast tumors and brain metastatic samples from 35 patients, of which there are 12 HER2-positive, 14 triple-negative and nine ER-positive tumors according to clinical diagnostic criteria (Supplementary information). Formalin-fixed, paraffin-embedded primary breast and metastatic brain tumor samples were obtained from the Pathology Department, University of Queensland Centre for Clinical Research (Queensland, Australia). Tissues were collected with approval by human research ethics committees at the Royal Brisbane and Women's Hospital (2005/022) and the University of Queensland (2005000785). For tissue microarray (TMA) construction, tumor-rich regions (guided by histological review) from each case were sampled using 1 mm cores. All the archival paraffin-embedded tumor samples were coded with no patient identifiers.

2.14 Immunohistochemistry (IHC) and Immunofluorescence (IF)

Standard IHC staining was performed as done previously[28]. Briefly, after de-paraffinization and rehydration, 4 μm sections were subjected to heat-induced epitope retrieval

(0.01 M citrate for PTEN). Slides were then incubated with various primary antibodies at 4°C overnight, after blocking with 1% goat serum. Slides underwent color development with DAB and hematoxylin counterstaining. Ten visual fields from different areas of each tumor were evaluated by two pathologists independently (blind to experiment groups). Positive IBA1 and Ki-67 staining in mouse tumors were calculated as percentage of positive cells per field (%) and normalized by the total cancer cell number in each field. TUNEL staining was counted as average positive cells/field (10 random fields). We excluded necrotic areas in the tumors from evaluation. Immunofluorescence (IF) was performed following the standard protocol recommended by Cell Signaling Inc. Briefly, after washing with PBS twice, cells were fixed with 4% formaldehyde. Samples were blocked with 5% normal goat serum in PBS for 1 hour before incubation with a primary antibody cocktail overnight at 4°C, washed, then incubated with secondary antibodies before examination using confocal microscope. Pathologists were blinded to the group allocation during the experiment and when assessing the outcome.

2.15 Bioinformatics and statistical analysis

Publicly available GEO datasets GSE14020 and GSE19184, GSE2603, GSE2034 and GSE12276 were used for bioinformatics analysis. The top 2×10^4 verified probes were subjected to analysis. Differentially expressed genes between metastases from brain and other sites (primary or other metastatic organ sites) were analyzed by SAM analysis in R statistical software. The 54 commonly down-regulated genes in brain metastases from GSE14020 and GSE19184 were depicted as heat map by Java Treeview. For power analysis, we calculated sample size using Pwr package in R environment. For staining of patient samples, we calculated the correlation by Fisher's exact test. For survival analysis of GSE2603, the patient samples

were mathematically separated into PTEN-low and -normal groups based on K-means (K=2). Kaplan-Meier survival curves were generated by survival package in R. Multiple group IHC scores were compared by Chi-square test and Mantelhaen test in R. All quantitative experiments have been repeated using at least three independent biological repeats and are presented as mean ± s.e.m. or mean ± s.d.. Quantitative data were analyzed either by one-way analysis of variance (ANOVA) (multiple groups) or *t*-test (two groups). $P < 0.05$ (two-sided) was considered statistically significant.

Chapter 3: Microenvironment-induced PTEN Loss by Exosomal microRNA Primes Brain Metastasis Outgrowth

Most of this work has been published in:

Zhang L, Zhang S, Yao J, Lowery FJ, Zhang Q, Huang WC, Li P, Li M, Wang X, Zhang C, Wang H, Ellis K, Cheerathodi M, McCarty JH, Palmieri D, Saunus J, Lakhani S, Huang S, Sahin AA, Aldape KD, Steeg PS, Yu D. ***Microenvironment-induced PTEN loss by exosomal microRNA primes brain metastasis outgrowth***. **Nature**. 2015 Nov 5;527(7576):100-4.

3.1 Specific PTEN loss in brain metastasis is induced by brain microenvironment

3.1.1 Dramatic PTEN loss in clinical and experimental brain metastases

To obtain insights into how quiescent tumor cells acquire essential traits from metastatic organ microenvironment for their relapse, we set to start from integrated analysis of public available gene expression profiles from both clinical metastatic tumors and well-controlled experimental metastasis models (Fig. 7a-7c). Clinical metastases of breast cancer profile (Massague dataset, GSE14020) exhibited that 650 genes were significantly down regulated in brain metastases compared with bone and lung metastases (Fig.7b). We next compared the global gene expression level between experimental metastases and matched primary tumor derived from three differential cancer cell lines (human breast cancer cell MDA-MB-231.Br3, human prostate cancer cell PC14.Br4 and human melanoma cell A375SM) (Lee dataset, GSE19184). Compared with their respective primary site, 2161 genes were commonly down regulated in the brain metastases (Fig.7c). *PTEN* was one of the 54 down regulated genes commonly observed in both microarray analyses (Fig. 7a-7c). In order to confirm our finding from dataset analysis, we firstly collected 139 cases of primary breast tumors and 131 cases of brain metastasis tumor. In this cohort, primary breast tumors and brain metastasis tumor were unmatched. Our immunohistochemistry (IHC) analyses of PTEN expression confirmed a significantly higher rate of PTEN loss (defined by an immunoreactive score (IRS) of 0–3) [214] in brain metastases (71%) than in unmatched primary breast cancers (30%) (Fig. 8a). We also collected 35 cases of primary breast tumors with matched brain metastasis tumor in the second patient cohort. PTEN loss was also detected at a significantly higher frequency in brain metastases (71%) than in matched primary breast cancers (37%) in this cohort (Fig. 8b). The

result was consistent with the observation of the first unmatched cohort. Above observations showed PTEN dramatically low-expressed in clinical brain metastases and reveled a unique role of PTEN in the brain-specific metastasis process.

Figure 7

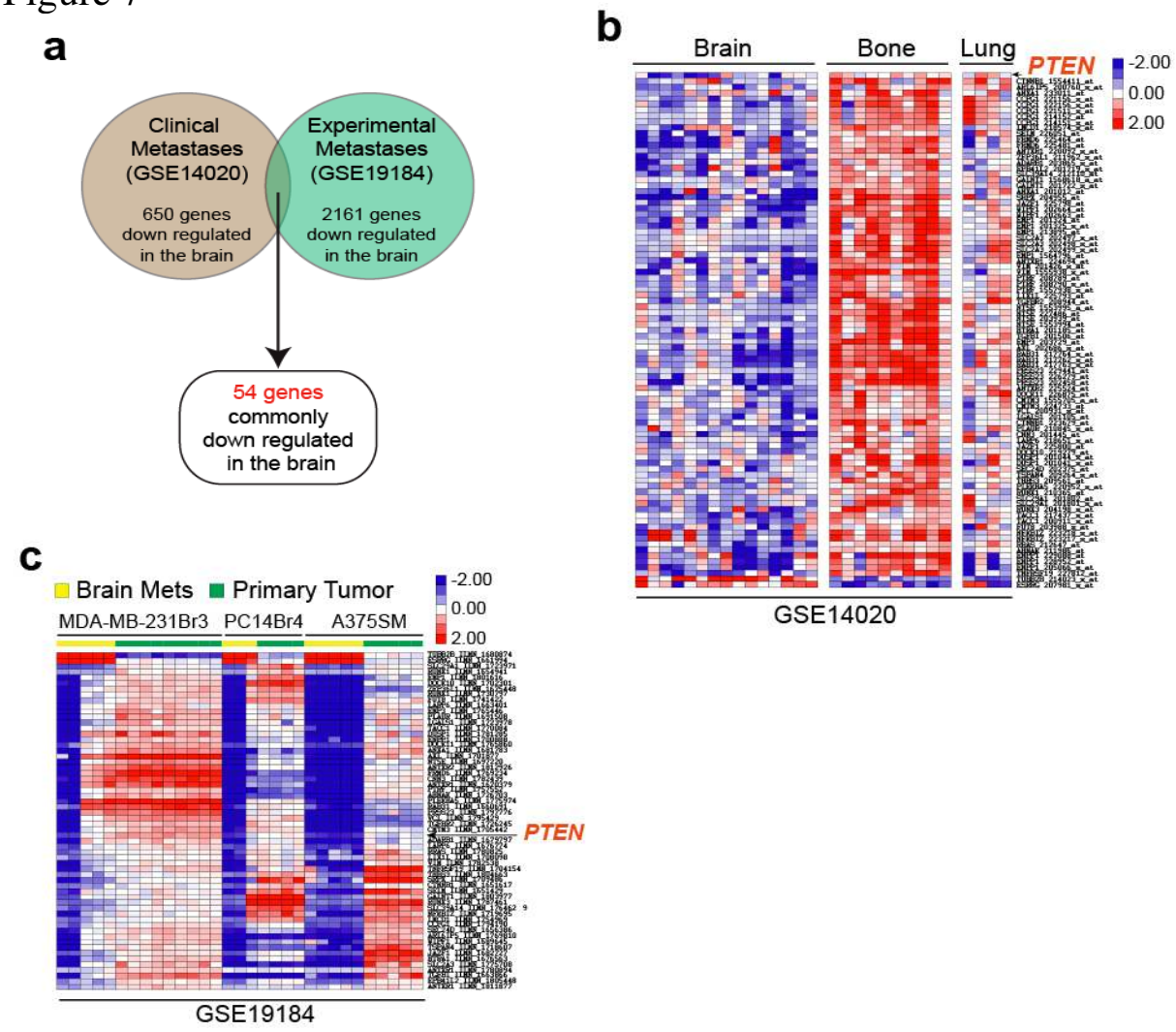


Figure 7. | Organ-specific loss of PTEN in brain metastases. a, Schematics of microarray analyses. Patients' brain metastases exhibited a discrete gene expression profile with 650 genes significantly down-regulated compared to bone or lung metastases (GSE14020). Brain metastases derived from three cancer cell lines exhibited 2161 commonly down-regulated genes compared to their respective primary tumors (GSE19184). PTEN is one of only 54 commonly down-regulated genes in brain metastases of both datasets. **b,** Heat-maps showing expression of 54 commonly down- regulated genes (see **a**) in clinical brain metastases versus lung metastases and bone metastases. **c,** Heat-maps showing expression of the 54 genes commonly down- regulated genes (see **a**) in cell line-induced primary tumors versus experimental brain metastases. *Collaborated with Siyuan Zhang and Jun Yao.*

Figure 8

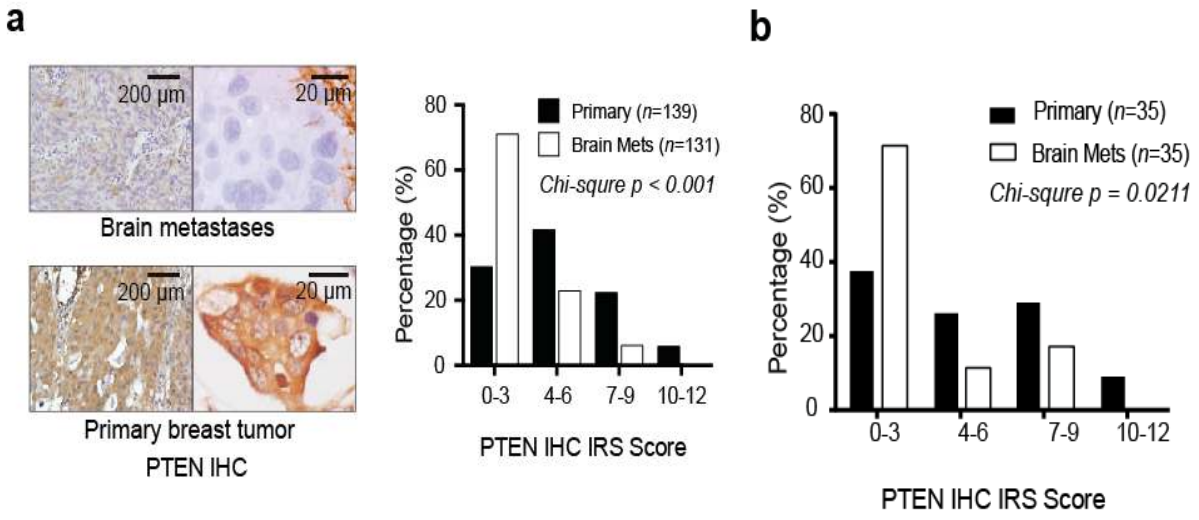


Figure 8. | IHC analysis of PTEN expression in clinical samples. a, Representative IHC staining and histograms of PTEN protein levels in primary breast tumors and unmatched brain metastases (Chi-square test). **b,** Histograms of PTEN protein levels in primary breast tumors and matched brain metastases from 35 patients (Chi-square test). *Collaborated with Hai Wang and Qingling Zhang.*

3.1.2 PTEN loss in the subtypes of breast cancer brain metastases

Clinically, HER2+ and triple negative breast cancers (TNBC) are two major types of breast cancer that more frequently metastasize to the brain[230]. Thus, it is conceivable that these two types (HER2+ and TNBC) constitute the majority of brain metastasis cases studied here. We stained 35 primary breast tumor and matched brain metastasis tissue samples with ER, PR, and HER2. Then, we analyzed PTEN expression levels by IHC in each subtype of breast cancers in this cohort (Fig. 9a). Based on IHC staining of ER, PR, and HER2, the 35 cases were divided into three subtypes: HER2+, triple negative, and hormone receptor (HR)+. Most of the primary breast tumors and matched brain metastases were of the same subtypes, but 5 cases of

brain metastases subtypes were different from their primary breast tumors (Fig. 9b). In the HER2+ and triple negative groups, PTEN-loss was more prominent in brain metastases (66.7% in HER2+ brain metastases vs. 16.7% in HER2+ primary breast cancer; 92.9% in triple negative brain metastases vs. 40% in triple negative primary breast cancer). In the HR+ subtype, PTEN-loss cases in brain metastases were not significantly different to in the primary breast tumor (Fig. 9a). Although this matched cohort was small, data from the extensive statistical analyses of subtypes of patients consistently demonstrated that PTEN-loss in brain metastases was significantly more than in primary breast tumors, especially in the HER2+ and triple negative subtypes, which generally have higher incidences of brain metastasis.

Figure 9

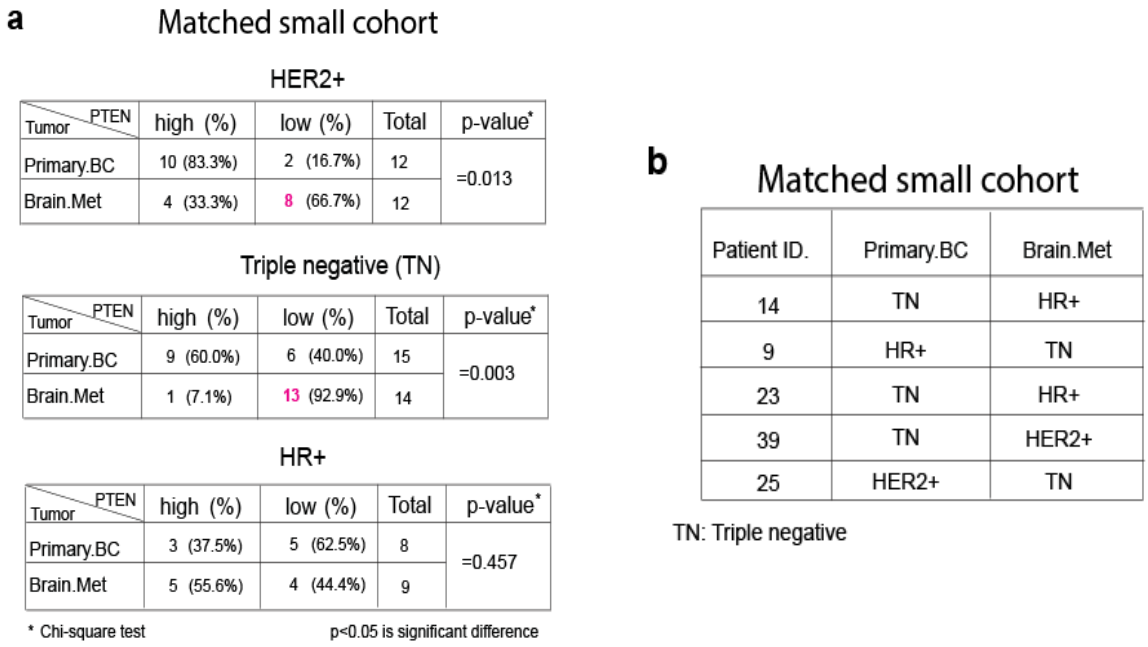


Figure 9. | PTEN expression in clinical subtypes of matched small cohorts. a, The parameter contingency tables showing the patient tumor distribution (matched small cohort in Fig.8b). Chi-square test was used to compare IHC score in primary breast tumor and matched brain metastases. $p<0.05$ is significant difference. **b,** The clinical subtype change from primary tumor to brain metastases in the matched small cohort. *Collaborated with Jodi Saunus and Qingling Zhang.*

3.1.3 Specific PTEN loss in brain metastases, not other metastatic organs

To test the hypothesis that PTEN-loss promotes the brain-specific metastasis, we knocked down PTEN in breast cancer cells before intracarotid injection to model brain metastasis. To our surprise, knocking down PTEN did not enhance the overall brain metastasis incidence nor do brain metastases size (Fig. 10a). Furthermore, patients with PTEN-normal or PTEN-loss primary tumors had comparable levels of brain-metastasis-free survival, and patients with or without brain metastases had similar PTEN levels in their primary tumors (Fig. 10b-10c). Thus, the observed PTEN loss in brain metastases was unlikely to be derived from PTEN-low primary

tumors. This perplexing observation led us to further decipher the intriguing role of PTEN in brain metastasis. Accumulating evidence suggests that metastasis tumor microenvironment (TME) might regulate gene expression of tumor cells [231]. To investigate whether PTEN loss in brain metastasis is a secondary non-genetic event imposed by the brain microenvironment, we injected five PTEN-normal breast cancer cell lines (MDA-MB-231, HCC1954, BT474, MDA-MB-435, 4T1) either into mammary fat pad (MFP) to establish primary xenograft tumor or intracarotidly to induce brain metastases respectively. The tumors derived from the same source of cells exhibited a striking difference in PTEN expression in the different microenvironments (Fig. 11a). The mammary tumor expressed a similar level of PTEN as the original cultured cells before injection, whereas there was a notable PTEN down regulation in brain metastases tumor compared to the respective MFP tumors, indicating disseminated tumor cells with normal PTEN expression might lose their PTEN expression at brain TME. This was also observed in the 4T1 syngeneic mouse model (Fig. 11b). It indicated that the specific PTEN-loss in brain metastases was independent of immune responses. We also injected MDA-MB-231 and 4T1 (mouse breast cancer cell line) into tail veil to generate lung metastases (Fig. 11b). The lung metastases expressed the similar level of PTEN as primary tumor, which showed PTEN down regulation specifically in brain metastases, not in other metastatic organs.

Figure 10

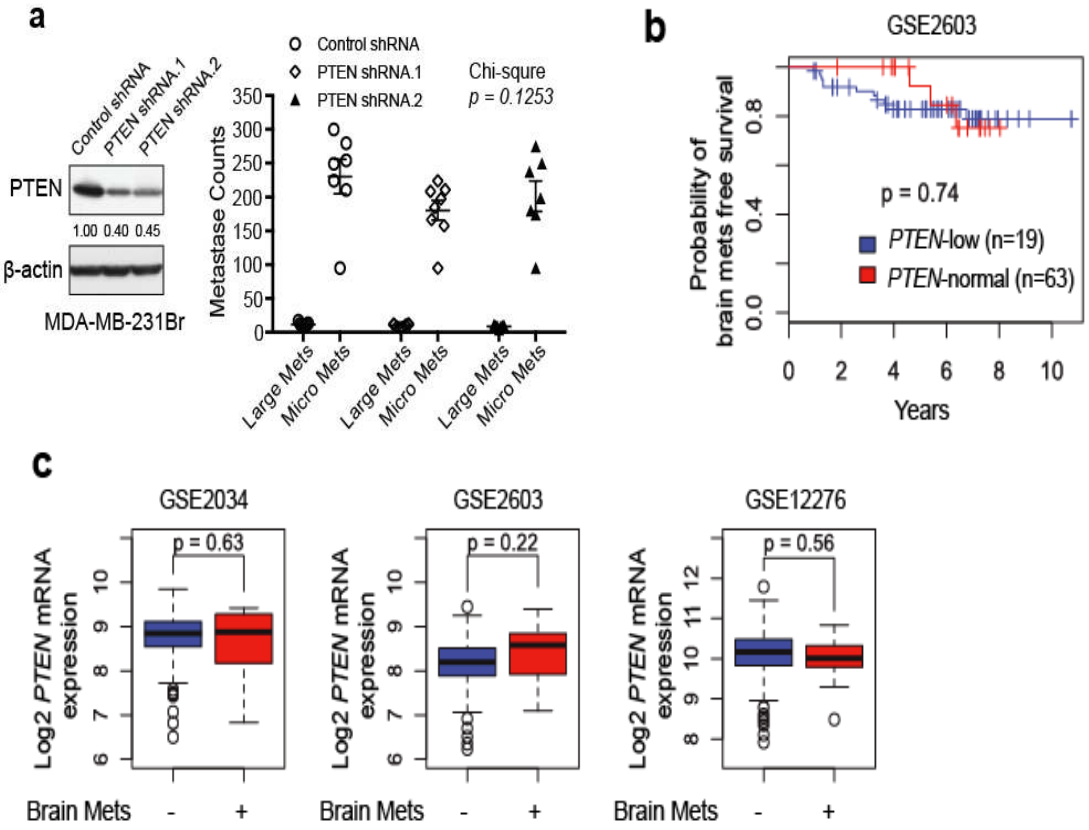


Figure 10. | PTEN loss in brain metastasis is not derived from PTEN-low primary tumors. **a,** PTEN western blots (WB, left) and brain metastasis counts 30 days after intracarotid injection (right) of MDA-MB-231Br cells transfected with control or PTEN shRNAs. Large Mets: >50 μm in diameter (mean ± s.e.m., Chi-square test). **b**, Kaplan–Meier survival analyses showing no significant differences in brain metastasis-free survival between breast cancer patients with primary tumors expressing normal PTEN or low PTEN mRNA in GEO cDNA microarray set GSE2603. **c,** PTEN mRNA levels detected in primary breast tumors from patients with or without brain metastasis relapse. Three GEO cDNA microarray datasets (GSE2034, GSE2603 and GSE12276) with clinical annotation were analyzed. Relative PTEN expression levels were compared by *t*-test. *Collaborated with Siyuan Zhang and Jun Yao.*

Figure 11

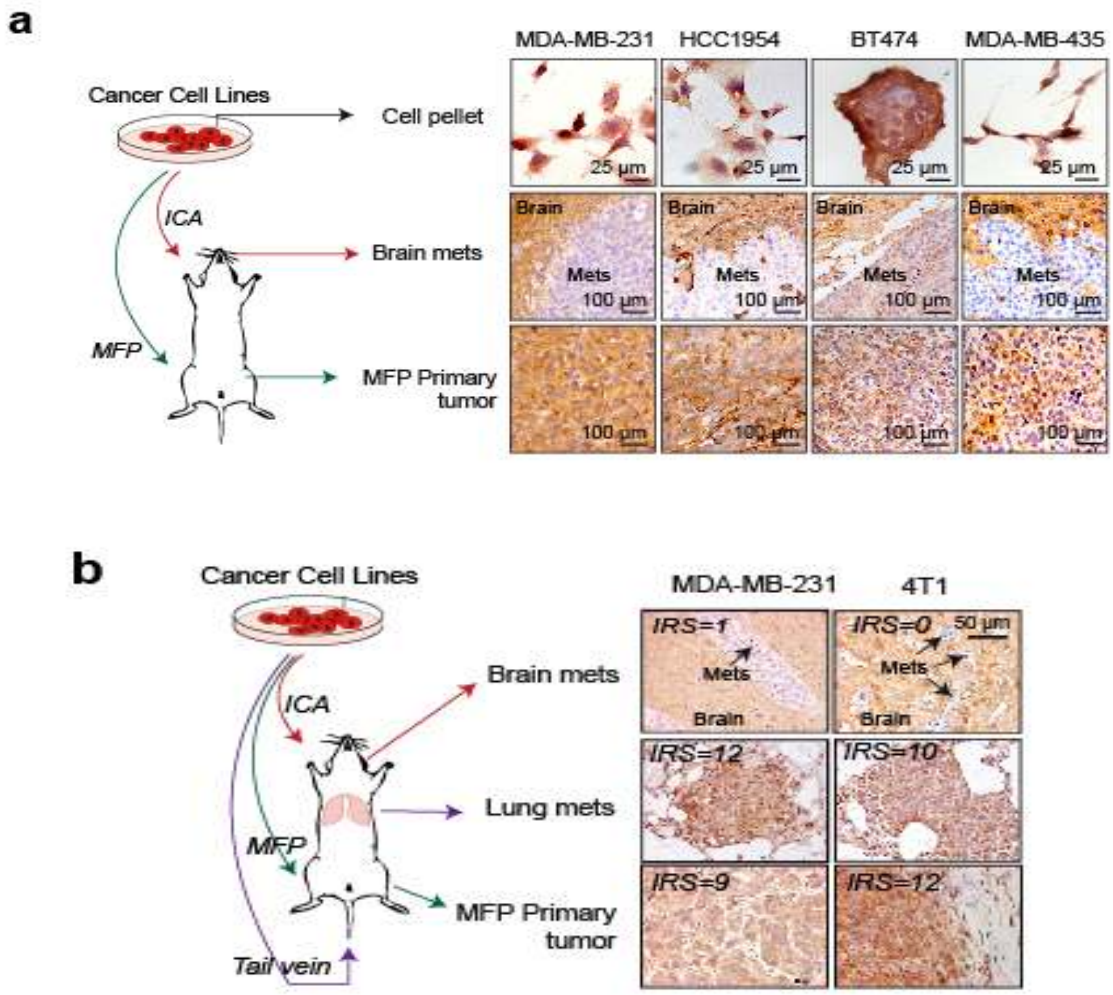


Figure 11. | PTEN expression in different metastatic organ microenvironments and *in vitro* culture condition. a, Breast cancer cell lines (MDA-MB-231, HCC1954, BT474, and MDA-MB-435) were cultured and injected either to mammary fad pad (MFP) to form primary tumor or intracarotidly to form brain metastases. Cells pellets and tumor tissues were stained for PTEN expression by immunohistochemistry using anti-PTEN antibodies. **b,** IHC staining of PTEN in brain metastases, paired lung metastases and primary tumor derived from either MDA-MB-231 or 4T1 cells. PTEN expression level was analyzed based on IRS scoring system. *Collaborated with Siyuan Zhang, Hai Wang and Qingling Zhang.*

3.1.4 Adaptive PTEN-loss at the brain TME

Breast cancers are heterogeneous. To test whether the marked PTEN-loss in the brain metastases resulted from rare PTEN-low cells pre-existed among the heterogeneous cell populations, we repeated the injections with cells clonally expanded from single PTEN-normal tumor cells. Remarkably, the brain metastases from intracarotid artery (ICA) injection lost PTEN expression while MFP tumors remained PTEN-normal (Fig. 12a), suggesting that PTEN-loss brain metastases were not selected from pre-existing PTEN-low cells in the primary tumors. To investigate the mechanism of this brain-specific PTEN-loss, we isolated PTEN-low cells from brain metastases and established brain-seeking sub-lines in culture (1° Br cells). Unexpectedly, the cultured 1° Br cells regained PTEN-normal expressions (protein and mRNA) similar to their parental cells before ICA injection (Fig. 12b). Analogously, two *in-vivo*-selected brain-seeking sublines exhibited similar PTEN levels to their matched parental cells *in vitro* (Fig. 12c). These indicate that the brain TME induced the specific PTEN-loss in brain metastasis *in vivo*. To verify this PTEN-loss was not due to gain of genetic alterations during the metastasis process, we re-injected the cultured PTEN-normal primary brain sublines (1° Br cells) back to mice ICA to establish secondary brain metastases (2° Brain Mets) or MFP for primary tumors (Fig.13a). Again, we detected a distinct PTEN-loss in the 2° Brain Mets but not in 2° MFP tumors (Fig. 13b). Then, we re-established 2° Br cell strains from 2° Brain Mets and PTEN mRNA level was fully restored in cultured 2° Br cells (Fig. 13b). We repeated this experiment in two models, HCC1954 and MBA.MD.231. The results were consistent (Fig. 13c). This reversible PTEN expression indicated a non-genetic PTEN loss in the brain tumor microenvironment (TME).

Figure 12

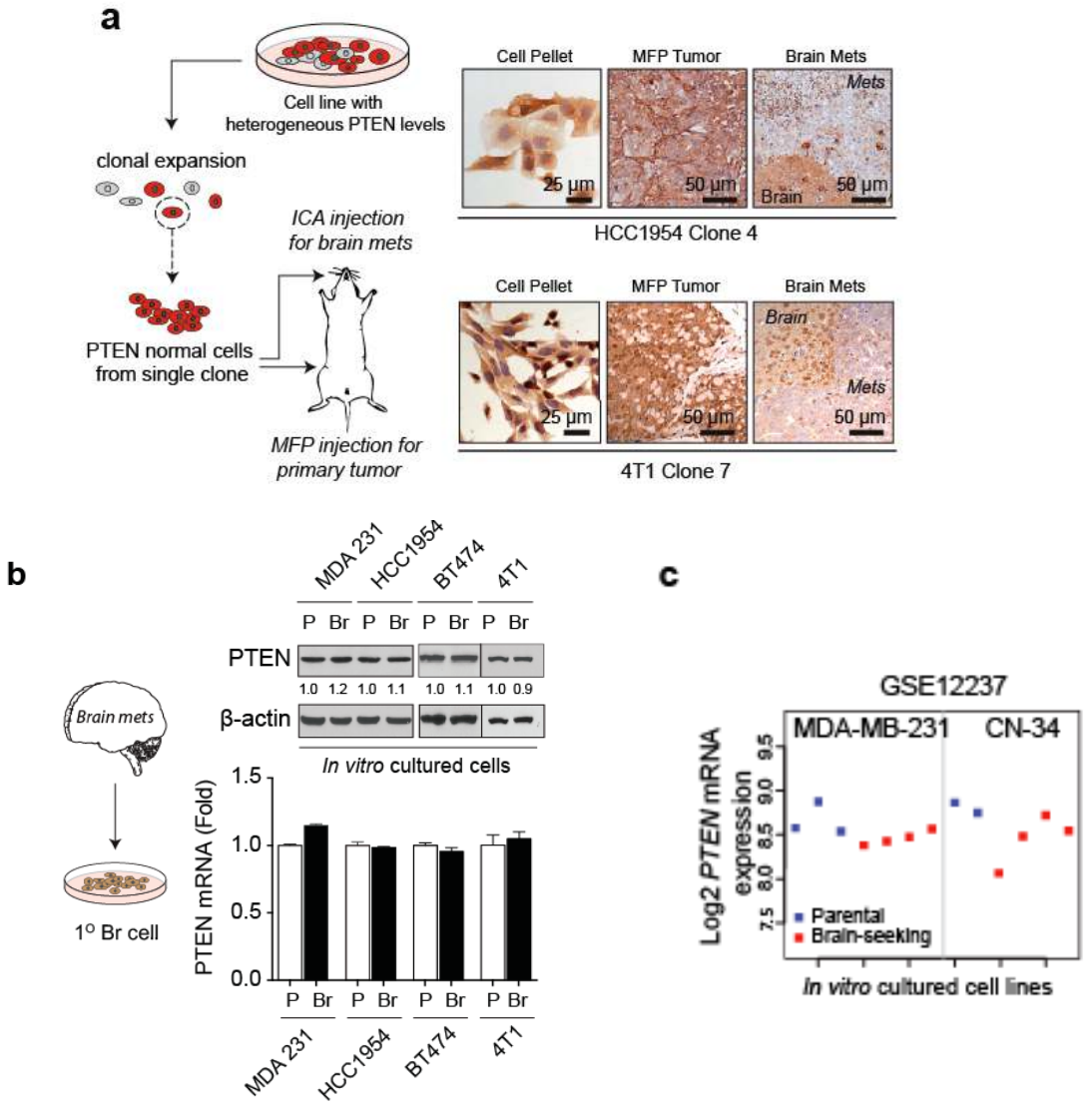


Figure 12. | PTEN loss in brain metastasis happens *in vivo*, not *in vitro*. a, PTEN IHC staining of tumors derived from clonally expanded of PTEN-normal sublines. ICA: intracarotid artery; MFP: mammary fat pad. **b,** WB and qRT-PCR of PTEN expression in the indicated parental (P) and brain-seeking (Br) cells under culture. **c,** PTEN mRNA levels between parental MDA-MB-231 and CN-34 breast cancer cell lines (blue) and their brain-seeking sublines (red). Normalized PTEN-specific probe intensity values were extracted from cDNA microarray dataset GSE12237. Dot plot shows the mean probe intensity derived from independent RNA samples. *Collaborated with Siyuan Zhang.*

Figure 13

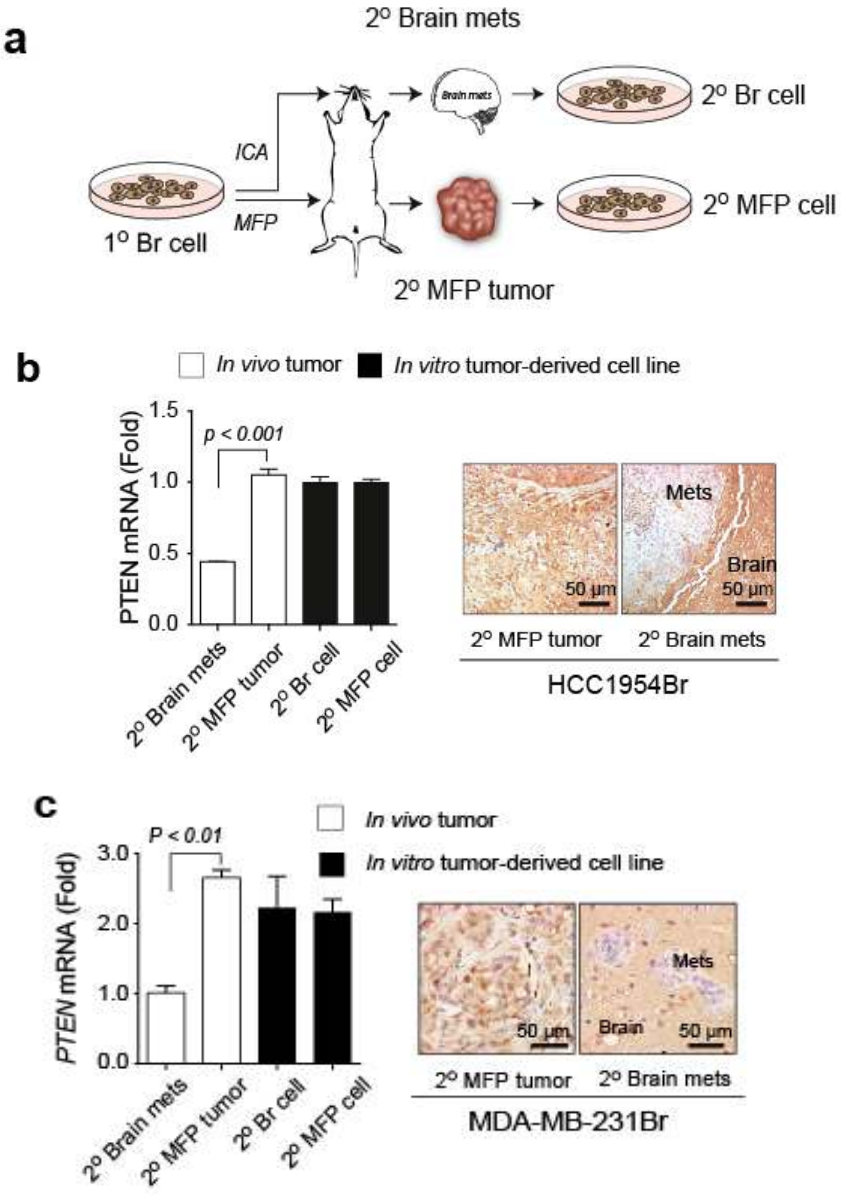


Figure 13. | PTEN loss in brain metastasis is reversible when tumor cells leave brain microenvironment. a, Schematic of *in vivo* re-establishment of secondary brain metastasis, MFP tumor, and their derived cell lines. **b,** PTEN qRT-PCR (mean ± s.e.m., *t*-test) and IHC in HCC1954Br secondary tumors and cultured cells. **c,** PTEN qRT-PCR (mean ± s.e.m., *t*-test) and IHC in MDA.MB.231Br secondary tumors and cultured cells. *Collaborated with Siyuan Zhang.*

3.2 Astrocyte-derived exosomal miR-19a silences PTEN expression in tumor cells

3.2.1 Astrocyte is response to PTEN loss in metastatic tumor cells

To explore how the brain TME regulates PTEN in the metastatic tumor cells, we co-cultured tumor cells with primary glia (>90% astrocytes) derived from neonatal mouse brain, cancer associated fibroblast (CAF) isolated from mammary tumors of MMTV-Neu mice which express an activated rat *c-neu* oncogene under the mouse mammary tumor virus (MMTV) promoter, or NIH3T3 fibroblasts. Co-culture with glia led to a significant decrease of *PTEN* mRNA and PTEN protein (Fig. 14a-14b) in all four tumor cells (MDA-MB-231, HCC1954, 4T1 and B16BL6), compared to under co-culture with CAF or NIH3T3, or tumor cells cultured in media only without any fibroblasts. In this experiment, it also showed that co-cultured with the same number ($2x10^5$) of fibroblasts (CAF or NIH3T3) as glia did not have a higher PTEN level compared to tumor cells cultured in media only without any fibroblasts, so it excluded one possible that CAF cells maintained PTEN expression in tumors outside brain. We also have extracted primary CAFs from MDA-MB-231 xenograft tumors grown in SCID mice which were lack of functional T cells and B cells. Co-culture of tumor cells with these CAFs from primary tumors did not significantly change *PTEN* mRNA expression (Fig. 15a), and PTEN protein level in tumor cells (Fig. 15b), but co-culture of tumor cells with primary glia significantly suppressed expression of PTEN at both mRNA and protein levels (Fig. 15a-15b). These data were consistent with previous observations using CAFs from MMTV-Neu mice, further strengthening our conclusion that glia but not CAFs produce inhibitory factors that significantly down-regulate PTEN expression in metastatic tumor cells.

The brain stromal cells contain astrocytes, microglia and oligodentrocytes. We have isolated astrocytes by specific GFAP biomarker, which is predominately expressed in reactive

astrocytes, but not in mature oligodendrocytes nor in microglia, [232, 233]. We also isolated microglia by surface marker CD11b+ beads, oligodentrocyte by A2B5 beads from primary glial cells [234, 235]. We then cultured MDA-MB-231 breast cancer cells in media only, with CAFs, or with the isolated different cell populations of the brain microenvironment and detected PTEN expression in MDA-MB-231 breast cancer cells four days later. PTEN protein levels in tumor cells had minimal changes when co-cultured with microglia, or with oligodendrocyte (as with CAFs), in contrast to the 50% decrease when co-cultured with astrocytes (Fig. 16). The data indicated that our observed PTEN loss phenotype was primarily contributed by astrocytes.

Figure 14

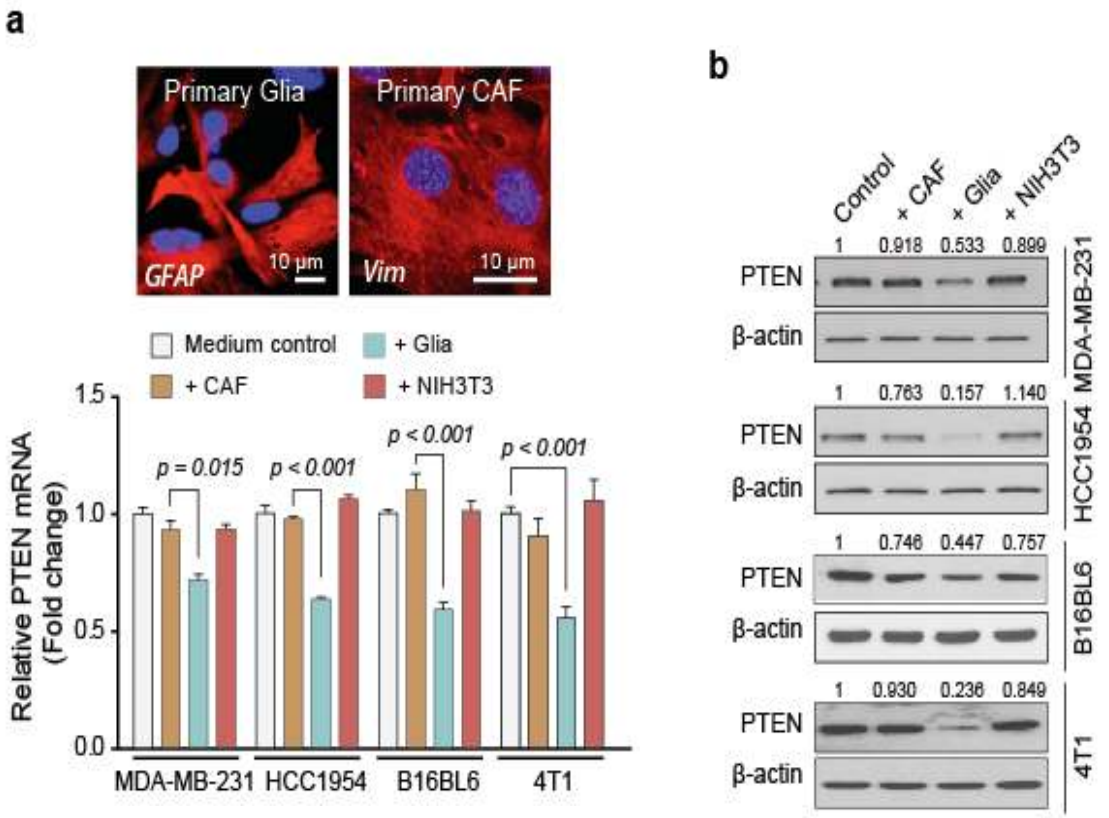


Figure 14. | Co-culture with glia silences PTEN in tumor cells. **a,** PTEN mRNA in the indicated tumor cells after 2-5 days co-culture with GFAP-positive primary glia or vimentin (Vim)-positive primary cancer-associated fibroblasts (CAFs) or NIH3T3 fibroblasts (mean ± s.e.m., *t*-test). **b,** WB of PTEN protein under co-culture as in (a). *Collaborated with Siyuan Zhang and Ping Li.*

Figure 15

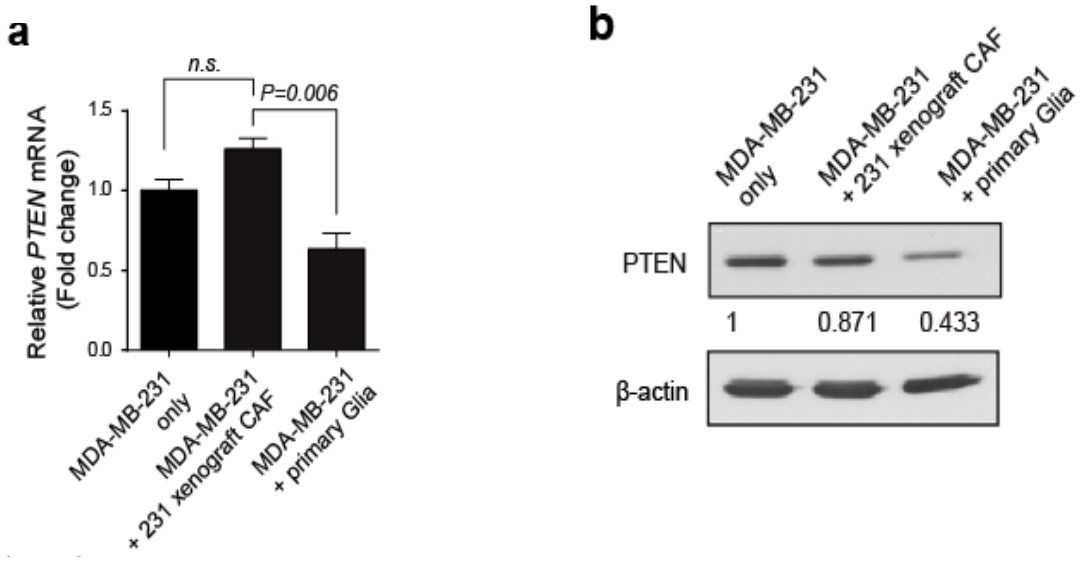


Figure 15. | MDA-MB-231 xenograft CAF does not silence PTEN in tumor cells. a-b, qRT-PCR (**a**) and western blot (**b**) analysis of PTEN mRNA expression (mean ± s.e.m., *t*-test) or protein expression in MDA-MB-231 cells after co-culture with either primary mouse CAFs isolated from MDA-MB-231 xenograft tumors or primary mouse glia isolated from mouse brain.

Figure 16

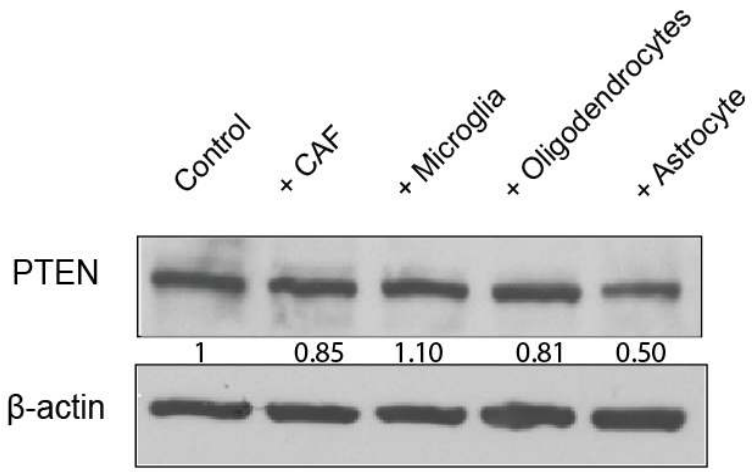


Figure 16. | Other stromal brain cells, except astrocytes, do not induce PTEN loss in tumor cells. WB of PTEN protein expression in MDA-MB-231 cells after 4 days co-culture with primary cancer-associated fibroblasts (CAFs), microglia, oligodendrocytes and astrocytes isolated from primary glial cells.

3.2.2 Astrocyte-derived microRNA silences PTEN in metastatic tumor cells

PTEN mRNA can be regulated by transcription [236] or microRNA (miRNA)-mediated degradation [237, 238]. Since PTEN promoter methylation and activities in cancer cells were similar under glia versus CAF co-cultures (Fig.17a-17b), this prompted us to examine whether astrocytes reduce *PTEN* mRNA stability through microRNAs (miRNAs).

Figure 17

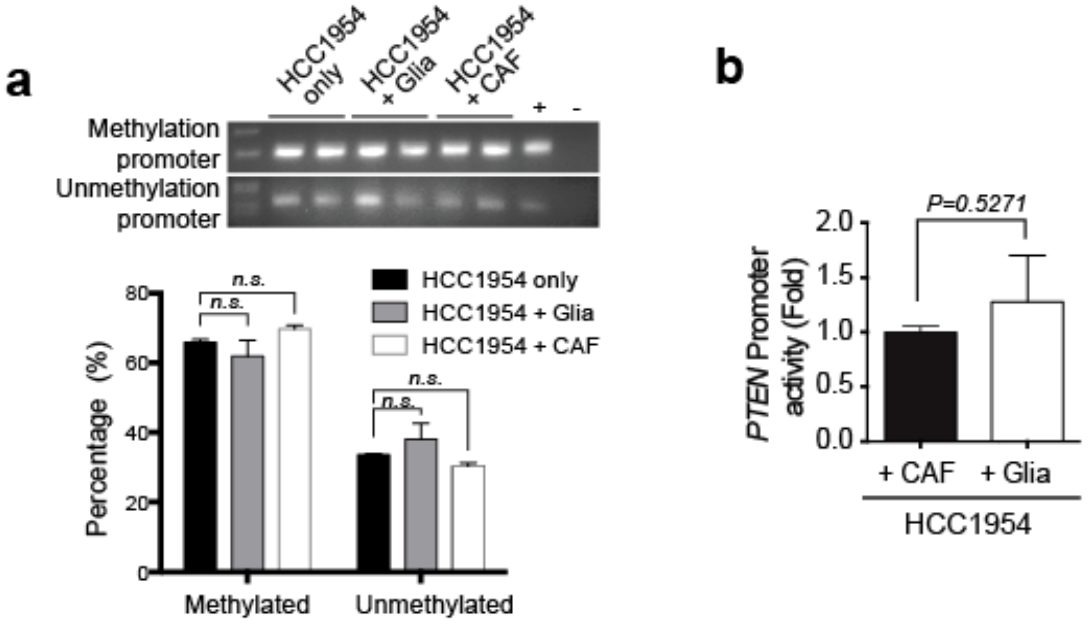


Figure 17. | PTEN promoter methylation and activation do not change under glia co-culture. a, Representative methylation-specific PCR of PTEN promoter and quantification under co-culture with glia or CAF (mean ± s.e.m., *t*-test). **b,** PTEN promoter activity measured by luciferase reporter in HCC1954 cells after co-culture with either CAF or glia cells for 48 hours (mean ± s.e.m., *t*-test).

Initially, we did look into all miRNAs that potentially regulate PTEN in miRDB (http://mirdb.org/miRDB/), which predicted that 189 miRNAs may target PTEN. However, among top-ranked predicted miRNAs, very few have been validated functionally. To narrow down to important miRNAs that functionally regulate PTEN in our cell system, we have applied a 3-tier filtering approach using the following criteria: 1) miRNAs that have been functionally validated to regulate PTEN in a biological system in prior literature; 2) miRNAs that have at least two consensus binding sites on the 3'UTR of PTEN gene; 3) miRNAs that have available knockout mouse model allowing us to test our hypothesis *in vivo*. Base on the above three criteria, miR17-92 cluster emerged as the top hit. Five miRNAs (miR-17, miR-19a, miR-19b, miR-20a and miR-92) in the miR-17~92 cluster were functionally demonstrated to target *PTEN* [239-242], and *Mirc1*$^{tm1.1Tyj}$*/J* mice have a floxed miR-17~92 allele[243]. We knocked out the miR-17~92 allele *in situ* in*Mirc1*$^{tm1.1Tyj}$*/J* mice by intracranial injection of astrocyte-specific Cre adenovirus (Ad-GFAP-Cre), then intracarotidly injected syngeneic mouse melanoma B16BL6 cells to form brain metastases (Fig. 18a). Astrocyte-specific depletion of *PTEN*-targeting miRNAs blocked PTEN downregulation (Fig. 18b) in the brain metastasis tumor cells and significantly suppressed brain metastasis growth compared to the control group (Fig. 18b, 18c), indicating a tumor cell non-autonomous PTEN downregulation by astrocyte-derived *PTEN*-targeting miRNAs. Astrocyte-specific depletion of *PTEN*-targeting miRNAs also suppressed intracranially injected tumor cell outgrowth (Fig.19a-19e).

To examine which *PTEN*-targeting miRNA primarily mediates the PTEN loss in tumor cells when co-cultured with astrocytes, the luciferase activities of the wild-type and mutated *PTEN* 3′-untranslated region (UTR) (containing various miRNA binding site mutations) in tumor cells were assessed (Fig.20). Compared with CAF co-culture, astrocyte co-culture inhibited

luciferase activity of wild-type *PTEN* 3′-UTR, which was rescued by the miR-19a binding site mutation (position 1), but not by other mutations, indicating the major role of miR-19a in astrocyte-mediated *PTEN* mRNA downregulation in tumor cells. At same time, we compared the expression of several miR-19a targets reported for other tumor sites (e.g., AMPK, Bim, and E2F1) in brain metastatic tumors of mice with or without miR-17~92 cluster knockout. We did not detect significantly different expression of these miR-19a targets in brain metastasis *in vivo* (Fig. 21), suggesting miRNA-19a's targets in different tumor environment may be different.

Furthermore, we infected primary astrocytes from Mirc1$^{tm1.1Tyj}$/J mouse with Cre-expressing adenovirus (Ad. GFP.Cre) to knockout the miR-17-92 cluster or with βGLuc-expressing adenovirus (Ad-βGLuc) as controls (Fig. 22a). Compared to co-culture with Ad-βGLuc-treated astrocytes, co-culture with Ad.GFP.Cre-treated astrocytes significantly decreased miR-19a in both HCC1954 and MDA-MB-231 breast cancer cells (Fig. 22b-22e), and consequently, increased PTEN mRNA and protein levels (Fig. 22b-22e). These data indicated that miRNAs, especially miR-19a, in the astrocytes were dynamically transferred into neighboring tumor cells and effectively down-regulated PTEN.

Figure 18

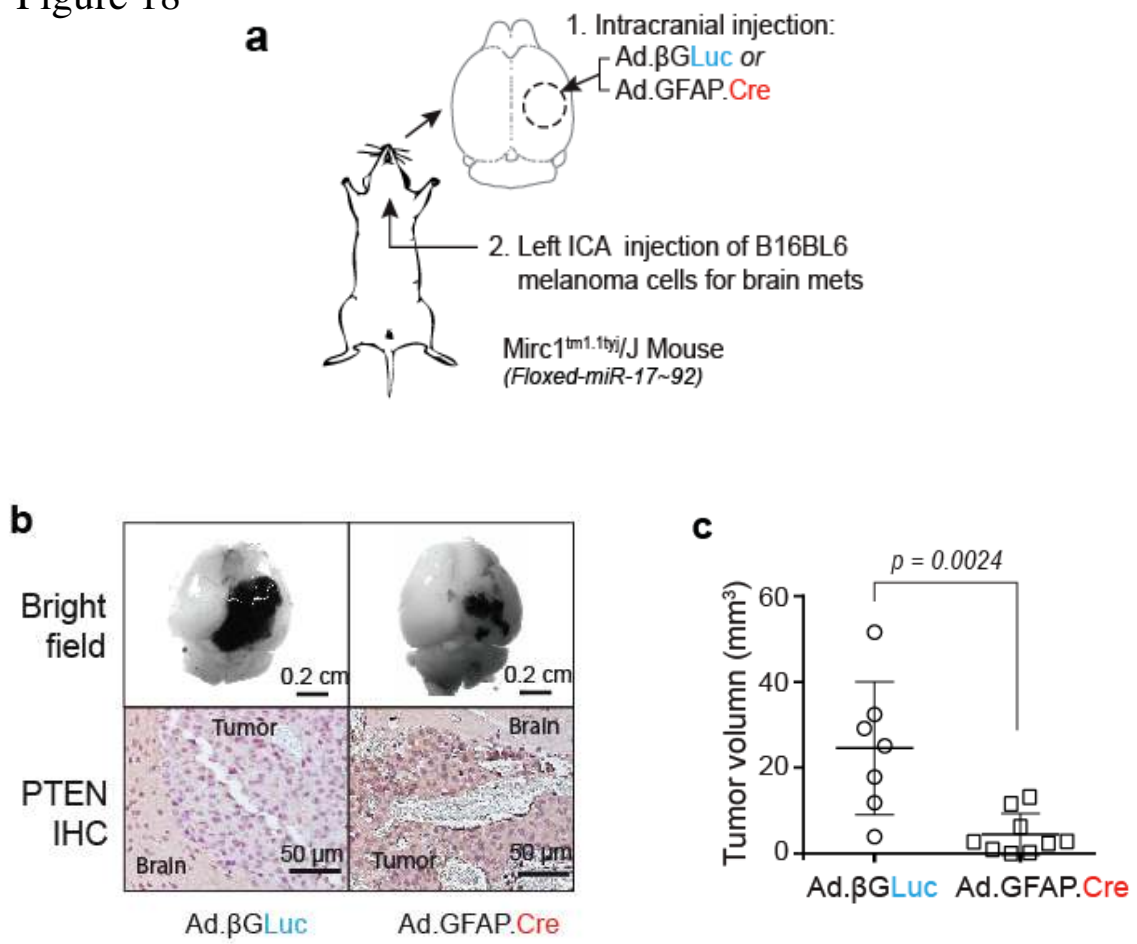


Figure 18. | Astrocyte-deplete miR-17-92 restores PTEN in tumor cells and inhibits tumor outgrowth *in vivo*. a, Schematic of astrocyte-specific miR-17-92 deletion by GFAP-driven Cre adenovirus (Ad.GFAP-Cre) in Mirc1$^{tm1.1Tyj}$/J mice. Injection of adenovirus (Ad-βGLuc) was control. **b,** Representative image of tumor sizes and PTEN IHC of brain metastases. **c,** Quantification of brain metastases volume (mean ± s.d., *t*-test). *Collaborated with Qingling Zhang and Kenneth Ellis.*

Figure 19

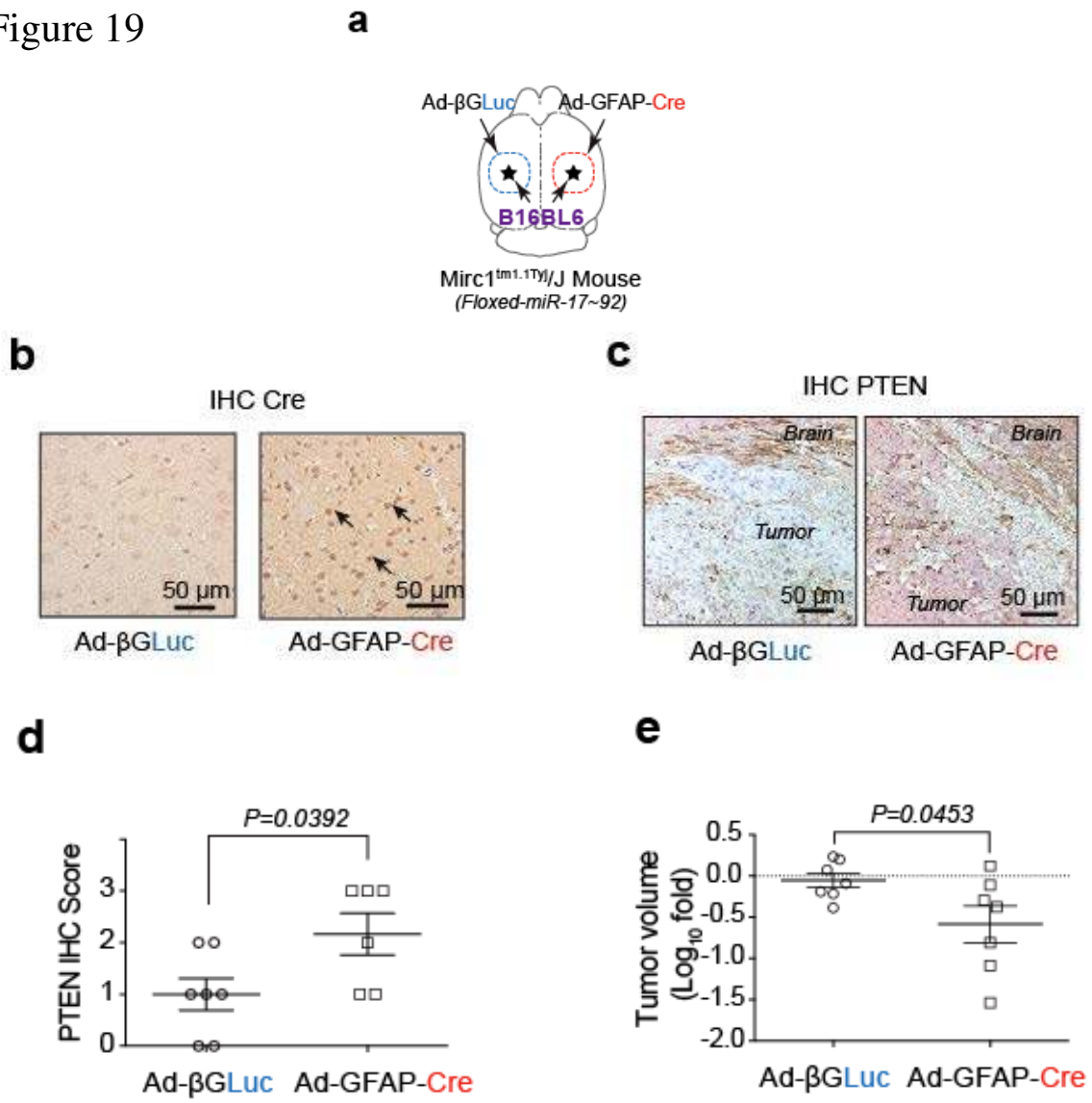


Figure 19. | Astrocyte-deplete miR-17-92 suppresses intracranially injected tumor cells outgrowth and restores PTEN expression in tumor cells. **a,** Schematic of experimental design. GFAP-Cre adenovirus (Ad-GFAP-Cre) was injected intracranially to the right hemisphere of the Mirc1 mouse, and the control adenovirus (Ad-βGLuc) was injected intracranially to contralateral side of the brain. Then B16BL6 cells were injected intracranially to both sides. **b,** IHC analysis of Cre expression in the brain astrocytes. **c,** IHC analysis of PTEN expression in the tumor cells. **d,** Quantifications of PTEN expression in tumor cells (mean ± s.d., *t*-test). **e,** Quantification of intracranial tumor outgrowth by volume (mean ± s.e.m., *t*-test). *Collaborated with Qingling Zhang and Kenneth Ellis.*

Figure 20

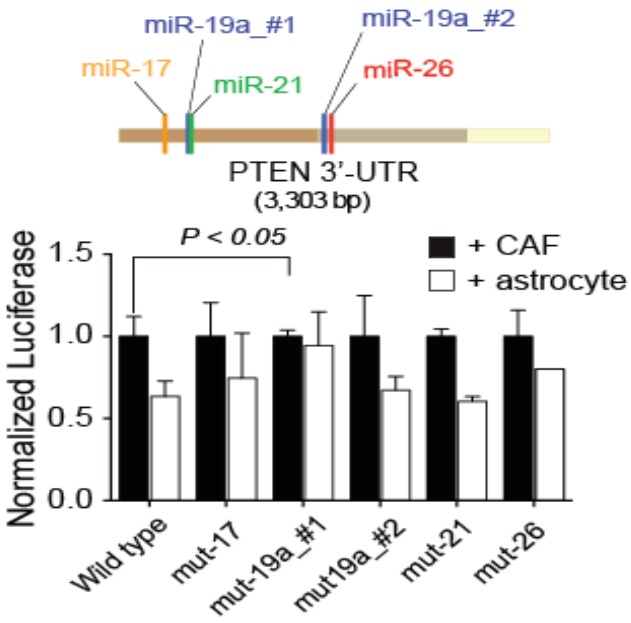


Figure 20. | miR-19a binding site 1 mediates *PTEN* mRNA downregulation in tumor cells. PTEN 3'-UTR- luciferase activity after co-culture (mean ± s.e.m., *t*-test). *Collaborated with Siyuan Zhang.*

Figure 21

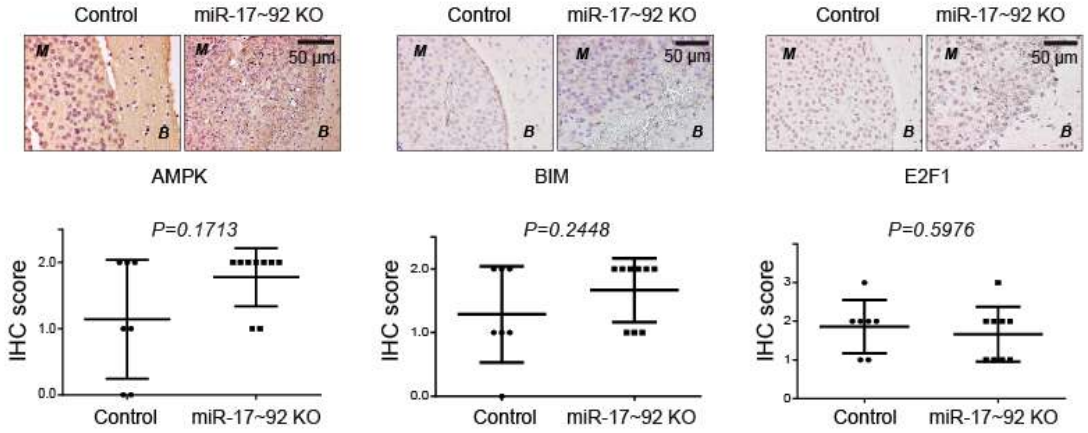


Figure 21. | Other miR-19a targets, except PTEN, are not changed after depletion of miR19-72 in astrocytes. a, IHC analyses of AMPK, Bim, and E2F1 expression (mean ± s.d, *t*-test) in brain metastasis tumors with/without pre-knocking out (KO) miR-17-92 cluster in brain microenvironment. M: brain metastases; B: brain tissue. *Collaborated with Qingling Zhang.*

Figure 22

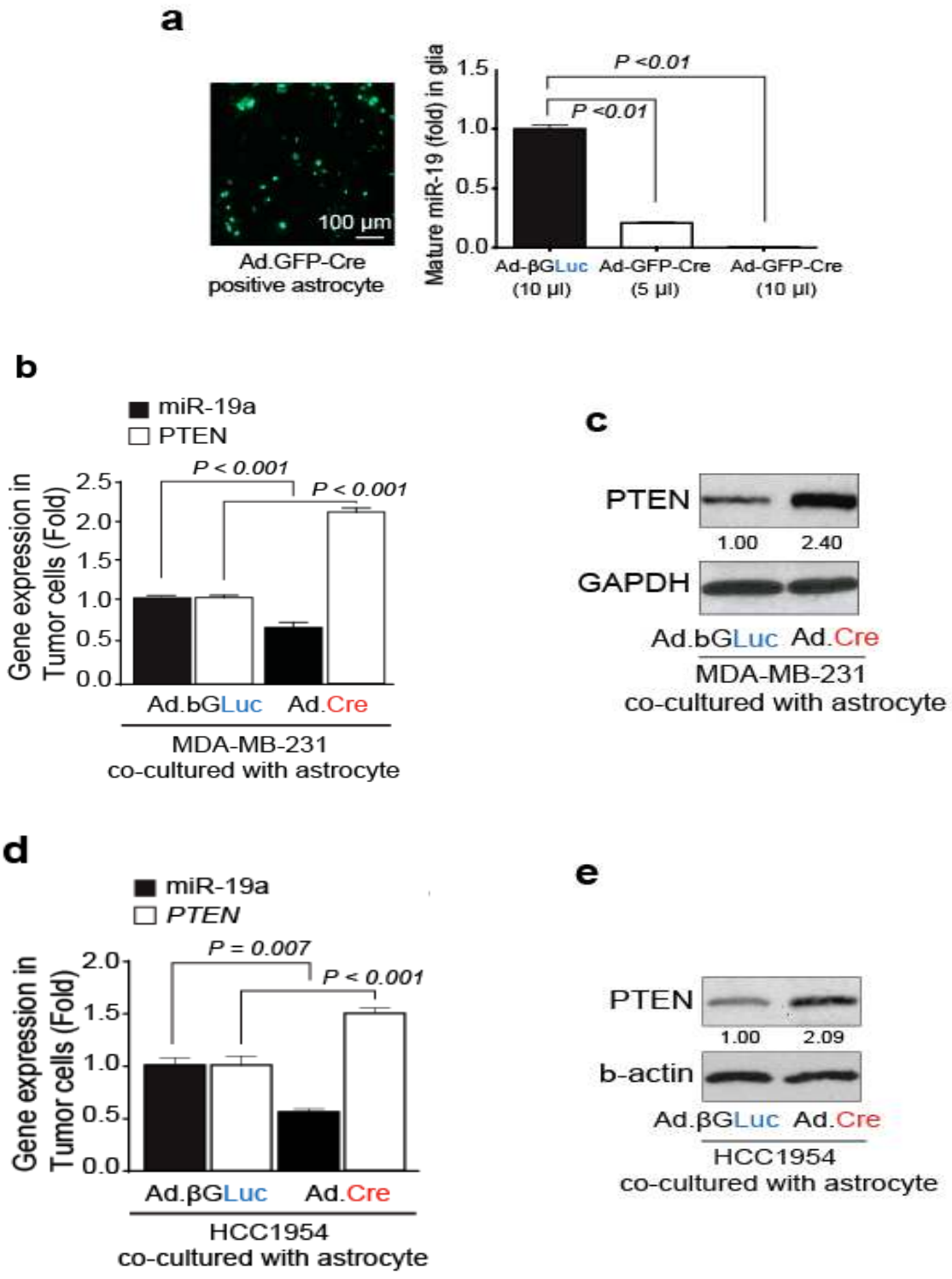


Figure 22. | miR-19a is responsible for PTEN loss in tumor cells *in vitro*.

Figure 22. | miR-19a is responsible for PTEN loss in tumor cells *in vitro*. a, Knockout of miR-17-92 allele in cultured primary astrocytes. miR-17~92 cluster is flanked by loxP site in Mirc1$^{tm1.1Tyj}$**/J** mouse. Primary astrocytes were isolated from Mirc1 mouse brain then infected by adenovirus encoding for βGLuc or GFP.Cre protein. Concentrated adenovirus particles of indicated volume (same MOI ~10^8 units/mL) encoding βGLuc or GFP.Cre proteins were added to 10^6 astrocytes. Left, representative photo showing the infection efficiency. Right: bar diagram showing the relative miR-19a expression (one of the five miRNA genes in the miR-17-92 cluster) three days after adenovirus infection (mean ± s.e.m., *t*-test). **b,** qRT-PCR analyses of miR-19a and PTEN mRNA in MDA-MB-231 cells after 48 hour co-culture with primary astrocytes from Mirc1$^{tm1.1Tyj}$**/J** mice pre-infected (48 hours) by adenovirus (Ad-βGLuc or Ad.GFP.Cre) (mean ± s.e.m., *t*-test). **c,** WB of PTEN protein in MDA-MB-231 cells co-cultured as in (**b**). **d,** qRT-PCR analyses of miR-19a and PTEN mRNA in tumor cell HCC1954 after 48 hour co-culture with primary astrocytes from Mirc1$^{tm1.1Tyj}$**/J** mice pre-infected (48 hours) by adenovirus (Ad-βGLuc or Ad.GFP.Cre) (mean ± s.e.m., *t*-test). **e,** WB of PTEN protein in the indicated tumor cells co-cultured as in (**d**).

3.2.3 Astrocyte-derived exosome transfers miR-19a to metastatic tumor cells

The astrocyte-dependency of PTEN down-regulation in tumor cells by miR-19a prompted us to test whether PTEN down-regulation in tumor cells resulted from intercellular transfer of PTEN-targeting miRNAs. We co-cultured HCC1954 tumor cells with Cy3-labelled miR-19a-transfected primary astrocytes. Amazingly, the Cy3-miR-19a in the EpCAM-negative astrocyte transferred into EpCAM-positive tumor cells after co-culture, as detected by fluorescence imaging (Fig. 23a). Compared with CAF co-cultures, there was a significantly more $Cy3^+$ epithelial cell adhesion molecule (EpCAM)-positive tumor cells under astrocyte co-culture over time than under CAF co-culture by FACS analyses (Fig. 23a-23b), suggesting that miR-19a was intercellularly transferred from astrocytes to tumor cells. miRNAs are transferable between neighboring cells through gap junctions or small vesicles[244, 245]. Treating tumor cells with a gap junction channel inhibitor, carbenoxolone disodium salt, had no significant effect on miR-19a intercellular transfer (data not shown), while adding astrocyte-conditioned media to tumor cells led to an increase in miR-19a levels and a subsequent PTEN downregulation (Fig. 24a-24d). The result indicated that it was likely that the miR-19a transfer from astrocytes was not cell-cell contact-dependent, but mediated by small vehicles.

Figure 23

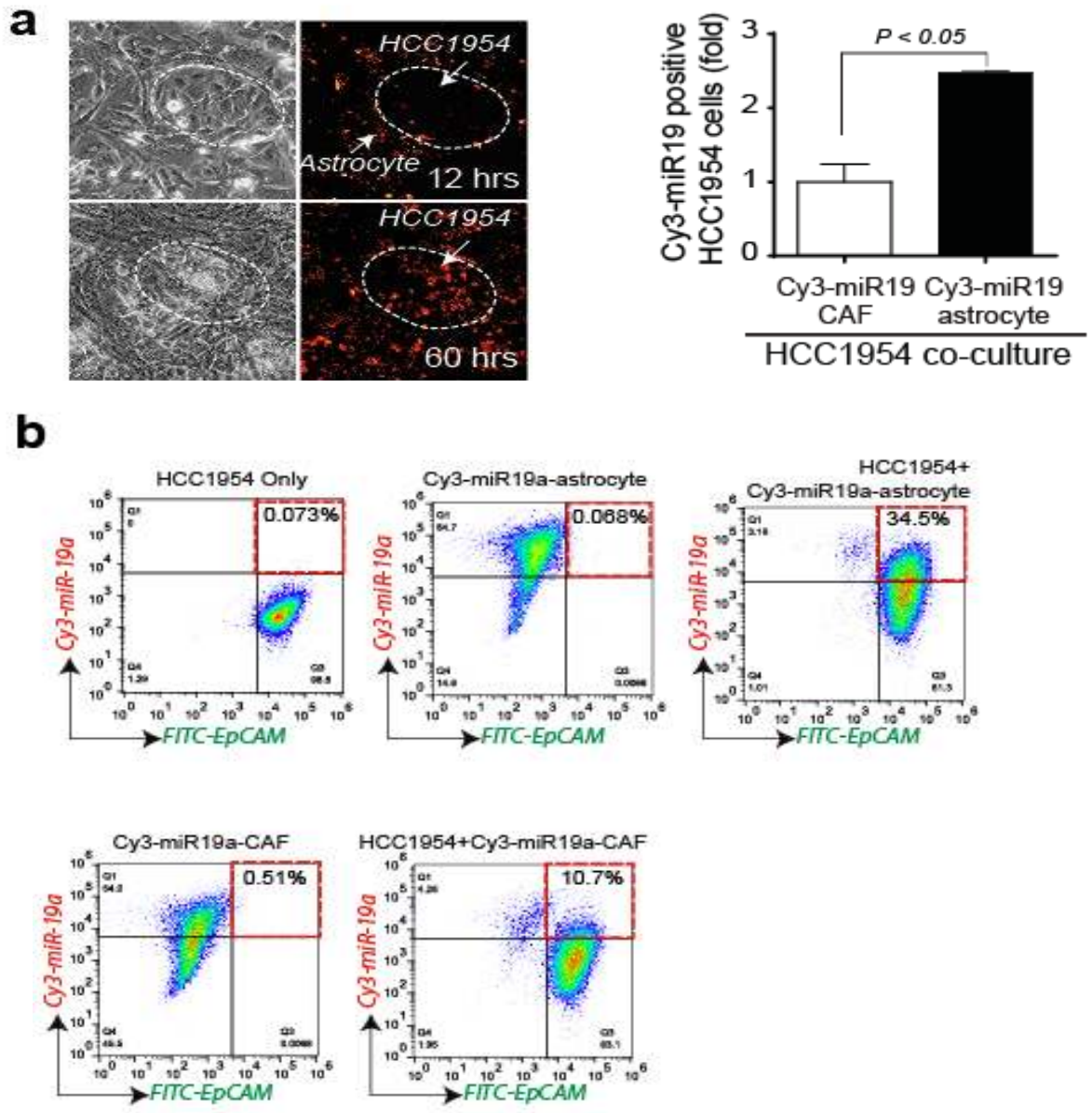


Figure 23. | Intercellular transfer of PTEN-targeting miR-19a to tumor cells. a, Intercellular transfer of miR-19a. Left: light microscopy and fluorescent images of HCC1954 cells 12 and 60 hours after co-cultured with astrocytes loaded with Cy3-labeled miR-19a. Right: flow cytometry analysis of Cy3-miR-19a in tumor cells 60 hours after co-culture (mean ± s.e.m., *t*-test). **b,** Flow cytometric detection of Cy3-miR-19a and FITC-EpCAM in tumor cells 60 hours after co-culture with Cy3-miR-19a-transfected astrocytes and CAFs. *Collaborated with Siyuan Zhang.*

Figure 24

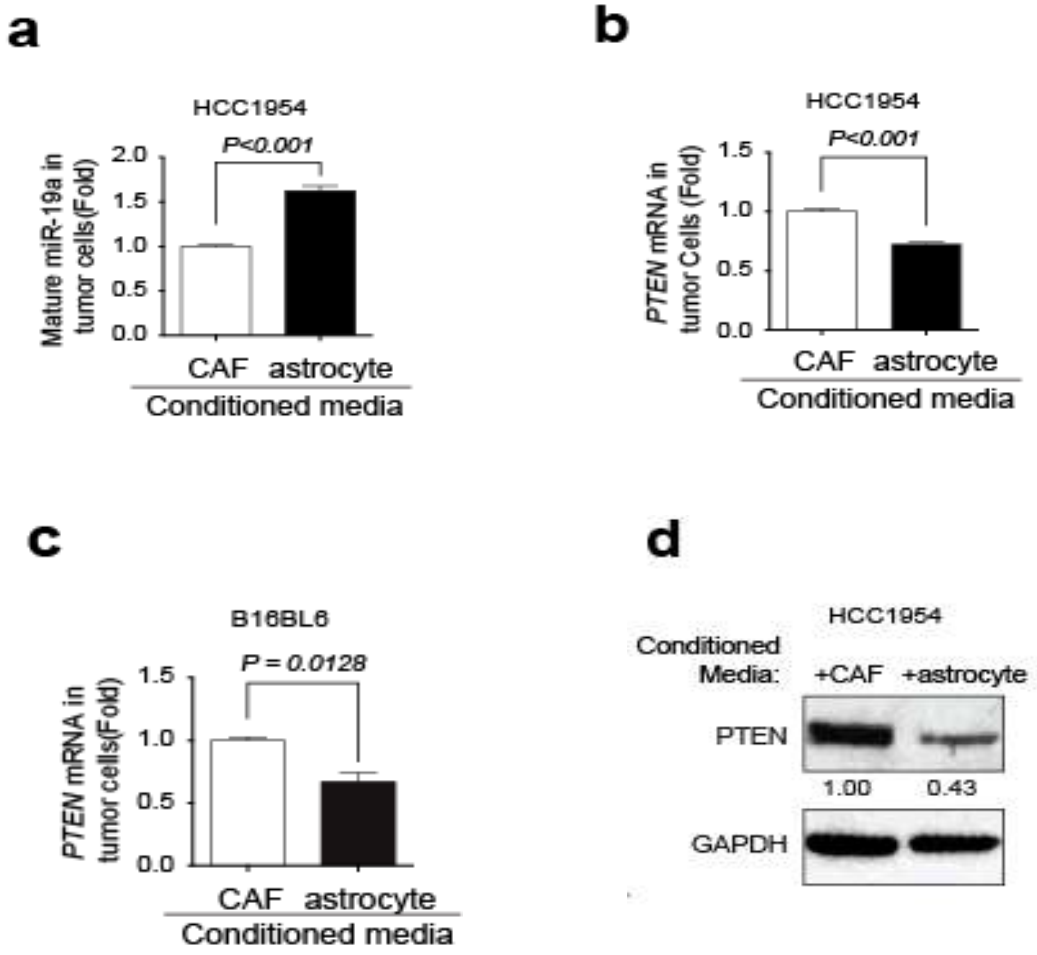


Figure 24. | miR-19a transfer from astrocyte to tumor cell is cell contact-independent. **a-c,** Tumor cells were co-cultured with conditioned media from astrocytes or CAFs for 60 hours. RT-PCR analyses of PTEN-targeting miR-19a level (**a**) and PTEN mRNA level in HCC1954 cells (**b**) and B16Bl6 cells (**c**) (mean ± s.e.m., *t*-test). **d,** Western blot detecting PTEN protein levels in HCC1954 cells after culture with conditioned media from either astrocytes or CAFs for 60 hours.

The next imposing question is how miR-19a was inter-cellular transferred from astrocytes to tumor cells. Recognizing exosomes' involvement in neuronal function and hypoxic response during glioma development [246], we postulated that exosome may mediate miR-19a transfer from astrocytes to tumor cells. Indeed, transmission electron microscopy detected spherical, membrane-encapsulated particles between 30 and 100 nm, typical of exosome vesicles, in astrocyte-conditioned media [247] (Fig. 25a). Additionally, the astrocyte-conditioned media contained significantly more $CD63^+$, $CD81^+$and $TSG101^+$ exosomes[247] than the CAF-conditioned media (Fig. 25b). FACS analysis and exosome protein measurement demonstrated that the astrocyte conditioned media contained significantly higher amount of CD63+ exosomes [247] than the CAF-conditioned media (Fig. 25c-25d). Moreover, the exosomes from astrocytes contained 3.5-fold higher levels of miR-19a than those from CAFs (Fig. 25e). In order to test miR-19a transferring is dependent on exosome, we firstly labelled miR-19a with Cy3 and transfected Cy3-miR-19a into astrocytes. Adding exosomes purified from conditioned media of Cy3-miR-19a-transfected astrocytes to tumor cells led us to detect Cy3 in the tumor cells, which means miR-19a transferred into cultured tumor cells by exosomes (Fig. 26a). Furthermore, treating tumor cells directly with astrocyte-derived exosomes led to a dose-dependent increase of miR-19a and a subsequent decrease of *PTEN* mRNA in tumor cells (Fig. 26b), indicating that astrocyte-released exosomes contained miR-19a and can transfer miR-19a into tumor cells to inhibit PTEN expression. To determine whether astrocyte-released exosomes are required for miR-19a transfer and PTEN down-regulation, we treated astrocyte with an inhibitor of exosome release, dimethyl amiloride (DMA), which reduced exosome secretion into conditioned media (Fig.27a). Compared to vehicle-treated astrocytes, DMA-treated astrocytes decreased miR-19a transfer into tumor cells (~20%) and, consequently, restored PTEN mRNA level under co-culture

conditions (Fig. 27b). Similarly, blocking exosome secretion in astrocytes by knocking-down Rab27a, a mediator of exosome secretion [146], with two siRNAs (Fig. 27c-27d) also led to decreased miR-19a transfer and recovered PTEN mRNA level in co-cultured tumor cells compared to control siRNA-transfected astrocytes (Fig. 27e). Collectively, astrocyte-derived exosomes functionally transferred miR-19a into tumor cells leading to PTEN down-regulation.

Figure 25

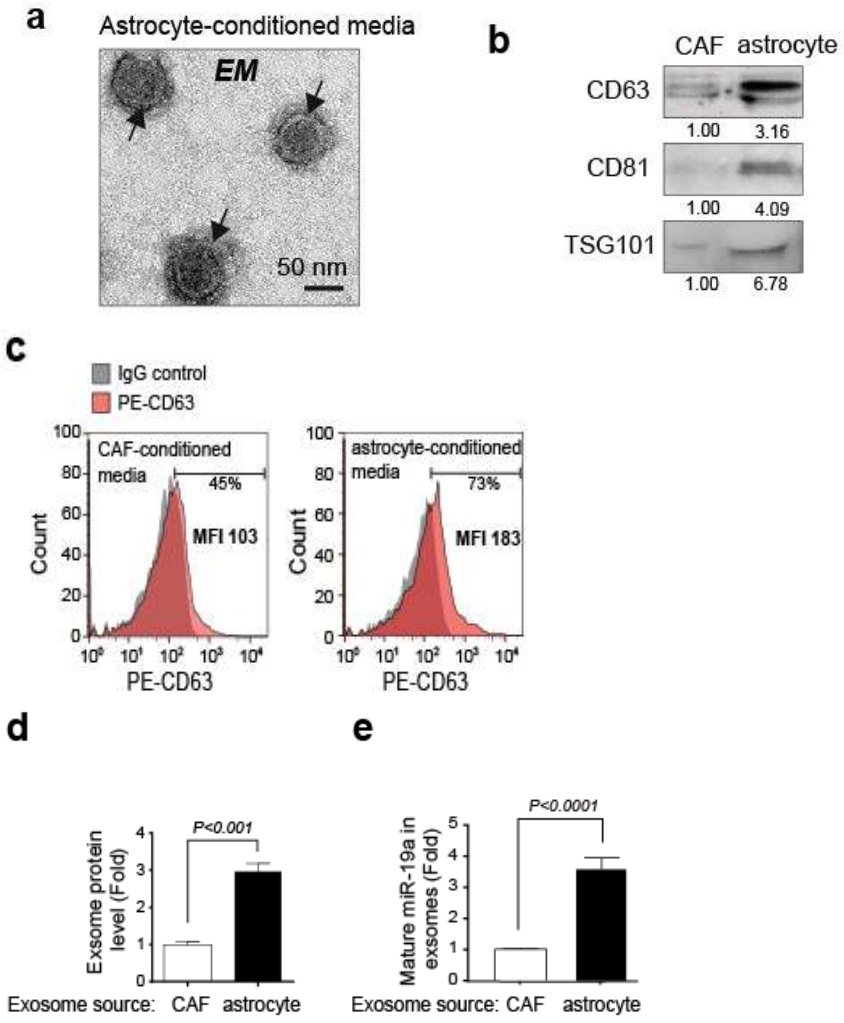


Figure 25.| Astrocyte conditioned media contains more exosomes than CAF conditioned media. a-b, Transmission electron microscopy (**a**) of exosome vesicles in astrocyte-conditioned media that are confirmed by western blot for CD63, CD81, and TSG101 exosome markers released by 1×10^6 of CAFs or astrocytes (**b**). **c,** Flow cytometry detecting CD63+ exosomes extracted from CAF- or astrocyte-conditioned media. **d,** Histogram showing the exosome protein level detected from CAF- and astrocyte-conditioned media normalized by cell number (mean ± s.e.m., *t*-test). **e,** RT-PCR analyses of miR-19a level in exosomes extracted from CAF or astrocyte conditioned media normalized by equal cell numbers (mean ± s.e.m., *t*-test).

Figure 26

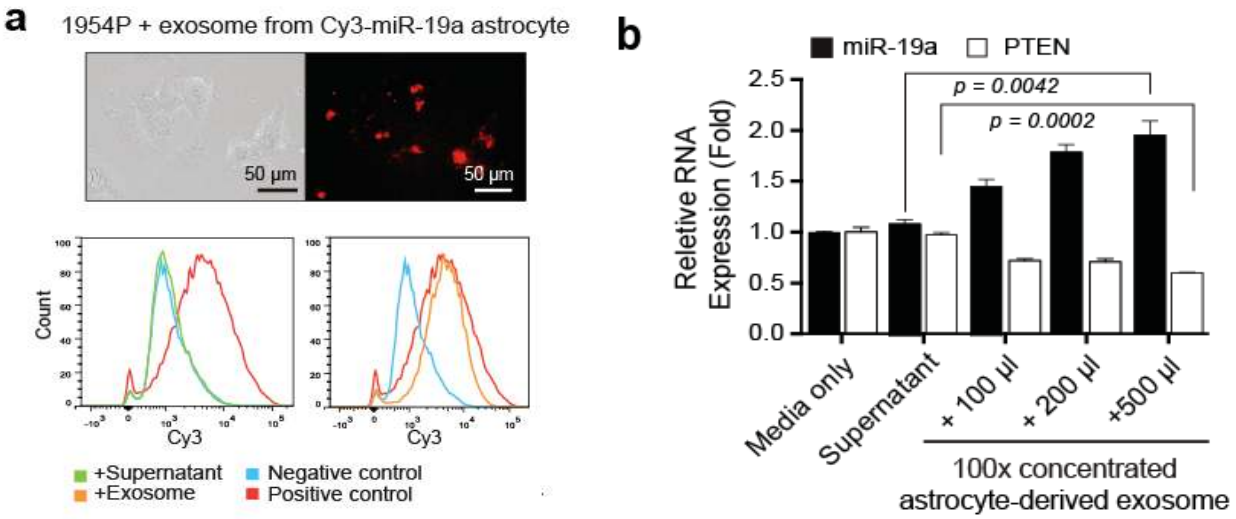


Figure 26. | Intercellular transfer of miR-19a to tumor cells via astrocyte-derived exosomes. a, Representative data showing presence of Cy3-miR-19a in HCC1954 breast cancer cells after adding exosomes purified from Cy3-miR-19a transfected astrocytes for 24 hours. Bottom panel: flow cytometry analysis of Cy3-miR-19a-positive HCC1954 cells after treatment with supernatant (without exosomes), or exosomes purified from Cy3-miR-19a-transfected astrocytes. Negative control is HCC1954 cells without treatment. Positive control is Cy3-miR-19a-transfected astrocytes. **b,** Histograms of miR-19a and PTEN mRNA in HCC1954 cells 48 hours after addition of media, astrocyte supernatant, or exosomes purified from astrocyte-conditioned media (mean ± s.e.m., *t*-test).

Figure 27

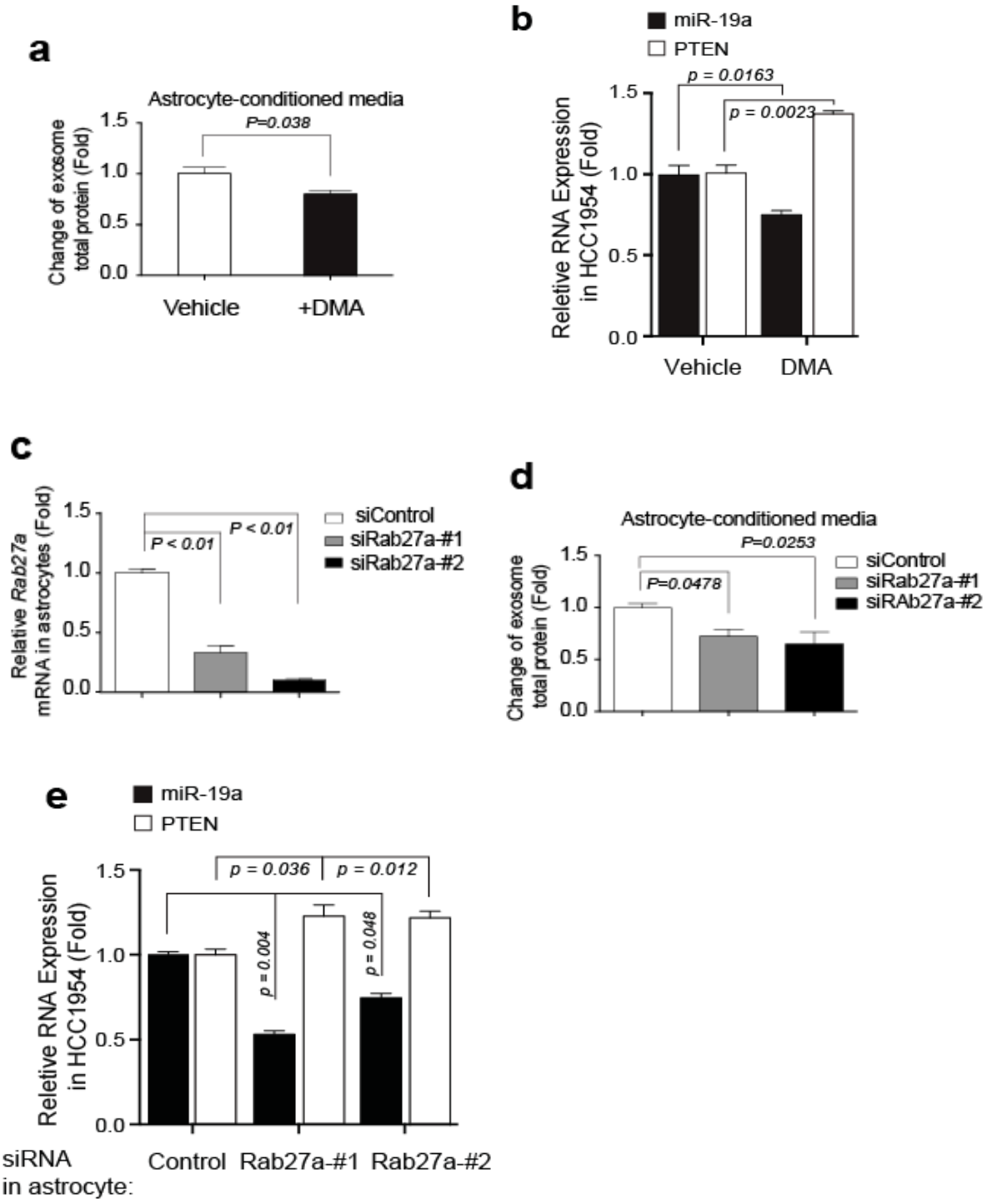
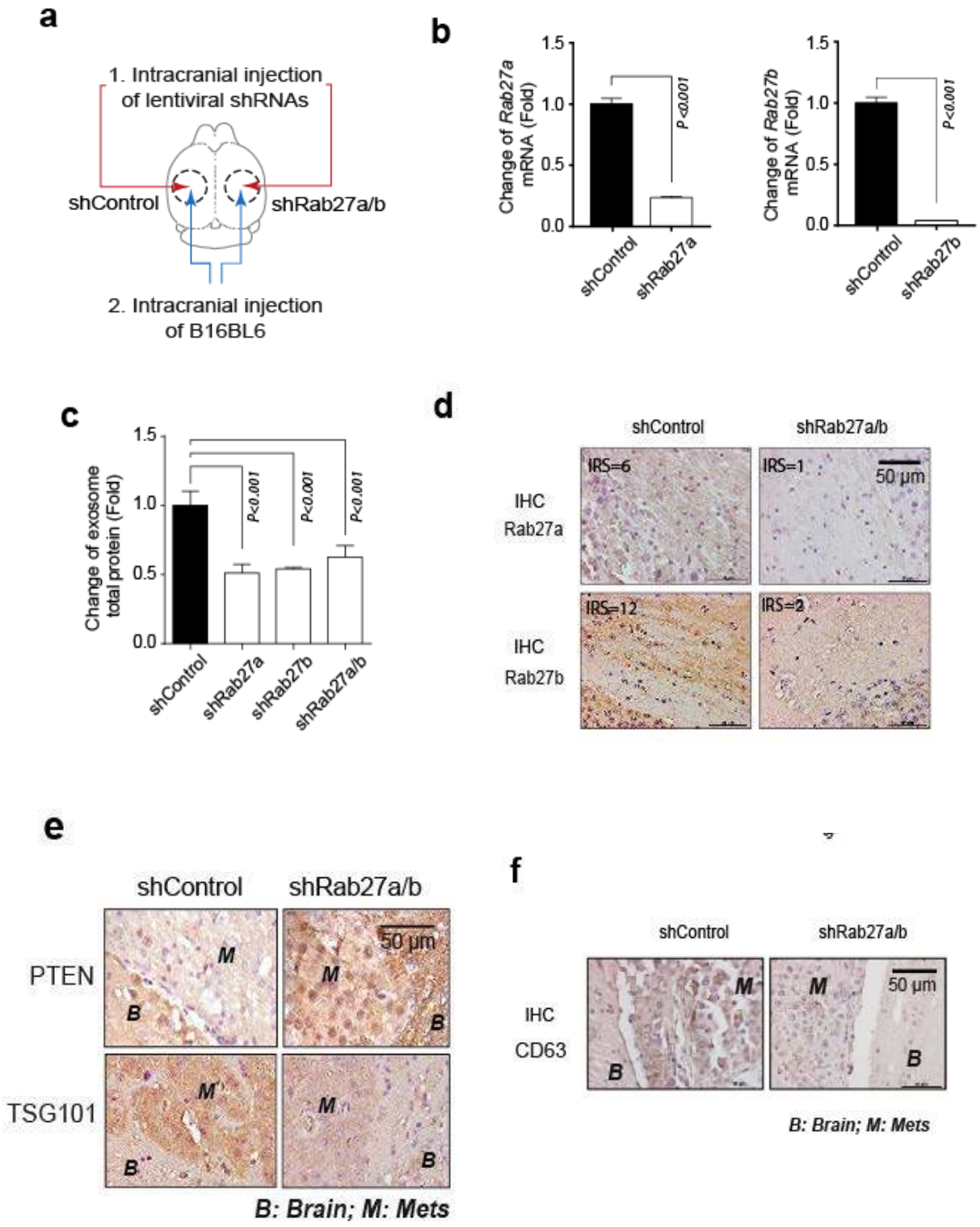


Figure 27. | Inhibition of exosome release by DMA or Rab27a siRNAs restores PTEN expression in tumor cells *in vitro*.

Figure 27. | Inhibition of exosome release by DMA or Rab27a siRNAs restores PTEN expression in tumor cells *in vitro*. a, Exosome-releasing inhibitor (DMA) treatment reduced exosome secretion from astrocytes compared to vehicle treated astrocytes. Astrocytes were treated with DMA (25μg/ml) or vehicle for 4 hours; exosomes were concentrated from astrocyte-conditioned media and total proteins from exosomes were examined by BCA assay (normalized to total cell numbers) (mean ± s.e.m., *t*-test). **b,** Histograms of miR-19a and PTEN mRNA in HCC1954 cells after 48 hour co-culture in conditioned media from vehicle- or DMA-treated (4 hours) astrocytes (mean ± s.e.m., *t*-test). **c,** Knockdown of Rab27a in astrocytes by siRNA. Two siRNAs targeting mouse Rab27a were transiently transfected into astrocytes and mRNA level of Rab27a was examined by RT-PCR 48 hours after transfection (mean ± s.e.m., *t*-test). **d,** Knocking down Rab27a in astrocytes inhibited exosome release. 48 hours after Rab27a-targeting siRNAs were transfected, exosomes were collected from astrocyte-conditioned media and total proteins from exosomes were examined by BCA assay (normalized to total cell numbers) (mean ± s.e.m., *t*-test**).** **e**, Histograms of miR-19a and PTEN mRNA in HCC1954 cells after 48 hour co-culture in conditioned media from control- or Rab27a-siRNA- transfected (48 hours) astrocytes (mean ± s.e.m., *t*-test).

Furthermore, we intracranially injected *Rab27a/b* short hairpin RNA (shRNA) lentiviruses to block exosome secretion in mouse brain parenchyma (brain metastasis stroma), and then inoculated B16BL6 melanoma cells to the same sites (Fig. 28a). Inhibiting Rab27a/b (Fig. 28b) reduced exosome release *in vitro* (Fig. 28c). IHC staining at *in vivo* samples also showed that knock-down Rab27a/b (Fig. 28d) inhibited $TSG101^+$ and $CD63^+$ exosomes (Fig. 28e- 28f), blocked PTEN downregulation in tumor lesions (Fig. 28e), and significantly decreased tumor outgrowth (Fig. 28g).To test the key effect of exosome on PTEN expression, we intracranially injected tumor cells with astrocyte-derived exosomes in mouse brain parenchyma where exosome secretion was inhibited by *Rab27a/b* shRNA (Fig. 29a-29b). Co-injection of tumor cells with astrocyte-derived exosomes rescued PTEN downregulation in tumor cells (Fig. 29c- 29d) and metastatic outgrowth (Fig. 29e). Collectively, exosome-mediated miR-19a transfer from astrocytes to tumor cells was critical for tumor PTEN downregulation and aggressive outgrowth in the brain.

Figure 28


a
1. Intracranial injection of lentiviral shRNAs
shControl
shRab27a/b
2. Intracranial injection of B16BL6
b
Change of Rab27a mRNA (Fold)
P<0.001
shControl
shRab27a
Change of Rab27b mRNA (Fold)
P<0.001
shControl
shRab27b
c
Change of exosome total protein (Fold)
P<0.001
P<0.001
P<0.001
shControl
shRab27a
shRab27b
shRab27a/b
d
shControl
shRab27a/b
IHC Rab27a
IRS=6
IRS=1
50 μm
IHC Rab27b
IRS=12
IRS=2
e
shControl
shRab27a/b
PTEN
TSG101
M
B
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f
shControl
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B: Brain; M: Mets

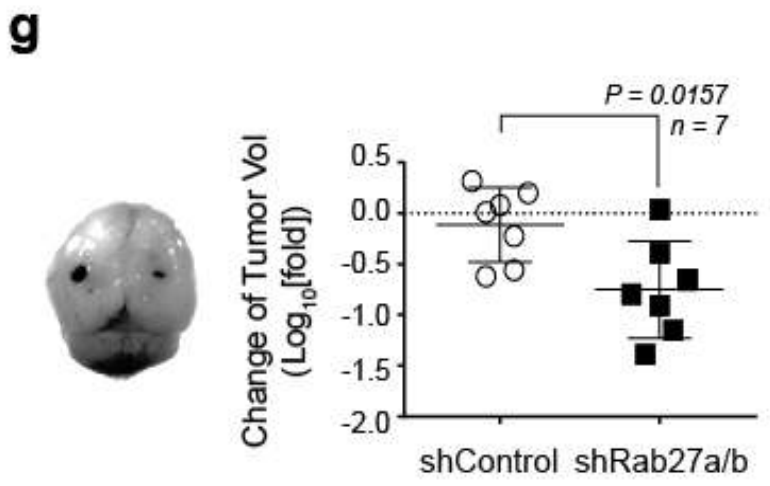


Figure 28. | Inhibition of astrocyte-derived exosomes restores PTEN expression in tumor cells and inhibits tumor outgrowth *in vivo*. a, Schematics of *in vivo* experiments. **b,** Histogram showing relevant changes of *Rab27a* and *Rab27b* mRNA level in primary astrocytes infected with pLKO.shRab27a or pLKO.shRab27b virus (mean ± s.e.m., *t*-test, $P<0.001$, 3 biological replicates, with 3 technical replicates each). **c,** Change of exosome protein level detected in astrocyte-conditioned media from cells infected by pLKO.shRab27a or pLKO.shRab27b virus by BCA assay (normalized to total cell numbers) (mean ± s.e.m., *t*-test, $P<0.001$, 3 biological replicates, with 3 technical replicates each). **d-g,** IHC analysis showing the expression level of Rab27a, Rab27b (**d**) ,PTEN, exosome marker expression TSG101(**e**), CD63 (**f**) , and changes of tumor volume (**g**) (mean ± s.d., *t*-test) in the brain tissue derived from mice injected with control lentivirus or Rab27a/b shRNA lentiviruses and subsequently intracranially injected with B16BL6 cells. *Collaborated with Min Li, Qingling Zhang and Kenneth Ellis.*

Figure 29

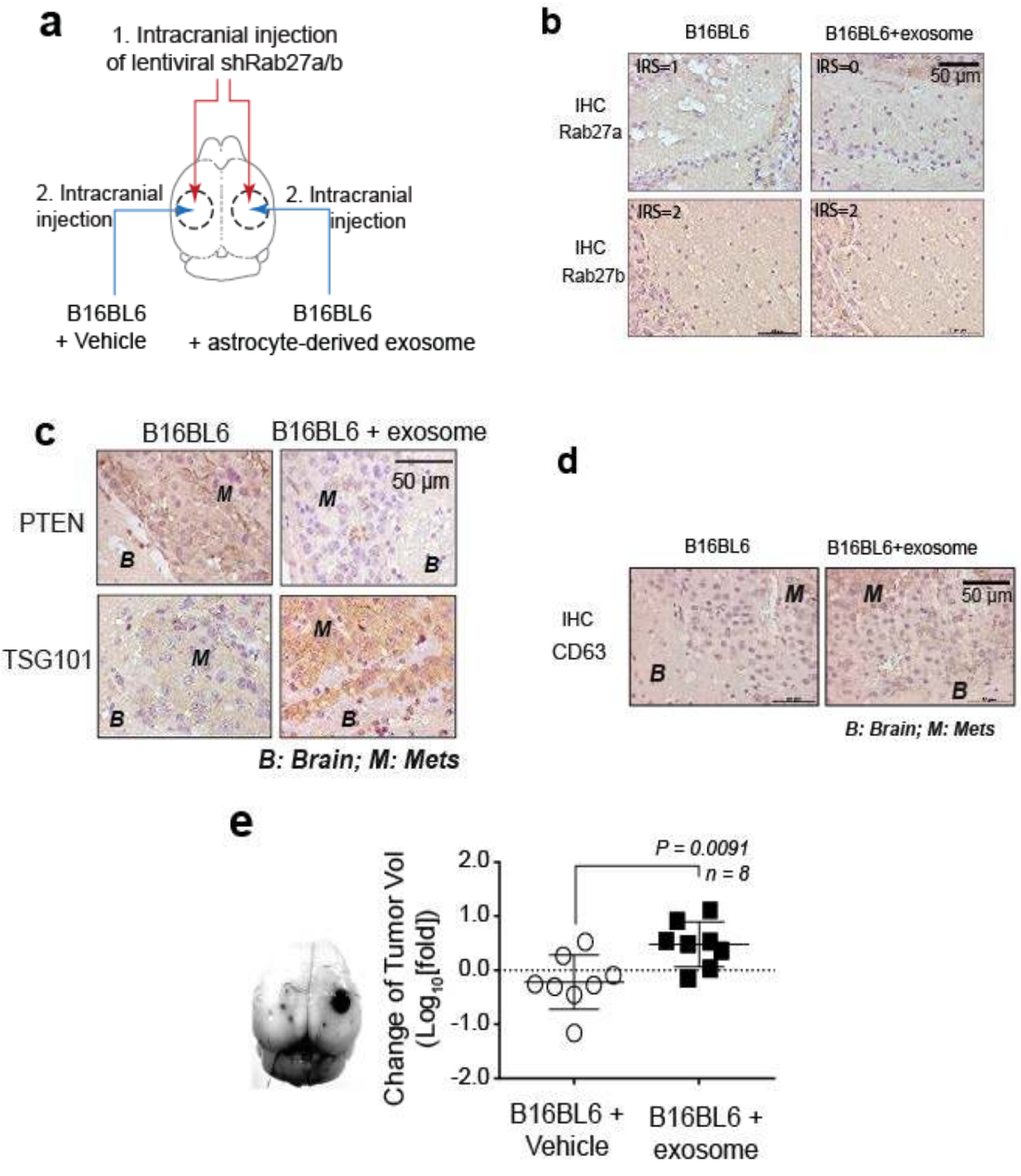


Figure 29. | Astrocyte-derived exosome induces PTEN loss in tumor cells and promotes tumor outgrowth *in vivo*.

Figure 29. | Astrocyte-derived exosome induces PTEN loss in tumor cells and promotes tumor outgrowth *in vivo*. a, Schematics showing *in vivo* rescue of exosome effect by pre-incubation of tumor cells with astrocyte-derived exosomes. **b,** IHC analysis showing the expression level of Rab27a and Rab27b. **c-e,** IHC analyses of PTEN and exosome marker expression TSG101 (**c),** CD63 **(d**) and changes of tumor volume (**e**) (mean ± s.d., *t*-test) in the brain tissue derived from mice injected with Rab27a/b shRNA lentiviruses and subsequently intracranially injected with B16BL6 cells with vehicle or B16BL6 cells with astrocyte derived exosomes. *Collaborated with Min Li, Qingling Zhang and Kenneth Ellis*.

3.3 PTEN-loss promotes metastatic tumor outgrowth by secreting CCL2, which recruits myeloid cells

3. 3.1 PTEN-loss promotes metastatic tumor outgrowth

To explore the biological significance of the PTEN-loss in brain metastasis, we first transfected subclones of human breast carcinoma cells that selectively metastasize to the brain (MDA-MB-231Br) with doxycycline (Dox)-inducible PTEN.shRNAs that significantly down-regulated PTEN with Dox-treatment but fully restored PTEN expression five days after Dox-withdraw (Fig. 30a). The inducible PTEN.shRNA.red fluorescent protein (RFP) cells and control.shRNA.green fluorescent protein (GFP) cells were mixed (1:1), treated with Dox for 24 hours in culture, and injected into ICA of Dox-fed mice. PTEN knockdown did not alter tumor cell extravasation into the brain parenchyma (Fig. 30b-30c), suggesting PTEN-loss does not promote tumor cell extravasation. This also suggested that the observed PTEN-loss in brain metastasis tumors (Figs. 7 and 8) happened after tumor cells extravasated into the brain microenvironment.

To test whether restoring PTEN expression after tumor cell extravasation inhibits metastatic outgrowth, we transfected MDA-MB-231Br stably with either a doxycycline-inducible *PTEN*-coding sequence without the 3′-UTR miRNA binding sites, or RFP controls (Fig. 31a-31b). RFP or PTEN expression were induced by feeding mice with Dox daily beginning at day 7 post-intracarotid injection (Fig. 31c), which induced strong expressions of RFP (Fig. 31b right) and PTEN in brain metastatic tumors *in vivo*. Restoring PTEN after extravasation of tumor cells markedly extended the overall survival of brain metastases-bearing mice ($p<0.001$, log-rank test) (Fig. 31d-31e). Collectively, PTEN loss primes brain metastasis outgrowth after tumor cell extravasation and PTEN restoration suppresses the outgrowth.

Figure 30

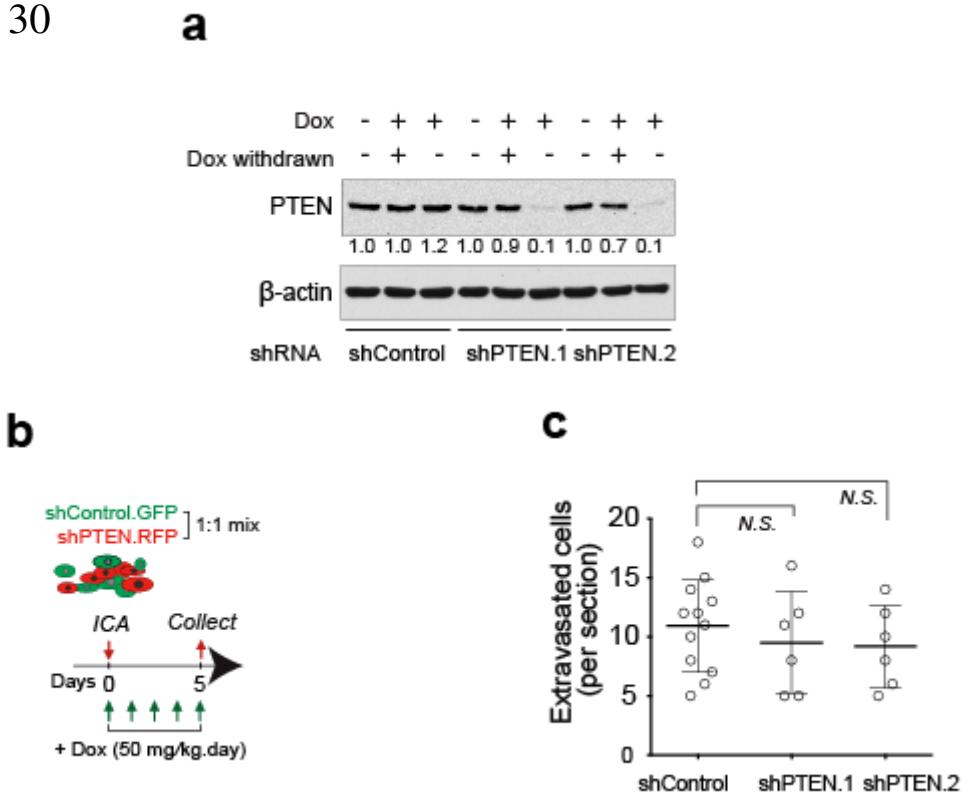


Figure 30. | PTEN knockdown does not alter tumor extravasation into brain parenchyma. **a,** Western blot showing PTEN expression levels after treating MDA-MD-231 cells with doxycycline (Dox). MDA-MD-231 cells were stably infected with inducible shRNA expression vectors (pTRIPZ-Control.sh.GFP as control and pTRIPZ.shRNA.RFP for PTEN shRNA). Dox (1 μg/ml) was added to induce shRNA expression for five days. As indicated, Dox were withdrawn in some samples for another five days before analysis. **b,** Schematics of *in vivo* extravasation assay. shControl.GFP and shPTEN.RFP cells were mixed at 1:1 ratio. Total 200,000 cells were injected intracarotidly into mice. Dox (50 μg/kg) was given to mice intraperitoneally daily. Brains were collected 5 days after injection. ICA: intracarotid injection. **c,** Dot-plot of extravasated cell counts five days after intracarotid injection of indicated MDA-MB-231 sublines. Tumor bearing brains were collected and sectioned into 100 μm brain coronal slices. The extravasated tumor cells were counted under the fluorescence microscope (mean ± s.d., *t*-test). *Collaborated with Siyaun Zhang.*

Figure 31

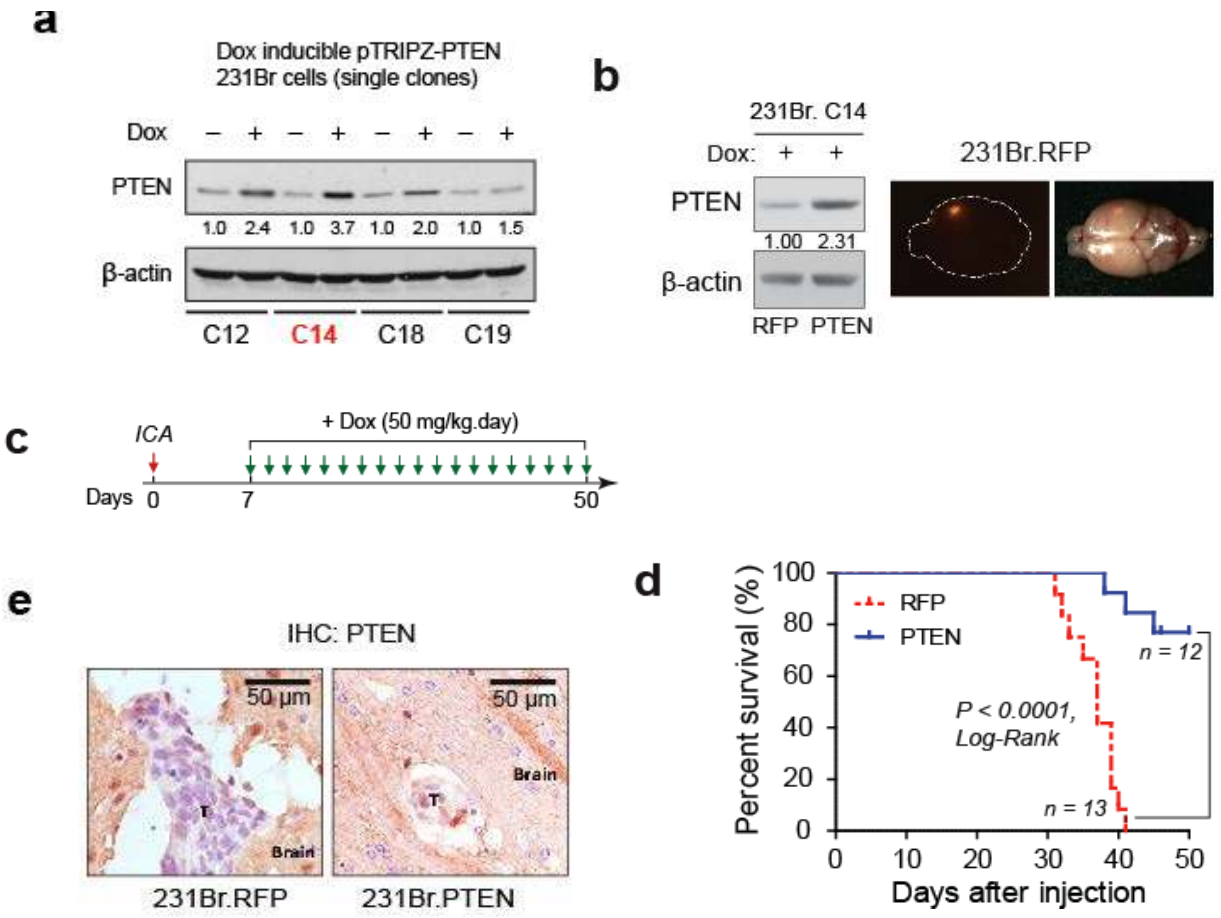


Figure 31. | The survival of mice bearing brain metastases is prolonged by restoration of PTEN expression after extravasation. a, MDA-MB-231Br single cells were expanded into subclones (C12, C14, C18, and C19) which were transfected with Dox-inducible pTRIPZ.RFP or pTRIPZ.PTEN. 48 hours after Dox (1μg/mL) treatment, PTEN induction was tested by western blotting. C14 clone were used for further *in vivo* assays. **b,** Dox-inducible RFP (left) and PTEN expression (right) in 231Br cells. **c,** Schematics of brain metastasis assay with dox-induced RFP or PTEN expression. **d,** Overall survival of mice bearing brain metastases of 231Br cells with induced PTEN re-expression or RFP expression (Log-Rank test). **e,** Induced PTEN expression in brain metastases. IHC staining of PTEN expression levels in brain metastases derived from mice injected with MDA-MB-231Br (231Br. RFP or 231Br. PTEN) cells. T: brain metastasis tumors at day 30 post intracarotid injection. *Collaborated with Siyaun Zhang.*

3.3.2 CCL2 is a downstream effector of PTEN loss

The interactions between disseminated tumor cells and their metastasis microenvironment through auto/paracrine signaling play decisive roles in metastasis seeding and outgrowth [51, 248, 249]. To dissect how PTEN-loss enhanced brain metastasis outgrowth, we explored PTEN-loss-induced changes of secreted factors from tumor cells into brain microenvironment using cytokine-array. Although PTEN restoration only led to a trend of reduced Akt and P70S6K phosphorylation (pAkt and pP70S6K, respectively (Fig. 32), cytokine array analyses revealed markedly reduced CCL2 secretion in PTEN-expressing tumor cells compared to controls (Fig. 33a), whereas PTEN knockdown increased CCL2 expression (Fig. 33b) in multiple human breast cancer cell lines.

Various cell types in the brain express CCL2. However, CCL2 expression in brain tissue is relatively low based on global gene expression comparison study (Fig. 34a)[250]. Brain metastasis tumors expressed a very high level of CCL2 whereas brain tissue positive for the neuron-specific marker MAP2 had barely detectable CCL2 levels (Fig. 34b). Together, the data indicated that tumor-derived CCL2 was the major source of CCL2 in brain metastasis tumors.

Figure 32

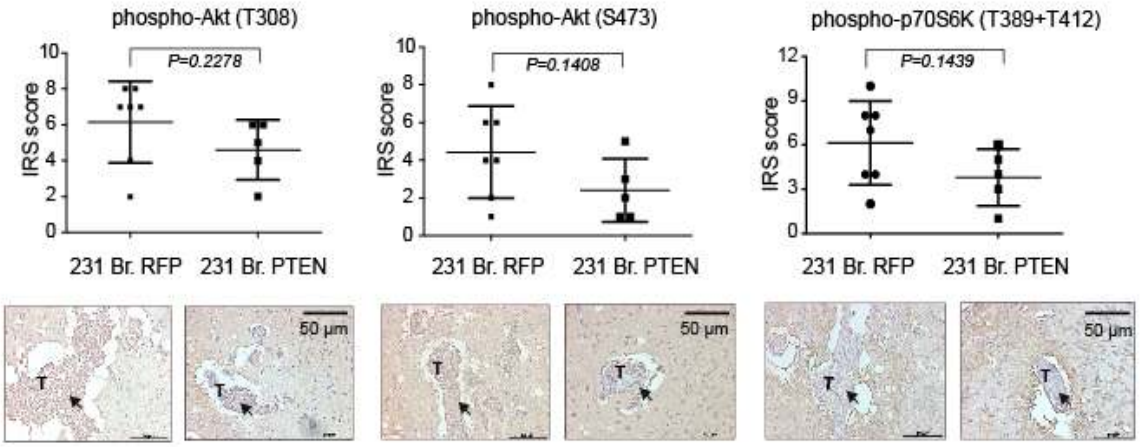


Figure 32. | PTEN restoration leads to a trend of reduced PTEN downstream signaling pathway. IHC analysis of phospho-Akt(T308), phospho-Akt(S473) and phospho-P70S6K(T389+T412) in brain metastases from mice injected with 231Br.RFP versus 231Br.PTEN cells. Top, dot-plot of IHC data quantification by immuno-reactive score (IRS) (mean ± s.d., *t*-test); bottom, representative IHC staining data. T: brain metastasis tumors at day 30 post intracarotid injection. *Collaborated with Qinging Zhang*.

Figure 33

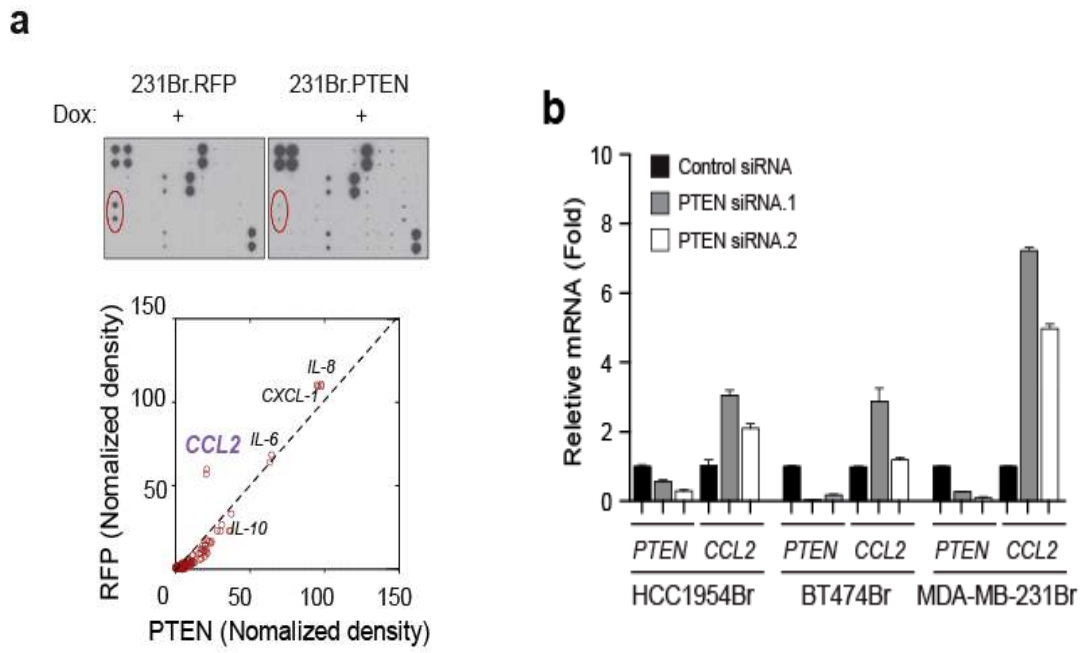


Figure 33. | CCL2 is a downstream effector of PTEN loss. a, Cytokine array of 231Br cells with dox-induced RFP or PTEN expression. **b,** Histograms of PTEN and CCL2 mRNA levels (mean ± s.e.m., *t*-test) in indicated cancer cell lines 48 hours after transfection with control or PTEN siRNAs. *Collaborated with Min Li.*

Figure 34

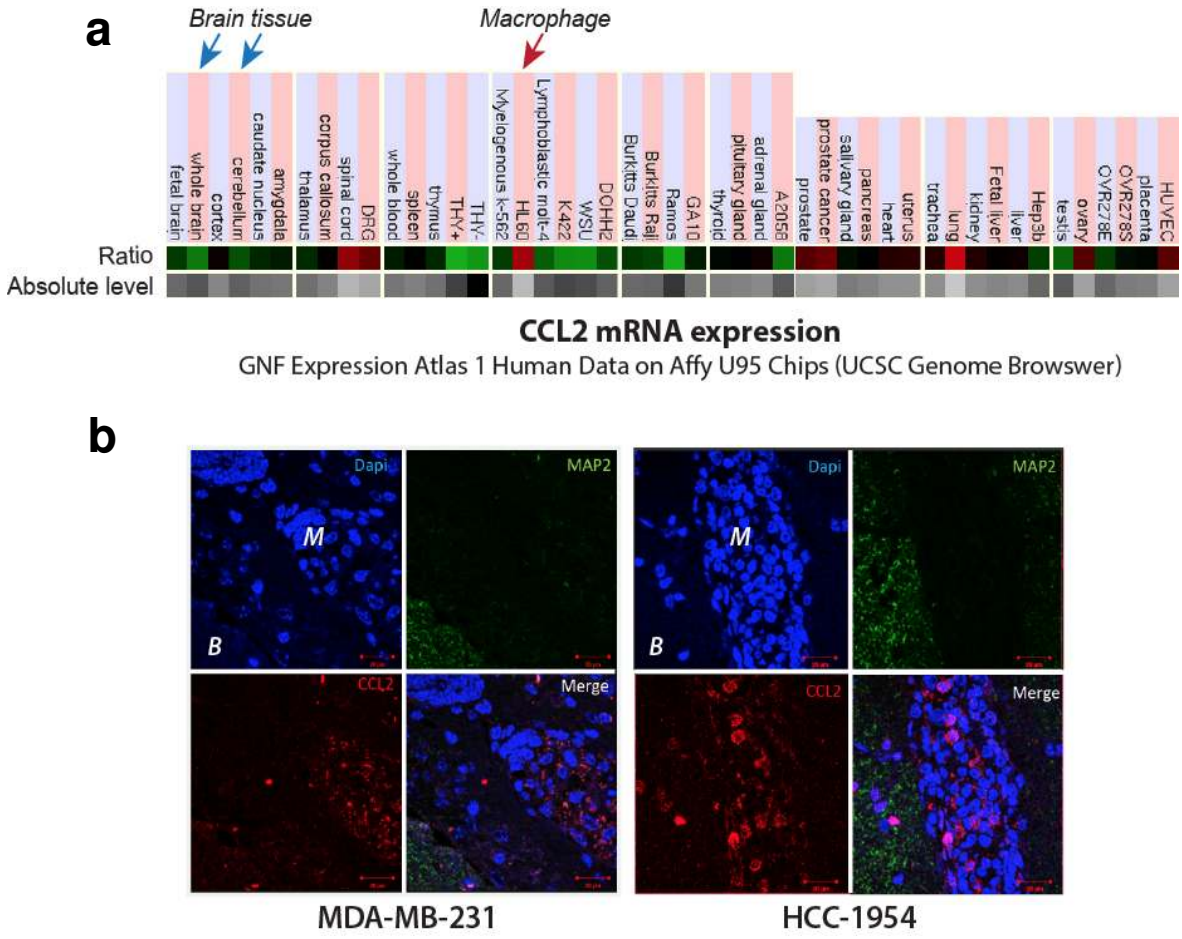


Figure 34. | CCL2 expression in normal brain tissue and brain metastatic tumor cells *in vivo*. a, Heat map of relative CCL2 expression across different normal tissue types. **b,** Immunofluorescence staining of brain metastasis tissue derived from MDA-MB-231 and HCC-1954 cells. M: metastases; B: brain tissue. MAP2, neuron specific marker: Green. CCL2: red. *Collaborated with Siyuan Zhang.*

Considering the important role of CCL2 in lung metastasis [51], we further tested whether PTEN down-regulation-induced CCL2 up-regulation enhances outgrowth of extravasated tumor cells. Dox-inducible CCL2 knockdown MDA-MB-231 sublines were established (Fig. 35a) and injected into ICA of mice for *in vivo* brain metastases. Knocking-down CCL2 significantly extended the overall survival of brain metastases-bearing mice (Fig. 35b-35c), decreased proliferation and increased apoptosis of brain metastasis tumors compared to controls (Fig 36a- 36b). Mechanistically, PTEN induction decreased NF-κB p65 phosphorylation (Fig. 37a-37b) by RPPA analysis, whereas PTEN knockdown by shRNAs increased p65 nuclear translocation, an indicator of NF-κB activation, and CCL2 expression (Fig. 37c-37d). p65 nuclear translocation is inhibited by the treatment of Akt inhibitor MK2206, indicating NF-κB activation in our system is partly through Akt activation (Fig. 37e). Furthermore, *CCL2* mRNA and CCL2 protein expression in brain-seeking tumor cells was inhibited by the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) (Fig. 38a-38c), indicating that NF-κB activation is crucial for PTEN-loss-induced CCL2 upregulation.

CCL2 is a chemo-attractant during inflammation[251]. CCL2 receptor (CCR2)-expressing brain-derived ionized calcium-binding adapter molecule 1-positive ($IBA1^+$) primary myeloid cells and BV2 microglial cells (Fig. 39a-39b) migrate towards CCL2, which was blocked by CCR2 antagonists[51] (Fig. 39c-39d). Functionally, co-culturing with BV2 cells enhanced proliferation and inhibited apoptosis of breast cancer cells (Fig. 40a-40b). *In vivo*, CCL2-knockdown brain metastases had decreased $IBA1^+/CCR2^+$ myeloid cell infiltration (Fig. 40c), corresponding to their reduced proliferation and increased apoptosis (Fig. 36a, 36b).

Figure 35

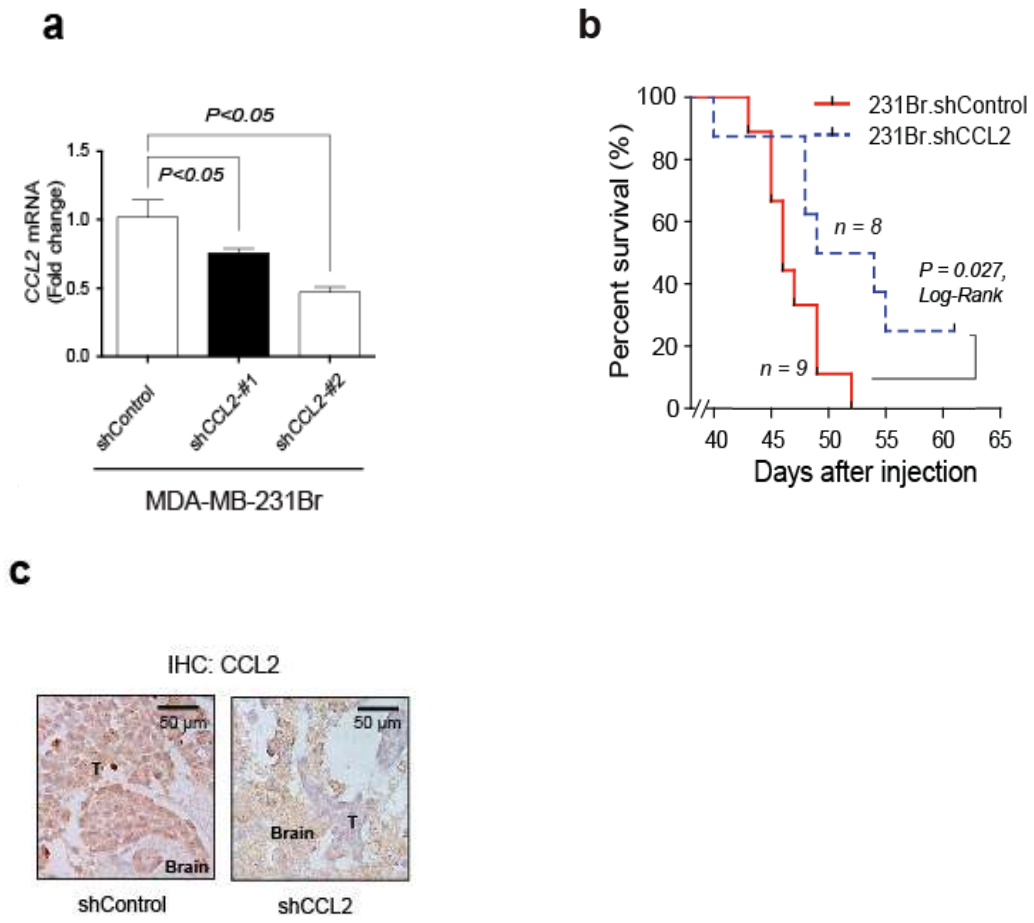


Figure 35. | CCL2 knockdown prolongs the survival of mice bearing brain metastases. a, Histogram showing the inducible CCL2 knockdown. MDA-MB-231Br cells were stably infected with pTRIPZ inducible CCL2 shRNAs. 48 hours after Dox (1μg/ml) treatment, CCL2 mRNA was examined by RT-PCR (mean ± s.e.m., *t*-test). **b,** Overall survival of mice bearing brain metastases of 231Br cells transfected with control or CCL2 shRNAs (Log-Rank test). **c,** Dox-induced CCL2 knockdown in brain metastases. Mice were ICA injected with MDA-MB-231Br cells harboring control or CCL2 shRNAs. Dox (50 μg/kg) was given to mice intraperitoneally daily after injection. IHC staining of CCL2 expression levels in brain metastases derived from MDA-MB-231Br cells. T: brain metastasis tumors at day 30 post intracarotid injection. *Collaborated with Frank Lowery and Qingling Zhang.*

Figure 36

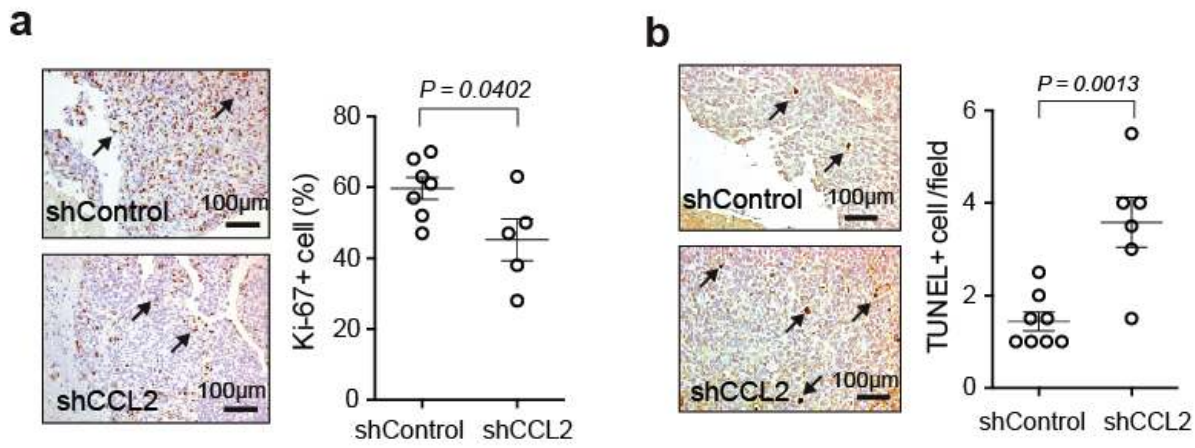
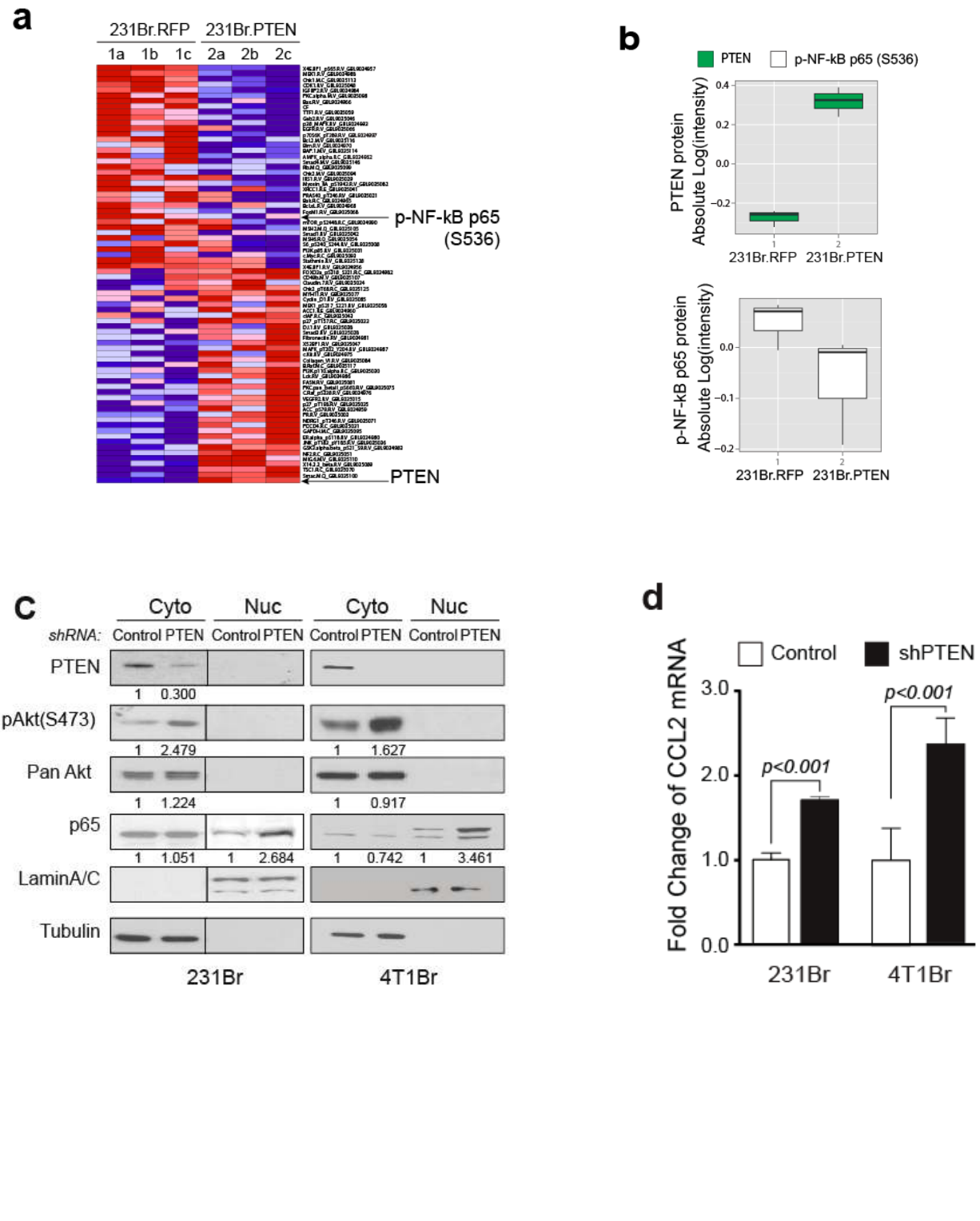


Figure 36. | CCL2 knockdown reduces proliferation and increases apoptosis of tumor cells *in vivo*. a-b, IHC analyses showing decreased proliferation (a: Ki-67) and increased apoptosis (b: TUNEL staining) in brain metastases after shRNA-mediated CCL2 knockdown *in vivo* (mean ± s.e.m., *t*-test). *Collaborated with Hai Wang*.

Figure 37


a
231Br.RFP
231Br.PTEN
1a 1b 1c 2a 2b 2c
p-NF-kB p65 (S536)
PTEN
b
PTEN
p-NF-kB p65 (S536)
PTEN protein Absolute Log(intensity)
231Br.RFP 231Br.PTEN
p-NF-kB p65 protein Absolute Log(intensity)
231Br.RFP 231Br.PTEN
c
Cyto Nuc Cyto Nuc
shRNA: Control PTEN Control PTEN Control PTEN Control PTEN
PTEN
1 0.300
pAkt(S473)
1 2.479 1 1.627
Pan Akt
1 1.224 1 0.917
p65
1 1.051 1 2.684 1 0.742 1 3.461
LaminA/C
Tubulin
231Br 4T1Br
d
Control shPTEN
Fold Change of CCL2 mRNA
p<0.001
p<0.001
231Br 4T1Br

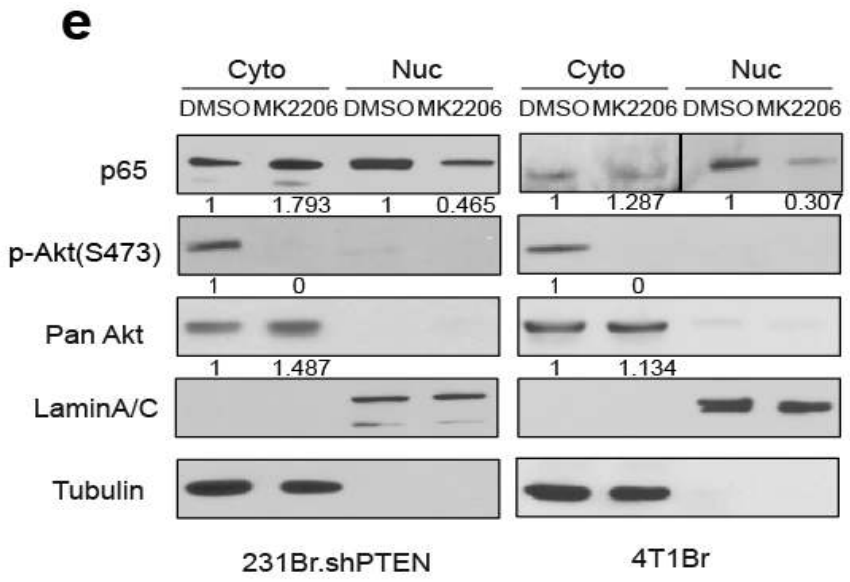


Figure 37. | PTEN-regulated CCL2 expression is through NF-κB pathway. a, Heatmap showing differentially expressed protein markers of Reverse Phase Protein Array (RPPA) analysis. Dox-inducible pTRIPZ.RFP or pTRIPZ.PTEN cells. MDA-MB-231Br were stably infected with pTRIPZ.RFP or pTRIPZ.PTEN (231Br. RFP or 231Br. PTEN) and induced by Dox (1μg/mL) for 48 hours. **b,** Box chart showing the absolute intensity of PTEN and NF-κB p65 (S536). **c,** WB analysis of NF-κB p65 nuclear translocation after knocking down PTEN. **d,** Histogram showing CCL2 mRNA level after PTEN knockdown detected by qPCR (mean ± s.e.m., *t*-test). **e,** Western blot analysis of NF-κB p65 nuclear translocation after Akt inhibitor (MK2206) treatment. Cells were treated with MK2206 (10 μg/mL) 24 hours before being separated into cytosol (Cyto) and nuclear (Nuc) fraction. *Collaborated with Siyuan Zhang, Xiao Wang and Ping Li.*

Figure 38

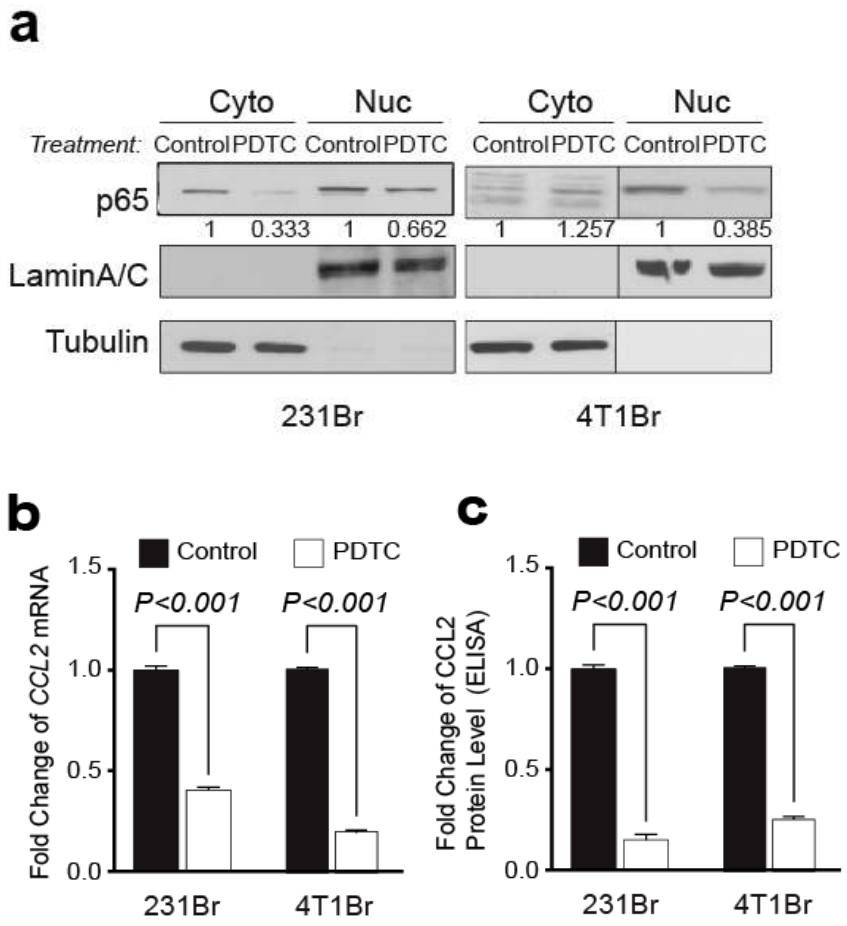


Figure 38. | NF-κB activation is crucial for PTEN-loss-induced CCL2 upregulation. **a,** Western blot analysis of NF-κB p65 nuclear translocation after NF-κB inhibitor PDTC treatment. Cells were treated with PDTC (0.2 mM) 16hours before being separated into cytosol (Cyto) and nuclear (Nuc) fraction. **b,** Relative CCL2 mRNA expression after NF-κB inhibitor PDTC treatment analyzed by qPT-PCR (mean ± s.e.m., *t*-test). Cells were treated with PDTC (0.2 mM) 16 hours.**c,** Relative CCL2 protein expression after NF-κB inhibitor PDTC treatment analyzed by ELISA (mean ± s.e.m., *t*-test). Cells were treated the same as (**b**). *Collaborated with Xiao Wang*.

Figure 39

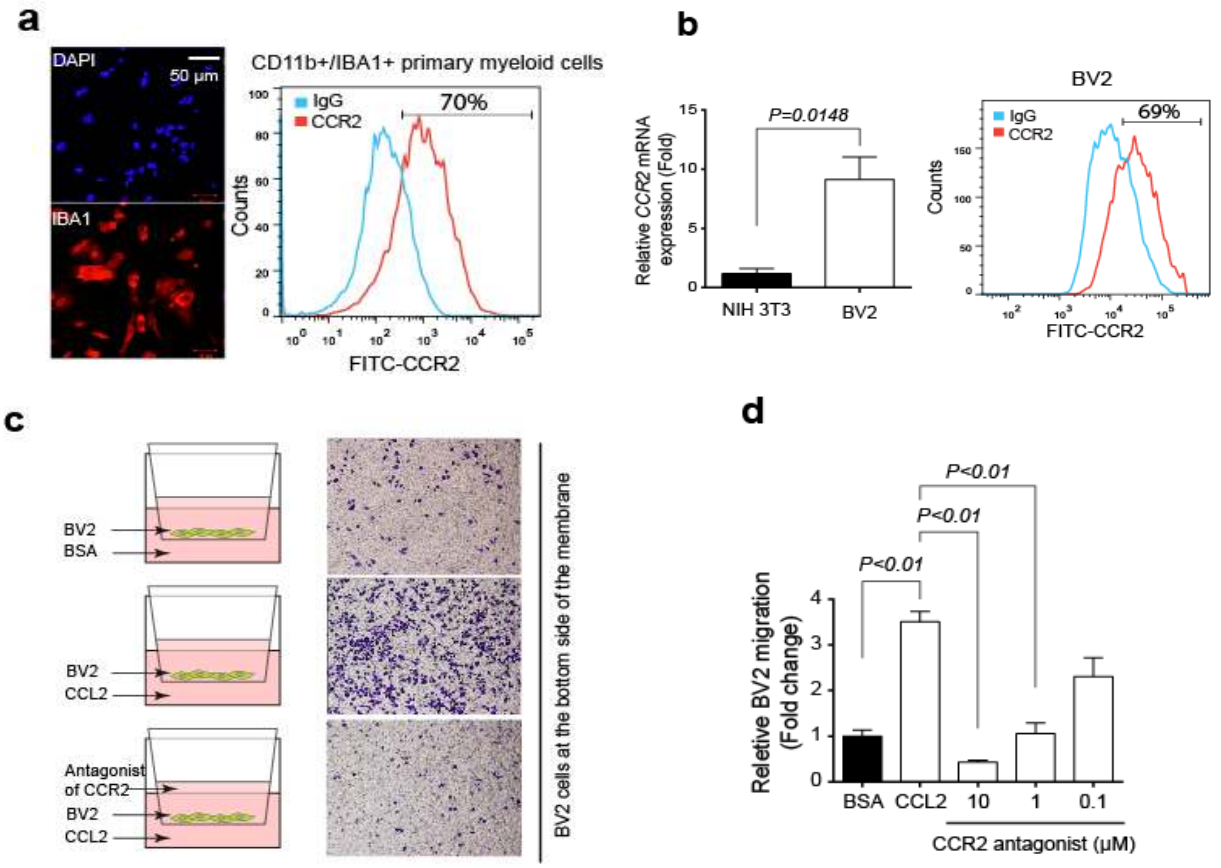


Figure 39. | CCL2-CCR2 mediates IBA1+ myeloid cell directional migration *in vitro*. a, Co-expression of IBA1 and CCR2 on myeloid cells freshly isolated from mouse brain by CD11b beads. Representative picture of immunofluorescence staining of IBA1 (left). FACS analysis of CD11b-positive cells for CCR2 expression. **b**, Relative CCR2 expression in the BV2 microglia cell line compared with NIH 3T3 fibroblasts. CCR2 mRNA level was analyzed by qRT-PCR (mean ± s.e.m., *t*-test) (left) and protein expression was analyzed by FACS (right). **c,** Transwell migration assay examining the directional migration of BV2 cells toward CCL2. 10^5 BV2 cells were seeded in the top chamber of the transwell units and CCL2 or BSA (20ng/ml) was added into serum-free media in the bottom chamber. The migrated cell numbers were counted at 24h. Next, CCR2 antagonists with different concentrations (10uM, 1uM, 0.1uM) were added into the top chamber with BV2 cells, and CCL2 (20ng/ml) was added into serum-free media in the bottom chamber. The migrated cell numbers were counted at 24h. **d,** Quantification of BV2 cell migration assay (mean ± s.e.m., *t*-test). *Collaborated with Xiao Wang.*

Figure 40

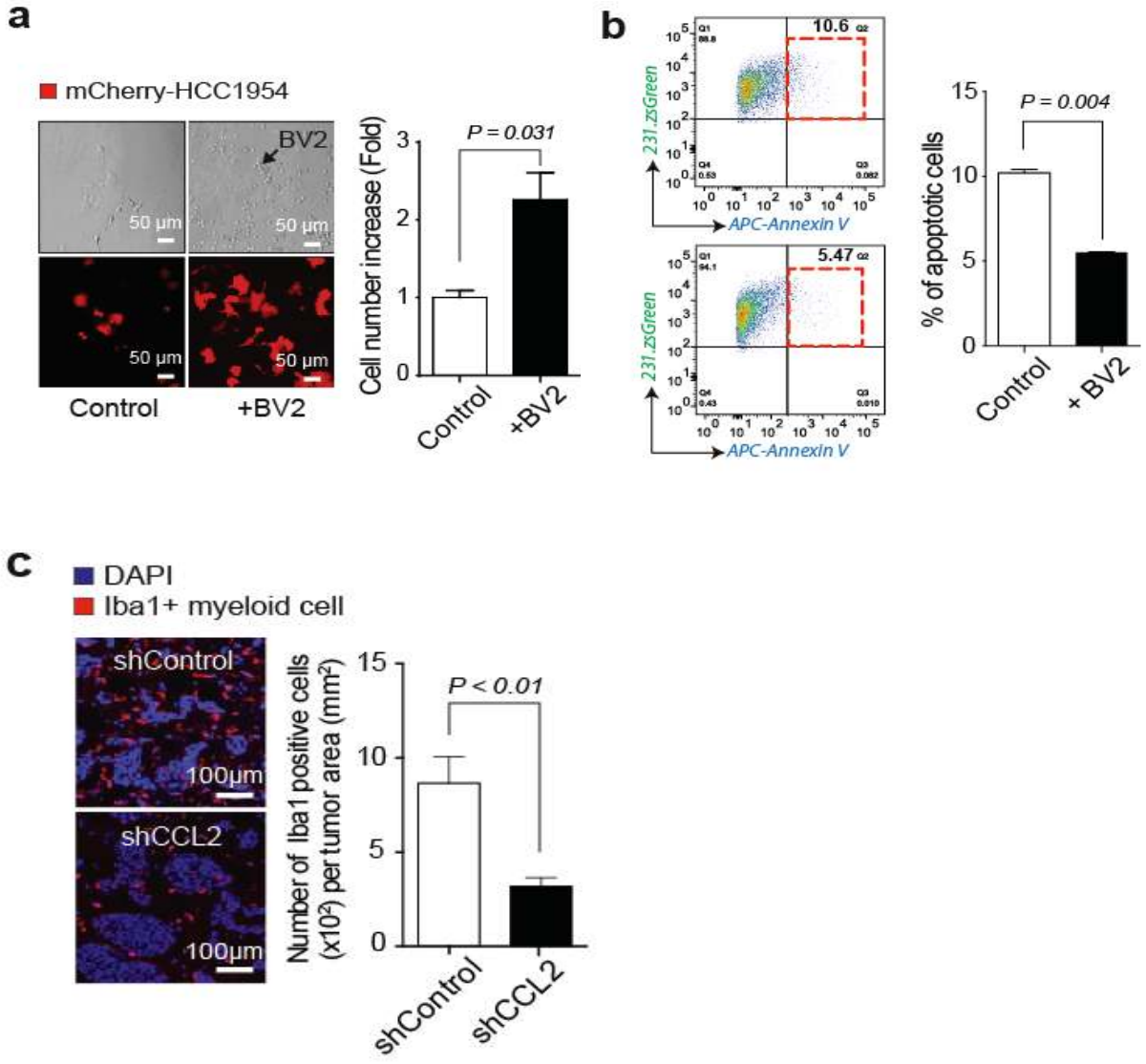


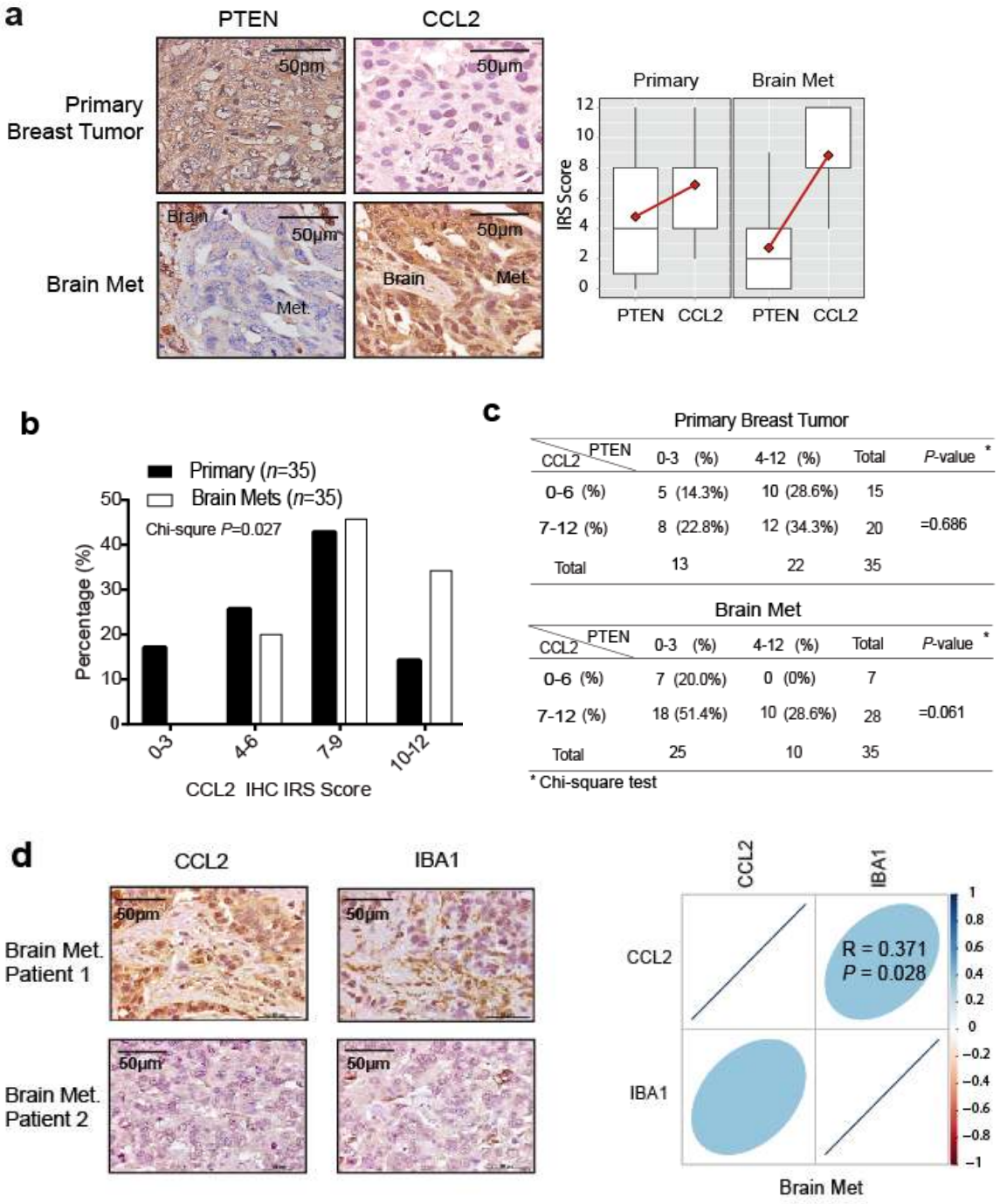
Figure 40. | Co-culturing with BV2 cells enhances proliferation and inhibits apoptosis of tumor cells. a, Light and fluorescent microscopy images and quantification of mCherry-labeled tumor cells with or without BV2 microglia co-culture under 2-day serum starvation (mean ± s.e.m., *t*-test). **b,** FACS analyses of annexinV+ apoptotic zsGreen-labeled 231Br cells under doxorubicin treatment with or without BV2 microglia co-culture (mean ± s.e.m., *t*-test). **c,** Immunofluorescence (IF) staining of IBA1+ myeloid cells in brain metastases of 231Br cells containing control or CCL2 shRNAs (mean ± s.e.m., *t*-test).

3.3.3 The correlation between PTEN, CCL2 and CD63 expression in patients' brain metastases and matched primary breast tumors

IHC staining of human primary breast tumors and matched brain metastases for PTEN and CCL2 (Figs 8b and 41a respectively) revealed a significantly ($P = 0.027$) higher CCL2 expression in brain metastases than in primary tumors (Fig. 41b). Importantly, severe PTEN loss in brain metastases corresponded to higher CCL2 expression (Fig. 41c), which significantly correlated with IBA1$^+$ myeloid cell recruitment (Fig. 41d), validating that PTEN downregulation in brain metastatic tumor cells contributes to CCL2 upregulation and IBA1$^+$ myeloid cell recruitment in clinical brain metastases.

Furthermore, we have explored the correlation between exosomes and CCL2 in matched breast tumors and brain metastases from patients. We stained them for the CD63 exosome marker, as well as CCL2 by IHC (Fig. 42a). We found that CD63 and CCL2 expressions are significantly higher in the brain metastases, compared with primary breast tumor (Fig. 42b). Importantly, CD63 and CCL2 high expression are significantly positively correlated with each other (Fig. 42c). We also have already validated that exosomes isolated from primary astrocytes contain more miR-19a than that in the exosomes from equal numbers of CAF's (Fig. 25e). Together these data validated the human relevance of our findings that brain stromal exosome/microRNA (miR-19a) down-regulates PTEN expression in tumor cells leading to CCL2 up-regulation.

Figure 41



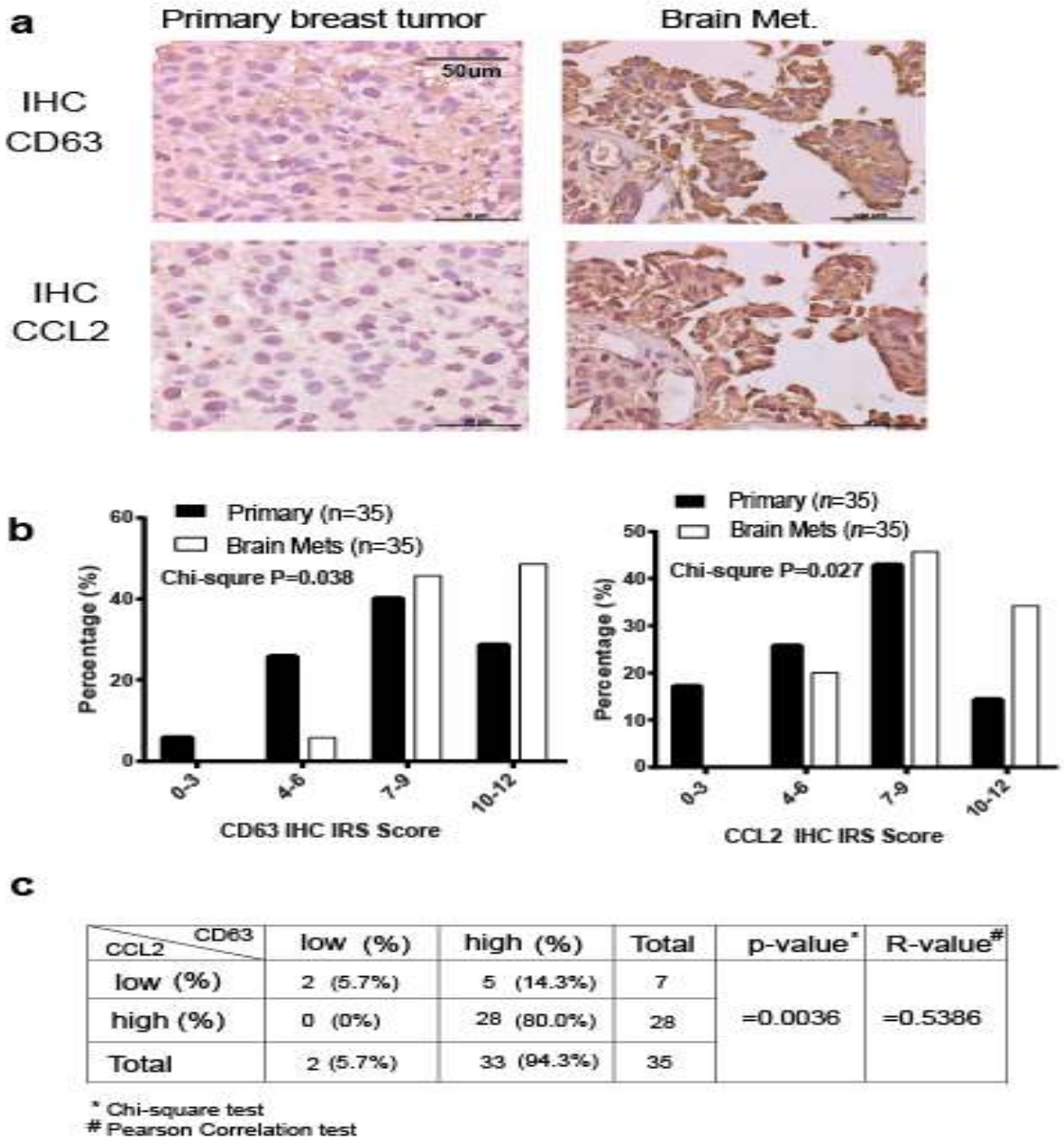
Primary Breast Tumor				
CCL2 \ PTEN	0-3 (%)	4-12 (%)	Total	*P*-value *
0-6 (%)	5 (14.3%)	10 (28.6%)	15	
7-12 (%)	8 (22.8%)	12 (34.3%)	20	=0.686
Total	13	22	35	

Brain Met				
CCL2 \ PTEN	0-3 (%)	4-12 (%)	Total	*P*-value *
0-6 (%)	7 (20.0%)	0 (0%)	7	
7-12 (%)	18 (51.4%)	10 (28.6%)	28	=0.061
Total	25	10	35	

Figure 41. |The association between PTEN, CCL2 expression and recruitment of IBA-1+ myeloid cells in patients' brain metastases and matched primary breast tumors.

Figure 41. |The association between PTEN, CCL2 expression and recruitment of IBA-1+ myeloid cells in patients' brain metastases and matched primary breast tumors. a: PTEN and CCL2 expression in matched primary breast tumors and brain metastases. Left: representative IHC staining of PTEN and CCL2. Right: quantification of PTEN and CCL2 expression in 35 cases of matched primary breast tumors and brain metastases (mean ± s.d.). **b.** Summary histogram of CCL2 protein levels in primary breast tumors and matched brain metastases from 35 patients. Chi-square test was used to compare IHC score in primary breast tumors versus matched brain metastases. $p<0.05$ is defined as significantly different. **c**, Tables showing IHC scores of PTEN and CCL2 expression in primary breast tumors and matched brain metastases. **d,** Representative IHC staining of CCL2 proteins and IBA1+ myeloid cells in patients' brain metastases, and the correlation plot showing the Pearson correlation between CCL2 and IBA1 staining in patients' brain metastases. *Collaborated with Qingling Zhang.*

Figure 42



CCL2 \ CD63	low (%)	high (%)	Total	p-value*	R-value#
low (%)	2 (5.7%)	5 (14.3%)	7	=0.0036	=0.5386
high (%)	0 (0%)	28 (80.0%)	28		
Total	2 (5.7%)	33 (94.3%)	35		

* Chi-square test
# Pearson Correlation test

Figure 42. |The correlation between CD63 and CCL2 expression in patients' brain metastases and matched primary breast tumors. **a,** Representative IHC staining of CD63 and CCL2 protein levels in primary breast tumors and matched brain metastases. **b,** The summary histogram of CD63 and CCL2 protein levels in primary breast tumors and matched brain metastases.(Chi-square test. $p<0.05$ is significant difference) **c,** The positive correlation between CD63 and CCL2 in brain metastases. *Collaborated with Qingling Zhang.*

Chapter 4: Summary

Taken together, our data unveiled a complex reciprocal communication between metastatic tumor cells and their TME, which primes the successful outgrowth of cancer cells to form life-threatening metastases (Fig. 43). Our study revealed that the apparent PTEN-loss in brain metastasis tumors directly resulted from brain microenvironment-induced adaptation of tumor cells, but not pre-selection of PTEN-deficient disseminated tumor cells. This PTEN down-regulation depended on miRNAs derived from astrocyte-derived exosomes in the brain microenvironment and led to increased tumor cell secretion of CCL2 that increased myeloid cells recruitment to promote metastatic tumor cells' proliferation, survival, and consequently, outgrowth. Our findings bring forward a fundamental concept that metastasis outgrowth results from tumor cell and microenvironment reciprocal cross-talks that enable their co-evolution, partly by exosome-mediated intercellular miRNA transfer and cytokine secretion. Meanwhile, the secondary PTEN-loss at the brain microenvironment and the disseminated PTEN-deficient tumor cells from primary site may co-exist in patients' brain metastases because PTEN-deficient cells pre-existed in primary tumors can also give rise to brain metastasis.

Beyond a tumor cell autonomous view of metastasis, our findings highlighted an important plasticity and tissue-dependent nature of metastatic tumor cells, and a bi-directional co-evolutionary view of the "seed and soil" hypothesis. Recently a lot of research related to "seed and soil" hypothesis has focused on the effect of tumor cells modulating the metastatic microenvironment, but the reciprocal function of the organ microenvironment on tumor cells has been overlooked. However, our project indicated brain microenvironment has an essential effect on the tumor cells for promoting their colonization and outgrowth. Brain metastasis frequently manifests in the late stages, and a long period of dormancy often precedes relapse. This implies

Figure 43

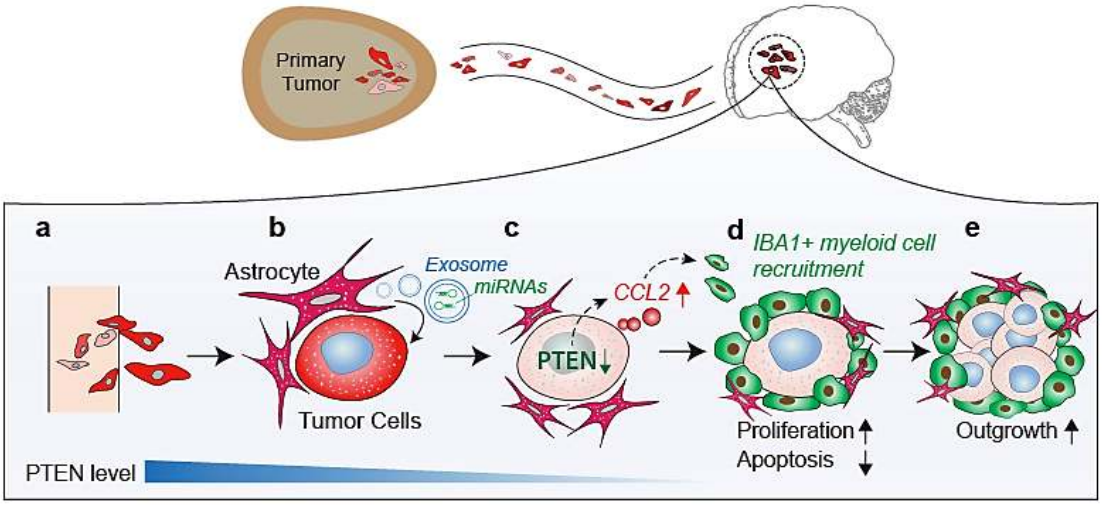


Figure 43. | PTEN loss induced by astrocyte-derived exosomal microRNA primes brain metastasis outgrowth via functional cross-talk between disseminated tumor cells and brain metastatic microenvironment. Top: Disseminated tumor cells extravasate into the brain. Bottom: **a-c**, Exosomes secreted by astrocytes in the brain microenvironment transfer PTEN-targeting miRNA into extravasated brain metastatic tumor cells leading to PTEN down-regulation in tumor cells. **c-d,** PTEN loss in brain metastatic tumor cells increases their CCL2 secretion, facilitating recruitment of IBA1+/CCR2+ myeloid cells at the micrometastasis site. **d-e,** The recruited IBA1+ myeloid cells enhance proliferation and inhibit apoptosis of metastatic tumor cells, and promote metastatic outgrowth. *Collaborated with Siyaun Zhang.*

that additional epigenetic regulations imposed by the metastatic microenvironment are essential for metastatic outgrowth. The question “when and how dormant cancer cells develop the ability of colonizing in secondary foreign organs?” has been a puzzle for a long time. In this project, we found one mechanism to answer this question: astrocyte-derived miR-19a helps tumor cells break dormancy and reinitiate their growth by down-regulating PTEN expression. It shows that

dormant metastatic cancer cells acquire essential traits from the metastatic microenvironment for their colonization and growth.

Especially, we also revealed the functions of recruited astrocytes and IBA1+ myeloid cells on brain metastasis. As mentioned in the introduction, astrocytes and immune cells have both anti- and pro-tumorigenic functions. In our system, both of them showed pro-tumorigenic effects: astrocytes cause PTEN downregulation in tumor cells, while recruited IBA1+ myeloid cells provide tumor cells growth advantage by increasing proliferation and inhibiting apoptosis. Under physiological or pathological conditions, astrocytes can release exosomes containing neuroprotective cargo to support neuronal survival [246]. Strikingly, our study is the first to report that astrocytes transfer exosomes to metastatic tumor cells to regulate tumor gene expression. It is very impressive to explore the role of astrocyte-derived exosomes in brain metastasis.

We identified CCL2 as an important downstream effector of PTEN-loss in the brain metastasis tumors, which has several implications. First, PTEN-loss-mediated secretome alteration of tumor cells implied a dynamic cross-talk between tumor cells and their immediate microenvironment. Our data signified the importance of studying the complex biology of metastasis in the context of its *in vivo* microenvironment. Second, the up-regulated CCL2 resulting from PTEN-loss may serve as a potential therapeutic target using CCL2-targeting agents [252]. Although clinical application of CCL2 inhibitor for metastasis treatment requires careful design [253], our findings highlight the potential of anti-CCL2 agents as preventive or treatment options for metastatic disease.

Chapter 5: Discussion and Future directions

Our study reveals a novel mechanism in which astrocytes cause the loss of PTEN in disseminated tumor cells by transferring exosomal miR-19a, subsequently priming tumor growth. It is a brain metastasis-specific mechanism and represents one of mechanisms that activated astrocytes or brain stromal cells can use to promote brain metastasis outgrowth. Other genes could also be downregulated by the similar mechanism. Data analysis (Fig. 7) showed that 54 genes were specifically downregulated in brain metastasis, compared to primary tumor and other metastatic organs. I hypothesize that the expression of other genes among those 54 genes, in addition to PTEN, might also be inhibited by activated astrocytes. Additionally, I also propose that activated astrocytes can induce the expression of some genes in metastatic tumor cells. In order to test my new hypothesis, I will design RNA sequencing to measure the gene expression changes of tumor cells under co-culture with activated astrocytes, compared to co-culture with CAF, NIH3T3 and tumor cells only. RNA sequencing result will show the upregulated and downregulated genes in tumor cells under co-culture with activated astrocytes. Combining RNA sequencing result with previous data analysis (Fig.7), I can identify the genes that are specifically induced or inhibited by activated astrocytes, and further explore the mechanism.

The next question is how the activated astrocytes regulate the gene expression in tumor cells. As I reported, exosomes can transfer bio-information between astrocytes and tumor cells. miR-19a is one mediator in astrocyte-derived exosome to promote brain metastasis. In order to study other mediators in astrocyte-derived exosomes that could regulate gene expression in metastatic tumor cells, I can isolate exosomes from activated-astrocytes for RNA sequencing or next generation sequencing to measure the enrichment of RNAs or microRNAs in the astrocyte-derived exosomes, compared with exosomes from CAF or tumor cells. I hypothesize that other microRNAs, in addition to miR-19a, long non-coding RNAs, mRNAs or proteins would be

enriched in astrocyte-derived exosomes and might regulate other gene expressions in tumor cells. Other than releasing exosomes, astrocytes can release cytokines [82-84] to regulate the signalling in tumor cells. Cytokine array can show us the cytokines released by astrocytes. The mechanism of activated astrocytes promoting tumor growth is intriguing and needs to be further studied in the future.

In my project, I focused on brain metastasis of breast cancer and melanoma. I hypothesize that the similar mechanism of reversible PTEN loss in brain metastasis might also operate in the brain metastases from other primary tumors, such as lung cancer, colon cancer, and primary brain tumors such as glioma. Glioma frequently loses *PTEN* (70% LOH) with 44% mutation (Table 1). Interestingly, miR-26a, a miRNA targeting PTEN, is high expressed in glioma (Table 1). It was reported that miR-26a is amplified in glioma [237]. However, I wonder whether it is possible that miR-26a is transferred from astrocytes to tumor cells in addition to miR-26a gene amplification. Primary brain tumors and brain metastases might share some mechanisms to prime tumor growth in the brain. The key experiments will be co-culture of glioma cells with astrocytes. If miR-26a level increased and PTEN expression downregulated in glioma cells when co-cultured with astrocytes, it would support my hypothesis. Furthermore, I will test whether depleting miR-16a in astrocyte can rescue the PTEN expression in glioma cells *in vitro* and *in vivo*. To study if the similar mechanism applies in brain metastasis from lung cancer, or colon cancer, I can do ICA injection of lung cancer cells or colon cancer cells to mice to establish brain metastasis. I will check PTEN expression in brain metastasis and examine whether PTEN loss of brain metastasis is partly induced by brain microenvironment.

Furthermore, PTEN is required for multiple aspects of neuronal maturation and function [254]. PTEN loss has been found in neurological diseases, such as the PTEN

hamartoma, Lhermitte–Duclos disease, Parkinson's disease, and Alzheimer's disease [255, 256]. My findings on tumor cells' PTEN loss by astrocyte-derived miRNAs may also provide a new paradigm of PTEN loss in general neurological diseases via similar cross-talk between neurons and brain microenvironment. The best model is astrocyte-specific miR-19a knockout mouse, which can be generated by Mirc1$^{tm1.1Tyj}$/J mice breeding with transgenic mouse which expresses Cre recombinase under the control of GFAP promoter. Observing the biology phenotypes of neurologic disorder and measuring the PTEN expression in brain should give me the hints whether astrocyte-derived miR-19a also causes PTEN loss in neurological diseases. This mouse model is also good for validating the whole mechanism of my project in which astrocytes induce PTEN loss in metastatic tumor cells.

In addition, I have several questions raised from my thesis, which need to be further explored in the future.

Q1: How do tumor cells induce astrocytes to secrete exosomes containing miR-19a?

In my experiment, direct co-culture of tumor cells with astrocytes decreased PTEN expression in tumor cells (Fig. 8). However, co-culture of tumor cells with the conditioned media of astrocytes also increased miR-19a expression and decreased PTEN expression in tumor cells (Fig. 18). Meanwhile, exosomes containing miR-19a were detected in the conditioned media of astrocytes (Fig. 19). These data indicated that even without tumor cells, astrocytes also can secrete exosomes. In this project, I did not compare the difference of exosomes released from astrocytes in physiological condition to those in pathological condition. I hypothesize that metastatic cancer cells can induce gliosis and stimulate astrocytes to secrete more exosomes than those under its physiological status. Additional experiments can be designed to quantitate

exosomes released by active astrocytes co-cultured with tumor cells, and compare them to those by the astrocyte only or tumor cells only. In order to separate the exosomes of astrocytes from those of tumor cells, it is better to label exosomes in astrocytes firstly.

Furthermore, it would be interesting to further examine how activated astrocytes induce the releasing or package of exosomes. As mentioned in the introduction, the Rab family controls exosome release, especially RAB27a and RAB27b. In my *in vitro* experiment, knockdown of RAB27a in astrocytes significantly inhibited exosome production (Fig. 21). However, I found RAB27a expression was low, while RAB27b expression was relatively high in normal brain tissue by IHC staining. Therefore, I decided to knock down both RAB27a and RAB27b *in vivo*, which resulted in effective inhibition of CD63+/ TSG101+ exosomes (Fig.22). At same time, it indicates that the brain has a specific mechanism to regulate exosome release. It is an exciting topic to detect the specific molecular mechanism of exosome releasing from astrocytes. For example, I am curious to explore the exact function of RAB27b in controlling exosome release from astrocytes *in vivo*. Astrocyte-specific RAB27b knockout mice can help me get the answer. In order to study other genes regulating the exosome biogenesis, formation, and secretion in activated astrocytes, I will do loss-of-function screen by CRISPR-Cas9 system or siRNA library to detect what other genes control exosome packaging or releasing in astrocytes, besides rab27b.

Q2: Can you further validate microRNA carried by exosome, not other EVs or other carries in your system?

My protocol for exosome purification is adapted from previous publications [146, 257]. Briefly, astrocytes or CAFs were cultured in media with exosome-depleted FBS for 48-72 hours and exosomes were collected from their culture media after sequential ultracentrifugation. As we

described in Chapter 2, “Cells were harvested, centrifuged at 300×g for 10 minutes and the supernatants were collected for centrifugation at 2000×g for 10 minutes, 10,000×g for 30 minutes and 100,000×g for 70 minutes. The pellet was washed once with PBS and purified by centrifugation at 100,000×g for 70 minutes again.” However, other EVs, large protein complexes and lipoprotein have also been reported to be able to deliver miRNAs [258, 259], as exosomes do. In order to further validate the role of exosomes in transferring miR-19a in our system, I plan to improve the protocol for isolation and purification of exosomes. After centrifugation, I will perform sucrose gradient and immuno-isolation to isolate purer exosomes for *in vitro* experiments. To validate our previous conclusion/result, I have designed two new experiments to test whether exosomes are responsible for the transferring of miR-19a to tumor cells.

I will isolate astrocytes from Mirc1$^{tm1.1Tyj}$/J mice which have a floxed miR-17-92 allele. Endogenous miR-19a in the astrocytes will be depleted by the transfection with Ad5.GFAP.Cre virus (transfection with Ad5.RSVβ-Gluc virus as control). Then I will isolate exosomes by improved protocol from the astrocytes with miR-19a depleted or control astrocytes. The purified exosomes from the two groups will be added into tumor cells and then miR-19a and PTEN expression in tumor cells will be measured. The result of this experiment will uncover whether transferring miR-19a to tumor cells is mediated solely by exosome, or other EV/proteins-mediated transfer and reveal whether miR-19 that is transferred by exosomes is the key to down regulate PTEN expression in tumor cells.

At the same time, I will re-transfect Cy3-labeled miR-19a into the astrocytes with endogenous miR-19a depletion. Purified exosomes from these astrocytes by improved protocol will be added to tumor cells and then Cy3 signal expression in tumor cells, as well in purified

exosomes, will be measured. The result will further confirm whether miR-19a is transferred by exosome.

However, I have not excluded that other EVs, large protein complexes, and lipoprotein can transfer miRNAs to tumor cells. By the several advanced technologies, I can identify these components more clearly. To elucidate the function of EVs or lipoprotein in the cell communication is an exciting project warranting further investigation.

Q3: Why does clinical application of CCL2 inhibitor for metastasis treatment require careful design?

My data showed CCL2 knockdown reduced microglial cell infiltration, inhibited brain metastases proliferation, and prolonged overall survival of brain metastasis-bearing mice. These data clearly serve as proof-of-concept that targeting CCL2 can be exploited clinically as a new therapeutic option for brain metastasis patients. CCL2 inhibitors are currently being assessed as anti-inflammatory agents in a phase II clinical study [260]. Therapies targeting the tumor microenvironment (TME) represent a novel treatment for cancer. Anti-CCL2 employs the strategy of targeting metastatic microenvironment, and especially in combination with targeting tumor cells, potentially may be more effective for treating genetically highly unstable metastatic tumors.

However, I think that clinical application of CCL2 for metastasis treatment should applied carefully for the following reasons: 1) humanized monoclonal anti-CCL2 antibody (CNTO888) is ineffective to suppress metastatic prostate tumor progression in phase II clinical study [261], likely due to the feedback of CCL2 expression. 2) Bonapace and the colleagues reported that although anti-CCL2 treatment inhibited tumorigenesis and metastasis in mouse

models, **discontinuation** of anti-CCL2 treatment increased lung metastasis events from primary breast tumor [253]. Their study neither refuted the anti-metastasis effect of anti-CCL2 treatment nor demonstrated any effect of anti-CCL2 on enhancing brain metastasis. Although this study design is different from the experiments in my study, one should be careful of the potential clinical complications. Additionally, my findings here should motivate future efforts on developing better anti-CCL2 agents, optimization of clinical treatment dosing, scheduling, combinations, and application strategy. Therefore, these should be further pursued as a follow-up study.

Another possible strategy is to block CCR2. CCR2 is expressed only in several cell types, e.g. monocytes, myeloid cells, and activated NK cells. One phase II clinical trial showed that CCR2 antibody (ClinicalTrials.gov ID: NCT01015560) has therapeutic effects on metastatic cancer patients[262]. It is also a potential treatment to block CCR2 for brain metastasis by reducing recruited myeloid cells.

Q4: Can we use exosome-derived miR-19a as a biomarker of brain metastasis?

There is a report indicating the strong diagnostic and prognostic potential of detecting specific proteins or RNAs in exosomes by liquid biopsy, as mentioned in the introduction [153]. In my model, exosomes containing astrocyte-derived miR-19a also might be a good specific biomarker for diagnosis and prognosis of brain metastasis, complemented with imaging approach. However, I found that miR-19a was transferred to tumor from surrounding astrocytes. These exosomes from astrocytes might not enter the blood circulation, but might be detected in cerebrospinal fluid (CSF). The challenge to use astrocyte-derived exosomal miR-19a as a biomarker for early detection or prognosis of brain metastasis is how to discern specific

exosomes derived by astrocytes from those by tumor cells. My hypothesis is that the cargos in astrocyte-derived exosomes are different from those in tumor cell exosomes. Comparing the complete set of miRNAs and proteins in astrocyte-derived exosomes with that in tumor cell-derived exosomes, I can discern the astrocyte-derived exosomes by specific protein markers. I can also identify other miRNAs or proteins, in addition to miR-19a, in astrocyte-derived exosomes, which are relevant to the colonization and outgrowth of brain metastasis. Then I will use the specific protein markers of astrocyte-derived exosome combined with miR-19a expression as biomarkers for brain metastasis. To investigate the positive correlation between astrocyte-derived exosomal miR-19a and progression of brain metastasis, I will extract CSF at different stages of brain metastasis in mouse models, purify astrocyte-derived exosomes from CSF by specific protein markers and common exosome biomarkers, and isolate RNA from exosomes to measure miR-19a expression, compare that with controls. Studying circulating exosome in CSF or blood is a novel direction. It could improve the sensitivity and specificity of brain metastasis diagnosis when combined with imaging screening. However, as mentioned in the introduction, there are still many technical limitations for studying exosome as biomarkers *in vivo.*

Q5: How to distinguish bone-marrow derived myeloid cells from resident microglia?

In my thesis, I found that PTEN loss induced the secretion of CCL2, which recruited bone-marrow derived IBA1+ myeloid cells, mainly monocytes, because one subgroup of monocyte expresses CCR2, the specific receptor of CCL2. However, this result raised new questions about discriminating infiltrating monocytes and resident microglia in our system, because they both express IBA1.

The source of microglia has been debated for a long time. The consensus held that microglia come from the myeloid lineage, but recent studies showed microglia originate in the yolk sac in the early stage of development, and reside in the CNS until adulthood [263]. Microglia express several biomarkers, including CD11b/CD45 ^{lo}, IBA1, CXCR3, CD68, and F4/80, which overlap with several biomarkers of macrophage or monocyte. Therefore, microglia is categorized as a specific subgroup of macrophages/monocytes.

Monocytes are the precursor of macrophages and dendritic cells. Monocytes can be separated into two groups: resident monocytes (CD14+/CD16- in human, Ly6C^{lo} CX3CR1+ CCR2- in mouse), and inflammatory monocytes CD14+/CD16+ in human, Ly6C^{hi} CX3CR1- CCR2+ in mouse) [264, 265]. Saederup and the colleagues [266] generated CCR2-red fluorescent protein knock-in mice and revealed that under pathological condition, e.g. experimental autoimmune encephalomyelitis, most of infiltrated monocytes are Ly6C^{hi}, CCR2+ in the inflammatory brain parenchyma. However, microglia express CX3CR1, rather than CCR2, in the healthy or pathological brain [266, 267].

In fact, it is still a challenge to delineate the resident macrophages from infiltrating monocytes or other myeloid cells. A previous study by Saederup and the colleagues [266] showed that, of these infiltrated Ly-6C^{hi} monocytes, most express CCR2, but a sub-population express both CCR2 and CX3CR1, or CX3CR1 only. This finding indicated that infiltrated monocytes might mimic the function of active microglia in pathological conditions. As new genetic tools became available, the neuroscience field recently has begun to discern the subtle differences of each subtype of phagocytic myeloid cell in CNS. The current studies showed two groups of monocytes (CX3CR1+ CCR2- and CX3CR1- CCR2+), serve the distinct functions in

healthy or pathological conditions. In contrast to the well-studied CCR2-CCL2 pathway, the regulation and function of CX3CR1-CX3CL2 pathway remains to be further studied.

In my project, I showed recruited IBA+1 myeloid cells promoted tumor growth by inhibiting apoptosis and increasing proliferation. However, the detailed mechanism of how myeloid cells help tumor cell growth remains elusive. It is possible that recruited myeloid cells release cytokines to activate tumor growth and inhibit immune reactions of macrophages and microglia. It will be worthwhile to explore the mechanism by which infiltrated immune cells and microglia promote brain metastasis in the future. I can utilize CCR2-knockout mouse or CX3CR1 knockout mouse to further check the function of monocytes or microglia in brain metastasis respectively.

In summary, this project focused on the co-evolution of disseminated tumor cells and the brain microenvironment. It also revealed the roles of the exosome-mediated miRNA transfer, brain inflammation, and brain immune reaction in brain metastasis growth. More importantly, the study showed the potential to directly benefit patients with brain metastasis. To further study the detailed mechanism of each step in our system and test our novel mechanism in other cancer types would contribute to the clear understanding of the brain metastasis process and benefit more patients with brain metastasis or primary brain tumors.

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Vita

Lin Zhang was born in Shanghai, China on September 27th, 1974. She graduated from Southeast University, Medical School in Aug, 1998, with a Bachelor's of clinical medicine. From 1998-2004, she worked as an oncologist in the Zhongda Hospital (Nanjing, China). Lin Zhang had further studied biochemistry at the Lamar University (TX, USA) from January 2005 to December 2006, and worked as a research associate in a biochemistry lab of Lamar University from October 2007 to August 2008. In August of 2011, she entered the Graduate School of Biomedical Science, the University of Texas Health Science Center at Houston to pursue her doctoral degree in Dr. Dihua Yu's laboratory in M.D. Anderson Center.

Current address:

7407 Norwood Point Ct.

Richmond, TX 77407