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MDA-9/Syntenin: From Glioblastoma Pathogenesis to Targeted Therapy

Timothy P. Kegelman
Virginia Commonwealth University

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A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University

by

Timothy P. Kegelman
Bachelor of Science, University of Notre Dame, Notre Dame, IN. 2007

Advisor: Paul B. Fisher, M.Ph., Ph.D.
Professor and Chair, Department of Human and Molecular Genetics
Director, VCU Institute of Molecular Medicine
Thelma Neumeyer Corman Endowed Chair in Cancer Research
Massey Cancer Center

Virginia Commonwealth University
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List of Abbreviations and Symbols

α ..... Alpha

β ..... Beta

m ..... Milli

n..... Nano

μ ..... Micro

Cdk..... Cyclin-dependent kinase

CNS..... Central nervous system

Cre..... Cre Recombinase

D..... Aspartic acid

DMEM ..... Dulbecco's modified eagle medium

DMSO ..... Dimethyl Sulfoxide

DNA ..... Deoxyribonucleic Acid

EGFR ..... Epidermal Growth Factor Receptor

EMT ..... Epithelial-mesenchymal transition

EphA2 ..... Ephrin type-A receptor 2

ER ..... Endoplasmic reticulum

ERK..... Extracellular signal-regulated kinase

F ..... Phenylalanine

FACS ..... Fluorescence-Activated Cell Sorting

FBDD ..... Fragment Based Drug Discovery

FBS ..... Fetal Bovine Serum

Fl ..... Floxed

G ..... Glycine

GBM ..... Glioblastoma Multiforme

GFAP ..... Glial Fibrillary Acidic Protein

GEMM ..... Genetically modified mouse model

GSK3 ..... Glycogen synthase kinase 3

H ..... Histidine

HCC ..... Hepatocellular carcinoma

HP ..... Hippocampus

IDH ..... Isocitrate Dehydrogenase

IGF ..... Insulin-like growth factor

IHC ..... Immunohistochemistry

IκB ..... Nuclear Factor kappa-light-chain-enhancer of activated B cells Inhibitor

IKK ..... IκB Kinase

I.P. ..... Intraperitoneal

H-Ras ..... Harvey rat sarcoma viral oncogene homolog

K-Ras ..... Kirsten rat sarcoma viral oncogene homolog

Luc ..... Luciferase

M ..... Molar (mol/L)

MAPK ..... Mitogen-activated protein kinase

MDA-9 ..... Melanoma differentiation associated gene-9

mg/kg ..... milligram/kilogram

miR ..... microRNA

mm ..... millimeter

MTT ..... 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N ..... Asparagine

NF-κB ..... Nuclear Factor kappa-light-chain-enhancer of activated B cells

OA ..... Oligoastrocytoma

ODG ..... Oligodendroglioma

PBS ..... Phosphate Buffered Saline

PI3K Phosphotidyl-inositol-3-kinase

PDGF ..... Platelet-Derived Growth Factor

PTEN Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase

PHFA ..... Primary Human Fetal Astrocytes

Rb ..... Retinoblastoma gene

REMBRANDT ..... Repository of Molecular Brain Neoplasia Data

RNA ..... Ribonucleic acid

RCWM ..... Rostral Caudal White Matter

S ..... Serine

SCLC ..... Small Cell Lung Carcinoma

SD ..... Standard deviation

SDCBP.....Syndecan Binding Protein

SH .....Src homology domain

siRNA .....Small interfering RNA

Stat3 ..... Signal transducer and activator of transcription 3

SVZ.....Subventricular Zone

T ..... Threonine

TCGA..... The Cancer Genome Atlas

TGF-B ..... Transforming growth factor beta

TNF ..... Tumor necrosis factor receptor

TMZ ..... Temozolomide

VEGF .....Vascular endothelial growth factor

WT ..... Wild Type

Y..... Tyrosine

Abstract

MDA-9/SYNTENIN: FROM GLIOBLASTOMA PATHOGENESIS TO TARGETED THERAPY

By Timothy P. Kegelman, B.S.

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Advisor: Paul B. Fisher, M.Ph., Ph.D.
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Massey Cancer Center

The most common malignant glioma, glioblastoma multiforme (GBM), remains an intractable tumor despite advances in therapy. Its proclivity to infiltrate surrounding brain tissue contributes greatly to its treatment failure and the grim prognosis of patients. Radiation is a staple in modern therapeutic regimens, though cells surviving radiation become more aggressive and invasive. Consequently, it is imperative to define further the cellular mechanisms that control GBM invasion and identify promising novel therapeutic targets. Melanoma differentiation associated gene-9 (MDA-9/Syntenin) is a highly conserved PDZ domain-containing scaffolding protein that promotes invasion and metastasis in human melanoma models. We show that MDA-9/Syntenin is robustly expressed in GBM cell lines and patient

samples, and expression increases by tumor grade. These findings are confirmed through database analysis, which revealed MDA-9/Syntenin expression correlates with shorter survival times and patient tumors high in MDA-9/Syntenin have a worse prognosis when undergoing radiotherapy. Modulating MDA-9/Syntenin levels produced changes in invasion, angiogenesis, and signaling, indicating MDA-9/Syntenin enhances glioma pathogenesis. Overexpression of MDA-9/Syntenin enhances invasion, while knockdown inhibits invasion, migration, and anchorage-independent growth in soft agar. MDA-9/Syntenin increases activation of c-Src, P38MAPK, and NF-κB, leading to elevated MMP2 expression and IL-8 secretion. Through an orthotopic tumor model, we show that sh*mda-9* tumor cells formed smaller tumors and had a less invasive phenotype *in vivo*. Knockdown of MDA-9/Syntenin radiosensitizes GBM cells and significantly reduces post-radiation invasion gains through abrogation of radiation-induced Src and EphA2 activity. In efforts to pharmacologically inhibit MDA-9/Syntenin, we describe the effects of a novel small molecule, PDZ1i, which targets the PDZ1 domain of MDA-9/Syntenin and successfully reduces invasion gains in GBM cells following radiation. While it does not effect astrocyte radiosensitivity, PDZ1i radiosensitizes GBM cells. PDZ1i inhibits crucial GBM signaling including FAK and mutant EGFR, EGFRvIII, and can negate gains in secreted proteases, such as MMP2 and MMP9, following radiation. In a model of glioma, PDZ1i treatment combined with radiation results in less invasive tumors and extends survival. Our findings indicate that MDA-9/Syntenin is a novel and important mediator of GBM pathogenesis, and further identify it as a targetable protein that enhances radiotherapy for treatment in glioma.

Chapter 1 - Introduction to MDA-9/Syntenin

Since its discovery, MDA-9/Syntenin has been linked to an expanding list of cellular functions, including cell-cell and cell-matrix adhesion, signal transduction, as well as intracellular and secreted lipid trafficking. The average number of citations per year that reference “MDA-9” or “syntenin” more than doubled in 2012-2013 compared to the previous seven years. Central to its cellular roles is its ability to bind numerous intracellular molecules, including proteins, glycoproteins, and lipids. These promiscuous binding interactions positions MDA-9/Syntenin in an abundance of complexes that regulate a range of activity in many cell types. A common theme among these intracellular activities is their direct or indirect relation to cancer invasion. Here, we explore the properties of MDA-9/Syntenin in the setting of tumor cell invasion, a complex process dependent on the integration of a myriad of signaling pathways.

I. Discovery and Cloning

MDA-9/Syntenin was cloned through a subtraction hybridization approach that was designed to identify genes differentially regulated between untreated melanoma cells, and their terminally differentiated counterparts (1-4). Briefly, human melanoma cells were treated with a combination of fibroblast interferon (IFN-β) and mezerein, a protein kinase C activating antileukemic agent, to induce a terminally differentiated state (1, 5-12). Comparing the gene expression of the resulting temporally spaced, subtracted libraries at various time points led to

the discovery of a number of important genes, termed "melanoma differentiation associated" (mda) genes. MDA-9/Syntenin was unique in this group in that it did not have a sustained induction pattern, rather it displayed biphasic kinetics with a peak at 8-12 hours post treatment followed by a return to baseline expression, which suggested a dissociation from a growth suppression role (13). Follow-up studies indicated that MDA-9/Syntenin was induced in human melanoma cells by treatment with interferon-gamma (13). Additional yeast-two hybrid studies showed that MDA-9/Syntenin is an interacting partner of the syndecan family of heparan sulfate proteoglycans, cell surface molecules involved in cell-cell and cell-matrix adhesion, signal transduction, trafficking of lipoproteins and cell surface receptors, as well as activity as co-receptors (14).

II. Structure and Regulation

A. The PDZ Domains

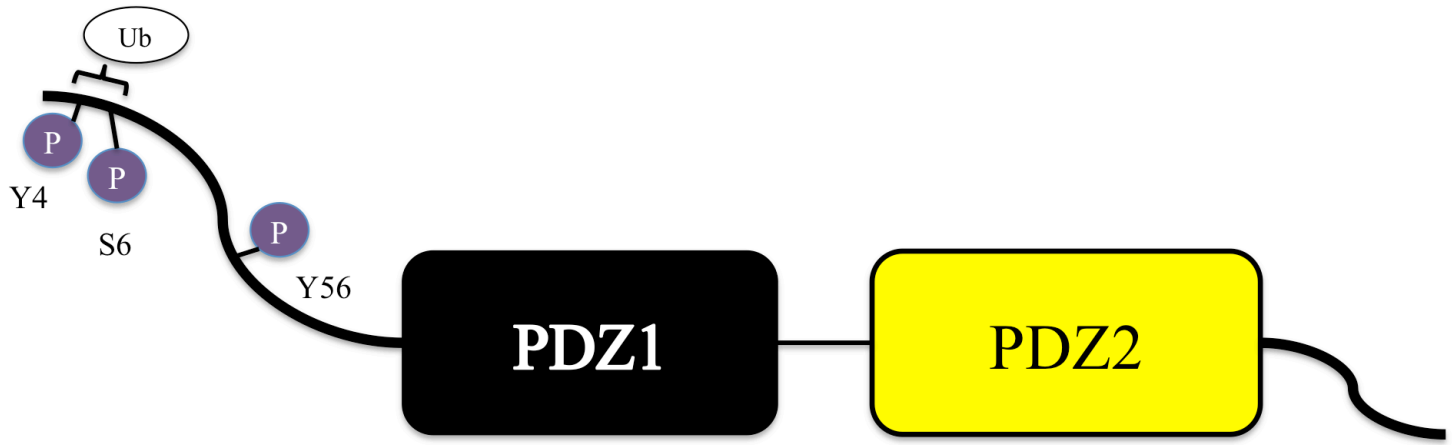
MDA-9/Syntenin is a 2.1-kb gene located on 8q12 with an ORF of 894-bp, encoding a 298-aa protein of about 33-kDa (13-15). Cross-species analysis shows that MDA-9/Syntenin highly conserved with homologues in rat, mouse, zebrafish, and *Xenopus* (16-18). A distinguishing feature of MDA-9/Syntenin is its inclusion in the family of proteins with PDZ domains. This motif, (so named for its discovery in post-synaptic density protein **P**SD95/SAP90, drosophila tumor suppressor **D**LGA, and tight junction protein **Z**O-1) are well-described regions of 80-100 residues organized into six β strands and two α helices that form compact, globular domains of 25-30-Å. PDZ domains often mediate the assembly of multiprotein complexes by binding the C-terminal of their targets at the plasma membrane as well as intracellular membranes (19-21). Target peptide sequence divides the PDZ proteins into 3 groups: I (-S/T-X-

Φ), II (Φ-X- Φ), and III (D/E-X- Φ), of which MDA-9/Syntenin has been shown to bind class I, II, and other groups with a low to moderate affinity (22, 23).

During syndecan binding, MDA-9/Syntenin's PDZ-2 motif serves as a high-affinity domain, whereas PDZ-1, though necessary for binding, acts as a complementary, low-affinity domain (24). This pattern is also observed in the binding pattern of MDA-9/Syntenin to c-Src (25).

B. The N- and C-terminals

While the majority of activity as a scaffolding protein occurs through the PDZ domains, the amino- and carboxy-terminals of MDA-9/Syntenin influence its structure, stability, and have recently been implicated in a growing number of unique functions (Figure 1.1). The N-terminal of MDA-9/Syntenin has been linked notably to recruiting transcription factor SOX4 as well as eukaryotic translation initiation factor 4A (EIF4A) to signaling complexes (19, 26, 27). A focus on possible phosphorylation sites in the N-terminal has resulted in the discovery of a number of interactions and layers of regulation. Phosphorylation at tyrosine sites was shown to prevent interaction with receptor type protein tyrosine phosphates (rPTPnu) CD148 (28), and recent work pointed to an interesting interaction with ubiquitin (Ub), regulated by the N-terminal (29).



N-Terminal

- Recruits Sox4
- Binds unique Ub site
- Phosphorylated by Ulk-1 (Ser6)
- Involved in chemotaxis through Src phosphorylation (Tyr4)
- Autoinhibitory action: turned off by Tyr56 phosphorylation

PDZ Domains

- Binds numerous partners:
  - Syndecans
  - c-Src
  - PIP_2
- Cooperative Binding
  - PDZ2 → Preferential Binding
  - PDZ1 → Domain Stabilization

C-Terminal

- Interacts/stabilizes PDZ tandem
- Important for membrane localization
- Potential phosphorylation sites inhibiting membrane interaction

Figure 1.1 MDA-9/Syntenin Domain Structure. Recent studies have shed light on the various activities and functions of the domains comprising MDA-9/Syntenin. Domain structure is depicted and select activities listed beneath each.

The N-terminal of MDA-9/Syntenin binds Ub and may have an important role in the Ub-dependent sorting of transmembrane cargo (29). MDA-9/Syntenin was also shown to interact with Ub with an affinity (K_D) of 27.3 μM, relatively tight compared to most Ub interactions. A conserved LYPSL sequence in the N-terminus of MDA-9/Syntenin binds a unique site on the C-terminus of Ub, interacting equally well with Lys^{48}- or Lys^{63}-linked poly-Ub chains. These studies implicate MDA-9/Syntenin in binding a set of ubiquitylated proteins that it links to its transmembrane partners, thus forming "Ub-based molecular hubs." This is particularly important because several transmembrane interacting partners of MDA-9/Syntenin are regulated through Ub-dependent endocytic trafficking including syndecan-4 (30), GlyT2 (31), and IL5R (32).

This interaction with Ub requires the C-terminus of MDA-9/Syntenin to be intact, and is regulated through MDA-9/Syntenin dimerization, as dimerization-defective mutants of MDA-9/Syntenin failed to bind Ub. The head to tail dimerization of MDA-9/Syntenin mediated by its PDZ domains allows it to bind two Ub molecules, or two ubiquitinated proteins, in its dimerized state. Further, the principal binding site on Ub for deubiquitinating enzymes overlaps with MDA-9/Syntenin's binding. Thus, monoubiquitylated partners in complex with MDA-9/Syntenin could be shielded from deubiquitination, thereby leading to prolongation of MDA-9/Syntenin-dependent pathways (29). This has interesting implications of mediating the interactions of disparate proteins, as well as amplification of cellular pathways regulated by this interaction (Figure 1.2).

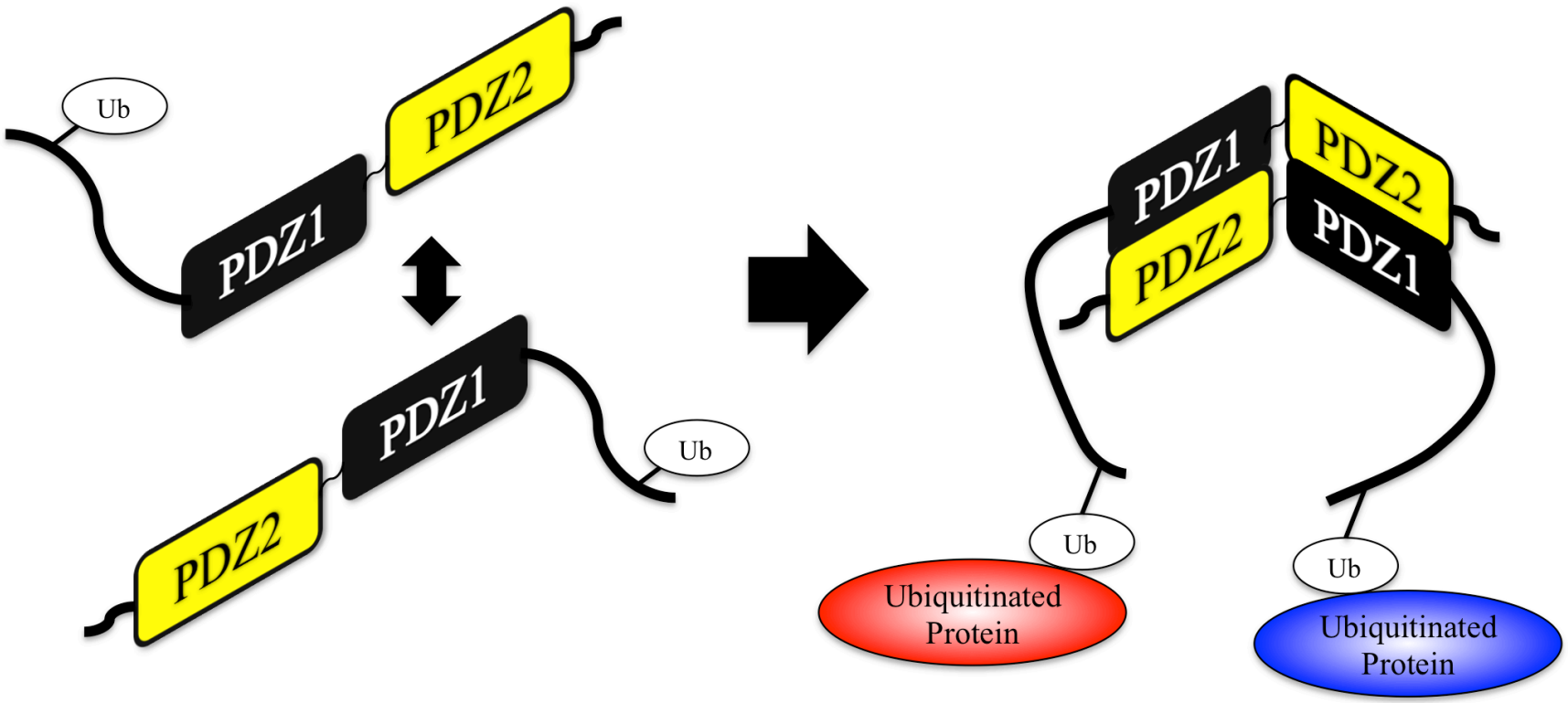


Figure 1.2 MDA-9/Syntenin-Ubiquitin Binding. The N-terminal of MDA-9/Syntenin has been shown to bind a unique site on Ubiquitin (Ub) (Rajesh et al., 2011). As described in this work, the dimerization of MDA-9/Syntenin enhances Ub binding. The possible ramification of this insight is another method by which MDA-9 can facilitate interactions, now between two Ub-bound proteins.

Ulk1, an interacting partner and S/T kinase with roles in autophagy and regulating both clathrin-dependent and clathrin-independent endocytosis, phosphorylates MDA-9/Syntenin on the N-terminal. When Ulk-1 phosphorylates Ser(6) in the N-terminal LYPSL sequence, this prevents MDA-9/Syntenin interaction with Ub. When MDA-9/Syntenin mutants were expressed that lose the ability to bind Ub, they demonstrated reduced co-localization with CD63, a marker for late endosomes and lysosomes (29).

Further evidence is emerging supporting the importance of the previously unexplored N-terminal domain of MDA-9/Syntenin and its involvement in cell motility programs. Recently, a study showed that MDA-9/Syntenin was a crucial element for immune cell polarization and chemotaxis (33). MDA-9/Syntenin was vital for forming polarized actin structures as seen in the leading edge and contact zone with antigen presenting cells (APCs). It accomplishes this after phosphorylation by Src at Tyr^{4}, leading to the activation of small GTPase Rac by specific association with myosin phosphatase Rho interacting protein (M-RIP). Integrin and chemokine receptor activation is crucial in T-cell migration and producing functional asymmetry in these cells in forming areas of cell-cell contact referred to as the "immunological synapse" (33).

Further investigation of the N-terminal region suggests that MDA-9/Syntenin exists in equilibrium between a closed and open state, possibly regulated by the phosphorylation of an auto-inhibitory domain in this region (34). N-terminal deletion mutants of greater than 57 amino acids lead to an enrichment of this mutant form at the plasma membrane (16, 34). Additionally, a mutant mimicking phosphorylation at Tyr^{56} (Y56E) was strongly enriched at the plasma membrane, indicating that N-terminal phosphorylation negates autoinhibition and leads to enhanced plasma membrane association (34).

Data shows that the C-terminal domain is also functionally important in MDA-9/Syntenin, as NMR results indicate that the C-terminal domain includes structural segments that interact in tandem with PDZ domains (35). A deletion mutant lacking the entire C-terminal completely lost plasma membrane localization, while positively charged residues were found to be important for promoting membrane targeting. Further, a mutant mimicking phosphorylation in the C-terminal showed reduced plasma membrane association (34).

C. Regulation of Expression

Genetic regulation of mda-9 has not been thoroughly elucidated and is likely a complex, multifactorial process, but some clues have been uncovered. Early work showed that MDA-9/Syntenin was inducible through IFN treatment, and TNF-α treatment can generate expression as early as 10 minutes post treatment in umbilical arterial endothelial cells (36). Nonetheless, a precise picture of the transcriptional regulation of MDA-9/Syntenin has not been established.

MDA-9/Syntenin expression and PKCα activity were shown to be interdependent following fibronectin (FN)-induced PKCα activation (37). Inhibition of PKCα suppressed both endogenous and FN-induced MDA-9/Syntenin expression, therefore a positive feedback loop incorporating PKCα activation may be involved, but the detailed mechanism for this has not been revealed (37). Recently, Raf kinase inhibitor (RKIP) was found to be strongly downregulated in multiple metastatic melanoma cell lines and inversely related to MDA-9/Syntenin expression. Co-immunoprecipitation experiments demonstrated that RKIP could bind MDA-9/Syntenin and when overexpressed, inhibited MDA-9/Syntenin's interaction with cSrc/FAK complexes leading to decreased invasion, less anchorage independent growth, and reduced ability to seed lung tissue *in vivo* (38).

D. Roles in Development and Neural Function

In studies of developmental expression, including those of mouse fetal development, significant expression was noted in the fetal kidney, liver, lung, and brain as well as the placenta, adult spleen, and heart (2, 16, 39). MDA-9/Syntenin was recently shown to be essential for normal development in zebrafish, in which it has two homologues: syntenin-a and syntenin-b (18). Lack of syntenin-a expression resulted in the death of over 80% of embryos 24 hours post fertilization. Zebrafish embryos that did survive displayed a markedly shorter body axis (18). This is similar to an earlier finding in *Xenopus* that found knockdown of syntenin homologues resulted in a shorter body axis (17). The studies in zebrafish found that syntenin-a was crucial for a central process to gastrulation, epiboly – the spreading and thinning of blastoderm to cover the yolk cell and close the blastosphere in fish embryos. Through interaction with syndecans, notably syndecan-4, PIP2 and small GTPase ADP-ribosylation factor 6 (Arf6), MDA-9/Syntenin regulates epiboly progression through actin cytoskeleton rearrangement (18). Syntenin-a is also upregulated in the zebrafish spinal cord following injury. Knockdown of syntenin-a results in reduced regrowth of descending axons from brainstem neurons and significant inhibition of locomotor recovery 6 weeks post-injury (40).

Synaptic function is tightly regulated and dysfunction can be associated with a range of neurological pathology, including neurodegenerative states such as Alzheimer's, and psychiatric disorders such as schizophrenia (41). The matrix of proteins underlying synaptic membranes is rich in scaffolding proteins, including MDA-9/Syntenin. MDA-9/Syntenin has roles in maintaining stable synaptic structures through interaction with adhesion molecules such as SynCAM, neurexin, and neurofascin (19, 24, 42-44). The polarized protein composition at the synaptic plasma membrane can be stabilized by MDA-9/Syntenin's recruitment of a number of

intracellular regulators and formation of multimeric complexes through interaction with ERC2/CAST1, a cell surface molecule (45). Additionally, MDA-9/Syntenin promotes an increase in the numbers and branching patterns of neurites. In PC12 cells, a model for neuron-like cells, Akt inhibition, either through dominant negative (DN) expression or pharmacological inhibition, led to an increase in MDA-9/Syntenin expression and improvement in neurite outgrowth (46). Taken together, these findings serve as foundation for a key role of MDA-9/Syntenin in directional cell movements during early development and regeneration.

Among the receptors that interact with MDA-9/Syntenin at the synaptic cleft are glutamate receptors, involved in the transport of the main excitatory neurotransmitter in the CNS (42, 47). Exogenous expression of MDA-9/Syntenin leads to an increase in the number of dendritic protrusions in both young and mature neurons. This supports the view that MDA-9/Syntenin is involved as a key effector of glutamate-induced membrane protrusions, which establish connections in the developing brain (47). Additionally, MDA-9/Syntenin can bind with Unc51.1 and Rab5 to initiate axon outgrowth through scaffold formation and endocytic machinery. Unc51.1 is a serine/threonine kinase shown to be important in neurite extension, while Rab5 is a member of the Ras-like small GTPases and found in early endosomes (48). While further studies are needed, including the consequences of MDA-9/Syntenin downregulation, MDA-9/Syntenin's multifaceted roles in membrane-associated activities and actin cytoskeleton rearrangement are evident in developmental processes such as these.

E. Localization

MDA-9/Syntenin is commonly found in areas of cell-cell contact, co-localizing with F-actin, syndecan-1, E-cadherin, β-catenin, and α-catenin (16). In fibroblasts, MDA-9/Syntenin is localized to focal adhesions and stress fibers. MDA-9/Syntenin is also involved in regulating the

rearrangement of the actin cytoskeleton as its overexpression leads to the formation of distinct structures such as ruffles, lamellipodia, fine extension, and neurite-like structures (16). The variety of interactions with important adhesion proteins provides the foundation for MDA-9/Syntenin's involvement in invasion regulation. Activation of cellular programs leading to altered cell-cell and cell-matrix interaction is necessary for cancerous cells to invade and migrate away from the primary tumor. Further, cellular locomotion relies heavily on actin cytoskeleton rearrangement, another area of MDA-9/Syntenin influence.

MDA-9/Syntenin is associated with membranes throughout the cell, anchored to the plasma membrane by interaction with PIP2 and phospholipase Cγ (16). It also localizes to the early secretory pathway: the endoplasmic reticulum, intermediate compartment, cis-Golgi, as well as apical endosomes while facilitating the trafficking of cell-surface molecules (20, 49-51).

F. Syntenin-2

Two isoforms of MDA-9/Syntenin have been identified: syntenin 2α and 2β, cloned from a library of fetal human brain cDNA (19). Syntenin 2α shares greater than 70% homology in the PDZ domains as MDA-9/Syntenin, while syntenin 2β is a shorter isoform of 2α that lacks 85 residues at the N-terminal (19). To date, relatively few studies have focused on this isoform, yet like MDA-9/Syntenin, it has also been shown to interact with PIP2 (52). One study found lower expression of syntenin-2 in colorectal cancer as compared to normal tissue, but was not found to be a significant prognostic marker (53). In other work, syntenin-2 was found in higher amounts in bile from biliary stenosis related to malignant causes, such as pancreatic adenocarcinoma or cholangiocarcinoma, compared to nonmalignant samples such as chronic pancreatitis or biliary stones (54). Initial studies suggest roles for syntenin-2 in cell division, nuclear PIP2 organization, and cell survival (52), though these interesting areas remain relatively unexplored.

III. Involvement in Tumor Progression and Invasion

A. Clinical Correlations

Tumor cell invasion and metastasis is a complex process requiring the cell's successful execution of numerous essential steps (55). MDA-9/Syntenin has repeatedly been found to be expressed at higher levels in more invasive, metastatic cell lines of multiple cancer types compared to their less invasive, less aggressive counterparts (20) (Table 1.1).

Furthermore, genetic manipulation of cancers forcing elevated expression in cells with lower baseline levels of MDA-9/Syntenin consistently leads to increased migration and invasion. Along with these observations come reports of more polarized distribution of F-actin and increased pseudopodia formation when MDA-9/Syntenin is overexpressed (14, 15, 47).

This is clearly outlined in clinical examples of melanoma invasion. As melanoma progresses, the prevailing hypothesis is that it develops from a benign nevi to a radial growth phase primary melanoma, followed by a vertical growth phase primary melanoma as it invades downwards through the dermis, eventually to become a metastatic melanoma. MDA-9/Syntenin expression increases as these phases advance (15, 56).

Table 1.1 MDA-9/Syntenin in Cancer: Observed Clinical Correlations

Cancer	MDA-9 Enhanced Invasion?	Tumor Grade	Survival Correlation	Years	References
Melanoma	Yes	↑with ↑ MDA-9	Not Reported	2004-2013	(15, 25, 57-59)
Glioma/GBM	Yes	↑with ↑ MDA-9	↓with ↑ MDA-9	2012 2014	(50, 60)
Breast	Yes	↑with ↑ MDA-9	↓with ↑ MDA-9	2002 2013	(51, 61, 62)
UCC	Yes	↑with ↑ MDA-9	Not reported	2013	(49)
SCLC	Yes	↑ MDA-9 in advanced disease	Not reported	2014	(63)
Uveal Melanoma	Yes	↑ MDA-9 in recurrent cases	↓with ↑ MDA-9	2012	(64)
Gastric	Yes	Only compared to control tissue	Not reported	2002	(61)

MDA-9/Syntenin has been shown to be a marker of higher aggression and tumor grade in numerous cancers. In melanoma, breast cancer, glioma, and urothelial cell carcinoma (UCC) MDA-9/Syntenin correlates with advancing tumor grade (20, 49-51, 60), is overexpressed in gastric cancer (61), and in breast cancer and glioma, higher MDA-9/Syntenin expression portends shorter survival in patients (50, 62). In addition to being a marker of higher tumor grade in breast cancer, MDA-9/Syntenin was also more highly expressed as estrogen receptor expression is lost (51). Furthermore, silencing MDA-9/Syntenin led to an accumulation of cells in G1 along with enhance p21 and p27 expression. Separate work in breast cancer showed that MDA-9/Syntenin expression correlated positively with tumor size, lymph node metastasis, and tumor recurrence. Furthermore, overall survival and disease-free survival was shorter in patients with high MDA-9/Syntenin tumor expression (62).

In UCC, MDA-9/Syntenin was found to be associated with advanced stages and higher grades of tumors, which can rapidly progress to invade surrounding muscle tissue. Ectopic overexpression in nontumorigenic cells enhanced proliferation and invasion, while MDA-9/Syntenin inhibition led to fewer lung metastases in an *in vivo* model (49). MDA-9/Syntenin also appears to play a role in uveal melanoma, the most common intraocular tumor in adults and a particularly aggressive cancer with survival times of about 5-7 months following metastasis. This tumor expresses elevated levels of MDA-9/Syntenin, and even higher levels in recurrent cases (64). Uveal melanoma metastasizes to the liver in nearly 50% of patients, and patients with higher expression of MDA-9/Syntenin showed significantly shorter disease-free survival. Additionally, knockdown of MDA-9/Syntenin *in vitro* inhibited HGF-induced invasion in matrigel (64). Further, MDA-9/Syntenin was identified in secretomes of uveal melanoma in patients with metastatic tumors (65).

With results similar to other studies, MDA-9/Syntenin was found to be an important regulator of invasion in small cell lung cancer (SCLC). SCLC is another particularly aggressive cancer, with a median survival of 8 months, and a 2-year survival of less than 5% and is known to be highly invasive and metastatic. In these patients high expression of MDA-9/Syntenin correlated with more advanced and extensive disease at diagnosis (63).

B. Binding partners/Cellular Mechanics

Central to MDA-9/Syntenin's ability to induce invasive behavior in tumor cells is its interaction with key binding partners. Among these are known oncogenic proteins as well as those that have well-demonstrated activity in cell motility and invasion patterns. Studying the mechanisms of these interactions will allow us to understand the full scope of MDA-9/Syntenin's involvement in tumor invasion (Figure 1.1).

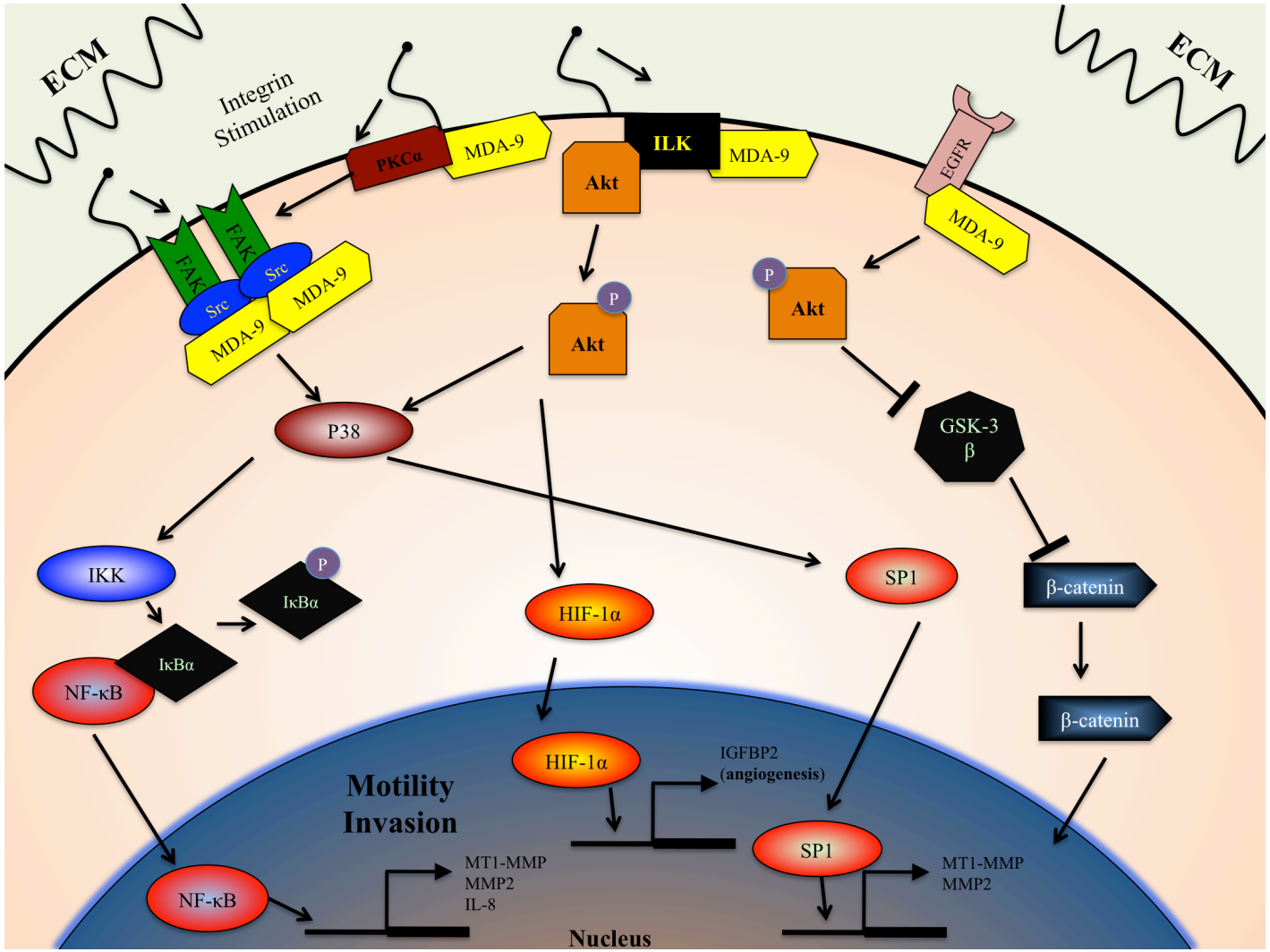


Figure 1.3 MDA-9/Syntenin invasion signaling. A summary of important signaling pathways affected by MDA-9/Syntenin that impact cancer progression, particularly motility, invasion, and angiogenesis.

Src

c-Src is a member of the Src family tyrosine kinases (SFKs), known to have fundamental roles in cell motility, invasiveness, and survival and have been implicated in the pathology and progression of numerous cancer types including breast, prostate, glioma, and melanoma (66-69). MDA-9/Syntenin co-localizes with Src in metastatic melanoma, especially in areas corresponding to focal adhesion (57), and interacts with c-Src in glioma (50). MDA-9/Syntenin-Src complexes have also been found diffusely within the cytoplasm as well as in and near the nucleus (3, 4, 70), suggesting a heterogeneity of complexes in which they participate and that MDA-9/Syntenin-Src may be involved in promoting transcriptional activities (3, 4, 20, 70). c-Src is a crucial component of MDA-9/Syntenin-induced invasion. Overexpression of MDA-9/Syntenin can induce invasion in normal or weakly metastatic tumor cell types of diverse tissue origins, yet these invasion gains are lost when c-Src is inhibited either genetically or pharmacologically (50, 57).

c-Src interaction is mediated through the PDZ domains of MDA-9/Syntenin, as deletion mutants lacking PDZ1 or PDZ2 dramatically reduce c-Src binding (25). PDZ-2 was found to be essential for c-Src binding, while PDZ-1 was not (19, 71). This is consistent with experimental data that describe PDZ functional units mutually chaperoning each other, enabling the full function of tandem PDZ domains that are necessary to mediate specific interactions with binding partners (3, 24, 72). The PDZ1 and PDZ2 domains of MDA-9/Syntenin have been shown to be structurally associated and undergo cooperative denaturation (22). Thus, PDZ-2 can primarily bind c-Src while PDZ-1 promotes proper folding such that MDA-9/Syntenin successfully assembles into a more stable, multimeric complex (20).

Focal Adhesion Stimulation

Integrin stimulation leads to the autophosphorylation of focal adhesion kinase (FAK) at Tyr^{397}, which creates a binding site for SFKs. This leads to the formation of FAK-c-Src dual kinase complexes, further phosphorylating FAK and resulting in the coordination of signaling through multiple pathways that influence the regulation of migration, tumor growth, and invasion (68, 73). Increased MDA-9/Syntenin expression correlates with higher levels of FAK-c-Src complexes as well as active, phosphorylated FAK in melanoma cells (15). Following MDA-9/Syntenin siRNA treatment in highly invasive melanoma cells, p-FAK levels are decreased (3, 4, 15, 25, 57). Dominant negative (DN) FAK expression (FRNK) significantly reduces the MDA-9/Syntenin induced migration of weakly metastatic melanoma cells on FN-coated plates (15).

Another protein involved in focal adhesion activation, PKCα, plays important roles in migration and is essential for integrin-mediated signaling, especially through association with integrin β1 associated complexes (73-75). For example, integrin α5β1 binding to FN activates PKCα. Inhibiting PKCα in this scenario suppresses focal adhesion formation and cell migration, critical steps in cancer cell invasion. Both knockdown of MDA-9/Syntenin or DN PKCa expression abrogates FN-induced FAK phosphorylation in metastatic breast and melanoma cells (37). Additionally, FN-stimulation also increases FAK association with β1 integrins, c-Src, and MDA-9/Syntenin, which can be counteracted by MDA-9/Syntenin siRNA treatment or PKCα DN expression (37). Furthermore, FN stimulation led to increased plasma membrane association of MDA-9/Syntenin and PKCα, while knockdown of MDA-9/Syntenin reduced membrane targeting of PKCα (37). Since MDA-9/Syntenin also binds recognized membrane occupants such as syndecan-4 (24) and PIP2 (76) through its PDZ domains, it may be responsible for

facilitating binding of PKCα to PIP2 by forming a complex at the plasma membrane following FN attachment.

Downstream Effectors

c-Src/FAK signaling leads to the activation of the NF-κB pathway, which has been repeatedly demonstrated to be involved in invasion-related transcription activity. The NF-κB p50-p65 complex is normally maintained in an inactive state bound to IκBα in the cytoplasm. Upstream activation leads to IκB-kinase (IKK)-mediated phosphorylation of IκBα, targeting it for degradation. Thus, liberated NF-κB translocates to the nucleus, where it binds target DNA sequences in the promoter of an array of genes, enhancing their transcription (77). The p38MAPK pathway is a known activator of NF-κB, and MDA-9/Syntenin inhibition can reduce the levels of phosphorylated p38MAPK in melanoma and glioma (25, 50).

A key step in tumor cell invasion is degradation of the ECM via matrix metalloproteinases (MMPs), and MDA-9/Syntenin–related signaling has been shown to lead to such expression in multiple cancer indications. MMP-2 is a crucial member of the MMP family often involved in tumor cell invasion (78). MDA-9/Syntenin signaling leads to enhanced MMP2 transcript and protein expression in melanoma and GBM (15, 50). MMP2 activation occurs through the activity of MT1-MMP, a transmembrane-bound MMP that cleaves and activates pro-MMP2 in conjunction with the activity of tissue inhibitor of metalloproteinase-2 (TIMP-2) (15, 20). Both of these processes can be initiated by NF-κB signaling downstream of MDA-9/Syntenin-c-Src activation and p38MAPK signaling in multiple tumor types (15, 50). Nonetheless, there is evidence that MDA-9/Syntenin can be involved in additional tissue-specific signaling.

While FN engagement and integrin stimulation were found to be important for MDA-9/Syntenin activation of invasion in melanoma and glioma (15, 50), Ras, Rho, Rac, and PI3K/Akt signaling mediated MDA-9/Syntenin actions in HEK293T cells (20). In SCLC, MDA-9/Syntenin led to the activation of p38MAPK, Akt, and production of MT1-MMP and MMP2. Additionally, the transcription factor specificity protein-1 (SP1), which can promote MT1-MMP and MMP2 production, was activated by MDA-9/Syntenin, adding to the growing number of pathways that MDA-9/Syntenin influences (63).

Collagen binding in breast cancer cells was also found to prompt MDA-9/Syntenin-related signaling events. MDA-9/Syntenin was shown to regulate the action of Akt by facilitating integrin linked kinase (ILK) adapter function during breast cancer cell adhesion to type-I collagen(79). Inhibition of MDA-9/Syntenin disrupted the translocation of both ILK and Akt to the plasma membrane, while collagen-I stimulation increased the association of ILK and MDA-9/Syntenin at the plasma membrane. Thus, MDA-9/Syntenin was found to control the membrane targeting of the ILK-Akt complex, thus mediating the activation of Akt during collagen-I adhesion (79). Further, ILK is also involved in a signaling platform for integrins along with PINCH1 and α-parvin (making up the IPP complex) (80). Inhibiting MDA-9/Syntenin led to a decrease in plasma membrane translocation of the IPP complex upon collagen-1 binding, leading to decreased assembly of integrin β1-IPP signaling complexes (79). MDA-9/Syntenin-related effects on transmembrane proteins and Akt activation was also found in UCC cells, where MDA-9/Syntenin inhibition led to decreased EGFR and Akt activation as well as reduced expression of epithelial to mesenchymal transition (EMT) markers (49).

IV. Directing Cellular Traffic: Role as Intracellular Adapter

Adaptor molecules are often involved in crucial protein-protein interactions that lead to the assembly of multimeric complexes, which can play key roles in propagating extracellular signals to their designated intracellular targets (81). The PDZ family of proteins is known to control diverse, centrally important physiological processes (82), and MDA-9/Syntenin is no exception. It interacts with an impressive host of binding partners, regulating a plethora of molecular outcomes, which often can be related directly or indirectly to invasion signaling (20).

A. Syndecans

Syndecans are a family of abundant type I transmembrane proteins with heparan sulfate side chains on their extracellular domains. The MDA-9/Syntenin-syndecan interaction was the first characterized functional interaction of this protein (14) and was further defined when studies demonstrated MDA-9/Syntenin altered PIP2 binding led to trapping of syndecans in the perinuclear recycling endosomes. This highlights the importance of MDA-9/Syntenin in syndecan trafficking and recycling back to the plasma membrane (82). Syndecans can bind and influence the actions of numerous extracellular receptors, influencing their expression through regulation of uptake and trafficking. For example, integrin trafficking was influenced by the phosphorylation of syndecan-4 at Tyr^{180} by Src (83). This enhanced MDA-9/Syntenin binding, increased Arf6 suppression, stabilized focal adhesions, and promoted recycling of $\alpha V\beta 3$ integrin to the plasma membrane at the expense of $\alpha 5\beta 1$. Since the Tyr^{180} residue and the ability to bind MDA-9/Syntenin are conserved among syndecans, similar processes could be utilized in many cell contexts by any of the numerous syndecan-cell surface receptor interactions (83). A similar interaction is employed in the recycling of fibroblast growth factor receptor (FGFR), as it has

been shown to accumulate in syndecan-MDA-9/Syntenin-PIP2 endosomes in an FGF-dependent fashion (84).

MDA-9/Syntenin's interaction with the cytoplasmic domain of syndecan-2 leads to increases in cell migration. When this interaction is abolished, or MDA-9/Syntenin expression is inhibited, cell migration is reduced. Furthermore, MDA-9/Syntenin was shown to mediate Rac activation induced by syndecan-2 (85). Heparanase can cleave heparan sulfate and is shown to be involved in cancer invasion, angiogenesis and metastasis (86, 87). Its processing and activation is dependent on MDA-9/Syntenin's interaction with syndecan-1, and knockdown of MDA-9/Syntenin can inhibit heparanase processing by greater than 50% (88).

B. Exosomes

An emerging role for MDA-9/Syntenin function is a role in the regulation of exosome biology. Recent work has shown that syntenin can form a complex with ALIX, which can be recruited to the cytoplasmic tails of syndecans and subsequently support membrane budding (21). This leads to the formation of early endosomes, which can themselves undergo invaginations that lead to intraluminal vesicles (ILVs) in what are then termed multivesicular bodies (MVBs). These ILVs can be released as exosomes upon fusion of MVBs to the plasma membrane. MDA-9/Syntenin knockdown led to both reduced numbers and average size of exosomes detected, while overexpression of MDA-9/Syntenin could increase the number of exosomes approximately 2-fold in breast cancer cells. Formation of MDA-9/Syntenin exosomes requires ESCRTs (endosomal-sorting complexes required for transport), as well as syndecan oligomerization and cleavage (21). Exosome biology has wide applicability and can have implications in numerous processes that utilize extracellular signaling, including inflammation, cancer biology, and tumor invasion.

CD63 is a tetraspannin that is abundantly expressed on the plasma membrane and in late endocytic organelles (35) as well as a marker for exosomes (21). Tetraspannins are found in the plasma membrane and can associate with numerous receptors and cell surface molecules, including RTKs and integrins, and regulate their maturation, activity, and processing. MDA-9/Syntenin has been shown to bind the cytoplasmic tail of CD63 using biochemical and heteronuclear magnetic resonance spectroscopy (NMR), indicating that the interaction occurs through its PDZ domains (35). When MDA-9/Syntenin is overexpressed, the constitutive rapid internalization of CD63 is slowed, and an N-terminal-lacking deletion mutant of MDA-9/Syntenin blocked the internalization of CD63. Additionally, MDA-9/Syntenin can inhibit AP-2 dependent internalization by competing for binding in the CD63-AP-2 interaction (89). This demonstrates yet another role of MDA-9/Syntenin activity at membrane-associated structures within the cell.

In comparing exosomes of melanoma cells to normal melanocytes, MDA-9/Syntenin was reduced about twofold in exosomes from melanoma cells (90). This is a similar finding to that shown in analysis of melanoma secretomes, showing that MDA-9/Syntenin was downregulated in the secreted profile of more metastatic B16 melanoma cells compared to those that did not form lung metastases (91). Taken together, these findings suggest differential roles for intracellular vs. secreted MDA-9/Syntenin.

C. Notch Signaling and Regulation of Stemness

Analysis of human epidermal stem cells revealed that a more proliferative and adhesive population of stem cells marked by high delta-like 1 (DLL1), a binding partner of MDA-9/Syntenin, had over 13 fold higher MDA-9/Syntenin expression. Consistent with MDA-9/Syntenin's known actions, this group had a transcriptional profile that associated with active

endocytosis, integrin-mediated adhesion and receptor tyrosine kinase signaling (92). Research conducted in keratinocytes show that MDA-9/Syntenin interacts near cell-cell borders with delta1, a ligand of Notch that leads to Notch cleavage and translocation to the nucleus. In the mammalian epidermis, Notch signaling can have a tumor suppressor function that prompts adjacent cell differentiation (93). When the C-terminal of Delta1 is mutated in the region of its PDZ-binding motif, or if MDA-9/Syntenin expression is downregulated, Notch-driven transcriptional activation was dramatically increased (84). Both of these approaches decreased plasma membrane expression of delta1, thus indicating that normal MDA-9/Syntenin activity promotes a less differentiated state (84). This is consistent with the finding that MDA-9/Syntenin is highly expressed in the reservoir of interfollicular stem cells (84). However, the relationship between MDA-9/Syntenin and Notch signaling has not been fully explored in other settings, including breast cancer, in which this pathway can be a promoter of tumor stem cell activity and invasion (94, 95).

D. Ephrin Family Interactions

Ephrin receptors and their respective ligands have been characterized as crucial regulators of neuronal development. Moreover, they have been shown to be involved in cell-cell repulsion in both the developmental environment regulating axons (96) and in cancer cell repulsion, a first step in invasion (97). Additionally, ephrins are involved in the motility of neural crest cells, fusion of epithelial sheets that close the palate, as well as angiogenesis (98, 99).

During synaptic development, Ephrins can recruit a variety of adaptor and signaling complexes that support normal synaptic function, including SFKs, guanine nucleotide exchange factors (GEFs), and PDZ proteins (100). Ephrin-B is a transmembrane-bound ligand for EphB, and this pair can signal between dendrites and axons in a forward (in the receptor (EphB)-

expressing cell) or in reverse (the ligand (ephrin-B)-expressing cell). These signals are important for promoting assembly, maturation, and plasticity, as well as axon guidance, pruning, and presynaptic development. MDA-9/Syntenin and another PDZ protein, PICK1, were implicated in regulating the number of functional synapses. MDA-9/Syntenin was shown to be expressed in the developing hippocampus and was involved in ephrinB3-related reverse signaling that enabled dendrite pruning, synaptic maturation, and formation of neural circuits (101). Other reports support this role for MDA-9/Syntenin as EphB1 and EphB2 have been shown to bind the PDZ domains of MDA-9/Syntenin to enable synaptic development and inhibition of this partnership prevents presynaptic development (100).

E. Sox4 Activity

Sox4 is aberrantly expressed in many human tumors, but generally has a short half-life of less than 1 hour, being degraded by the proteasome in a polyubiquitin-independent fashion. MDA-9/Syntenin binds to the C-terminal of Sox4 and stabilizes its expression, providing a mechanism for the observation that DNA damage increases Sox4 protein expression independently of Sox4 mRNA levels (102). p53 binds to the C-terminal of Sox4 and Sox4 is critical for p53 stabilization. It is unknown if Sox4 C-terminal binding proteins, including p53, may act in parallel or compete with MDA-9/Syntenin in regulating stability or functions (102).

Furthermore, IL-5 interaction with its receptor, IL-5R, can result in Sox4 activation, regulating the development and differentiation of B-cells (103). The IL-5Rα subunit interacts with MDA-9/Syntenin and mediates IL-5-induced Sox4 activation (103), yet another demonstration of the wide variety of cell-specific actions that can be modulated through this important adapter protein.

F. Immune Cell Modulation and Viral Trafficking

MDA-9/Syntenin has especially high expression in the germinal centers of normal lymph nodes and is robust in follicular dendritic cells (FDCs). An *in vitro* proxy of FDC cells, HK cells, showed that knockdown of MDA-9/Syntenin reduced FAK activation, similar to observations in cancer-derived cell lines (104).

During dendritic cell-T-cell interactions, MDA-9/Syntenin was shown to be responsible for linking activated leukocyte cell adhesion molecule (ALCAM) to the actin cytoskeleton, stabilizing it as part of a supramolecular complex engaged to CD6 (105). Interestingly, ALCAM has also been shown to be involved in a multitude of interactions including neural and hematopoietic development, immune responses, and osteogenesis. Further, ALCAM has been implicated in the progression of breast cancer, bladder cancer, colorectal cancer, and melanoma. It is also involved in GBM tumor invasion and is expressed on glioblastoma progenitor cells (106).

MDA-9/Syntenin is recruited to the plasma membrane during HIV-1 attachment and associates with CD4, the main HIV-1 receptor. When MDA-9/Syntenin is knocked down in T-cells, actin polymerization is decreased, while PIP2 production is increased along with HIV-1 entry. Conversely, MDA-9/Syntenin overexpression reduces HIV-1 production and HIV-mediated cell fusion (107). Additionally, Nef, an HIV-1 accessory protein, was demonstrated to reduce the expression of MDA-9/Syntenin (108).

V. Angiogenesis and Inflammation

In addition to numerous examples of MDA-9/Syntenin as a mediator of invasive pathways, this protein has also been shown to be involved in inflammation and angiogenesis, a process that overlaps with invasion in numerous respects. In both melanoma and glioma, MDA-

9/Syntenin was shown to increase angiogenic potential in tumor cells (50, 59). In melanoma, MDA-9/Syntenin was found to induce angiogenesis by activating Akt, leading to HIF-1α induction and transcription of IGFBP-2. Subsequent secretion of IGFBP-2 induces endothelial cell production of VEGF-A and angiogenesis (59). In glioma, MDA-9/Syntenin induced NF-κB activation and the production of the prominent angiogenic chemokine IL-8 at both the transcript and protein expression level. Furthermore, knockdown of MDA-9/Syntenin reduced microvessel branching in *in vivo* assays, and reduced tumor vascularity in an orthotopic xenograft mouse model (50). MDA-9/Syntenin was also shown to help maintain the blood-brain barrier (BBB) integrity, as miR-155 targeting of MDA-9/Syntenin can lead to downregulation and higher measures of BBB permeability (109).

Inflammatory pathways can be important for numerous normal and pathogenic states, and MDA-9/Syntenin's role in regulating these processes could prove that it represents a highly useful therapeutic target. MDA-9/Syntenin partners with syndecan-4 regulating exosome-dependent secretion of Angiopoietin-2 (Ang2), a crucial Tie2 ligand that influences vascular integrity and inflammation. A recent study demonstrated that excessive Ang2 secretion could be rescued by syndecan-4 knockout or syntenin inhibition. Notably, knockdown of MDA-9/Syntenin, which can bind all syndecans, had a larger reductive effect in Ang2 secretion than single syndecan knockdown (110). MDA-9/Syntenin was found to be significantly elevated in the plasma of diabetic patients compared to healthy donors as well as upregulated in liver cells cultured in high glucose media (111).

VI. Summary

MDA-9/Syntenin displays an impressive diversity of interacting partners, indicating it has a number of flexible roles within the cell. It forms a variety of complexes, some specific to a

particular cell type and others to a subcellular compartment, and is involved in numerous intracellular pathways. As noted, MDA-9/Syntenin is frequently identified as being integral in regulating cell migration, invasion, and metastasis in a variety of cancer types. This presents the unique opportunity of developing novel and worthwhile cancer therapeutics that target MDA-9/Syntenin.

Chapter 2 - Introduction to Glioma

I. Overview

The nearly 70,000 patients estimated to be diagnosed with glioma in 2015 will look to treatments developed using the latest insights into the pathology of this devastating form of cancer (112). While expected survival for GBM patients remains vanishingly small, increases in our understanding of the biology of this tumor have been made in the last decade. GBM has emerged as the most extensively described cancer through genomic profiling (113), uncovering subtleties that may prove highly useful in development of novel therapies. To support this, the lag time from discovering a possible target to implementation of therapy has decreased (114). Dedication to a deep understanding of the molecular biology of GBM has been shown through marked progress in characterizing the genome (115, 116) and the transcriptome (117, 118) of a wide sampling of patient tumors. This process has provided a far more detailed topography of the GBM landscape; as well as unmasked what seemed to be a collection of highly related tumors to show a heterogeneous cast of distinct subtypes with unique tendencies and characteristics.

II. Malignant Glioma

The World Health Organization guidelines for classification of gliomas are still the most widely used categorization method for clinical grading (119). While this method remains rooted in morphological and histopathological findings, insights into molecular signatures of tumor grade has been supplemented and advocated for incorporation into updated standards (113, 120).

Glioma can include tumors defined as astrocytomas, oligodendrogliomas, or oligoastrocytomas, among others (119). Grade I gliomas have a limited proliferation profile and have the possibility of cure with surgical resection alone (119). While still considered low grade, grade II astrocytomas are known as "diffuse astrocytomas," show cytological atypia and invade into surrounding tissue as the name suggests. Gliomas are considered as high-grade at grade III, noted for high mitotic activity and anaplasia. Once microvascular proliferation and/or necrosis are observed, gliomas are categorized as grade IV, or glioblastoma (GBM) (119). 90-95% of GBMs arise as "primary GBM," while 5-10% develop from lower grade gliomas, predominately in younger patients, and are known as "secondary GBMs" (121, 122). These tumors show distinct molecular profiles as discussed below. Using just this classification system without molecular profiling, stratification for survival can be observed based on tumor grade(112, 119). As molecular characterization and diagnostics become more prevalent, future classification systems will undoubtedly include genomic profiling to delineate tumor subclasses (123).

Cancers of the central nervous system (CNS), like all other cancer types, have characteristic genetic instability with alterations in chromosome structure and/or copy number (113). The systemic analysis of genetic data from tumors has identified a number of new, common somatic copy number alterations (SCNAs) present in cancer (124). Many of the most recent advanced methods used to identify new, important targets in tumors were first used to investigate GBM, and later other cancer types, through the probing of numerous primary patient samples (113, 115). Such investigation into SCNAs resulted in a compilation of presumed targets of amplification (which includes *EGFR, MET, PDGFRA, MDM4, MDM2, CCND2, PIK3CA, MYC, CDK4,* and *CDK6*) and deletion (*CDKN2A/B, CDKN2C, PTEN,* and *RB1*) (125, 126). This was a precursor to the first of a number of consortium-based analyses of the cancer genome, The

Cancer Genome Atlas pilot study, dedicated to resolve the molecular picture of GBM through multiplatform profiling (112, 127). This project both confirmed earlier results and identified new molecular abnormalities, including amplification of *AKT3*, homozygous deletion of *PARK2* (112, 127). This project was able to quantify the dysregulation of major signaling pathways known to be important in GBM, while confirming previous studies (128-134). Although *TP53* was known to be a commonly deleted or mutated gene, its signaling was impaired by various mechanisms in 87% of analyzed samples. In addition to direct effects, this occurred through events such as *CDKN2A* deletion, and *MDM2* or *MDM4* amplification. Similarly, Rb signaling was inhibited in nearly 80% of samples, through either direct mutation or deletion of *RB1* or *CDK2NA,* or alternatively through amplification of *CDK4, CDK6,* and *CCND2*. Oncogenic signaling through drivers such as receptor tyrosine kinases (RTKs), RAS signaling, or PI3K activation was present in almost 90% of tumors.

III. Notable Aberrant Signaling Pathways in Malignant Glioma

Alterations in important checkpoint and driver pathways have been shown to contribute to development of gliomas. Enhanced signaling by RTKs (especially EGFR, PDGFR, and MET), PI3K pathway activation, signaling pathways left unchecked by decreased PTEN or NF1 activity, and effects of mutant IDH proteins play critical roles in the molecular biology of GBM (116-118, 127).

A. Activating Kinases

EGFR/EGFRvIII

EGFR is the flagship protein of the EGFR/ErbB receptor family, consisting of a ligand-binding extracellular domain connected by a hydrophobic transmembrane region to a

cytoplasmic domain with tyrosine kinase activity (135, 136). It is amplified in about half of primary GBMs and is associated with a poor prognosis (137-139). In cells with *EGFR* amplification, about half have a truncated variant, *EGFRvIII*, resulting from an intragenic gene rearrangement produced by the in-frame deletion of extracellular region exons 2-7. EGFRvIII expression portends a worse prognosis than wild-type EGFR expression alone (140, 141). EGFR can initiate its signaling through the Shc-Grb2-Ras pathway as well as through activation of PI3K (136, 142, 143). When overexpressed *in vitro*, EGFRvIII has been shown to display constitutive phosphorylation, enhanced cell proliferation, and superior tumorigenicity (142, 143), as well as modulation of pro-survival protein Bcl-xL expression leading to resistance to apoptosis by DNA-damaging agents (144). Unless EGF ligand is present at high concentration, wild-type EGFR overexpression cannot simulate the downstream effects of EGFRvIII (145). EGFR has been shown to translocate to both the mitochondria (146) and the nucleus (147). In the nucleus, EGFR and EGFRvIII are possibly acting through both transcriptional and signaling mechanisms to drive proliferation as well as induce DNA damage repair (147).

Expression of EGFRvIII in human tumors is in fact heterogeneous, often observed in only a subpopulation of cells (148). This subset of cells likely promotes its own growth and potentiates the proliferation of neighbor, wild-type EGFR cells, through the transmembrane glycoprotein gp130 (149). Downstream EGFR signaling has been shown to induce IL-8 production through NF-κB (150). Moreover, heterozygous deletion of *NFKBIA* (encoding for endogenous NF-κB inhibitor IκBα) displays a mutually exclusive pattern to *EGFR* amplification (151).

When cultured *in vitro*, most lines fail to maintain faithful *EGFR* amplifications or EGFRvIII expression. However, passage of cells using stem cell conditions or serial *in vivo*

xenograft harvesting has been shown to result in preservation of *EGFR* status (152, 153). While *EGFR* alterations are known to be common in other cancer types, the type of genetic alterations in GBM have been shown to be distinct from others such as non small cell lung cancer (NSCLC). For example, *EGFR* amplifications in glioma tend to be focal and at very high copy numbers (>20). Mutations in non-glioma cancers are often found in the intracellular domains of EGFR (154), while the majority of EGFR mutations in glioma, including the vIII mutation, are found in the extracellular domain (116, 155).

PDGFR

Platelet derived growth factor receptor (PDGFR) is another RTK with demonstrated importance in GBM signaling. *PDGFRA* is amplified in 15% of all samples, but enriched in the proneural subtype (discussed below) (117, 118). Of all samples with gene amplification, 40% harbor an intragenic deletion, PDGFRA$^{\partial 8,9}$, an in-frame deletion of exons 8 and 9 leading to a truncated extracellular domain (156). Additionally, an in-frame gene fusion of the extracellular domain of VEGFR-2 and the intracellular/kinase domains of PDGFRA has been identified. Both these mutations lead to constitutively active, transforming mutant proteins (116). *PDGFRB* expression appears to be restricted to proliferating endothelial cells within GBM tumors (157-160).

Other methods of activating the PDGFR signaling pathway include the production of endogenous ligands, PDGF(A-D), which are overexpressed in ~30% of glioma tissue samples and cell lines (161). Both autocrine and paracrine mechanisms are likely candidates responsible for potentiating PDGF/PDGFR signaling within a tumor. Work in animal models has shown that, much like EGFR/EGFRvIII, tumors can be comprised of diverse populations of cells, some of

which overexpress PDGF, and recruit cells which do not, leading to heterogeneous malignant gliomas (162, 163).

c-Met

A third RTK that is amplified in 5% of GBMs, though rarely mutated, is *c-Met* (116). It also is coactivated in cells with increased levels of EGFR/EGFRvIII (164-166). Activated EGFR can associate with c-Met, resulting in ligand-free activation of c-Met (167). The c-Met–EGFR relationship is further intertwined in that ligand for c-Met, HGF, can transcriptionally activate EGFR ligands TGF-α and EGF, leading to activation of EGFR (168). Additionally, blocking EGFRvIII activity through monoclonal antibody delivery (panitumumab) can lead to a switch to c-Met activation through HGF binding. This is a demonstration of inter-pathway cross talk that leads to obvious means for intrinsic drug resistance. Indeed, this relationship holds in lung cancer expressing mutated EGFR, where c-Met overexpression can lead to resistance to gefitinib, an EGFR-tyrosine kinase specific inhibitor (169). Therefore, it is imperative to apply a multifocal approach to RTK-specific inhibitors. Treatment targeted against EGFR (erlotinib), PDGFR (imatinib), and c-Met (SU11274) showed a significant improvement of GBM cell growth inhibition *in vitro* (165). However, when c-Met is amplified alone, crizotinib treatment, directed against ALK and c-Met activity (170), shows significant radiographic and clinical improvement in a case report (171).

Src-family kinases

Non-receptor kinases belonging to the Src family often mediate signaling from growth factor receptors and are widely expressed in GBM (172). Src has been implicated in numerous cellular processes and can mediate pro-oncogenic processes such as cell viability, migration, invasion, and metastasis(25, 57, 173). These proteins are often activated in GBM tumors and cell

lines (173, 174), and are effectors of oncogenic EGFR signaling (172). In 31 samples of GBM tumors, SRC was significantly activated in 61%. Inhibition via dasatinib inhibits viability and migration *in vitro*, as well as tumor growth *in vivo* (173). Src family kinases are among the most frequently observed tyrosine kinase activations in a study of 130 human cancer cells, along with: EGFR, fibroblast growth factor receptor 3 (FGFR3), and focal adhesion kinase (FAK) (173).

PI3K-related signaling

One of the most extensively studied and important dysregulated pathways in glioma signaling is the PI3K signaling cascade; driving survival, proliferation, migration, and invasion (175). PI3K signaling can be activated through GTP-bound Ras as well as RTK activity, recruiting PI3K to the plasma membrane where it catalyzes the phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PIP_2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). The major tumor suppressor PTEN reverses this reaction. PIP_3 recruits serine/threonine kinase AKT to the plasma membrane, where it is fully activated via phosphorylation at T308 by PDK1 and S473 by mTORC2 (176). Elevated AKT phosphorylation is noted in up to 85% of GBM patient samples and cell lines (177). Another important RTK, Ephrin A2 (EphA2), highly expressed in GBM (178) binds PI3K after ligand stimulation and induces GBM cell migration by activating AKT (179).

Aside from RTK-dependent activation, PI3K signaling can be activated via mutation or amplification of catalytic subunit isoform *PIK3CA* (116, 180, 181) or overexpression of *PIK3CD* (182). Important interactions with other proteins can also govern PI3K signaling. PI3K can interact with Src family kinase Yes, promoting CD95-driven invasion through activation of GSK3β and matrix metalloproteinase (MMP) expression (183). In GBM that lacks EGFR amplification, insulin-like growth factor-2 (IGF2) promotes aggressive growth via insulin-like

growth factor-1 receptor (IGF-R1) and phosphoinositide-3-kinase regulatory subunit 3 (PI3K-R3) (184). In the absence of functioning PTEN, resulting AKT activation can contribute to RTK inhibitor sensitivity (185, 186). Inhibitors directed against PI3K have proven to be cytostatic as opposed to cytotoxic, which may be due to arrest at different phases of the cell cycle (187, 188). Therefore, numerous combination studies have been undertaken, simultaneously inhibiting other targets such as mTORC1, mTORC2, and MEK G1 (187-190). Due to the variety of mechanisms by which downstream targets can be activated, inhibition of the PI3K pathway has proven difficult. RTK signaling can circumvent AKT through PKC signaling, activating mTORC1 independent of AKT (191). Additionally, PI3K activation can modulate phosphorylation and inactivation of the pro-apoptotic protein Bad through PDK1 and PKC, increasing cell survival (192). Overall, the PI3K cascade and feedback mechanisms, though widely studied, have proven complex and are not yet completely elucidated. Thus, inhibition strategies must be implemented as part of a combination treatment plan (193).

B. Tumor Suppressor Pathways

PTEN

Loss of *PTEN* function leads to high levels of PI3K activity and downstream signaling effects. *PTEN* is lost, mutated, or epigenetically inactivated in up to 50% of gliomas, and is one of the most frequently lost tumor suppressors in all cancers (194). Under normal conditions, PTEN stability is governed post-transcriptionally by GSK3β-mediated phosphorylation at T366 (195) and NEDD4-1-mediated ubiquitination leading to proteasomal degradation (196). Thus, PTEN function can be impeded without being genetically mutated or deleted. Since PTEN is part of a heterotrimeric tumor suppressor complex with NHERF-1 (Na^+/H^+ exchanger regulatory factor-1) and PHLPP-1 (pleckstrin homology domain leucine-rich repeat protein phosphatase-1),

upregulation of NEDD4-1 or a similar alteration to the stability of other members of the complex can lead to disrupted PTEN function (197). Overexpression of forkhead transcription factor, FoxM1B, frequently enhanced in glioma samples, is associated with NEDD4-1 overexpression (78, 198). It follows that the estimate of 50% of glioma samples showing *PTEN* mutation or deletion is an underestimate of the true number of tumors affected by altered PTEN function (113). PTEN is involved in additional, disparate functions in the cell, including drug resistance, protein stability and metabolism. *PTEN* loss was shown to increase the transporter protein ABCG2, implicated in drug efflux mechanisms (199). In other cancer types, increases in ENTPD-5 (ectonucleoside triphosphate diphosphohydrolase-5) in *PTEN*-null cells lead to an increase in growth factor levels, enhanced AKT-mediated anabolism, and higher aerobic glycolysis (Warburg effect) (200). While *PTEN* loss and mutation often affect the phosphatase activity of the protein, these distinctions are not necessarily functionally equal in all respects. PTEN has been shown to localize to the nucleus to enhance the tumor-suppressor activity of the APC-CDH1 complex in a phosphatase-independent manner (201). PTEN remains a crucial brake for diverse cellular functions and an especially crucial participant in gliomagenesis.

NF1

Neurofibromin, the protein product of *NF1*, is a Ras-GTPase activating protein (Ras-GAP) and is known to inhibit Ras – mTOR signaling in astrocytes (202). In gliomas, inactivation of NF1 can occur through deletion, mutation (127), or enhanced proteasomal degradation by PKC hyperactivation (116, 203). *NF1* loss can act through Ras-mediated overactivation of mTOR leading to increases in proliferation and greater migratory abilities in primary murine astrocytes (204). Other downstream effects include the activation of Stat3 through mTORC1 and Rac1 signaling thereby resulting in transcription of cyclinD1 in *NF1*-null cells (205). In total,

RTK/RAS/PI3K activation is found in 88% of tumors, which can occur through either activating mutations or loss of tumor suppressor function (116).

C. IDH mutations

Isocitrate dehydrogenase (IDH) proteins have emerged as important new targets in glioma, particularly in grade II/III gliomas and in secondary GBM. While only 3-7% of primary GBMs show evidence of *IDH1* mutation, 60-80% of grade II and III gliomas and up to 80% of secondary GBM harbor mutant *IDH1*, with the most common mutation being an R132H substitution (206-209). As an enzyme in the tricarboxylic acid (TCA) cycle, IDH1 catalyzes the $NADP^+$-dependent reduction of isocitrate to 2-oxoglutarate (α-ketoglutarate, α-KG), resulting in production of NADPH (210). However, mutant IDH1 catalyzes α-KG to an R-enantiomer of 2-hydroxyglutarate (2-HG), the same chemical built up in D-2-hydroxyglutaric aciduria (D-2-HGA), a rare autosomal recessive organic aciduria (113). It is possible that this and similar conditions could lead to increased risk for malignancies (211, 212). Both the wild-type and mutant IDH1 proteins can catalyze this reaction, but the mutant protein does so with much greater efficiency, leading to an excess of 2-HG (213). 2-HG can impair function of α-KG-dependent proteins, affecting a wide range of cellular functions including: DNA demethylation, histone demethylation, fatty acid metabolism, hypoxic state detection, and collagen modification among others (214). A global DNA hypermethylated state is observed in mutant IDH1 gliomas, as well as IDH1/2 mutant AML samples (215, 216). In normal astrocytes expressing mutant IDH1, this hypermethylated state can be replicated, possibly due to the TET2 enzyme, an α-KG-dependent enzyme (217, 218). The persistent hypermethylated state could favor a dedifferentiated state (216), supported by additional dysfunction in histone demethylases in IDH1 mutant cells (217, 219, 220). Presence of repressive histone methylation in IDH1 mutant

cells precedes observed DNA hypermethylation, and impairs differentiation (220). Additionally, 2-HG can alter the cell's ability to regulate physiological reactions to hypoxia, possibly by stabilizing HIF-1 (221), leading to a less robust HIF-1 response to hypoxia in mutant IDH1 cells (222).

When comparing patients with varying grades of glioma who have mutant IDH1 expression to those with wild-type IDH1, those with mutant IDH1 tend to present at a significantly younger age and have notably longer survival times compared to those with wild-type expression (127, 209, 223, 224). Such mutations could be a leading event in glioma progression, as a subset of patients having only IDH1 mutations at a preliminary biopsy acquired TP53 or 1p19q loss at subsequent biopsies (113). The IDH1 status of tumors has a clear clinical relevance and efforts have been made to develop non-invasive tests specific to this mutation. Although 2-HG is easily monitored in the serum of AML patients, its presence may be less specific in glioma (225). Other studies point to the possibility of imaging the brain to monitor 2-HG using magnetic resonance spectroscopy (MRS) (226). The morphological characteristics and histological findings are often indistinguishable. However, it has become clear that IDH1 mutant and wild-type GBM undergo disparate disease progressions.

D. Molecular classification of GBM

The heterogeneous nature of GBM has been revealed transcriptional profiling efforts to discover multiple subtypes with unique expression patterns through mRNA analysis of tumor samples (227). While these tumors may share histopathological and morphological characteristics as well as WHO tumor grade, recent studies have elucidated solid evidence for subtypes of GBM (117, 118). Phillips et al. (2006) analyzed expression of a panel of genes linked to differences in survival outcomes to define three subtypes of GBM: proneural,

mesenchymal, and proliferative. Alternatively, using unsupervised hierarchical clustering analysis, Verhaak et al. (2010) classified 200 TCGA samples of GBM into 4 different subtypes: proneural, mesenchymal, classical, and neural. Each of these subtypes had at least 210 genes defining it, and three of the four subtypes showed unique molecular alterations. Amplification of *PDGFRA, CDK6, CDK4,* and *MET,* were linked to the proneural subtype, as well as *PI3KCA/PIK3R1* mutation and mutation or loss of heterozygosity of *TP53*. Importantly, *IDH1* mutations were observed frequently in the proneural group, and it contained the highest number of young patients, corresponding with other *IDH1* data showing its prevalence in grade II and III gliomas and secondary GBM. The signature alteration in the mesenchymal group was *NF1* mutation, and other changes included mutation or loss of *TP53* and *CDK2NA*. *EGFR* amplification along with *PTEN* and *CDK2NA* loss that were common in the Classical group, while the neural group so far has no defining mutations.

Since the proneural and mesenchymal subtypes were created using different methods and sample sets, and thus are considered the most compelling and definitive (228). Using both methods, expression of *DLL3* and *OLIG2* were found to be strong in the proneural subtype. Expression of *CD40* and *CHI3L1/YKL-40*, a potential target for serum protein monitoring of GBM progression (229), were robust in the mesenchymal subtype in both studies. A subset of genes that are associated with the various subtypes of GBM are represented in a 9-gene panel shown to predict patient outcome, with expression related to a mesenchymal phenotype showing poor prognosis (230). However, not all biologically relevant data can be distilled from purely genomic data. Using unsupervised clustering of proteomic analysis, three groups defined by signaling pathways were developed: EGFR-related, PDGFR-related, or signaling coordinated with *NF1* loss (231). Analysis indicated these pathways were non-overlapping, as activation of

each signaling pathway was mutually exclusive. These results demonstrated a need for protein-based data to complement genomic profiling as tumors with high PDGF expression often had low PDGF mRNA and no amplifications. Therefore, a given signaling pathway could be undervalued based solely on transcription-based approaches. Further extensions in these efforts are being undertaken to uncover networks and signaling programs that lead to a particular transcriptional state. Computational network analysis is reverse engineering the identity of critical regulatory molecules and transcription factors unique to each subtype. For example, six transcription factors were identified as being largely responsible for the transcriptional state of the mesenchymal subtype, and validation of STAT3 and C/EBPβ showed that they were each required for successful growth of orthotopic xenografts (232). Further studies using similar methods aimed to discover other important regulators of the mesenchymal state. A group of important molecules were defined including YAP, MAFB, HCLS1, and TAZ, a transcriptional co-activator. TAZ was hypermethylated in proneural subtype tumors, and found to drive the mesenchymal phenotype when overexpressed, cooperating with PDGF-B to induce malignant progression (233). Continued investigation into the control of GBM subtype selection will assist in focusing the identification of novel treatment targets for enhanced clinical therapy.

E. Progression of Glioblastoma

Invasion

The majority of GBMs treated with current standard-of-care therapy recur within centimeters of the primary tumor mass (234, 235). Even in cases of grade II diffuse gliomas, evidence can be found of cells up to 2-cm from the primary tumor mass (236). Up to 20% of patients show evidence of macroscopic invasion upon presentation. These observations can be of multifocal disease, bihemispheric invasion along white matter tracts (also known as “butterfly

pattern" glioma), and invasion along the subependymal and subarachnoid spaces (235, 237). Early studies of glioma show up to 50% of untreated patients with histological findings demonstrating bilateral hemispheric involvement (238, 239). While magnetic resonance imaging (MRI) is more sensitive than computed tomography (CT) scanning in detecting small tumors, and can identify extratumoral involvement such as peritumoral edema, it is accepted that radiographic study is sure to miss the full extent of the disease area (240, 241).

Spread through lymphatic or vascular systems is exceptionally limited, unlike other cancer indications (242). Thus, the invasive nature of glioma is imposed on surrounding brain tissue. To successfully invade, tumor cells must detach from the primary tumor mass, adhere to the extracellular matrix (ECM), degrade the ECM, and activate motility programs to instigate movement (243). The composition of the ECM makes the brain parenchyma unique compared to extra-CNS tissue sites. Glia externa limitans, which coats the cortical surface and encloses the cerebral blood vessels, is a conventional collagen-rich basement membrane (131, 244, 245). Unique to the brain parenchyma is the perineuronal network, a lattice of predominately hyaluronan sulfate proteoglycans as well as chondroitin sulfate proteoglycans, link proteins, and tenascins (245, 246). The subventricular zone (SVZ), and area of neurogenesis, is more enriched for chondroitin and heparan sulfate proteoglycans (247).

Receptors for hyaluronan, immunoglobulin superfamily member CD44 and hyaluronan-mediated motility receptor (RHAMM) are overexpressed by GBM cells (248). These proteins are repressed by p53, pointing toward a linkage between cell checkpoints and migration/invasion abilities (113, 249, 250). The actions of integrins, especially $\alpha v\beta 3$ and $\alpha v\beta 5$ heterodimers contribute to adherence to the ECM through cytoskeletal rearrangement. This proceeds through

intracellular proteins including FAK (251, 252) and Pyk2 (253). Testing of an αvβ5 inhibitor, cilengitide, together with TMZ and radiation is underway in a phase III clinical trial (254).

After adhering to the extracellular matrix, cells must clear a path through degradation of the ECM meshwork. Matrix metalloproteinases (MMPs) are heavily involved in this process. MMPs 2 and 9 have been widely studied for their involvement in GBM invasion (255). CD95/FasR, the expression of which is enhanced at the leading edge of invasion, can activate AKT1, recruiting SFK, Yes and p85, upregulating both MMP2 and MMP9 (183). Migration has been shown to be increased by LRP1 (Low density lipoprotein receptor-related protein-1), acting through ERK to upregulate both MMP2 and MMP9 (256). FoxM1B is an important transcription factor, overexpressed in GBM and shown to transform immortalized human astrocytes into invasive cells. It can assert its activity through PTEN degradation and AKT activation, leading to MMP2 upregulation (198). Among the growing list of additional molecules involved in GBM invasion (257, 258), MMP1 has been implicated through an EGFR-dependent mechanism in which its expression is increased through EGFR-induced guanylate-binding protein (GBP1) (259). Cultured cell lines usually do not replicate the invasive growth seen in GBM patients. However, tumor initiating cells grown under neurosphere conditions have demonstrated the ability to recapitulate an invasion patterns similar to the tumors from which they were derived (260).

After gaining a foothold and beginning to degrade the surrounding ECM, invading tumor cells must engage cellular programs to induce locomotion. These programs will largely involve the rearrangement of the actin cytoskeleton. CD44 is cleaved by ADAM proteases and MMP9 leading to cytoskeletal reorganization and promotes motility (261-263). Girdin, an actin binding protein known to be involved in directing neural cell migration, is regulated by AKT and is

important in the invasiveness of tumor-initiating cells (264). Myosin II is also an important protein for invasion in GBM as a predominant initiator of cellular force. Interestingly, it enables GBM cells to invade through pores less than the width of their nuclear diameter, a vital advantage in the narrow ECM of the brain parenchyma (265). It will be important to test future inhibitors of invasion in models that accurately recreate the infiltrating nature of human GBM.

Angiogenesis

Angiogenesis is a hallmark of GBM and microvascular proliferation, along with necrosis, is a major characteristic that delineates WHO grade III from grade IV tumors (119). Glioma cells need blood vessels not only to replenish oxygen, deliver nutrients, and remove waste, but also to create a vascular niche to selectively support glioma stem cells (266, 267). Several mechanisms are used to accomplish this, including: angiogenesis, vasculogenesis, and tumor cell repurposing. Angiogenesis involves using the existing vasculature to create new blood vessels (268, 269). Alternatively, bone marrow-derived endothelial progenitor cells can be recruited to form new blood vessels in the absence of pre-existing ones in a process known as vasculogenesis. The newly formed vasculature can then expand and be pruned through angiogenesis (270). Tumor cells themselves can be recruited directly into the vascular wall, or may directly differentiate into vascular endothelium (271, 272). It is well known that the intratumoral vasculature formed through these processes is incomplete, highly aberrant, and tortuous, which leads to sections of acidosis, hypoxia, and the development of peritumoral edema (273).

The constant balance between pro- and anti-angiogenic signaling molecules determines the outcome of blood vessel formation, and these molecules can be highly dysregulated in the tumor microenvironment (243, 274). Vascular endothelial growth factor (VEGF)-related signaling is thought to be a crucial proangiogenic factor in tumor angiogenesis, and can be

induced by hypoxia inducible factor-1α (HIF-1α). VEGF largely acts through the VEGFR-2/KDR pathway, stimulating signaling in endothelial cells to promote proliferation, survival, migration, and permeability (275). Numerous other signaling factors have been implicated in stimulating angiogenesis in GBM including: PDGF, FGF, angiopoetin signaling (ANG/TIE system), Notch signaling, Integrins, Ephrins, and SDF-1/CXCL12 (276-278). Angiogenesis can be inhibited by endogenous factors such as: angiostatin, interferons, thrombospondins, endostatin, and tumstatin (279). Blood vessel formation is favored when pro-angiogenic signaling outweighs anti-angiogenic signaling.

Pharmaceutical angiogenesis inhibitors have been shown to be successful in treating GBM in patients, with Bevacizumab, an anti-VEGF antibody, undergoing accelerated approval for use in GBM (280). Most angiogenesis inhibitors have similar approaches, targeting the VEGF ligand (281), the VEGFR-2/KDR receptor (282), or the downstream signaling molecules. Bevacizumab has been the most successful inhibitory antibody used thus far and is widely used in patients. Two initial phase II trials combining bevacizumab with irinotecan, a topoisomerase I inhibitor, showed remarkable increases of the 6-month, progression-free survival percentage from 9-15% to 38-46%, median survival gains from 22-26 weeks increased to 40-42 weeks, as well as a 60% radiographic response rate (281, 283). Two subsequent studies showed more modest, but still significant treatment improvements (284, 285), with a phase III study in progress. However, inhibition of angiogenesis is known to decrease the peritumoral edema caused by permeable tumor vasculature, dramatically altering the appearance on MRI (286, 287). Moreover, tumors that recur after anti-angiogenic therapy tend to progress rapidly and rarely respond to additional chemotherapy (288, 289). We need to continue to understand these phenomena, as there is growing evidence that angiogenic inhibitors instigate invasion (290-292).

The use of relevant animal models will aid in understanding the complex interplay between treatment approaches.

IV. Introduction to Animal Modeling in Glioma

Animal modeling is vital to uncovering important mechanisms in any cancer setting, and glioma is no exception. To be useful in the modeling of human cancer, a particular model must strike a balance of faithfulness in recapitulating the human disease, and convenience of use to the researcher. The consummate model would display authentic histopathology observed in human disease, including the infiltration, angiogenesis, and distant spread so often seen in the human as tumors progress through similar pathological stages. Signaling pathways found to be significant in patient screens would be prominent, while the heterogeneity of human tumors, both cellular and molecular, also would be preserved. Stromal interactions should remain as faithful to human counterparts as possible, including recapitulating immune system influences. Once these stipulations are met, this ultimate model should develop tumors with high penetrance, preferably with short latencies and within a predictable window of time, all while possessing the means to reliably monitor tumor initiation and progression non-invasively. Of course, human tumors themselves do not adhere to these requirements, as wide heterogeneity and differences in progression are common. Each *in vivo* system has advantages and drawbacks whether in authenticity or convenience (Figure 2.1). Nonetheless, useful data can be generated from diverse models, and progression of new treatments through fruitful preclinical experimentation will lead to advances in treatment, especially for patients with aggressive disease that portends a grim prognosis.

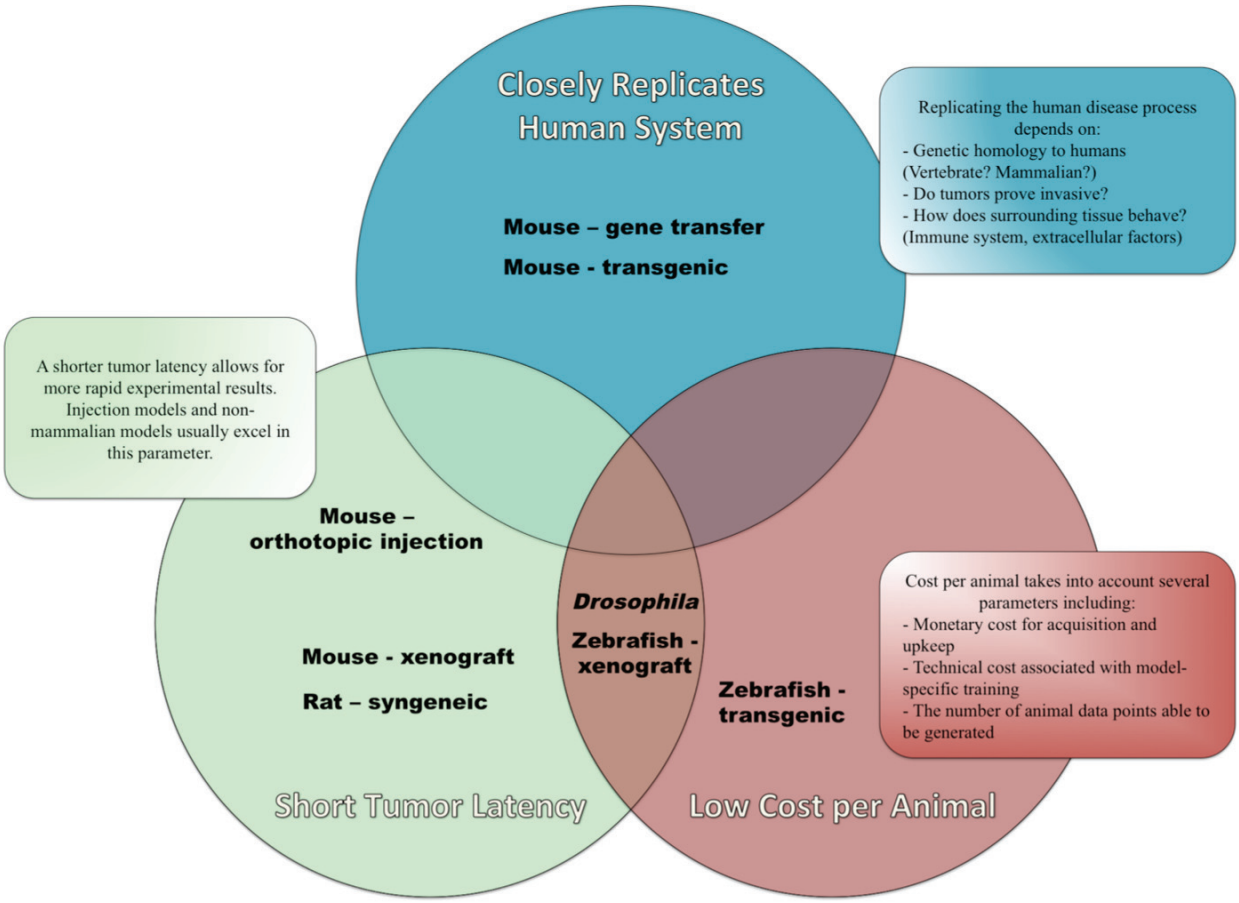


Figure 2.1 GBM modeling considerations. Three areas are represented: Human tumor recapitulation (top), tumor latency (bottom left), and cost on a per animal basis (bottom right).

While the catalogue of genetic abnormalities observed from sampling end-stage tumors has led to numerous advances in the understanding of glioma biology, modeling using an *in vivo* system allows us to scrutinize the role of specific alterations and their effects on gliomagenesis. Likewise, genetic profiling that has identified subtypes of GBM based on expression patterns has revealed intriguing differences about prognosis and resistance to standard therapies. By using animal modeling systems, we can further address which therapies will be most effective for each subtype and devise new, more targeted therapeutic strategies. The natural progression of human glioma often leads to advanced disease before symptoms arise, and due to this late presentation, there is a scarcity of information on early stage lesions. Animal modeling of glioma is built to confront these challenges in various ways. 1) Gliomagenesis: Animal modeling can identify which genetic aberrations are necessary to initiate a tumor. Additionally, models can help categorize specific activating lesions as necessary and others that may not be sufficient on their own, but are effective in accelerating the process. 2) Tumor progression: During the course of disease, tumors acquire mutations that endow advantageous properties such as enhancements in growth rate, angiogenic potential, or invasive ability. A tumor must adapt to what may be considered hostile conditions as it grows. It must evade immune surveillance, deal with inflammatory conditions, and grow despite hypoxic surroundings. Exploring the signaling pathways involved in these features gives insight into how the glioma cells survive and thrive in the brain. 3) Treatments: The ultimate goal of animal modeling is to improve and test treatments, efficiently moving effective new options into the clinical arena. Animal models must be able to select appropriate molecular targets. The knowledge acquired in uncovering essential tumor signaling pathways and aberrations that lead to an enhancement in tumor progression will yield novel therapeutic strategies that can be tested in animal models. The time to chemically develop

a new compound for testing is considerably shorter than the time it takes to verify its efficacy *in vivo*. Therefore, the animal models used must faithfully recapitulate the human disease in a manner that will permit effective testing of new compounds and the evaluation of drug toxicity. When time is a dominating factor in experimental planning, tumor latency must be carefully considered when choosing an appropriate animal model (Figure 2.2). Ideally, an animal model would help identify biomarkers that are able to demonstrate the efficacy of a tested drug. Moreover, addressing how tumors become resistant to therapy is a valuable aspect of *in vivo* disease modeling. This opens up the possibility of exploring combinations of treatments, from refining existing therapies to devising new, more effective fusions of targeted treatments. Modeling glioma *in vivo* provides the opportunity to examine the relative contributions of factors outside the neoplasm itself. One can address the significance of extracellular matrix (ECM) composition, supporting roles of normal brain tissues, and immune modulation, each of which may help or hinder glioma growth.

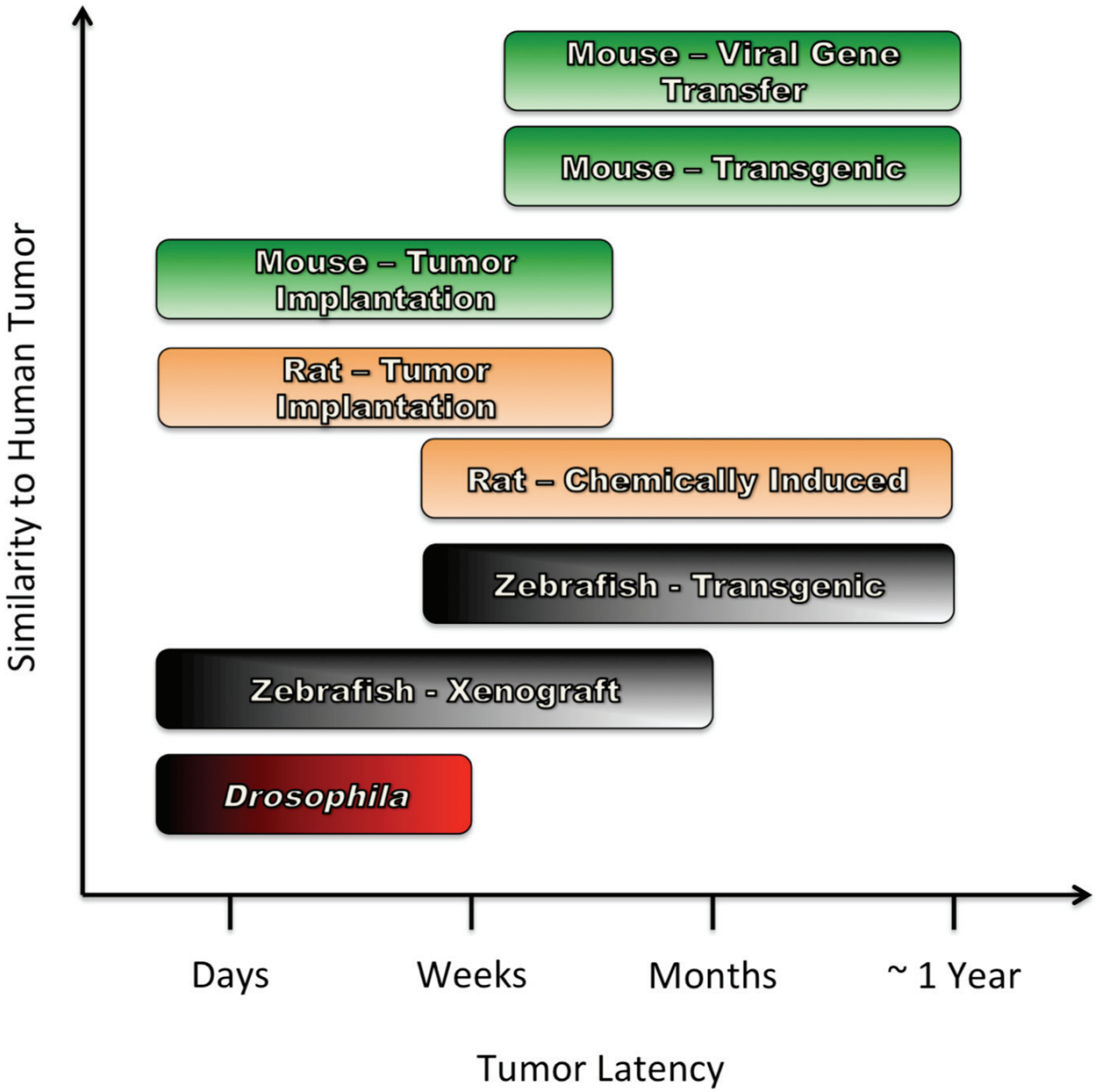


Figure 2.2 Tumor latency in models of GBM. Tumor latency can vary widely between, but also within animal models of glioma. Pictured is a representation of comparative tumor latencies for *Drosophila*, zebrafish, rat, and mouse models.

V. Non-mammalian models of glioma

A. Drosophila

While not as widely used as mammalian models, a system for the simulation of invasive glioma in the fruit fly has recently been developed. A *Drosophila* model could have significant advantages over *in vitro* experiments without the time investment of genetically engineered mouse (GEM) models. Often, cultured GBM cells lose key genetic and phenotypic features that drove their *in vivo* pathogenesis (293). EGFR amplification, and invasive growth upon reimplantation are commonly not retained after extended *in vitro* maintenance. Neural stem cell culture can propagate tumor cells in neurosphere form, thought to retain the most aggressive tumor initiating cells' characteristics, yet these can only be established from a subset of GBM tumors (294, 295). *In vitro* models are further restricted due to the lack of surrounding environmental cues normally present within the CNS stroma, including signals from reactive astrocytes, microglia, and vasculature (296). Even within the same tumor, the heterogeneity displayed *in vivo* is a crucial driver of proliferation and survival. In tumors with large populations of cells with amplified EGFR, a smaller population of cells expressing EGFRvIII secretes cytokines that promote growth and maintain the heterogeneity of the entire tumor (149). While transgenic mouse models have been indispensible in elucidating roles and signaling mechanisms of known glioma mutations, they are not as well suited for more high-throughput screening of the many mutations revealed by databases such as TCGA and REMBRANDT. The investment of time, money, and expertise needed to make such large-scale studies routine is prohibitive. Therefore, *Drosophila* systems may be able to find an important niche within GBM *in vivo* modeling.

Drosophila models have proven to be powerful tools in the study of human disease, including neurological disease and cancer (297, 298). Almost 70% of genes known to be involved in human disease have orthologs in *Drosophila* (297). Important genes with established involvement in glioma signaling are among highly conserved pathways including EGFR/RTK-Ras, PI3K, Notch, Wnt, Jak-STAT, Hedgehog, and TGF-β. Numerous components of these crucial signaling pathways were uncovered in studies of neural development and tumorigenesis in fruit flies (299). Observations made in fruit fly models on human pathogenesis have been shown to translate to mammalian models of disease (300-302). The ability of targeting individual cell types is crucial in the modeling of human disease, and is a strength of *Drosophila* systems.

Precise cell-specific alterations in gene expression have been demonstrated in fruit flies, including those in the CNS (reviewed in (303). Knockdown models are available for almost all genes with a sequenced and well-annotated genome. This allows for the exploration and identification of novel gene functions as well as the ability to delve into more complex, multigene interactions (304). Notably, *Drosophila* neural stem cells and neurons share many similarities with their vertebrate counterparts (305, 306). The remaining question is, would a fly model of glioma capture the crucial aspects of the human pathology, both genetically and phenotypically?

The loss of tumor suppressor-like genes has been shown to yield disruptions in the divisions and cell fate determination of neural progenitor cells (307). Alternatively, the study of oncogenic drivers has made recent advances. Since signaling pathways found to be important in glioma signaling are intact in the fruit fly, such as EGFR-Ras and PI3K pathways, development of a *Drosophila* glioma model has focused on these as targets for alteration. Using the repo-Gal4 glial-specific driver of transcription, orthologs of activated EGFR and PI3K (dEGFR and dp110

in *Drosophila*, respectively) were overexpressed in the brains of *Drosophila*, leading to glial neoplasia in developing larva (308, 309). This was accomplished through the concurrent expression of constitutively active variants, $dEGFR^{\lambda}$ and $dp110^{CAAX}$, at 50-100 fold above normal observations in glia (308). The neoplastic glia, first observed in post-embryonic stages, lose their normal glial morphology while becoming robustly proliferative and form multilayered aggregations in the brain, disrupting normal architecture (308). Likewise, overexpression of *Drosophila* orthologs of genes known to be important in glioma produced similar results. Overexpression of dAkt (or knockdown of dPTEN via siRNA), $dRas^{V12}$ (activated Ras), as well as orthologs for activated PDGFRα and FGFR led to neoplastic growth and patterns of inappropriate migration(308, 309). Cells from $dEGFR^{\lambda}$ and $dp110^{CAAX}$ mutant glia formed sizeable, invasive tumors when injected into the abdomen of host fruit flies, an established test of tumorigenicity. Notably, these tumors were adept at stimulating new trachea or commandeering existing trachea, or oxygen delivery tubules in *Drosophila* (308).

One advantage of fruit fly models is the ability to engage in forward genetic screens, uncovering the identity of other genes that suppress or enhance disease phenotypes when mutated or inhibited. Fruit fly models have proved successful in these screens and have identified novel genes and gene functions within cell processes and signaling pathways (301, 310, 311). This valuable approach to *in vivo* screening, utilized in "enhancer-suppressor screens," have uncovered core components and cell-type specific regulators of signaling pathways (310, 312, 313). Signaling transduction pathways in fruit flies, especially the RTK pathways, are highly conserved to the degree that mouse and human components can functionally replace *Drosophila* homologues (304, 314). One recent study demonstrating the advantages of such an approach used RNAi to interrogate almost the entire *Drosophila* kinome, the kinases within the

genome (315). 223 of the 243 kinases in the fruit fly genome were knocked down and 45 were found to modify the development of neoplasia in the $dEGFR^{\lambda}$; $dp110^{CAAX}$ model of glioma. Knockdown targeting dRIOK1 and dRIOK2, orthologs of mammalian RIO (right open reading frame) atypical kinases, resulted in especially robust inhibition of tumorigenesis. This was in turn validated via database and human cell line experimentation, showing that RIOK1 and RIOK2 were highly expressed in GBM and upregulated in cell lines expressing EGFRvIII compared to wtEGFR. Further, mouse *Pten*$^{-/-}$*;Ink4a/arf*$^{-/-}$ astrocytes overexpressing RIOK2 formed invasive, high-grade gliomas in 70% of intracranial injections, while control *Pten*$^{-/-}$*;Ink4a/arf*$^{-/-}$ astrocytes formed none. Knockdown of RIOK1 and RIOK2 led to apoptosis and chemosensitization in the presence of wild-type p53 (315). Such a study exemplifies the strength of an *in vivo* system capable of undergoing a large genetic screen, and still result in relevant output.

Overall, *Drosophila* modeling is a robust system with a high level of conserved cellular signaling in the nervous system. It has already been used to test known and potential treatments for GBM (309) as compounds can be directly fed to the fruit flies. Live imaging is possible in rapidly developing tumors and successful drug and drug targets can be verified in corresponding mammalian systems. The complexity and heterogeneity of GBM signaling is becoming apparent as interactions and crosstalk between pathways are being discovered. Therefore, the ability for this model to engage in larger scale genetic screens is a major advantage in decoding crucial targets for future therapies.

B. Zebrafish

Zebrafish (*Danio rerio*) systems have emerged as viable hosts for modeling human cancer pathology in recent years. In addition to the added physiologic and genetic homology to

mammals as a vertebrate model, a number of advantages are inherent in using zebrafish. Genetic manipulation is easily accomplished and animal maintenance is relatively inexpensive. Additionally, zebrafish have large numbers of offspring, embryos undergo rapid development, and their translucent nature readily allows for structure visualization (316). Studies of human tumor cells injected into zebrafish embryos have shown that these cells can survive and interact with zebrafish tissues, highlighting a potential for new modeling approaches to glioma. Geiger and colleagues injected U251 cells into the yolk sac at 2 days post fertilization (dpf) to successfully form tumors. There are important differences in zebrafish and human tissue culture. Zebrafish embryos are usually maintained at 28°C, and do not survive well at 37°C, a typical temperature for cell line maintenance (317). However, U251 cells showed no significant growth changes at temperatures above 28°C, and zebrafish fare well at temperatures of 30-35°C, so xenograft maintenance in this range is acceptable (316, 317).

The transgenic zebrafish model *fli1:EGFP* is frequently used to visualize zebrafish blood vessels and has been combined with xenograft approaches to study several aspects of glioma in recent years. Temozolomide and radiation therapy were used in combination showing that TMZ has a radiosensitizing effect on xenograft tumors in zebrafish, similar to what has been shown in mammals. Moreover, TMZ treatment alone showed no developmental effects to the embryo (316). Tumor angiogenic and invasive studies were also undertaken using the same *fli1:EGFP* model. The angiogenic potential of U87 tumors was evaluated after injecting cells into the yolk sacs of zebrafish embryos and measuring vessel formation and length in the ventro-lateral aspect of the yolk sac. Inhibiting JNK through pharmacological means decreased the observed angiogenic vessel growth (317). Invasion of glioma stem cells (GSCs), as designated by CD133 expression, were also evaluated using this model. Cells isolated from tumorspheres and injected

into the yolk sac invaded via vessels away from the injection site. Injections enriched for CD133 expression proved to be more invasive, invading to the edges of the yolk sac, and had higher expression of MMP9. However, after cells migrated away from the primary injection site, only 42.3% of the cells retained CD133 expression compared to 74% at the point of injection (318).

Transgenic models of zebrafish are also being used to explore *NF1* mutations, important in glioma formation. Knockouts of both *NF1* orthologs, *nf1a* and *nf1b*, were found to be developmentally lethal with aberrant proliferation and differentiation of OPCs (319). However, if one intact allele remained, as in $nf1a^{+/-}; nf1b^{-/-}$, loss of tumor suppressor function cooperated with p53 mutation to form tumors with 62% penetrance at 45 weeks post fertilization (wpf). This is significantly higher than the 28% penetrance at 66 wpf observed in *p53*-null zebrafish (320). Both brain and peripheral nerve sheath tumors developed, but brain tumors developed early (31-33 wpf) and showed features of human high-grade glioma (HGG), likely grade III due to lack of necrosis or vascular proliferation (319). Another recent study demonstrated that when a dominant active form of Akt1, DAAkt1, is overexpressed in zebrafish, gliomas of grades varying from I-IV develop in over 1/3 of fish by 6 months and almost 50% by 9 months. When an active form of Rac1, DARac1, is co-expressed in this model, the penetrance increases to 62% and 73%, with enhanced invasion, higher grade of tumors observed, and shorter survival times (321).

VI. Mammalian models of glioma

A. Rat

Until relatively recently, rat models were used more widely than mouse models for the study of glioma (322). Xenograft transplantation of tumor cells into immunocompromised rats has been used extensively (323), but the popularity of the syngeneic orthotopic approach is

unique to rat modeling of glioma. Development began in the 1970s using carcinogenic exposure to MNU (N-methylnitrosourea) or ENU (N-ethyl-N-nitrosourea), which led to the production of reproducible tumors. These tumors could then be transplanted into syngeneic hosts through stereotactic injection (324). Advantages of using rats include the size of the brain, roughly three times larger than that of a mouse, which tolerates larger volumes when injecting tumor cells (325). Additionally, thicker rat skulls can tolerate screws to implant tumor cells or deliver therapeutics, something not possible in the thinner skulls of mice (326). However, most rat models are not genetically engineered, therefore specific targeting of pathways is not as prevalent as with mouse models. Likewise, exploration into tumor initiating cell types or the effect of stromal factors on tumor development is more easily achieved with the genetic pliability of mouse models (327). Nonetheless, rat models have great utility and have been used to test numerous treatment modalities and imaging techniques over the years.

The C6 model was originally produced in outbred Wistar rats over 8 months through the repeated administration of MNU and eventually characterized as a glial tumor. Features include pleomorphic cells and occasional points of invasion into the surrounding brain. Mutations were shown in *Ink4a,* while *p53* remained unaltered (328, 329). Increased expression of notable genes involved in human brain tumors were demonstrated, such as *PDGFβ, EGFR, IGF-1 and Erb3* (330, 331). Although numerous therapies have been tested using this model, including anti-angiogenic therapy, radiation, and oncolytic viral therapy (325), no syngeneic host exists in which these cells can be maintained. The tumor line was created using outbred Wistar rats, therefore, even Wistar rats develop an immune response to the cells (332).

The most prevalent rat brain tumor model has been the 9L gliosarcoma, produced through MNU exposure over 26 weeks in Fischer 344 rats (333). They have a spindle-like, sarcomatoid

appearance and grow rapidly when implanted. *p53* is mutated in this line, but *Ink4a* and *Arf* remain normal (328, 329). *EGFR* is overexpressed in these cells as is *TGFα,* one of its ligands (334). 9L tumors have been shown to contain CSCs, which can grow as neurospheres in culture and express NSC markers Nestin and Sox2 (335). Studies using this cell line have yielded insight into transporting therapeutics across the BBB (336) as well as imaging techniques that utilize MRI and PET (337). Despite the fact that 9L cells can form tumors in allogeneic Wistar rats (338), the tumors they form in Fischer rats have been shown to be significantly immunogenic (325). T9 rat glioma cells may in fact be 9L gliosarcoma cells under a different name and show strikingly similar phenotypes (339, 340). Repeated MNU injections over 6 months also led to the development of CNS-1 glioma in an inbred Lewis rat (341). When implanted, these cells formed tumors that had many features of human HGG: invasive growth in a periventricular and perivascular pattern, nuclear atypia, necrotic foci, and some evidence of pseudopalisading arrangements, but not to the level observed in human samples (323). These features, coupled with the observation of infiltrating macrophages and T cells led to this model being useful for studying glioma invasion as well as tumor-stroma interactions (342-344).

RG2 and F98 glioma cells were produced concurrently in the same lab and originate from progeny of Fischer 344 rats treated with ENU on gestation day 20. They have a spindle-like appearance with fusiform nuclei present and are highly invasive *in vivo*, which make them more authentic representatives of human GBM (345). Both lines overexpress *PDGFβ, Ras,* and *EGFR* and RG2 has demonstrated a loss in the *Ink4a* locus (329, 334). Importantly, both RG2 and F98 tumors are weakly immunogenic (345, 346), making them useful models to study pathways relevant to glioma resistance to immunotherapy. The BT4C glioma was also developed through administration of ENU to a pregnant rat, in this case a Lewis inbred line, and subsequently

isolated *in vitro* after 200 days in culture (347). A somewhat heterogeneous population of cells resulted with some appearing flattened, others showing glial-like multipolarity, and sporadic giant cells (348). Tumors demonstrate signs of dilated, nonuniform blood vessels, irregular nuclei and areas of high proliferation and dense cellularity (349). The periphery of the tumor shows marked increases in VEGF, tPA, uPA, and microvessel density (325). Numerous studies have employed this tumor model, including investigations into combining VEGF inhibition with temozolomide and radiation (350).

While previously discussed models have been created through the use of carcinogen exposure, the RT-2 glioma line was established through the intracranial injection of Rous sarcoma virus in neonatal Fischer rats (351). These tumors appear to evoke a $CD8^+$ T-cell-mediated immune response, likely from virally encoded antigens (352). These cells have been used in wide-ranging studies, including cytotoxic gene therapy and its effect on radiosensitization (353, 354) as well as quantification of invasion into the surrounding brain through the use of GFP-tagged cells (355).

One transgenic model in rats was developed expressing a viral form of EGFR (v-erbB) under the control of the S100β promoter (356). Over 60% were found to develop tumors at a mean latency of 59 weeks (357). These were classified as malignant glioma, anaplastic oligodendroglioma, and low-grade oligodendroglioma, though there were reports of occasional lung metastases (356, 357). Most recently, this model was used to characterize the tumor-associated macrophages (TAMs) that infiltrate gliomas (358). Though the long latency and variability in tumor grade may not be ideal, future studies could be aided by adding transgenic rat models to glioma research, particularly future imaging investigations.

B. Mouse

Mouse models of human cancers remain the most widely used *in vivo* model for the study of tumor pathogenesis. The mouse genome has a high level of similarity to humans and is well characterized. A strong foundation of transgenic animals has been built and thoroughly described to establish a wide availability of tumor models of all kinds. Glioma modeling using murine systems varies widely in sophistication, speed, and authenticity of human tumor recreation. Each has unique advantages and disadvantages.

Tumor implantation techniques

The simplest approach to growing tumors *in vivo* is a subcutaneous xenograft in immunocompromised mice. These are the least challenging technically and this can result in quick tumors that can be monitored non-invasively through a caliper method. Additionally, one can inject tumors into both flanks on the same mouse to test for *'bystander'* effects of treatment (359, 360). Other advantages include the ease of using a variety of cell lines. Well-described, commercially available established lines are often used in subcutaneous xenografts, but cells isolated from patient samples are also used in injection techniques (152, 361). Drawbacks include the obvious: a CNS tumor growing under the skin will not encounter the same microenvironment as it would within the brain. Furthermore, a lack of blood brain barrier (BBB) precludes reliable analysis of therapeutics as the pharmaceutical kinetics are altered. Consequently, orthotopic implantation of tumor cells is a superior model for CNS tumors.

While technically more challenging, direct injection of tumor cells into the brain of immunocompromised mice leads to fast-forming tumors within a predictable timeframe and narrow window (362). This method carries all the advantages of using genetically altered cultured cells to test signaling pathways *in vivo*, whether using established cell lines, or primary

tumor cells at low passage (363). Disadvantages of this model include failure of some cells that grow robustly in culture to engraft tumors *in vivo* (364, 365). Cells that are successful in growing tumors can fail to replicate pathology often seen in human HGG. Specifically, human cell lines can be prone to forming tumors that are well circumscribed, compact, and have little infiltration of the surrounding brain parenchyma (362, 366). Multiple passages *in vitro* can lead to an alteration in genetic profile due to different selective pressures experienced by the cells (367). Brief culture under neurosphere conditions or harvesting the cells from a flank xenograft before intracranial injection has been shown to maintain the expression patterns of the primary tumor (153, 368-370). Cells exposed less to conditions outside of their normal *milieu* will more faithfully simulate the parental tumor in histological presentation, invasion ability, $CD133^{+}$ expression, and expression profile, thus yielding reproducible tumors (371, 372).

Syngeneic Mouse Models

An alternative to using human tumors for intracranial injection is to use mouse tumors harvested from spontaneous or induced methods. Immunocompromised mouse models do not completely recapitulate the clinical histopathology of human tumors, and remain unable to study tumor-specific immune responses (373). Therefore a syngeneic model of glioma in mice provides the control of an injection model, while the authenticity of an immunocompetent host. Binello and colleagues (2012) use the CT-2A cell line in such a model. These cells were derived in the same fashion as the C1261 cell line, harvested from induced gliomas resulting from the intracranial implant of 3- methylcholanthrene pellets into C57/B6 mice (374). CT-2A cells were collected from malignant astrocytoma formed after implantation of 20- methylcholanthrene pellets in C57/B6 mice (375). Genetically, these cells have wild-type p53, are PTEN deficient, and capture a number of features of HGG: a high cell density and mitotic index, nuclear

polymorphism, hemorrhage, pseudopalisading necrosis and microvascular proliferation (376). CT-2A cells were shown to readily form neurospheres and could be used for BTSC studies in the future(377).

Another example of syngeneic mouse modeling was utilized to investigate the inhibition of mTOR in combination with radiotherapy. The SMA (spontaneous murine astrocytoma)-560 cell line was used orthotopically to study temsirolimus (CCI-779) and radiation treatment. Since sub-lethal radiotherapy leads to increased invasiveness in glioma (378, 379), and relapsed GBM tends to evade treatment targeting EGFR and mTOR signaling, an initial treatment that combats the pro-invasive effects of radiation is needed. Weiler and colleagues showed that CCI-779 targeted RGS4, a driver of invasion in GBM, thereby inhibiting RT-induced invasion and leading to a survival benefit in mice with injected syngeneic tumors that received both CCI-779 with radiotherapy (380).

Genetically Engineered Mouse (GEM) Models

The well-described and often engineered murine genome allows for the generation of tumor models in immunocompetent mice, extensively used in glioma modeling (Table 2.1). Germline deletion of a tumor suppressor can determine its importance in glioma formation. Similarly, enhanced expression of potential oncogenes under a designated promoter can reveal roles in activating mutations for tumor formation (381). Yet, global approaches lead to simulation of tumor predisposition syndromes throughout a target organ, or significant developmental aberrations, some of which can be lethal to the animal even before a tumor forms. Some targeting is achieved by using specific promoters, however, these can depend on stage of development and may express in multiple types of cells (382).

Table 2.1 Genetically Engineered Mouse Models (GEMMs) of Glioma. Abbreviations: TG – transgenic; KO – knockout; OA – Oligoastrocytoma; ODG – Oligodendroglioma; SVZ – Subventricular Zone; HP – Hippocampus; RSWM* – Stereotactic injection to Rostral Subcortical White Matter (no promoter); TG – Transgenic; AdV – Adenovirus; RCAS – RCAS/TVA system; LV – Lentivirus; RV – Retrovirus; MADM – Mosaic Analysis with Double Markers.

Promoter	Genetics	Incidence/Latency	Morphology	Mechanism	Reference
Global	*Nf1*$^{+/-}$;*p53*$^{+/-}$	90% at 6 months	Variable grade Astrocytoma	Conventional KO	(383)
GFAP	*Nf1*$^{+/-}$;*p53*$^{+/-}$	100% at 5-10 months	Variable grade Astrocytoma	Conditional KO	(384)
GFAP	*Nf1*$^{+/-}$;*p53*$^{+/-}$;*Pten*$^{-/-}$	100% at 5-8 months	High grade Astrocytoma	Conditional KO	(385)
GFAP	*GFAP-T*$_{121}$	100% at 10-12 months	Low grade Astrocytoma	TG	(386)
GFAP	*GFAP-T*$_{121}$; *Pten*$^{-/-}$	100% at 6 months	High grade Astrocytoma	TG/Conditional KO	(387)
GFAP	*H-Ras (V*^{12}*)*	100% at 3 months	High grade Astrocytoma	TG	(388)
GFAP	*H-Ras (V*^{12}*);EGFRvIII*	100% at 3 months	High grade ODG	TG; AdV	(389)
GFAP	*H-Ras (V*^{12}*); Pten*$^{-/-}$	100% at 6 weeks	High grade Astrocytoma	TG; Conventional KO	(390)
GFAP	*p53*$^{-/-}$;*Pten*$^{+/-}$	70% at 9 months	High grade Astrocytoma/GBM	Conditional KO	(391)
GFAP	*PDGFB; p53*$^{+/-}$	40-70% at 6 months	High grade ODG/GBM	TG	(392)
GFAP	*c-myc*	50% at 1.5 months	High grade Astrocytoma	TG	(393)
GFAP	*K-Ras*$^{(G12D)}$	100% at 3 months	Intermediate grade Astrocytoma	TG	(394)
GFAP	*PDGFA*$_L$	80% at 1 year	High grade OA	TG	(395)
GFAP	*K-Ras*$^{(G12D)}$*; Ink4a/Arf*$^{-/-}$	33% at 3 months	Variable grade Glioma/Sarcoma	RCAS; Conventional KO	(396)
GFAP	*K-Ras*$^{(G12D)}$*; Akt; Ink4a/Arf*$^{-/-}$	40% at 3 months	Variable grade Astrocytoma	RCAS; Conventional KO	(396)
GFAP	*Akt; H-Ras (V*^{12}*); p53*$^{+/-}$	75%(SVZ)/100%(HP) at 6 months	High grade Glioma	LV; Conventional KO	(397)
GFAP	*p53*$^{+/-}$;*Pten*$^{-/-}$	30% at 8 months	Variable grade Astrocytoma	AdV-Cre in SVZ	(398)
GFAP	*PDGFB;Ink4a/Arf*$^{-/-}$	40% at 3 months	Variable grade ODG/OA	RCAS; Conventional KO	(399)
Nestin	*EGFR; Ink4a/Arf*$^{-/-}$	40% at 2.5 months	Variable grade Glioma	RCAS; Conventional KO	(400)
Nestin	*PDGFB; Ink4a/Arf*$^{-/-}$	60-95% at 3 months	Variable grade ODG	RCAS; Conventional KO	(399, 401)
Nestin	*PDGFB; Akt*	45% at 3 months	Variable grade Astrocytoma	RCAS	(402)
Nestin	*K-Ras*$^{(G12D)}$*; Pten*$^{-/-}$	60% at 3 months	Variable grade Astrocytoma	RCAS; Conditional KO	(403)
Nestin	*K-Ras*$^{(G12D)}$*; Akt*	25% at 3 months	Variable grade Astrocytoma	RCAS	(396)

Nestin	*K-Ras$^{(G12D)}$; Akt; Ink4a/Arf$^{-/-}$*	50% at 3 months	Variable grade Astrocytoma	RCAS; Conventional KO	(396)
Nestin	*Nf1$^{+/-}$;p53$^{-/-}$;Pten$^{+/-}$*	100% at 60 weeks	High grade Astrocytoma/GBM	Conditional KO (Nestin-Cre/AdV-Cre to SVZ)	(404)
Nestin	*Nf1$^{-/-}$;p53$^{-/-}$*	100% at 60 weeks	High grade Astrocytoma/GBM	Conditional KO (Nestin-Cre/AdV-Cre to SVZ)	(404)
Nestin/ NG2/GFAP	*Nf1$^{-/-}$;p53$^{-/-}$*	100% at 5 months	High grade Astrocytoma/GBM	MADM	(405)
S100β	*v-ErbB; Ink4a/Arf$^{+/-}$*	90% at 6 months	High grade ODG	TG; Conventional KO	(406)
S100β	*v-ErbB; Ink4a/Arf$^{+/-}$*	90% at 6 months	High grade ODG	TG; Conventional KO	(407)
CNP	*PDGFB*	33% at 3 months	Low grade ODG/Glioma	RCAS	(408)
RSWM	*PDGFB; Pten$^{-/-}$*	90% at 3 months	High grade Glioma/GBM	RV-Cre Injection to WM	(409)
RSWM	*PDGFB; Pten$^{-/-}$; p53$^{-/-}$*	90% at 1.5 months	High grade Glioma/GBM	RV-Cre Injection to WM	(409)

Efforts to target expression of a mutation are often achieved through a Cre-lox system, wherein expression of Cre recombinase aids in the knockout or expression of a gene of interest. To develop a transgenic mouse using this system, loxP is inserted, flanking a strong transcriptional stop site followed by the gene of interest. When Cre is present, the STOP cassette is removed, leading to gene expression in these cells. Alternatively, a conditional knockout mouse can be created by flanking a tumor suppressor with loxP sites, leading to its loss in cells expressing Cre (382, 410). Most commonly, transgenic Cre mice express the recombinase under the control of glial fibrillary acidic protein (GFAP), observed in mature astrocytes and subventricular zone glial progenitor cells, or Nestin, expressed in the neural progenitor compartment (411). Refined control over gene expression can be executed with an inducible Cre system using fusion Cre-estrogen receptor (ER) protein. After exposure to tamoxifen, Cre-ER translocates to the nucleus and initiates recombination (412).

As with any GEM model, multiple mutations within the same cell type can be achieved with appropriate breeding strategies. Tumors developed in this fashion show a range of latencies and tumor grades depending on the genes altered in each model (413). The downside to this approach can be the long times involved in tumor formation as well as the investment in money, time, expertise, and resources to design and develop genetically engineered mice.

Approaches to mouse modeling of glioma

Loss or inhibition of key tumor suppressors

Tumor suppressors that are typically targeted in the development of mouse models of glioma include p53, PTEN, Nf1, and Rb. Targeting multiple tumor suppressors under different settings has yielded variable tumorigenesis and penetrance. The mutation of one allele of Nf1 and p53 has led to the development of a wide range in grade of astrocytomas at a rate of 92% by

6 months. The majority of these tumors have lost heterozygosity at the remaining Nf1 and p53 alleles, which reside close to each other in the mouse genome. Of note, this model may be well suited to study more slowly developing astrocytic tumors such as secondary GBM, as older mice tend to have tumors of a higher grade. Outside of the CNS, sarcomas have been observed in this model (383). This extracranial tumor incidence is reduced by utilizing a floxed *Nf1* allele on a GFAP-Cre background. p53 remains mutated, but astrocytes now have two mutations. This leads to tumors occurring with 100% penetrance, but on a somewhat longer time scale: 5-10 months (384). Mutation to PTEN leads to a decreased latency in this model as well as an enhancement in the grade of tumors formed (385). When p53 is lost in astrocytes along with one copy of PTEN, grade III astrocytomas and GBMs develop by 15-40 weeks at a rate of over 70% (391).

Additionally, an effort was made to disentangle the respective contributions of *Ink4a* and its splice variant, *Arf*. Commonly, both may be deleted in glioma models, however, when using a PDGF-driven model of glioma, it was found that *Arf* loss contributed more to tumor development and enhancement of tumor grade than *Ink4a*, especially in glial progenitor cells (414). Expression of genes that often drive other cancer models also can initiate glioma in mice. For example, when mouse astrocytes express an activated, truncated form of SV40 T Antigen, T_{121}, which inhibits both the p53 and Rb signaling pathways, low-grade astrocytomas result within 300 days (386). Once again, additional mutation to PTEN leads to shorter tumor latency, more frequent higher-grade tumors, as well as increases in cellularity and mitotic activity (387).

Expression of oncogenes

Genetic analysis of human HGG reveals a wide range of activating mutations. Therefore, mouse modeling has attempted to address the relative importance of particular mutations and the combinations that consistently yield tumors *in vivo*. Constitutively active and oncogenic v-Src

induces growth and survival signaling, promoting glioma formation when overexpressed in mice (415, 416). Since the additional loss of p53 or Rb in this model did not enhance tumor formation, it can be concluded that these pathways were also impaired by v-Src expression (417), making it a difficult model in which to differentiate mutations that are necessary or sufficient for tumorigenesis.

Commonly, HGG exhibits strong RAS activation upon examination of human tumors. The transgenic expression of activated Ras, V^{12}Ha-Ras, is a common tactic to produce glioma in mice. Tumor formation and grade follows Ras-dosage, with homozygous animals reported to develop high-grade tumors sometimes with grade IV characteristics at two weeks of age (388, 418). However, these tumors show mutations in other pathways as they develop. Consistent formation of high grade lesions requires combination with other mutations, such as combination with EGFRvIII (389), which can both decrease latency and demonstrate oligodendroglial phenotypes, or PTEN knockout (390), which can increase tumor grade while hastening formation (396, 419, 420). While EGFR mutation is commonly observed in human GBM, Ras mutation is rare, possibly also due to its activation under RTK amplified signaling. Nonetheless, it must be a consideration when choosing a Ras-activated model for preclinical testing of a targeted inhibitor in GBM (411).

EGFR amplification, overexpression, and mutation are common in GBM, and can often be present along with INK4A/ARF deletion, which impairs both p53 and Rb (400, 421, 422). Conforming to data found in humans, the combination of PI3K signaling with Rb and p53 disruption leads to enhancement of tumor formation (116, 127). A transforming variant of EGFR, v-erbB, can be targeted to a subset of astrocytes under the S100β promoter, which is expressed in astrocytes that surround blood vessels and NG2-expressing cells (423). This results in

oligodendrogliomas of low grade at 60% of mice at one year. When either INK4a/ARF or p53 is knocked out, higher-grade lesions are observed and nearly 100% of mice develop tumors in 12 months (406). Mono-allelic knockout of either tumor suppressor locus yields intermediate effects.

Somatic gene transfer by viral vector

Even when gene alterations in GEM are controlled under cell-specific promoters, tumor initiation can occur in multiple locations, as opposed to the isolated focal nature that normally occurs in humans. To produce a model that more closely recapitulates this aspect of human tumorigenesis, transfer of genes to somatic cells can be accomplished using a viral vector, which only infects a limited number of cells in a targeted region of the brain. Genes can be activating oncogenes, or Cre can be delivered to excise a tumor suppressor with loxP flanking sites. Advantages of this approach are that it more closely represents what is observed in humans, one can deliver multiple genes of interest, as well as imaging markers, by implementing more than one virus, and one can circumvent some of the difficulties of creating transgenic or knockout mouse lines. At the same time, there can be some limits on the size of genes packaged within a virus, the technical aspect of intracranial injection can be challenging, and inflammatory conditions are created by the nature of the injection technique.

Retroviruses are a useful vector in which to package relevant genes of interest. A murine retrovirus, MoMuLV was injected into the forebrain of newborn pups and carried PDGF-B (424). 40% of mice developed tumors, which had a wide range of pathological features, including GBM characteristics, but also primitive neuroectodermal tumor (PNET) features. Another approach utilizes an avian virus coupled with transgenic mice expressing the receptor for the virus expressed under specific promoter control, GFAP or Nestin. The replication-competent avian sarcoma-leukosis virus long terminal repeat with splice acceptor (RCAS), which exclusively

recognizes the tv-a receptor, is normally foreign to mammalian cells. tv-a receptor was expressed in mice under the control of GFAP (Gtv-a), Nestin (Ntv-a) or CNPase, (present in oligodendrocytes progenitors) (Ctv-a), promoters(400, 408, 425). This targets the RCAS virus containing genes of interest to specific cells in a desired location of the brain, leading to focused tumor formation. Purified virus or infected chicken fibroblasts that shed RCAS can be injected intracranially. When constitutively active variants of Akt and KRas are delivered in combination, Ntv-a mice yield astrocytic tumors at a rate of 25% by 12 weeks (426). KRas delivery alone is sufficient when PTEN is deleted (403). PDGF-B delivered by RCAS in either Ntv-a or Gtv-a leads to a dosage-dependent formation of tumors with oligodendroglial or a mixed oligoastrocytic phenotype in up to 100% of mice (401). Much like other models, the loss of tumor suppressors like *INK4a/ARF* or *PTEN* yields more frequent tumors of a higher grade. Examples of this include improvement from 25% to 50% incidence with KRas and Akt delivery to mice with homozygous deletions of *INK4a/ARF* (396). Additionally, *INK4a/ARF* or *PTEN* loss decrease latency and enhance high-grade histology in RCAS-PDGF-B models (399). While the RCAS-tv-a system has shown the ability to produce tumors in adult cells, the virus only infects dividing cells and infection efficiency declines as the animal ages (427).

Lentiviral vectors can infect both dividing and non-dividing cells, and have been gaining in popularity as a somatic gene transfer vector. One model utilized lentiviral vectors to deliver activated H-RasV12 and Akt to GFAP-Cre mice (397). The oncogenes followed a cytomegalovirus immediate-early promoter (CMV)-loxP-red fluorescent protein (RFP)-loxP cassette, which, in the presence of Cre, would lead to transcription of the oncogene as well as a downstream GFP reporter. The injections were targeted to specific areas of the brain, and differential tumorigenesis was observed based on region. While lentiviral vectors with H-

RasV12 or Akt alone did not produce tumors, injecting both vectors simultaneously to the hippocampus led to 40% of mice developing high-grade tumors by 5 months. The same injections to the cortex produced no tumors, and the sub-ventricular zone (SVZ) yielded only one tumor out of 9 mice. When GFAP-Cre; $p53^{+/-}$ mice were used, H-RasV12 on its own produced tumors in 60% of mice injected in the hippocampus. The combination of H-RasV12 and Akt yielded tumors in 100% of mice injected in the hippocampus, 75% in the SVZ, and only 7% in the cortex. Tumors displayed many of the characteristics of GBM and occurred with much shorter latency in GFAP-Cre; $p53^{+/-}$ mice (397).

In another model, the lentiviruses themselves carried Cre driven by either a GFAP or CMV promoter. A combination of *INK4a/ARF*, *PTEN,* and *p53* were floxed in the mice, while mutated *K-Ras*^{V12} was under the control of a STOP-lox cassette, leading to KRAS production in the presence of Cre. Loss of *INK4a/ARF* and *K-Ras*^{V12} expression with and without concomitant *PTEN* loss led to 30-35% of mice developing tumors by 150 days when injected with a GFAP-Cre lentivirus. Additional *p53* loss raised the incidence to 80%, with over half categorized as grade IV lesions. When CMV-Cre lentiviruses were used, 100% of mice developed tumors with much shorter latencies, although less than 1/3 of them were grade IV tumors (428).

Through the use of the RCAS/Ntv-a model, TAZ altered the typically proneural phenotype observed in PDGF-B-driven tumors. TAZ is a transcriptional coactivator, shown to regulate mesenchymal differentiation *in vitro* through binding to the TEAD transcription factor (233). PDGF-B expression in $Nestin^{+}$ NPCs yielded grade II tumors and a median survival of about 11 weeks, similar to data shown in other studies (399). When TAZ or constitutively nuclear TAZ (4SA) were used alone in NPCs, no tumors developed in 90 days. In combination with PDGF-B, survival was reduced to less than 5 weeks with TAZ or 4SA expression. These

tumors were consistently grade III or IV and showed a more mesenchymal profile compared to a proneural profile of tumors from PDGF-B alone (233).

Discovery of a mutation resulting in a fusion protein present in ~3% of human GBM samples was validated through lentiviral infection in immunocompetent mice (429). The FGFR3-TACC3 fusion protein was found to be present in just 3 of 97 samples, but was shown to induce GBM formation, and more importantly, identified a potentially treatable subset of human GBM. FGFR3 is a member of the FGFR RTK family and TACC3 is a member of the TACC family, which mediates localization to the mitotic spindle and was shown to be oncogenic in several human tumors (430, 431). The expression of this fusion protein induced high rates of chromosome instability, aneuploidy, and enhanced proliferation in primary astrocytes. Injection of lentiviruses containing *FGFR3-TACC3* and sh*p53* led to high-grade glioma formation in 87.5% of mice within 240 days. Further study using orthotopic injection of *INK4a/ARF* $^{-/-}$ astrocytes expressing FGFR3-TACC3 showed that the resulting tumors could be inhibited by a FGFR inhibitor, AZD4547, prolonging survival by 28 days (429). This potential for treatment in a subset of GBM demonstrates the value of validating a rare mutation in relevant animal models.

Additionally, viral injection strategies can couple vectors with bioluminescent tracking. This method can provide insight on cooperative mutations and information on cells of origin for glioma. Drawbacks in injecting somatic genes include the inflammatory response to the trauma of injection, with subsequent immune cell recruitment possibly affecting results.

Investigating Glioma Pathology

Stromal Interactions

The numerous genetically modified mice available and the relative ease with which the mouse genome can be engineered leads to a multitude of options in exploring the interactions

between glioma and stromal cells. Specifically, progression can be shaped by the makeup of ECM proteins, cytokines, and other stromal factors encountered by neoplastic cells (432, 433). By selecting genetic backgrounds that alter the paracrine microenvironment, interactions involving a tumor and its surroundings can be investigated. While bi-allelic deletion of *Nf1* in astrocytes did not produce glioma in the presence of normal stroma (434), mice with an *Nf* +/- genotype produce optic pathway gliomas when the second *Nf1* gene is knocked out specifically in astrocytes (435). Additionally, by using CD38 null mice, Levy and colleagues demonstrated that CD38 was important in the tumor-supporting function of infiltrating microglia and macrophages (436). PDGF and its receptor PDGFRα, altered in GBM, have been shown to be involved in stromal autocrine or paracrine signaling promoting tumorigenesis (437), as well as in the recruitment of distant cells affecting the progression of high-grade tumors (162, 163). Notably, aberrant PDGF signaling introduced in neural progenitors can lead to development of oligodendrogliomas (399, 438) and astrocytomas of the proneural subtype (392, 409, 427, 439, 440).

Xenograft modeling has recently been utilized to understand why oncolytic virotherapy has been underwhelming in clinical trials. Conditionally replicative oncolytic herpes simplex virus (oHSV), has been shown to effectively lyse tumor cells in preclinical studies (441), but seems to fall short in early phase clinical trials (442). Using orthotopic models of GBM in SCID-γc^{null} mice, it was determined that natural killer (NK) cells impede on the efficacy of oHSV therapy through rapid viral clearance via cell-mediated killing and macrophage activation. This was dependent on the NK cell receptor NKp46, as sub-lethally radiated mice transplanted with *Ncr1*$^{-/-}$ NK cells showed more tumor inhibition after oHSV therapy than those transplanted with WT NK cells (443).

Cell of origin

Compelling evidence suggests that HGGs do not arise from only one cell type. The neural stem cell (NSC) has the ability to self-renew, proliferating through adulthood, and retaining multipotentiality. Due to its continued proliferation in adults, it is more susceptible to oncogenic mutations that could lead to glioma formation. The SVZ harbors numerous types of progenitor cells, and this heterogeneity could help to explain the heterogeneous nature of human glioma (444). Nonetheless, gliomas are not necessarily found adjacent to these proliferative niches within the brain. Often, they are found in the hemispheres, suggesting that transformed cells have enhanced migratory abilities on top of an already mobile phenotype (385, 404). In the context of mouse models, however, mutations introduced in areas of neural progenitors (e.g. the hippocampus and SVZ) are more efficient at transforming cells than those introduced to the cortex (397, 404, 427). Additionally, oligodendrocyte progenitor cells (OPCs) can generate gliomas in mice as evidenced by PDGFB expression in OPCs driving oligodendroglioma formation in mice (407, 408). A viral oncogenic form of EGFR, v-erbB, can also drive oligodendrogliomas with expression signatures similar to OPCs rather than NSCs. This particular model shows authentic oligodendroglioma formation along white matter tracts, much like in humans (407). OPCs were suggested to be the cell of origin in a model that develops proneural type GBM with PDGF stimulation combined with *p53* and *PTEN* deletion (409). The OPC-proneural link was further demonstrated in a study that revealed that only the OPCs derived from NSCs with mutations developed aberrant growth patterns, even when compared to the NSCs themselves, and eventually formed tumors with proneural features (405).

Glioma cells often express NSC-specific proteins such as Nestin and Sox2. However, this may be the result of defects in differentiation control rather than markers of a cell of origin, as

more differentiated cells can generate gliomas in mice (411). Substantial evidence exists that supports astrocytes as tumorigenic cells for glioma formation. In the absence of INK4a/ARF, expression of mutated EGFR transformed astrocytes and NSCs at a similar rate (145). EGFRvIII expression combined with p53 and PTEN deletion also leads to the transformation of astrocytes (445). However, in the absence of a driving mutation, but with PTEN, p53, and Rb deletion, only NSCs became tumorigenic (398). Finally, recent data shows that even mature neurons can give rise to GBM tumors in mice in addition to NSCs and astrocytes (446). These inconsistent results point toward the difficulty in ascertaining a definitive cell of origin for glioma, and even the sources for oligodendroglioma vs. astrocytoma. In light of the ability to reprogram fully differentiated cells into pluripotent stem cells, it is completely plausible that multiple cell types can lead to a spectrum of glioma tumors if given the necessary mutations.

One study in transgenic mice concurrently investigated identical mutations in different cells within the mouse brain. Oncogenic Ras ($Kras^{G12D}$) along with p53 loss ($p53^{fl/fl}$) were targeted to GFAP expressing cells, resulting in multifocal tumors arising in different parts of the brain. Depending on the origin of the tumor, the resulting genetic signature followed distinct patterns. Cells arising from the SVZ expressed a hallmark of neural stem/progenitor cells (NPSCs), while those that arose from the cortex or leptomeninges lacked this signature. Differences in resulting tumors were shown by harvesting and separating astrocytes and NPSCs from neonatal pups. Astrocytic tumors tended to be more aggressive, had strong GFAP expression, and matched a mesenchymal pattern, while NPSC-derived tumors retained their NPSC markers, were prone to differentiate, and had proneural expression patterns (447).

Intricate transgenic models can be of great use for defining cells responsible for tumor recurrence and exploring the existence of cancer stem cells in glioma. The model used by Chen

et al. (2010) had conditional deletions of *Nf1, p53*, and *Pten*, resulting in 100% penetrance of malignant gliomas likely deriving from NSCs of the SVZ (404). Building on this model, a transgene was added that expresses modified thymidine kinase (ΔTK) from herpes simplex virus (HSV) under the control of a Nestin promoter. This allows for the destruction of Nestin-expressing cells through systemic ganciclovir (GCV) administration. One to three days following TMZ treatment, most proliferating cells expressed Nestin. However, a loss of stem cell properties occurred around 7 days post-TMZ treatment. Subsequent GCV administration prolonged survival significantly compared to TMZ treatment alone, but not any longer than sole GCV treatment. This was postulated to be due to lack of a ΔTK transgene present in ventrally located tumors, separate from the dorsally growing tumors usually observed in this model (448). These findings show strong support for a quiescent CSC that avoids traditional chemotherapy, only to differentiate and self-renew, seeding recurring tumors.

Astrocytes, glial and oligodendrocyte precursors, and NSCs have been suggested to be the candidate cells for glioma origin. Recently, differentiated neurons were transduced via lentivirus to become oncogenic *in vivo* (446). Using mice expressing Cre under a Synapsin I promoter (SynI-Cre), specific to neurons, injection of a lentivirus that expresses shp53 and either H-Ras-V12 or shNF1 in the presence of Cre led to tumor formation in 20/20 mice within 6-10 weeks. A similar experiment with CamK2a-Cre mice, which targets mature neurons, led to tumors with a much longer latency of 9-12 months (446). The molecular signature of SynI-Cre tumors strongly correlated with a mesenchymal molecular subtype, and was very similar to tumors derived from GFAP-Cre mice. Alternatively, Nestin-Cre mice produced a neural subtype with these lentiviruses. These findings highlight the potential of mature cells to dedifferentiate

into a more stem-like state, and when in a supportive microenvironment, can both sustain the renewal of stem-like cells and differentiation of progeny cells (446).

Modeling Interplay between Standard and Novel Therapies

All animal models aim to advance the treatment of human disease, though some approaches skillfully combine standard treatments with novel approaches. This inclusion of conventional therapies more closely models typical clinical trial design, which evaluates the efficacy of new therapies against, or in combination with, standard of practice.

An interesting approach for advancing GBM treatment is the usage of therapeutic, engineered stem cells in combination with traditional treatments. A recent study utilized modified mouse NSCs expressing S-TRAIL, known to induce apoptosis in 50% of GBM (449, 450), in combination with surgical resection (451). U87 cells were injected into immunocompromised mice, formed tumors, and were resected 21 days after injection. A profound increase in survival was shown when therapeutic S-TRAIL NSCs encapsulated in a synthetic extracellular matrix (sECM) were applied to the resection cavity. 100% of these mice were alive 42 days post treatment, while mice with resection along with control NSCs had a median survival of 14.5 days post-treatment (451).

Along with surgical resection, radiation treatment for GBM is among standard practices. Another stem cell approach utilized the combination of radiation and umbilical cord blood derived mesenchymal stem cells (UCB-MSCs) expressing TRAIL. Radiation was shown to enhance the homing potential of stem cells by upregulating IL-8 production. Orthotopic tumor models in nude mice showed a radiation dose-dependent survival benefit of the combination of MSC-TRAIL treatment plus radiation with 80% of mice surviving more than 80 days post treatment, but only at the highest (10Gy) dose of radiation (452). This was significantly longer

than mock treated mice (median survival <30 days) and MSC treatment alone or in combination with 5Gy radiation (median survival <50 days) (452). Often, chemotherapy accompanies radiation in standard treatment of human gliomas. One such study in mice employed a model in which GBM was induced using Nestin-tva/*INK4a/ARF*$^{-/-}$*/PTEN* $^{loxp/loxp}$ mice injected with DF-1 (chicken fibroblast cells) infected with, and thus producing RCAS-PDGF-B and RCAS-Cre. The resulting model was used with a combination of chemotherapy and radiation to show gemcitabine can be a radiosensitizer in a proneural like PDGF-driven GBM subtype (453).

Another approach used a combination of viral introduction of oncogenes and syngeneic transplant to expose potential combination toxicity. The PDGF-IRES-Cre retrovirus was injected into mice with conditional deletions of *PTEN* and *Tp53* and resulted in 100% tumor penetrance and a median survival of 27 days post injection (409). The resulting tumors were used in a syngeneic model to test sunitinib, a small molecule RTK inhibitor, and radiation. Alone, sunitinib or high dose radiation provided a modest survival, and the combination of low-dose radiation with sunitinib provided a delay in tumor growth. However, combining high dose radiation with sunitinib resulted in a fatal toxicity to the animals (454).

Another intriguing new field encompasses the development of small molecules that can act as therapeutics as well as diagnostic markers. This can be especially difficult considering the challenge of the BBB in treating GBM. Using predictive computer modeling, the anti-tumor cytokine MDA-7/IL-24 was modified to add a diagnostic luciferase domain while maintaining the ability to induce its effects through IL-20Rα/β receptors (455). This modified protein, SM7L, was then delivered by mouse NSCs to an *in vivo* orthotopic GBM model using implanted U87 cells. Treatment with the mNSC-SM7L cells resulted in localized expression, specific to the

tumor tissue, and inhibition of tumor progression. When combined with S-TRAIL treatment through double-secreting mNSC-SM7L/S-TRAIL cells, the anti-tumor efficacy was further augmented.

VII. Summary

The molecular characterization of genetic alterations and creation of publically available information has accelerated our understanding of GBM within recent years. Numerous important signaling networks have been defined, advancing toward the goal of designing successful targeted therapy for this devastating disease. Animal models of glioma have been valuable tools for this progress, revealing tumor cell complexities and interactions within the CNS microenvironment that are difficult or impossible to accomplish *in vitro*. The continued focus on the most difficult aspects of GBM, invasion, angiogenesis, and therapeutic resistance, will ultimately guide future work in developing regimens with significant impact on survival and quality of life.

Chapter 3 - MDA-9/Syntenin is a Key Regulator of Glioma Pathogenesis

I. Introduction

Gliomas, named for the normal glial cell to which they bear the closest resemblance, encompass a wide histology of tumors and account for 80% of malignant primary CNS neoplasms. Over 75% of all gliomas are astrocytomas, which are composed of neoplastic astrocytes and range in grade from low-grade pilocytic astrocytoma (grade I), to high (grade IV, GBM) (456). GBM is a notoriously aggressive, proliferative, and invasive tumor that, despite recent advances in radiation and chemotherapies, has a 5-year survival rate of 5% (457). Furthermore, surgical resection to remove all of the tumor is difficult or impossible because of the vital nature of the surrounding tissue and is not curative due to the ability of single GBM cells to migrate from the primary tumor mass (257).

While all higher-grade astrocytic tumors demonstrate diffuse infiltration, GBM is particularly adept at invading the brain parenchyma (243). Rapid growth along myelinated portions leads to supratentorial bilateral extension of these tumors, notably along the fornicies towards the temporal lobes, and across the corpus callosum. In addition to tracking along myelinated structures, another avenue of invasion is observed within and along perivascular spaces(240). A detailed understanding of the involved and layered regulation of glioma invasion is only beginning to form (243). Nonetheless, crucial differences in how normal glial migration differs from glioma cell migration have yet to be uncovered (458).

To successfully invade the surrounding tissue, a glioma cell must detach from the primary mass, adhere to and degrade the ECM, and engage processes of cell motility and contractility (243). After breaking from the primary tumor, glioma cells adhere to the ECM most often through interactions utilizing integrins, transmembrane glycoprotein heterodimers. Integrins associate with an assortment of ECM proteins, including fibronectin, vitronectin, and fibrinogen, and increased expression of integrins can lead to increased cell motility in glioma cells (458). Next, invading cells use MMPs to degrade ECM, including MMP2 and MMP9, which can be regulated by the transcription factor NF-κB (458). Once a path is cleared, glioma cell locomotion relies on the function of actin, responsible for driving the leading processes of migrating cells, and the dynamics of myosin II, a major generator of contractile force (265).

MDA-9/Syntenin is an intriguing protein with involvement in a plethora of cellular functions (3, 4, 20, 459, 460). Earlier work on MDA-9/Syntenin revealed its influence as a positive regulator of the progression and metastatic potential of human melanoma through interactions with c-Src (15, 25, 57, 461). By facilitating active FAK/c-Src complexes, MDA-9/Syntenin amplifies signaling through a p38 MAPK-dependent pathway, ultimately activating NF-κB and leading to upregulation of MMP activity. In addition to melanoma, MDA-9/Syntenin was found at increased levels in multiple breast and gastric cancer cell lines and in a single uncharacterized human glioma cell line, contributing to cell migration (60, 61).

The current studies demonstrate that MDA-9/Syntenin expression is increased in multiple neuroepithelial tumors of the CNS. Focusing on glioma cell lines, we demonstrate that expression of MDA-9/Syntenin is elevated compared to controls and correlates with increasing tumor grade in patient samples. Additionally, we characterize MDA-9/Syntenin as a driver of

glioma motility and invasion, acting through a c-Src-dependent pathway leading to the activation of NF-κB.

II. Materials and Methods

Construction of plasmids, adenoviruses and stable cell lines

Small hairpin RNA for *mda*-9/*syntenin* (sh*mda-9*) has been constructed with pSilencerTM hygro expression vectors according to the manufacturer's protocol (Ambion Inc. TX). Specific hairpin siRNA oligonucleotides (sense 5′-GATCCGCGGATGGCACCAAGCATTTTCAAGAGAAATGCTTGGTGCCATCCGCTTTTTTGGAAA-3′ and antisense 5′-AGCTTTTCCAAAAAAGCGGATGGCACCAAGCATTTCTCTTGAAAATGCTTGGTGCCATCCGCG-3′) were annealed and ligated to pSilencer vector by T4 DNA ligase. The genomic sequence of *mda*-9/syntenin was amplified by PCR using genomic DNA as template and primers, sense: 5′-CTGCAAAAATGTCTCTCTATCC-3′ and anti-sense: 5′-GGTGCCGTGAATTTTAAACCTCAG-3′. The PCR product was cloned into pREP4 expression vectors from where it was digested and released with *Xho* and *Bam*H1 and subcloned into the pcDNA3.1 (+hygro) (Invitrogen, Carlsbad, CA). The DNA fragment (702-bp) containing the U6 promoter followed by oligonucleotide encoding shRNA designed for the *mda*-9/syntenin gene was isolated from *Eae*I-digested pSilencer-sh*mda-9* plasmid and then cloned into *Not*I site of pShuttle (pSh) plasmid generating pShmda-9-shuttle vector. To construct the shuttle vector pShCMV-*mda-9*, *Bam*HI and *Eco*RV DNA fragment (990-bp) containing the *mda*-9/syntenin gene was isolated from plasmid p0tg-CMV-*mda-9* 22 and cloned between *Bgl*II and *Eco*RV sites downstream of the CMV promoter in plasmid pShuttle-CMV. The shuttle plasmids were

recombined with genomic DNA of Ad.5/3.*Luc1* vector as we previously described (462) to derive plasmids pAd.5/3-sh*mda-9* or pAd.5/3-*mda-9*. The resultant plasmids were cleaved with PacI to release the recombinant adenovirus genomes and then transfected to HEK-293 cells to rescue the corresponding Ad.5/3-based vectors. The rescued viruses were upscaled using HEK-293 cells and purified by cesium chloride double ultracentrifugation using standard protocol and the titers of infectious viral particles were determined by plaque assay using HEK-293 cells as described (463).

Alternate siRNA sequences were obtained through Qiagen with the following sequences: 5'-TTGACTCTTAAGATTATGTAA-3' (si*mda-9* #3) and 5'-TGGGATGGTCTTAGAATATTT-3' (si*mda-9* #4). Ad.5/3 sh*mda-9* – resistant *mda-9* was constructed using the following primer sequences: forward 5'-GCCTGCTTTTATCTTTGAACATATTATTAAGCGAATGAAGCCTAGTATAATGAAAAG CCTAATGGACCACACCATTCCTGAG-3' and reverse: 3'-CGGACGAAAATAGAAACTTGTATAATAATTCGCTTACTTCGGATCATATTACTTTTC GGATTACCTGGTGTGGTAAGGACTC-5'.

Cell lines, cell culture, and human tissue samples

Human malignant glioma cells U87, U251, and T98G as well as grade III astrocytoma lines Sw1088 and Sw1783 were purchased from the American Type Culture Collection (Manassas, VA). Human primary GBM6 cells were described previously and were provided by Dr. C. David James (University of California, San Francisco, CA) (152). Cells were cultured in DMEM + F12 supplemented with 10% FCS in a 37°C incubator supplemented with 5% CO_2. Primary human fetal astrocytes (PHFA) were obtained from pre-term abortions as previously described with IRB approval, and h-TERT-immortalized primary human fetal astrocytes (IM-

PHFA) were produced and cultured as described (464). All cells were routinely screened for mycoplasmal contamination. Specimens of human primary brain tumors were collected from subjects who underwent surgical removal of their brain tumor at the Department of Neurosurgery, New York Presbyterian Hospital, Columbia University. All subjects were informed of the nature and requirements of the study and provided written consent to donate their tissues for research purposes. Tumor specimens of all histological types and grades of primary brain tumors were collected, snap frozen and stored in the tumor bank following the protocol approved by an institutional review board.

Preparation of whole-cell lysates and Western blotting analysis

Preparation of whole-cell lysates and Western blotting analysis was done as described (465). For densitometric evaluation, X-ray films were scanned and analyzed with ImageJ software (NIH).

Antibodies and reagents

Antibodies against IκBα, phospho-IκBα (Ser32/36), P38MAPK, phospho-P38MAPK (Thr180/Tyr182), Src, and phospho-Src (Tyr 416), were obtained from Cell Signaling (Beverly, MA). MMP-2, β-actin and α-Tubulin were obtained from Abcam (Cambridge, MA), and MDA-9/Syntenin from Abnova (Taipei, Taiwan). MDA-9/Syntenin used for IHC (HPA023840) was purchased from Sigma-Aldrich (St. Louis, MO). Fibronectin was purchased from Sigma-Aldrich (St. Louis, MO) and SB203580 was from Calbiochem (San Diego, CA). IKK inhibitor was kindly provided by Dr. Albert S. Baldwin, Jr. (UNC, Chapel Hill, NC).

Immunohistochemistry

A portion of the frozen tumor specimen was fixed in phosphate-buffered formalin and preserved as paraffin sections following standard procedure for the maintenance of histological structure. Paraffin-embedded sections were dewaxed and rehydrated through incubations in xylene and a gradient series of alcohol. Antigen retrieval was processed in 10 mM citric acid (pH 6.0) with microwave treatment for 20 min. Endogenous hydrogen peroxidase was quenched with 3% H_2O_2 for 20 min. After blocking non-specific binding sites with 5% normal sera, the sections were incubated overnight with antibody. The sections were incubated with appropriate biotinylated secondary antibody and subsequently with ABC-peroxidase (Vector Elite, Vector laboratories, Burlingame CA). Colorimetric reactions were developed by incubation in DAB substrate (0.02% DAB, 0.005% hydrogen peroxide), and counterstained with 10% Harris' hematoxylin. Hematoxylin & eosin staining was conducted following a standard protocol (15). The images were analyzed under the Olympus BX41 microscope system equipped with DP25 digital camera and software. Tissue microarray CNS2081 was purchased from US Biomax (Rockville, MD).

Upon analyzing MDA-9/Syntenin staining of normal brain and lymph node tissue (positive control), Dr. Christine Fuller scored the CNS2081 tissue microarray on a scale of 0-3. Average score for each of the 104 cores were used to calculate mean scores for each tissue group. Scores were compared to cancer adjacent tissue and normal brain tissue using Student's t-test.

Invasion and migration assays

Invasion was measured using 24-well BioCoat cell culture inserts (BD Biosciences, Bedford, MA) with an 8-μm-porosity polyethylene terephthalate membrane coated with Matrigel Basement Membrane Matrix (100 μg/cm^2). Briefly, the Matrigel was allowed to rehydrate for 2 h

at room temperature by adding warm, serum-free DMEM. The wells of the lower chamber were filled with medium containing 5% fetal bovine serum. Cells (5 x 10^4) were seeded in the upper compartment (6.25-mm membrane size) in serum-free medium. The invasion assay was done at 37°C in a 5% CO_2 humidified incubator for 18 h. At the end of the invasion assay, filters were removed, fixed, and stained with the Diff-Quick Staining kit (IMEB, San Marcos, CA). Cells remaining on the upper surface of the filters were removed by wiping with a cotton swab, and invasion was determined by counting the cells that migrated to the lower side of the filter using at least 5 fields per insert at 100x magnification. Wound healing scratch motility assays were done as described previously (15). Data from triplicate experiments were expressed as mean ± 95% confidence interval (CI).

Anchorage-independent growth assays in soft agar

Anchorage-independent growth assays were performed by seeding 5 × 10^4 cells in the CytoSelect™ Cell Transformation Assay (Cell Biolabs, San Diego, CA) in 24-well plates according to the manufacturers instructions. Colonies were counted 12 days after seeding and analyzed using ImageJ colony counter (NIH). The data from triplicate wells were expressed as mean ± 95% confidence interval.

Preparation of conditioned media (CM)

CM was harvested from an overnight culture of plated cells in serum-free DMEM + F12 media, filtered with 0.2 μM filters and further concentrated 10-fold on an Amicon Ultra centrifugal filter – 3K (Millipore, Billerica, MA).

Virus infections and reporter assays

Viral infection conditions and protocols were performed as delineated previously (15). Luciferase reporter assays were performed using 2×10^5 cells infected with either Ad.5/3-*vec* or Ad.5/3-*mda-9*. Twenty-four h post-infection, cells were transfected with a NF-κB–responsive luciferase reporter construct with LipofectAMINE 2000 as described(15, 57). Forty-eight h after transfection, cells were trypsinized and plated on fibronectin-coated surfaces (10 μg/mL) in serum-free medium for 1 h. Cell lysates were harvested and luciferase activity was measured using a Dual-Luciferase Reporter Assay system (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity was normalized by Renilla activity and data represent the average of triplicates $\pm$ S.D.

Human angiogenesis arrays and CAM Assays

Using conditioned media (CM), equal amounts of protein (500 mg) in 100 mL samples were assayed using Human Angiogenesis Antibody Arrays (R&D Biosystems, Minneapolis, MN) and processed according to the manufacturer's instructions. Chicken chorioallantoic membrane (CAM) assays were performed using 9-day-old chick embryos; sterile gauze with conditioned media was seeded on the CAM surface according to established protocols (466). One week after inoculation, the neovasculature was examined and photographed.

Xenograft studies in athymic nude mice

Subcutaneous xenografts were established in the flanks of athymic nude mice (4-5 week old female mice, from Charles River, Wilmington, MA) using 1×10^5 cells and followed for three weeks. Tumor volume was measured three times weekly with a caliper and calculated using the following formula: $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2. Post-sacrifice, the tumors were resected, weighed, and preserved for IHC staining.

Intracranial implant of GBM6 cells in nude mice

Athymic female NCr-nu/nu mice (NCI-Fredrick) weighing ~20 g were used for this study. Mice were maintained under pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U.S. Department of Agriculture, Washington, DC, the U.S. Department of Health and Human Services, Washington, DC, and the NIH, Bethesda, MD. At least 5 mice per group were utilized. Mice were anesthetized through i.p. administration of ketamine (40 mg/kg) and xylazine (3 mg/kg), and immobilized in a stereotactic frame (Stoelting, Wood Dale, IL). A 24-gauge needle attached to a Hamilton syringe was inserted into the right basal ganglia to a depth of 3.5-mm and then withdrawn 0.5-mm to make space for tumor cell accumulation. The entry point at the skull was 2-mm lateral and 1-mm dorsal to the bregma. Intracerebral injections of 1.5×10^4 cells in 2 μL per mouse were completed over 10 min using an automated injector (Stoelting, Wood Dale, IL). The skull opening was enclosed with sterile bone wax and the skin incision was closed using sterile surgical staples.

Database Mining

We surveyed two publically available databases: 1) The Cancer Genome Atlas (TCGA) that combined LGG (lower grade glioma) and GBM (glioblastoma) datasets (https://tcga-data.nci.nih.gov/tcga/), generated using Illumina HiSeq 2000 RNA Sequencing, and 2) the NCBI Gene Expression Omnibus (GEO) dataset GSE4290 (467) derived by using the Affymetrix HG U133 Plus 2 platform. The datasets were obtained through the NCBI-GEO and UCSC Cancer Genomics Browser (468) websites, respectively, and analyzed the data using the Gene-E (Broad Institute, Cambridge, MA) and JMP Pro 10 (SAS, Cary, NC) analytical/statistical programs.

Statistical analysis

The data are reported as the mean ± S.D. of the values from three independent determinations and significance analysis was performed using the Student's t test in comparison with corresponding controls. Probability values < 0.05 were considered statistically significant. Survival curves were analyzed using Cox Proportional Hazards Survival Regression (http://statpages.org/prophaz.html).

III. Results

Elevated mda-9/syntenin expression is common in neuroepithelial tumors of the CNS and correlates with astrocytoma tumor grade

Given the increased expression of MDA-9/Syntenin observed in human melanoma, we asked if similar trends were evident in primary brain tumors. To answer this question, we analyzed two publicly available glioma datasets: a) The Cancer Genome Atlas (TCGA) combining LGG (lower grade glioma) and GBM datasets and b) the NCBI Gene Expression Omnibus (GEO) dataset(467). As both datasets indicate (Figure 3.1A, B), the MDA-9/Syntenin transcription level, on average, increases as tumor grade progresses (ANOVA test, $p < 0.0001$ in both datasets). In the GSE4290 dataset, non-tumor cases were obtained from epilepsy patients. As of this writing, reference (normal) cases are not included in the TCGA LGG and GBM datasets.

Increased expression of MDA-9/Syntenin appears to be a clinically important phenomenon, as shown by analysis of TCGA patient survival segregated by expression (Figure 3.1C). Patients with gliomas expressing above median levels of MDA-9/Syntenin show a marked decrease in survival compared to those with below median MDA-9/Syntenin (Kaplan-Meir survival analysis, $p < 0.0001$ in both log-rank and Wilcoxon statistical tests). Cell lines used *in vitro* were then analyzed for MDA-9/Syntenin expression level. Barely detectable levels were observed in primary human fetal astrocytes (PHFA) and slightly higher levels were found in h-TERT immortalized PHFA cells (IM-PHFA), while robust expression was seen in GBM lines U87MG, T987G, and U251 (Figure 3.1D).

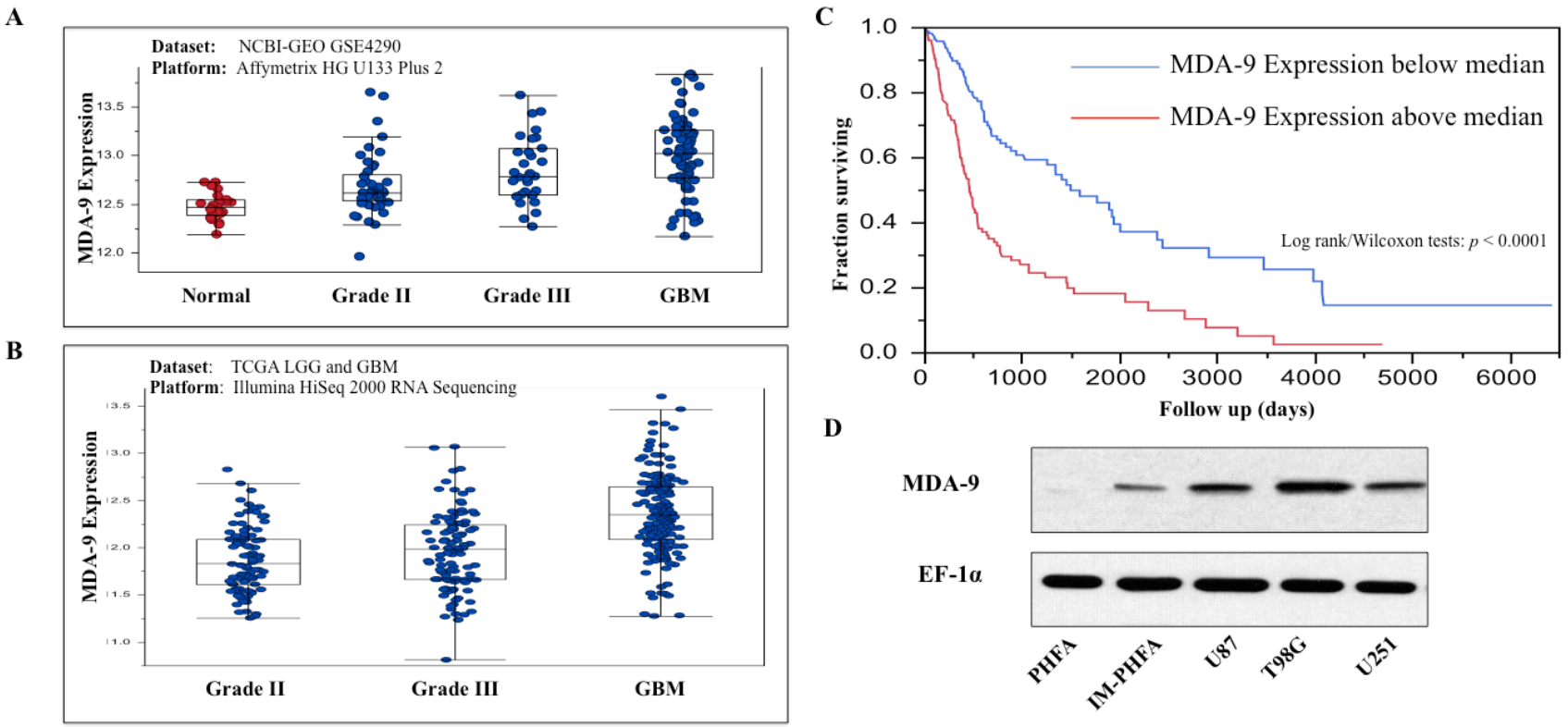


Figure 3.1 MDA-9/Syntenin is overexpressed in glioma clinical samples and cell lines. A, *mda*-9/syntenin expression in glioma by tumor grade compared to normal tissue using the NCBI-GEO GSE4290 dataset. **B**, *mda*-9/syntenin expression in varying grades of glioma tumors using the TCGA LGG and GBM dataset. **C**, Kaplan-Meier survival plot comparing survival time according to *mda*-9/*syntenin* expression. High expression – *mda*-9/*syntenin* levels above median, Low expression- *mda-9/syntenin* levels below median. **D,** MDA-9/Syntenin expression in various cell lines. PHFA – primary human fetal astrocytes, IM-PHFA – immortalized primary human fetal astrocytes.

To validate these observations from genomic datasets, we analyzed a tissue microarray consisting of 104 cores, including tumor, cancer adjacent normal, and normal tissues. In these samples, specimens are classified according to the WHO classification of brain tumors (456) and strength of MDA-9/Syntenin staining was scored (Table 3.1). We find that astrocytomas of all grades had significantly higher MDA-9/Syntenin expression than normal tissue counterparts (Figure 3.2), or normal lymph node sections, used as a positive control (Figure 3.3). Taken together, we determined that in human astrocytomas, similar to the pattern observed in human melanomas, elevated MDA-9/Syntenin was more common in high-grade tumors. Protein expression of MDA-9/Syntenin was verified by Western blotting in 46 specimens from surgically removed primary brain tumors. When compared to samples obtained from normal brain tissue, which expectedly show low but detectable levels, tumor tissue showed high expression of MDA-9/Syntenin more frequently (Figure 3.4).

Table 3.1 MDA-9/Syntenin Expression in Tumors of the CNS. Tumor sections from listed types were scored (0-3) for MDA-9/Syntenin expression. Right columns compare average tumor section scores to average scores for cancer-adjacent tissue (CAT) or normal brain tissue (NT).

	Average Score	Samples	p vs CAT	or NT
Astrocytoma Grade I	2.67 ± 0.52	6	0.007	0.077
Astrocytoma Grade II	2.82 ± 0.40	11	0.0002	0.007
Astrocytoma Grade III	3.00 ± 0.00	7	0.001	0.007
GBM	2.75 ± 0.46	8	0.001	0.028
Oligodendroglioma	3.00 ± 0.00	5	0.003	0.023
Malignant Oligodendroglioma	2.83 ± 0.29	3	0.028	0.120
Ependymoma	1.33 ± 0.58	3	0.608	0.491
Malignant Ependymoma	2.80 ± 0.45	5	0.008	0.061
Medulloblastoma	2.80 ± 0.42	10	0.0003	0.011
Cancer-adj. normal tissue (CAT)	0.94 ± 1.21	8	1.000	0.165
Normal brain tissue (NT)	1.79 ± 0.99	7	0.165	1.000

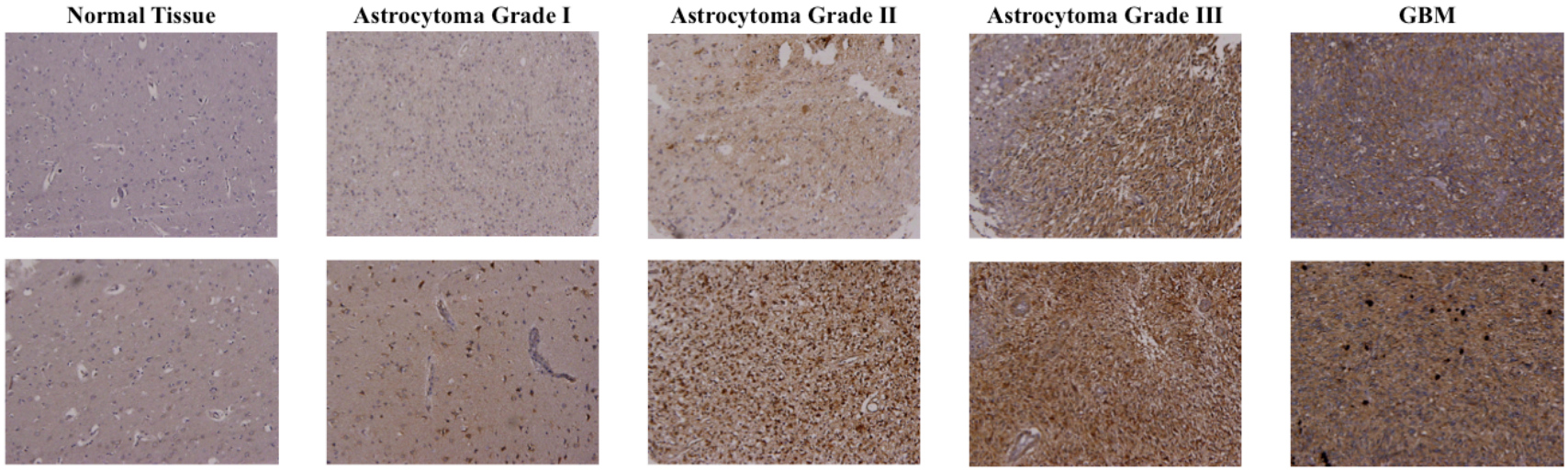


Figure 3.2 MDA-9/Syntenin expression correlates with tumor grade. Representative images of MDA-9/Syntenin expression in normal and tumor samples

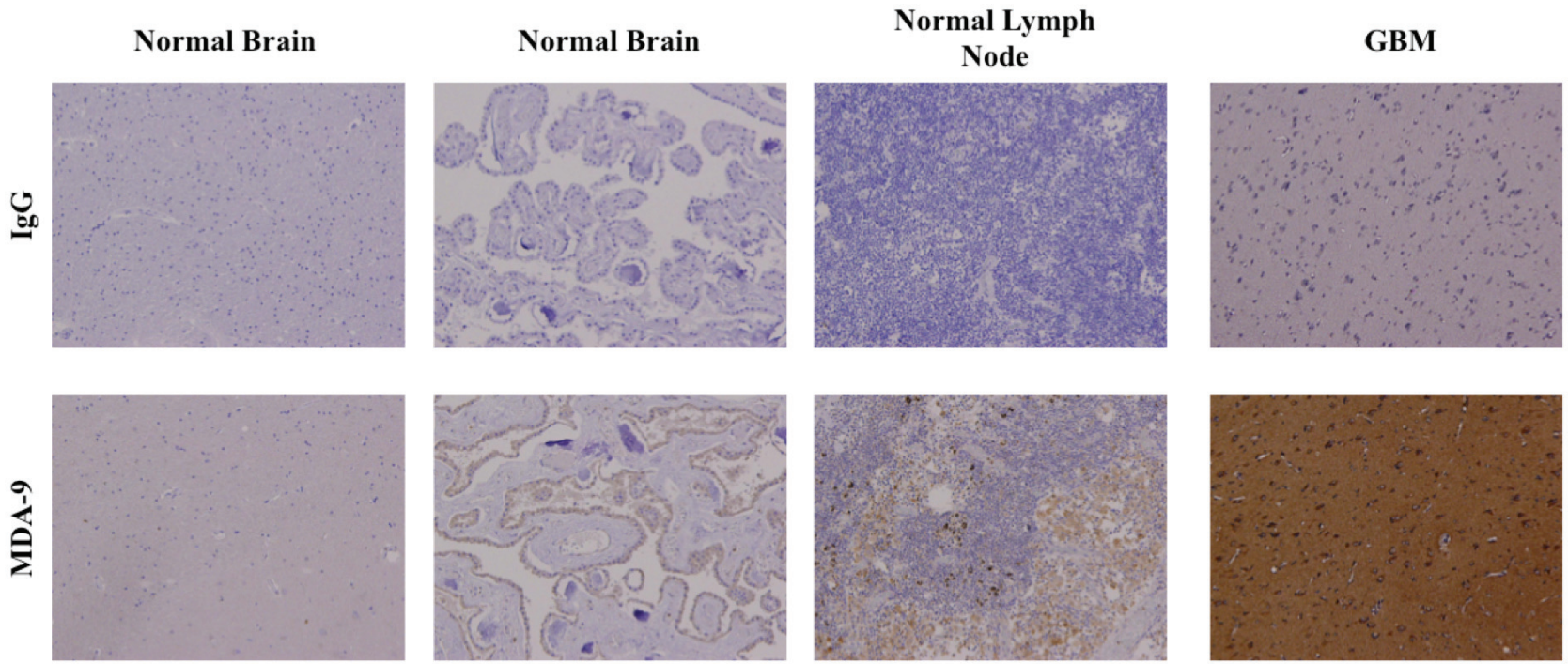


Figure 3.3 MDA-9/Syntenin antibody validation. Representative images used as antibody validation for MDA-9/Syntenin staining. Normal lymph node sections were used as positive control

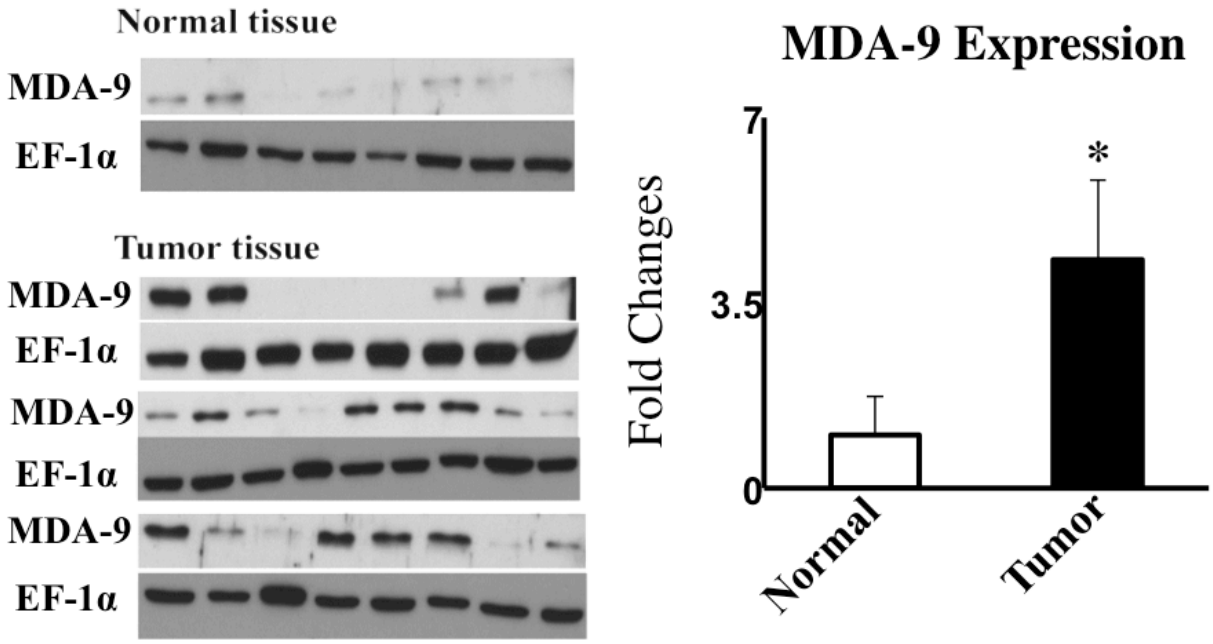


Figure 3.4 Analysis of normal and tumor tissue for MDA-9/Syntenin expression. (Left) Expression of MDA-9/Syntenin by Western blotting in both normal control brain tissue and primary tumor tissue. EF-1α is used as an internal control for protein loading. (Right) Quantification of protein expression using ImageJ.

MDA-9/Syntenin protein level correlates with changes in the invasive ability of astrocytoma cells

Next, we determined if manipulating MDA-9/Syntenin expression could lead to changes in invasion *in vitro*. Accordingly, MDA-9/Syntenin expression was reduced in GBM cell lines T98G and U87MG using an adenovirus expressing shRNA targeted to MDA-9/Syntenin. Cells treated with Ad.5/3-sh*mda-9* had approximately a 5-fold decrease in invasive ability compared to vector (Ad.5/3-con sh) treated cells (Figure 3.5A) ($p<0.001$). Conversely, MDA-9/Syntenin was overexpressed using adenovirus (Ad.5/3-*mda-9*) in three cell lines that normally express low levels: IM-PHFA, Sw1783, and GBM5. When compared to cells infected with vector alone (Ad.5/3-*vec*), cells that overexpress MDA-9/Syntenin after Ad.5/3-CMV-MDA-9 (Ad.5/3-*mda-9*) treatment invaded at significantly higher rates, with 2-3-5-fold increases (IM-PHFA, Sw1783, GBM5), respectively, in invasion (Figure 3.5B) ($p<0.001$). To account for the possibility of off-target effects, we used multiple alternative siRNA sequences for knockdown experiments, and an *mda*-9/syntenin construct designed to be resistant to Ad.5/3-sh*mda-9*. Similar results were observed when using alternative siRNA sequences. Protein levels and invasion were reduced at levels equivalent to those observed using Ad.5/3-sh*mda-9* (Figure 3.6). Additionally, a plasmid construct with sequences mutated to be resistant to the targeting of our adenoviral vector rescued invasiveness in the presence of Ad.5/3-sh*mda-9* (Figure 3.7).

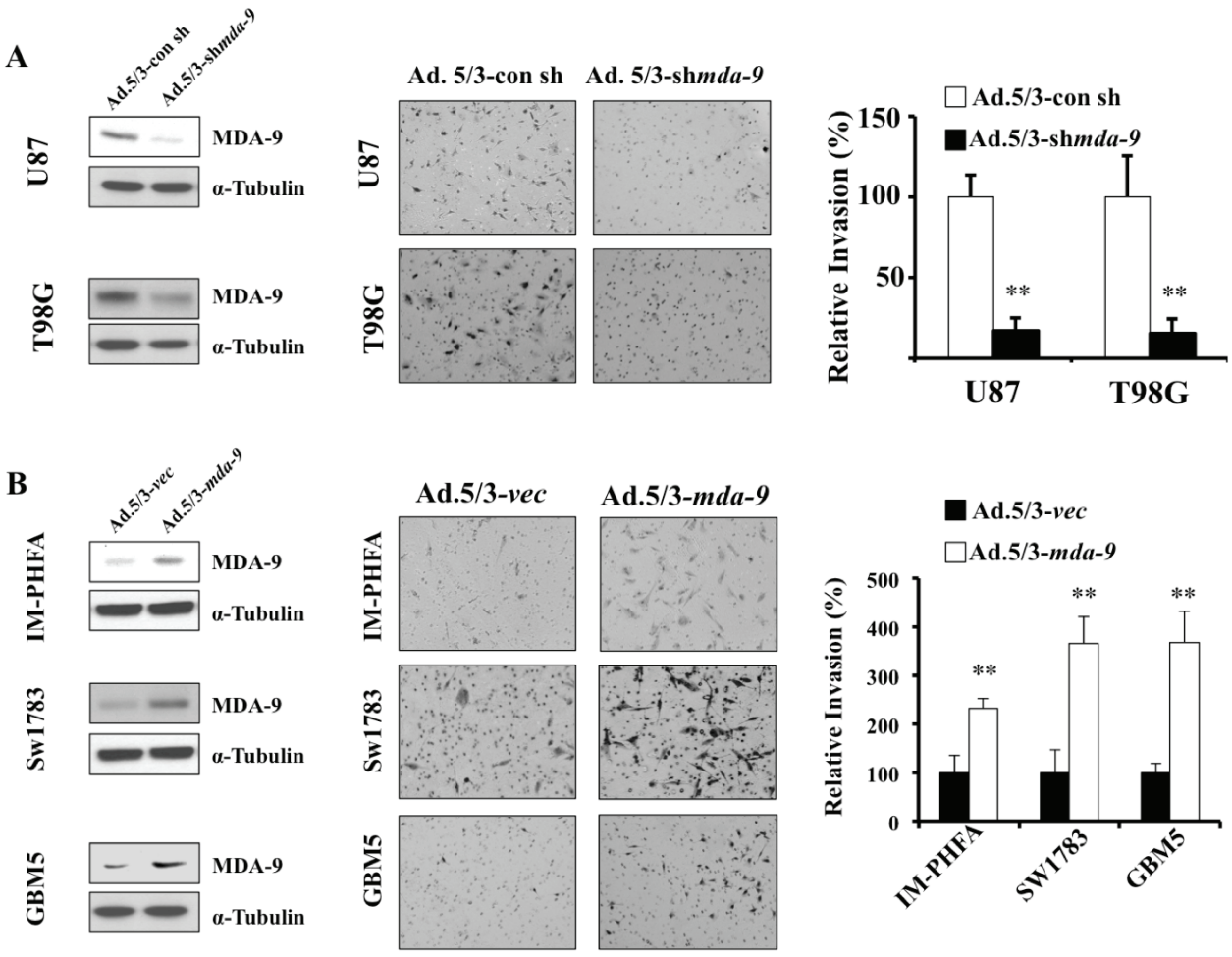


Figure 3.5 MDA-9/Syntenin regulates astrocytoma invasion. A, Knockdown of MDA-9/Syntenin leads to decreases in invasion. *(Left)* Protein expression of MDA-9/Syntenin after viral infection, 200 pfu/cell. *(Center)* Representative images of Matrigel invasion in GBM cell lines T98G and U87. Decreases in MDA-9/Syntenin expression correspond with inhibition of invasion. *(Right)* Invasion quantified using five fields per triplicate well. **B,** Overexpression of MDA-9/Syntenin leads to increases in invasion. *(Left)* Protein expression of MDA-9/Syntenin after viral infection, 100 pfu/cell. *(Center)* Representative images of Matrigel invasion in IM-PHFA, Sw1783, and GBM5 lines. Increases in MDA-9/Syntenin expression leads to enhanced invasion. *(Right)* Invasion quantified using five fields per triplicate well. **, $p < 0.01$.

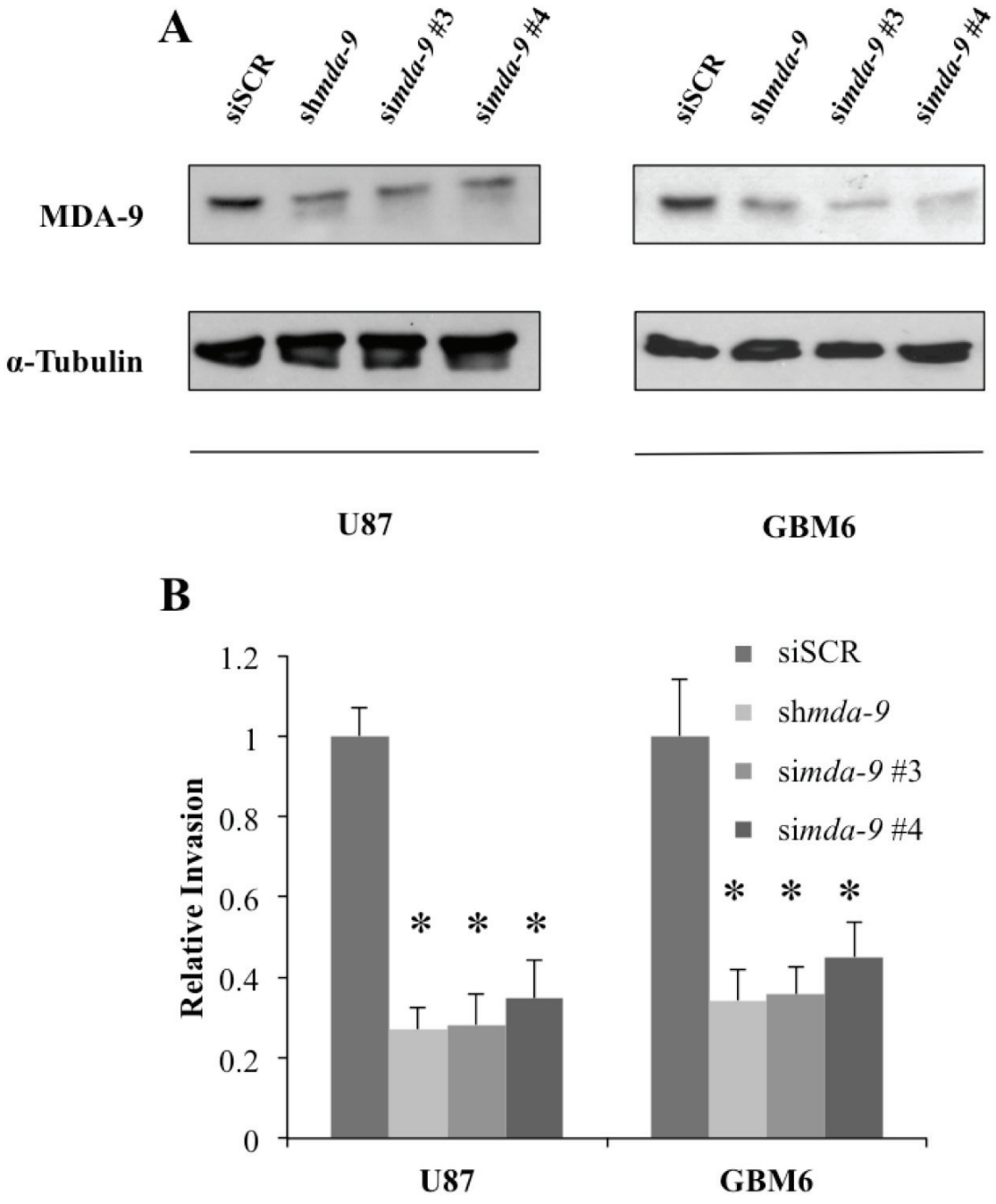


Figure 3.6 Alternate siRNA directed against *mda-9/syntenin* has equivalent effect. A, MDA-9/Syntenin expression after treatment with scrambled control siRNA (siSCR), sh*mda-9* plasmid, or siRNA directed against *mda-9*. α-tubulin is used as a protein loading control. **B,** Invasion quantified using five fields per triplicate well. *, $p < 0.01$.

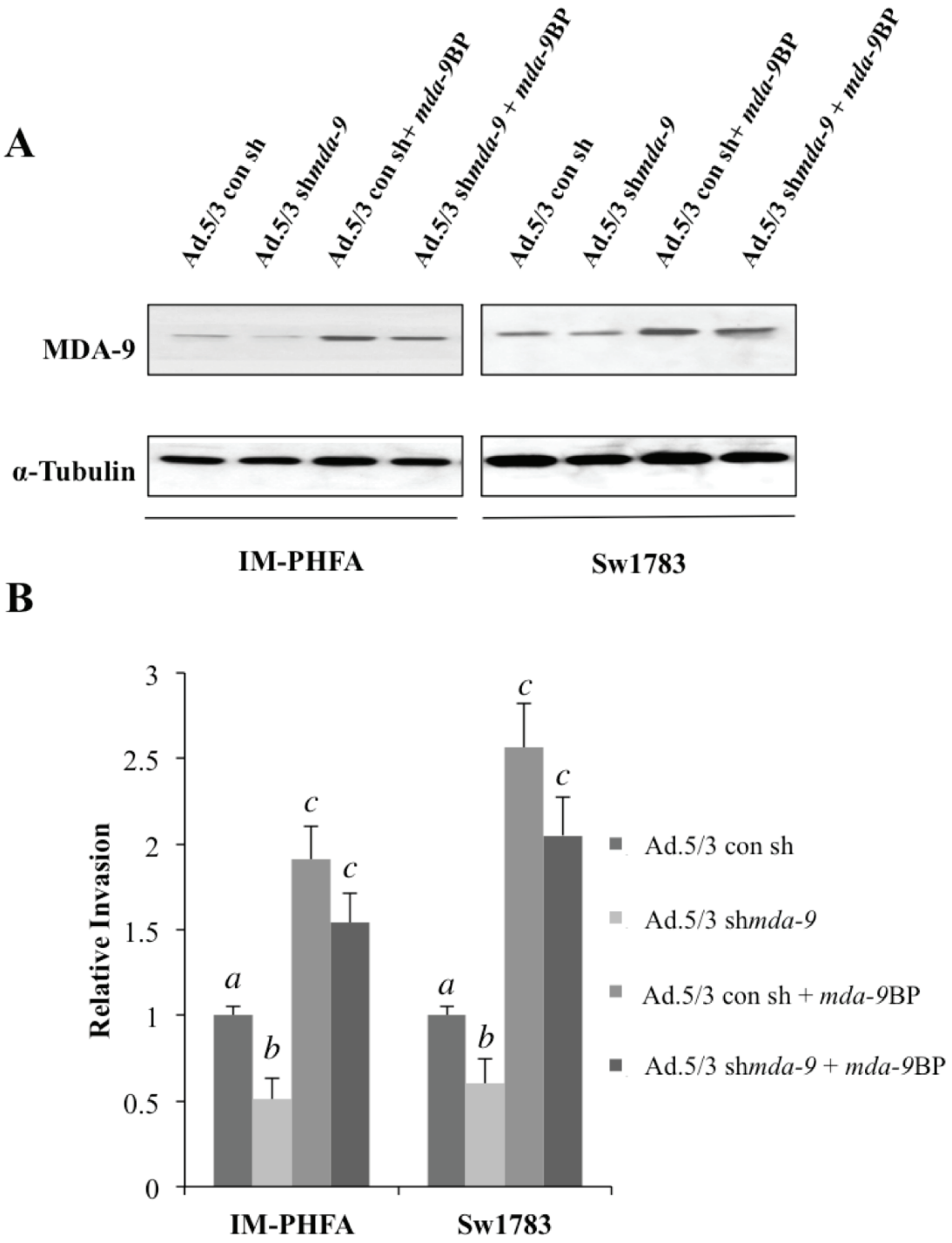


Figure 3.7 Ad.5/3 sh*mda-9* resistant *mda-9* plasmid (*mda-9*BP) abrogates invasion loss. A, MDA-9/Syntenin expression after treatment with control virus or Ad.5/3 sh*mda-9,* with or without *mda-9*BP. α-tubulin is used as a protein loading control. **B,** Invasion quantified using five fields per triplicate well. Within each group, *a* differs from *b* ($p < 0.01$) and *c* ($p < 0.01$).

MDA-9/Syntenin signaling is c-Src-dependent and activates NF-κB through the p38 MAPK pathway

To determine the mechanism by which MDA-9/Syntenin provokes changes in downstream signaling, we analyzed protein levels in astrocytoma cells after MDA-9/Syntenin manipulation (Figure 3.8A). In previous studies, c-Src was found to be an important interacting partner and effector of MDA-9/Syntenin signaling, contributing to a metastatic phenotype in human melanoma (15, 25, 57, 461). Indeed, overexpression of MDA-9/Syntenin in Sw1783 and GBM5 cells leads to increased levels of activated Src. Conversely, reducing MDA-9/Syntenin in T98G and U87MG cells lowers phosphorylated Src levels. Initiation of c-Src signaling can lead to activation of the p38 MAPK pathway and ultimately to NF-κB activation and the transcription of cellular genes involved in invasion. Phosphorylated p38 MAPK was increased upon MDA-9/Syntenin overexpression in Sw1783 and GBM5 cells. A decrease in activated p38 MAPK was also observed after knockdown of MDA-9/Syntenin. The phosphorylation of IκBα, which normally maintains NF-κB in an inactive state in the cytoplasm, targets it for degradation, leaving NF-κB free to translocate into the nucleus. When MDA-9/Syntenin is overexpressed, there is an increase in phosphorylated IκBα and a concomitant increase in MMP2, a known target of NF-κB. Likewise, infection with Ad.5/3-sh*mda*-9 reduced phosphorylated IκBα and MMP2.

To further investigate the activation of NF-κB following forced *mda*-9/syntenin expression, we transfected Sw1783 cells with a plasmid producing luciferase under the control of a promoter with NF-κB binding sites. Consequently, we expected increased luminescence in cells subsequently treated with Ad.5/3-*mda*-9 compared to those treated with Ad.5/3-*vec*. As

anticipated, examining cells with the reporter plasmid that overexpressed *mda*-9/syntenin, we observed >2.5-fold induction of NF-κB activity compared to vector-infected cells (Figure 3.8C).

As in previous studies performed in human melanoma (57), we found that MDA-9/Syntenin bound to Src in GBM cells by immunoprecipitation (Figure 3.10A). To determine if the increase in invasion correlating with MDA-9/Syntenin overexpression is dependent on c-Src, we transfected plasmids encoding wild type or dead kinase Src, which acts as a dominant negative protein, along with a control plasmid or MDA-9/Syntenin into GBM5 cells. In this experiment the enhanced invasion we observe when overexpressing MDA-9/Syntenin was mitigated when co-expressing the dominant negative c-Src protein (Figure 3.9A, Figure 3.10A).

Finally, we employed pharmacological inhibitors *in vitro* to confirm the pathway through which *mda*-9/syntenin exerts its effects. Using IM-PHFA and Sw1783 cell lines, cultures were infected with an Ad.5/3-*vec* or Ad.5/3-*mda*-9. These cells were then treated with DMSO, I-kappa kinase (IKK) inhibitor, or SB203580, a P38 MAPK inhibitor, and subsequently analyzed for invasive potential. As expected, overexpression of *mda*-9/syntenin leads to significant increases in invasion compared to vector-treated cells (Figure 3.9B, Figure 3.11). We would expect if MDA-9/Syntenin performed its invasion signaling through a primary pathway outside of the P38MAPK-NF-κB axis, pharmacological inhibition of these proteins would have minimal effect. Notably, both the IKK inhibitor and SB203580 abrogated the invasion gains in cells overexpressing *mda*-9/syntenin.

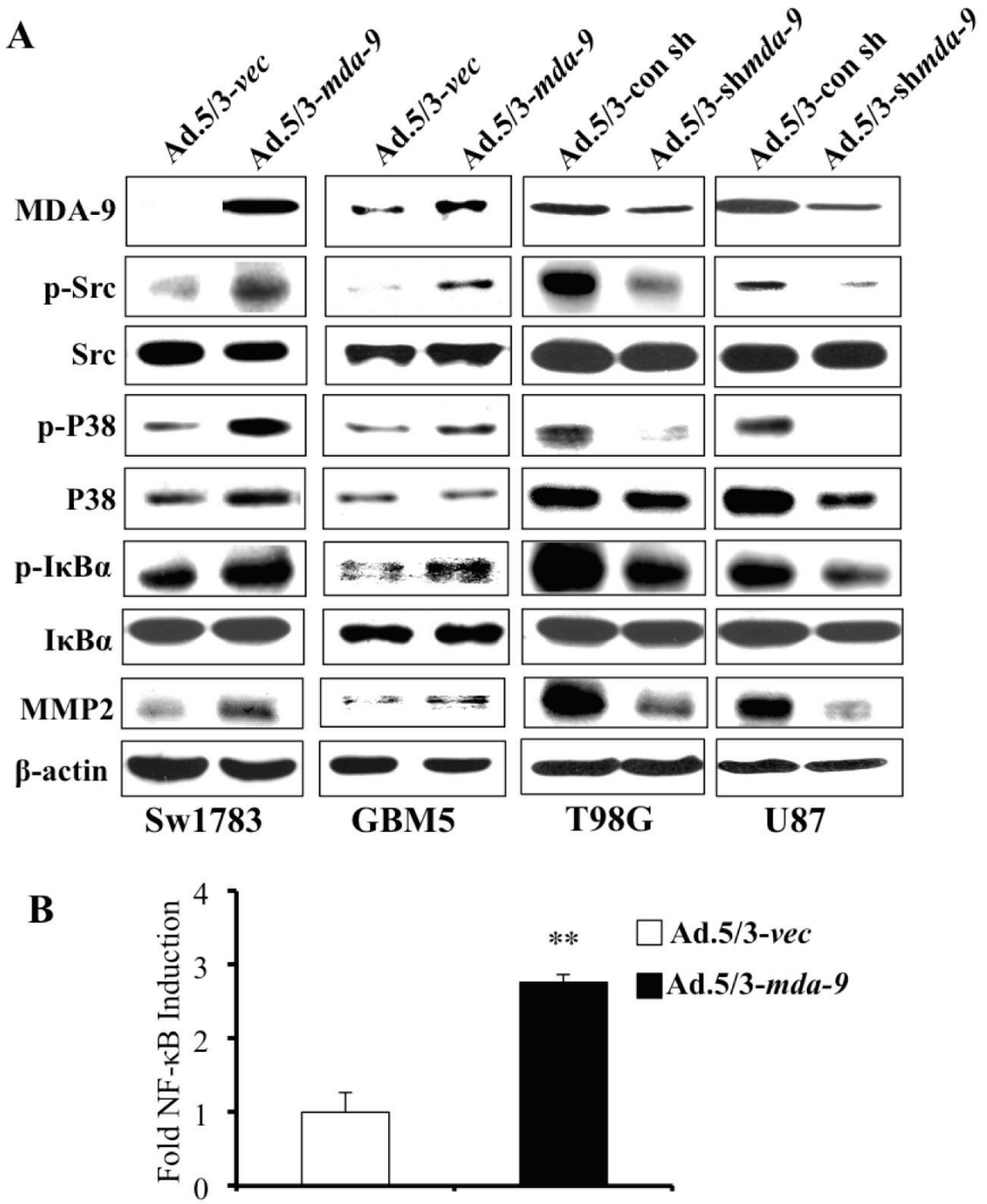


Figure 3.8 MDA-9/Syntenin activates NF-κB through a Src, P38MAPK-dependent pathway. A, Protein expression following overexpression *(left columns)* or knockdown *(right columns)* in the indicated cell lines. β-actin is used as an internal control for protein loading. **B,** Fold NF-κB induction in Sw1783 cells after MDA-9/Syntenin overexpression compared to vector control. NF-κB induction measured using 3κB-Luc transfection, a plasmid containing three tandem NF-κB binding sites upstream of the luciferase gene. **, $p < 0.01$

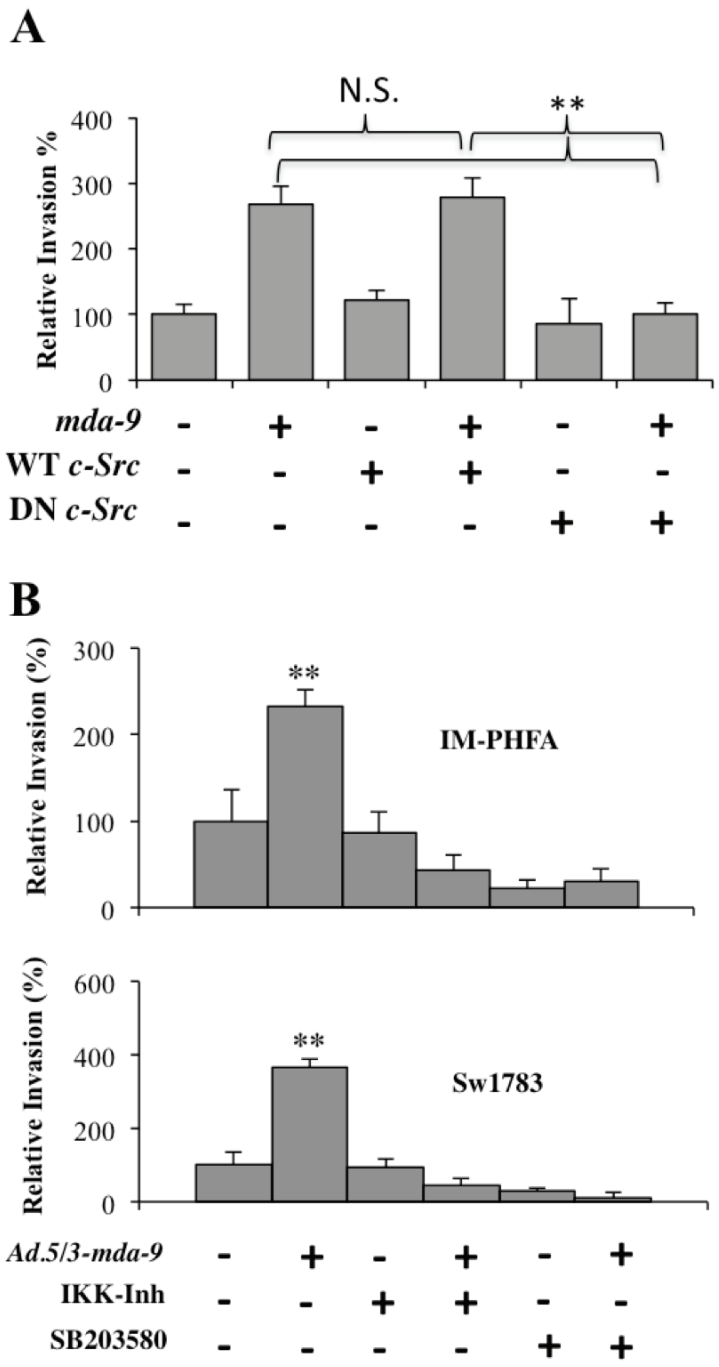


Figure 3.9 MDA-9 – induced invasion acts through Src-P38MAPK-NFκB. A, Results from Matrigel invasion in GBM5 cells following Ad.5.3-*vec* or Ad.5/3-*mda-9* infection and transfection with vector, wild-type Src (WT), or dominant negative (DN) dead kinase Src. Invasion quantified using five fields per triplicate well. *a* differs from *b* ($p < 0.01$). **B,** Results from Matrigel invasion in IM-PHFA and Sw1783 cells following Ad.5.3-*vec* or Ad.5/3-*mda-9* infection and treatment with DMSO, IKK-inhibitor, or SB203580 (P38MAPK inhibitor). Invasion quantified using five fields per triplicate well. **, $p < 0.01$.

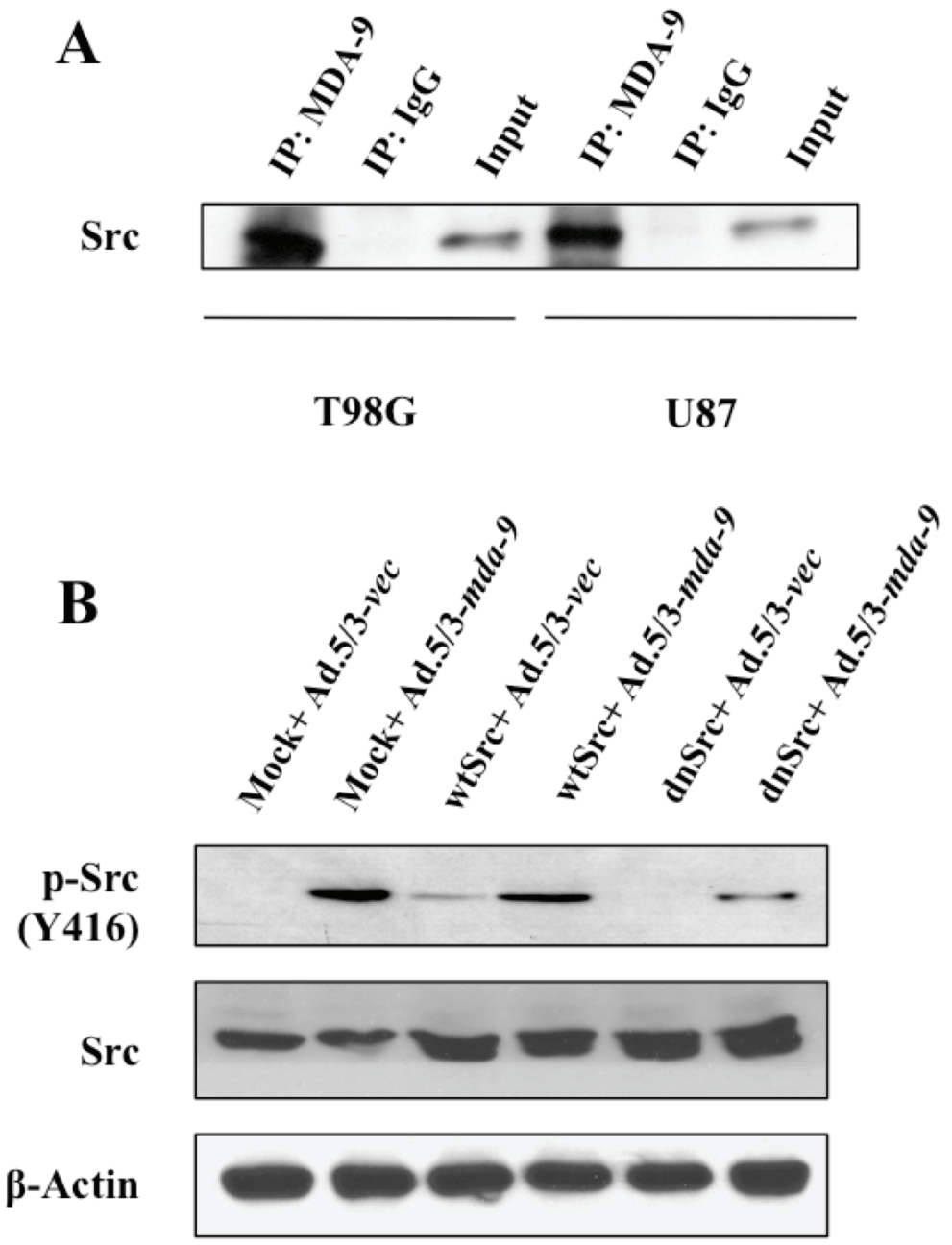


Figure 3.10 Interaction with MDA-9 enhances Src signaling. A. Src was detected after immunoprecipitation of MDA-9/Syntenin using T98G and U87 cells. Lane 3 of each represents 10% input. **B,** activated and total Src levels following Ad.5/3-*mda*-9 treatment with vector, wild type Src (wtSrc), or dominant negative Src (dnSrc). MDA-9/Syntenin interacts with Src.

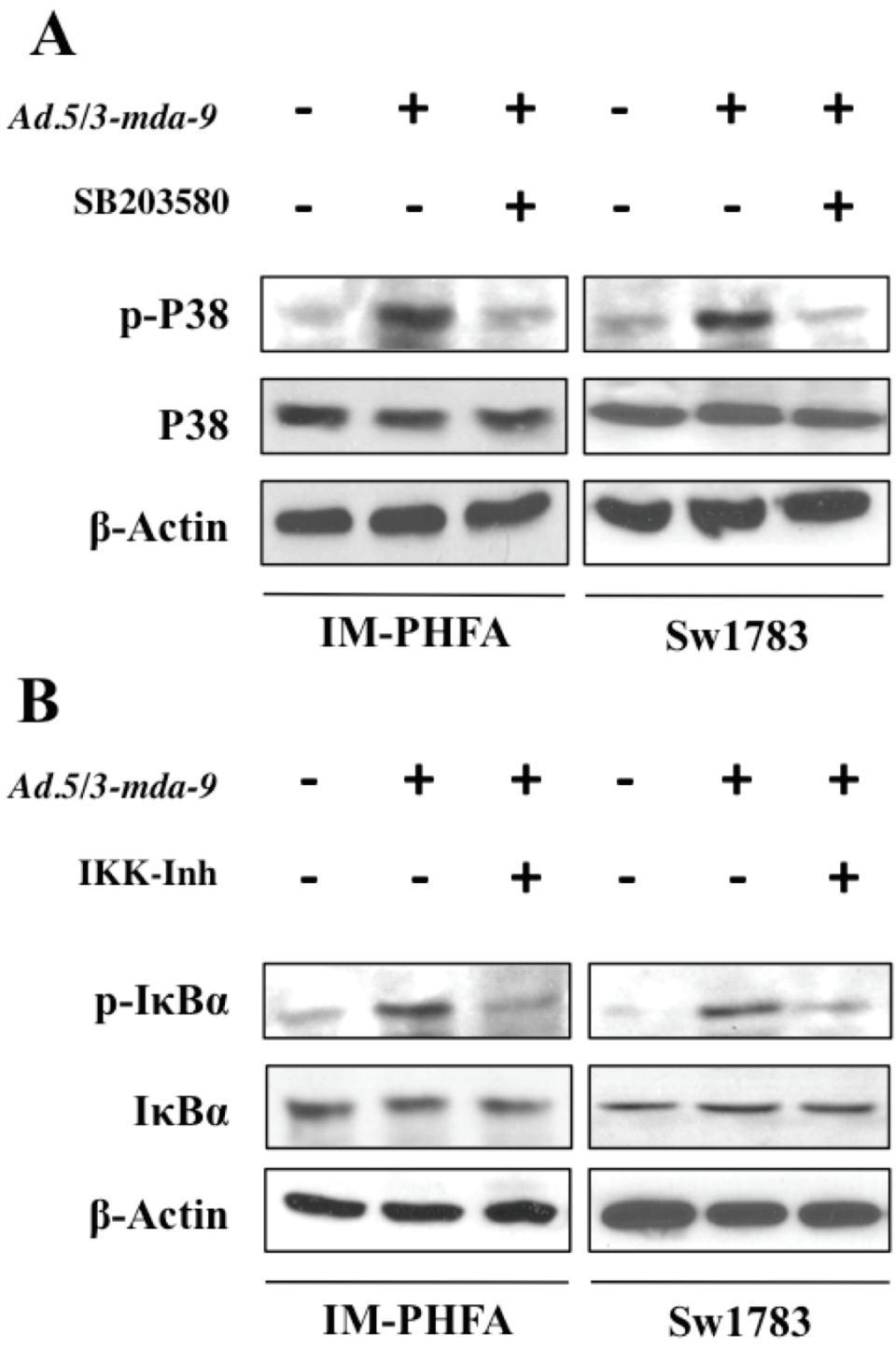


Figure 3.11 Efficacy of pharmacological inhibitors. A, activated and total p38MAPK expression following Ad.5/3-*mda*-9 treatment with or without SB203580, a p38MAPK inhibitor. **B,** phosphorylated and total IκBα expression following Ad.5/3-*mda*-9 treatment with or without I-kappa-kinase inhibitor. β-actin used as loading controls.

GBM cells expressing shmda-9 show marked reductions in invasion, tumorigenesis, and angiogenesis

To further explore the effects of MDA-9/Syntenin knockdown, clones that stably express sh*mda-9* were produced in GBM6 cells, with substantial knockdown achieved in several of the isolated colonies (Figure 3.12A). Compared to the normally invasive control clones, GBM6-sh*mda-9* clones exhibited 2.5- to 4-fold decreases in invasive ability (Figure 3.12B).

The invasive capacity of GBM cells depends on both digestion of extracellular matrix (ECM) as monitored by Matrigel invasion assays, and the locomotive ability of these cells once a path is cleared. Thus, the migration rates of GBM6-sh*mda-9* clones were analyzed by quantifying gap closure after 18 and 24 h. As a result of reduced MDA-9/Syntenin levels, gap closure was reduced by as much as 50% in GBM-sh*mda-9* cells compared to GBM6-control (Figure 3.13).

Anchorage-independent growth is an established marker for transformation and a tumorigenic phenotype in specific cancer cell types. To determine the effect of MDA-9/Syntenin knockdown on the ability of transformed cells to grow in an anchorage-independent environment, GBM6-control and sh*mda-9* clones were seeded in a soft agar transformation assay. Control GBM6 cells readily formed large and numerous colonies after 8 days of growth in soft agar. Reduced levels of MDA-9/Syntenin in GBM6-sh*mda-9* cells led to a decrease in the number of colonies formed and the size of the colonies (Figure 3.14)**.** Consequently, MDA-9/Syntenin has a significant effect on the ability of transformed GBM cells to grow in an anchorage-independent environment *in vitro*.

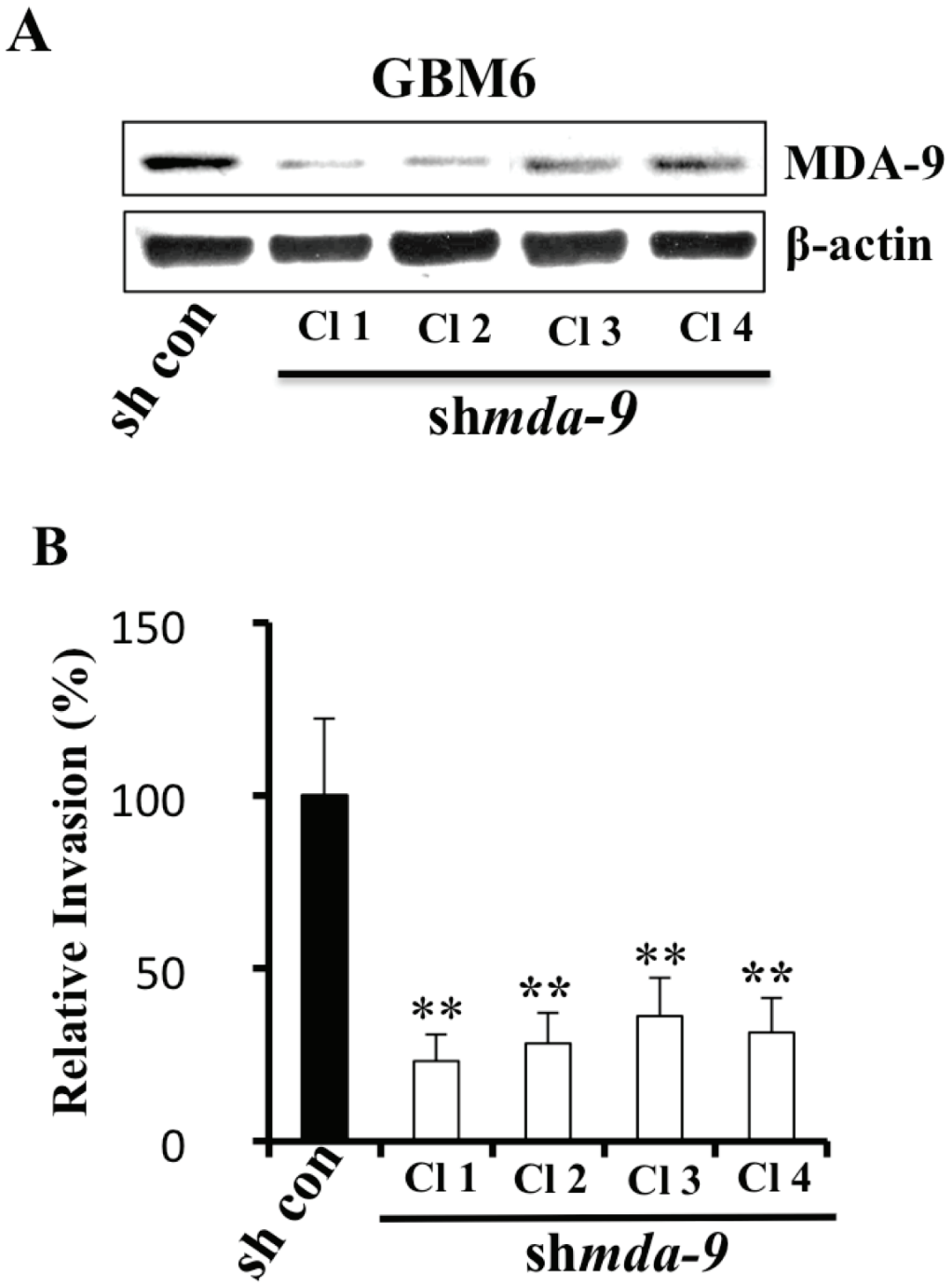


Figure 3.12 Stable MDA-9/Syntenin knockdown in GBM results in decreased invasion. A, MDA-9/Syntenin protein levels were decreased by stable expression of sh*mda-9* in GBM6 cells. **B,** Results from Matrigel invasion of GBM6-sh*mda-9* clones. Invasion quantified using five fields per triplicate well.

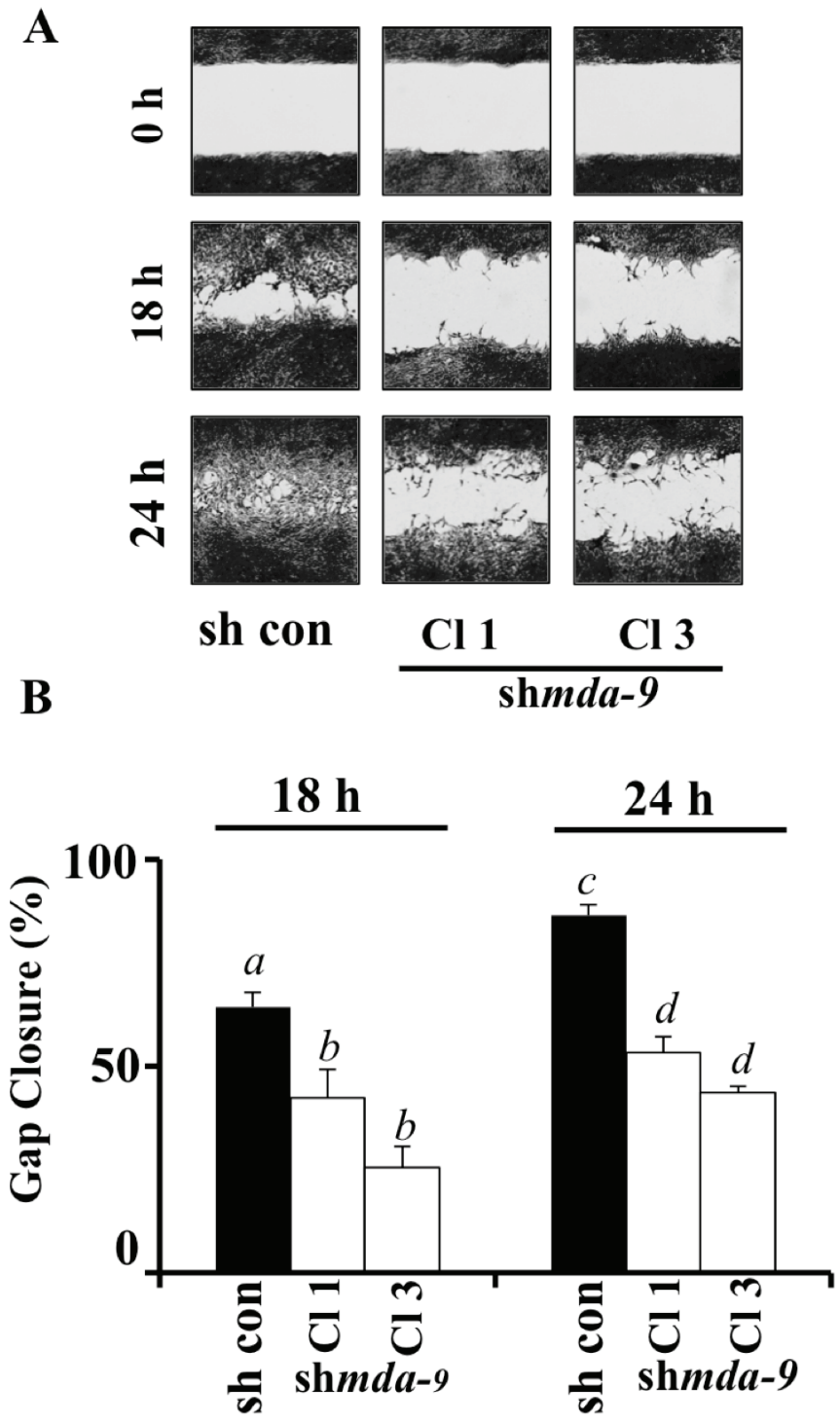


Figure 3.13 Stable MDA-9/Syntenin knockdown in GBM results in decreased migration. A, Wound healing assay after 18 and 24 h in control and sh*mda*-9 GBM6 Cl 1 and Cl 3. **B,** Quantification of triplicate wound healing scratch assays as quantified using ImageJ. *a* differs from *b* ($p < 0.01$), and *c* differs from *d* ($p < 0.01$).

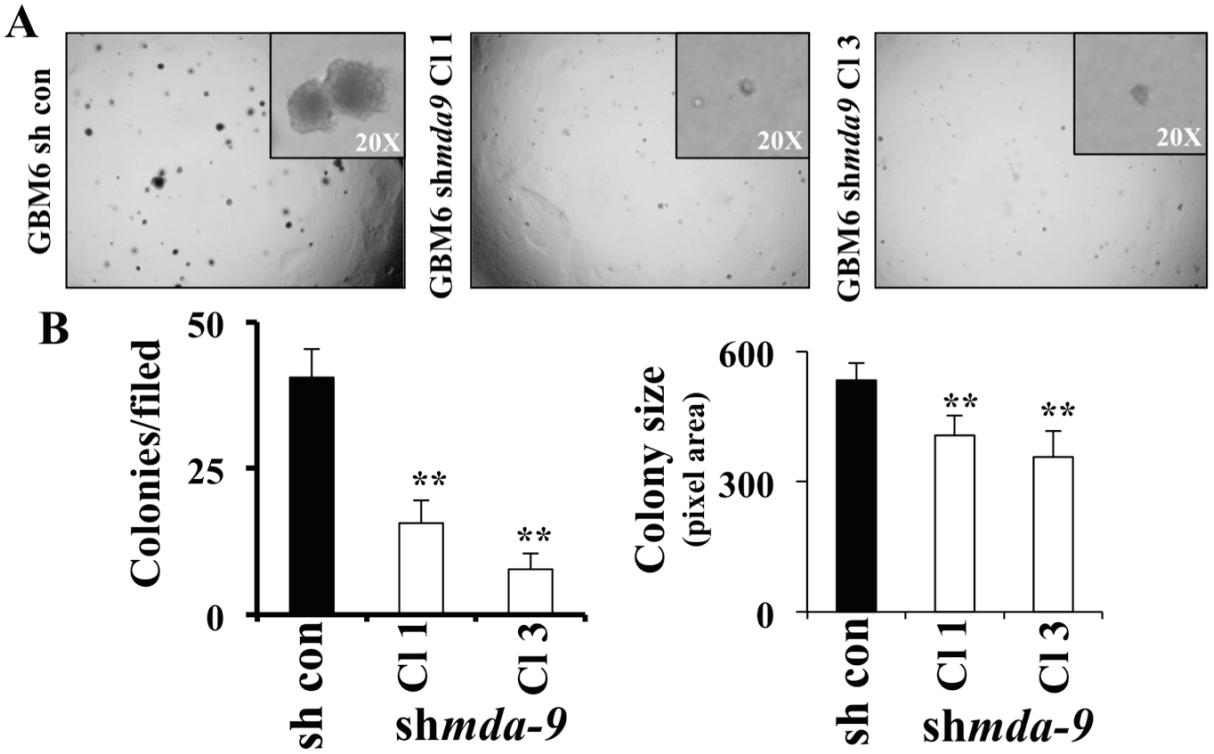


Figure 3.14 Stable MDA-9/Syntenin knockdown in GBM inhibits anchorage-independent growth. **A,** Representative images of anchorage independent growth in soft agar using GMB6-control and sh*mda-9* clones. *(Inset)* Higher magnification illustrating the differences in colony size. **B,** Quantification of colony number and size using ImageJ. **, $p < 0.01$.

Tumorigenic capacity *in vivo* was then analyzed by injecting 10^5 GBM6-control or GBM6-sh*mda-9* cells subcutaneously into athymic nude mice. Tumors were measured three times weekly, and after 21 days, the mice were sacrificed and the tumors resected and weighed. GBM6-control cells formed consistently larger tumors by volume and weight compared to their MDA-9/Syntenin-depleted counterparts (Figure 3.15). Because GBM6-sh*mda-9* clones show no differences in proliferation rate *in vitro* (Figure 3.16), differences in tumor size could be due to changes in angiogenic ability caused by the knockdown of *mda*-9/syntenin. To test this hypothesis, we knocked down *mda*-9/syntenin by Ad.5/3-sh*mda*-9 viral infection of T98G GBM cells and harvested conditioned media. By means of a chicken egg chorioallantoic membrane (CAM) assay, we observed marked differences in blood vessel development when exposed to control GBM conditioned media (T98G cells infected with Ad.5/3-*vec*) compared to media from Ad.5/3-sh*mda-9* infected T98G cells. Less microvessel development and fewer branch points were evident in membranes exposed to media from knockdown cells (Figure 3.17A). Upon analysis using an angiogenic protein array, substantial reductions in the levels of secreted IL-8 were observed (Figure 3.17B). This data is corroborated by similar trends in mRNA levels of IL-8 in T98G cells, as well as increased mRNA levels in IM-PHFA cells overexpressing MDA-9/Syntenin (Figure 3.17C). Moreover, secreted IL-8 levels were increased in the conditioned media of IM-PHFA cells infected with Ad.5/3-*mda-9* as measured by ELISA. Likewise, decreased IL-8 was detected from MDA-9/Syntenin-depleted T98G cells (Figure 3.17D). In total, these *in vitro* and *in vivo* data demonstrate the crucial role MDA-9/Syntenin plays in tumor formation and angiogenesis.

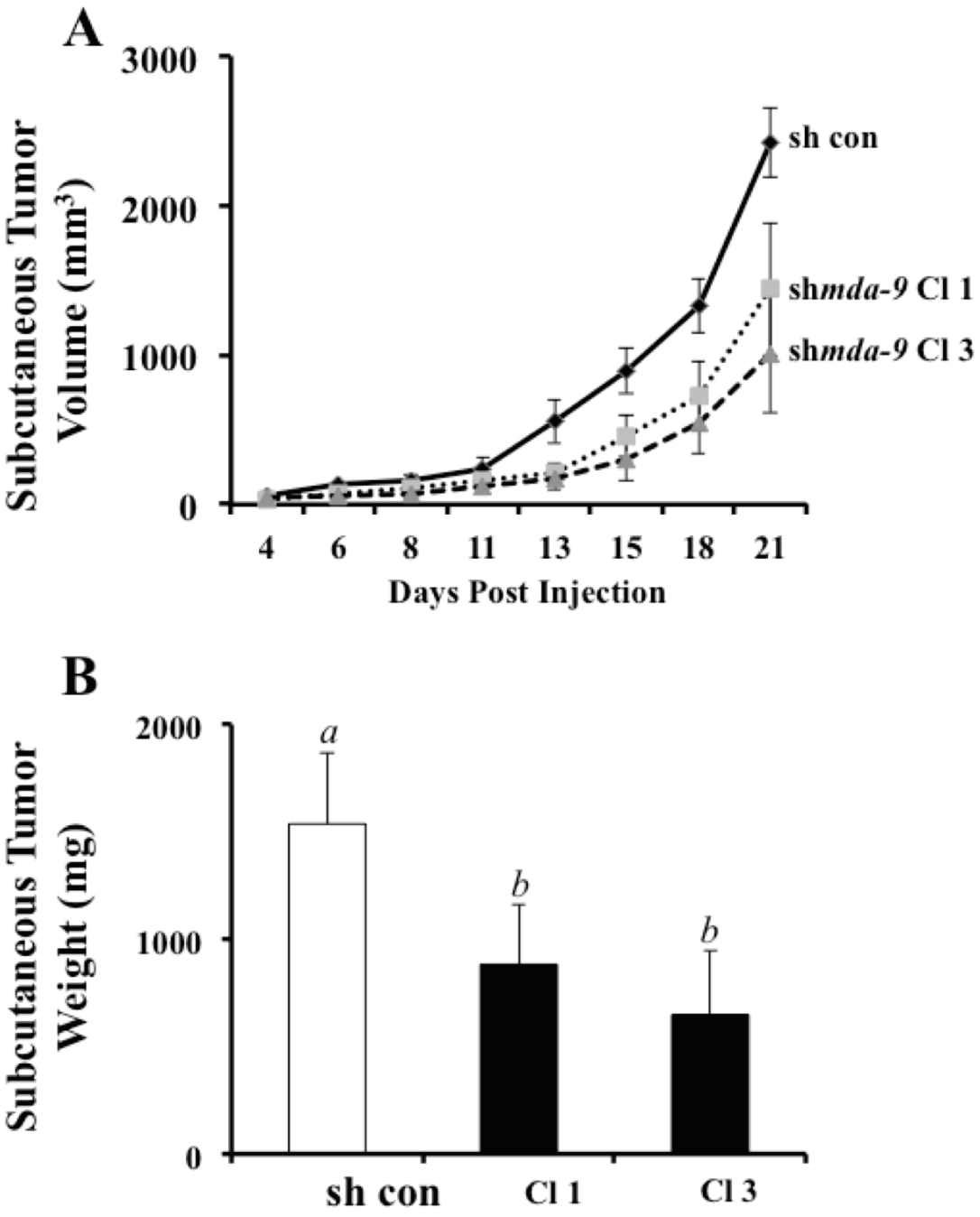


Figure 3.15 MDA-9/Syntenin knockdown decreases tumor size *in vivo.* **A,** Tumor volume from subcutaneous injection of GBM6 control and sh*mda-9* expressing clones. **B,** Weight of resected subcutaneous tumors after 21 days. *a* differs from *b* ($p < 0.01$)

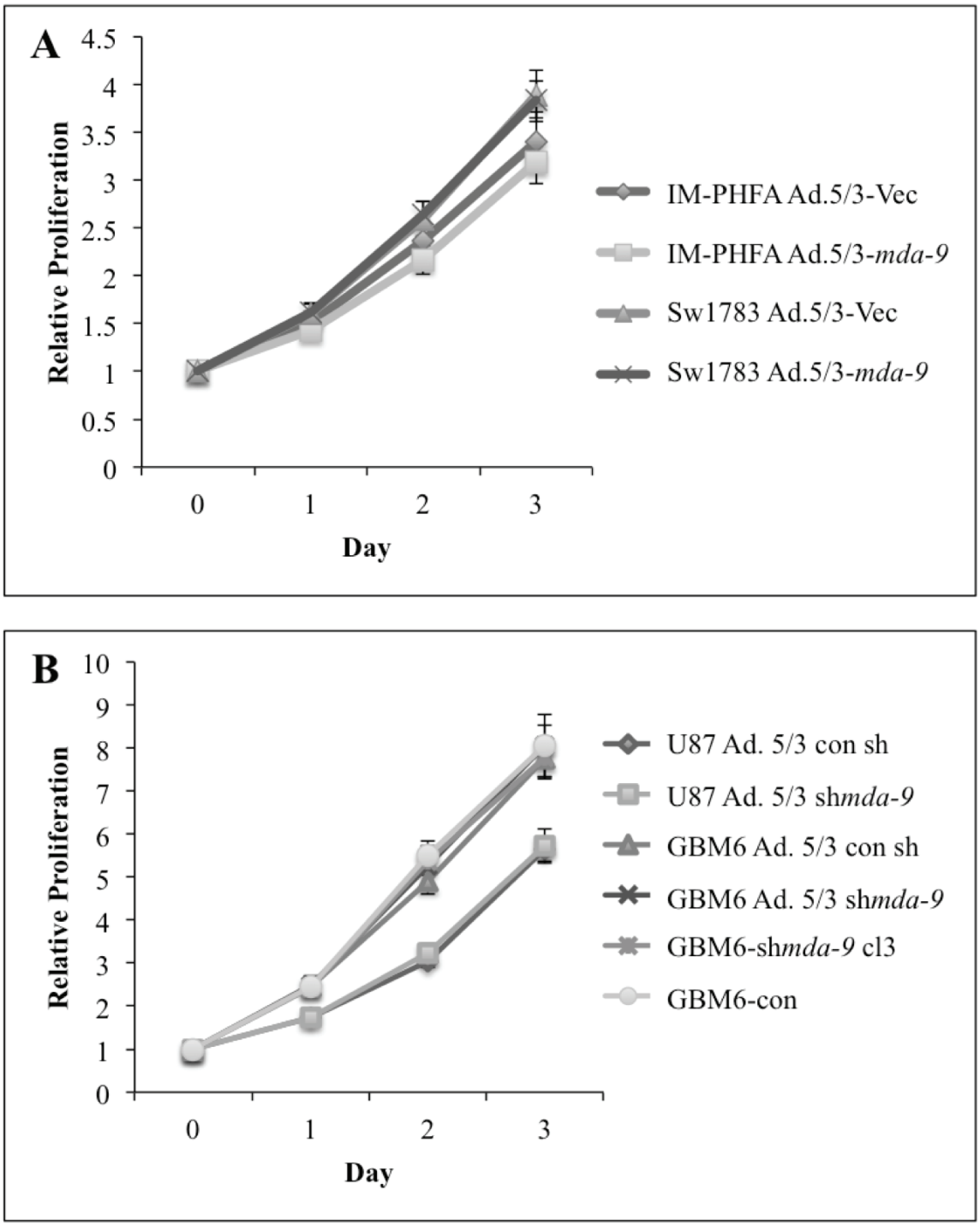


Figure 3.16 Altering MDA-9/Syntenin levels does not affect proliferation. A, Results from 3 days of MTT assays indicating treatment with Ad.5/3 *mda-9* does not alter proliferation of IM-PHFA nor Sw1783 cells. **B,** Results from 3 days of MTT assays indicating treatment with Ad.5/3 sh*mda-9* does not alter proliferation of U87 nor GBM6 cells. Additionally GBM6-con and GBM6-sh*mda-9* cells do not differ in their proliferation rates. Error bars = S.D. from 3 independent experiments.

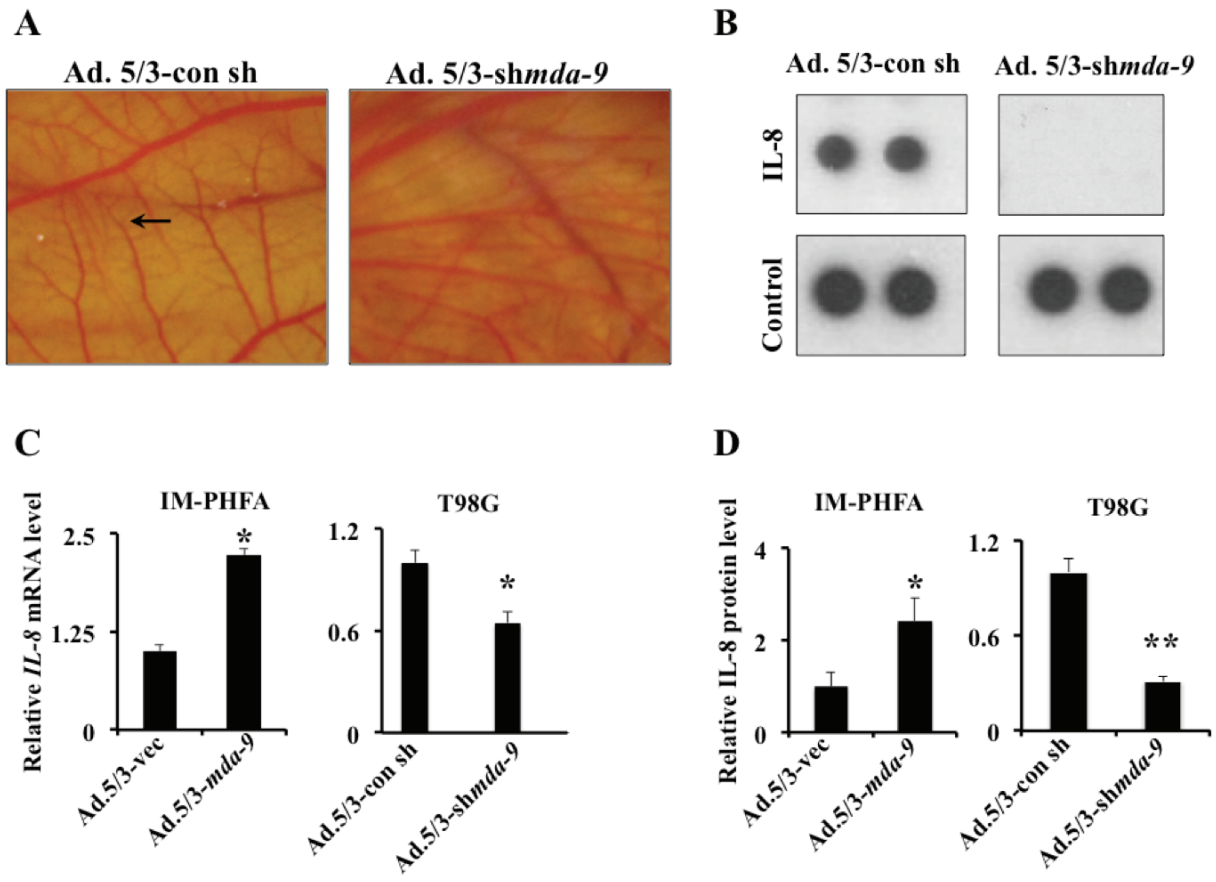


Figure 3.17 MDA-9/Syntenin knockdown decreases tumor angiogenesis *in vitro.* **A,** Representative images from chicken chorioallantoic membrane assay using T98G cells treated with vector or Ad.5/3-sh*mda-9*. The proliferation of the smallest vessels *(arrow)* is decreased with knockdown of MDA-9/Syntenin. **B,** IL-8 expression in conditioned media from treated T98G cells as measured by angiogenesis protein array. **C,** Real-time PCR for IL-8 mRNA using IM-PHFA and T98G lines following the indicated treatments. **D,** ELISA quantification of IL-8 protein in IM-PHFA and T98G conditioned media. Media from Ad.5/3-sh*mda-9* treated cells is normalized to Ad.5/3-con sh treated cells. *, $p < 0.05$, **, $p < 0.01$.

Knockdown of MDA-9/Syntenin leads to changes in tumor properties in an orthotopic tumor model of glioma

To provide a more authentic tumor microenvironment and to further explore the role of MDA-9/Syntenin in invasion, we employed an orthotopic xenograft model in which 15,000 cells were stereotactically injected intracranially into athymic nude mice. The mice were monitored for neurological symptoms such as paralysis, seizures, weight loss, and lethargy and were sacrificed according to an IACUC-approved protocol. The survival times for the GBM6-sh*mda-9* group were significantly longer when compared to the GBM6-control group by chi square analysis ($p=0.015$) (Figure 3.18A)**.** Post-mortem, brain and tumor tissue were collected and sectioned for immunohistochemical staining. GBM6-*shmda-9* tumors retained a lower level of MDA-9/Syntenin expression, although both control tumors and GBM6-*shmda-9* tumors showed localized increases in MDA-9/Syntenin at the tumor borders (Figure 3.18B). Additionally, GBM6-control tumors exhibited substantially greater CD31 expression compared to GBM6-*shmda-9* tumors, supporting a role for MDA-9/Syntenin in GBM angiogenesis (Figure 3.18B). Furthermore, noteworthy differences were found in the invasion patterns of tumors formed from GBM6-control cells and tumors from GBM6-sh*mda-9* cells. GBM6-control tumors were notable for finger-like projections and tumor nests separate from the main body of the tumor. Additionally, control tumors displayed prominent infiltration along Virchow-Robin spaces and conspicuous leptomeningeal invasion. (Figure 3.19A, B). Alternatively, tumors formed from GBM6-sh*mda-9* cells had minimal focal tumor invasion into surrounding brain parenchyma, much less than that observed in control tumors, with the majority of tumor having well-demarcated margins (Figure 3.19C, D). This provides additional

data relative to the critical role of MDA-9/Syntenin in GBM invasion and points to it as a possible target to reduce infiltration of GBM cells into surrounding brain tissue.

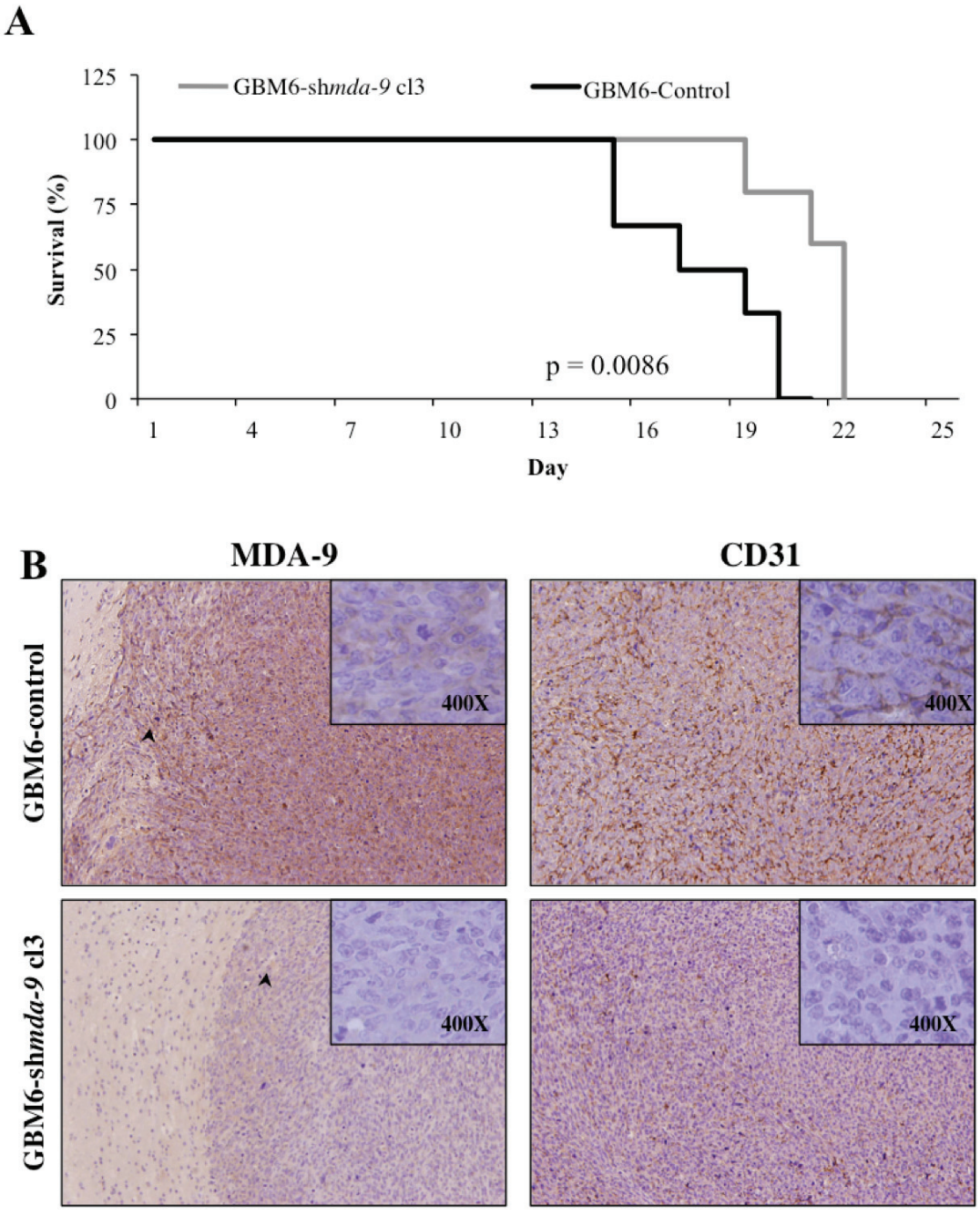


Figure 3.18 MDA-9/Syntenin knockdown reduces survival, invasion, and angiogenesis *in vivo*. A, Survival time for mice injected with GBM6-control or GBM6-sh*mda-9* Cl 3 (p = 0.0086). **B,** MDA-9/Syntenin and CD31 protein levels from GBM6-control tumors *(Left)* and GBM-sh*mda-9* tumors *(Right)*. Insert 400X magnification.

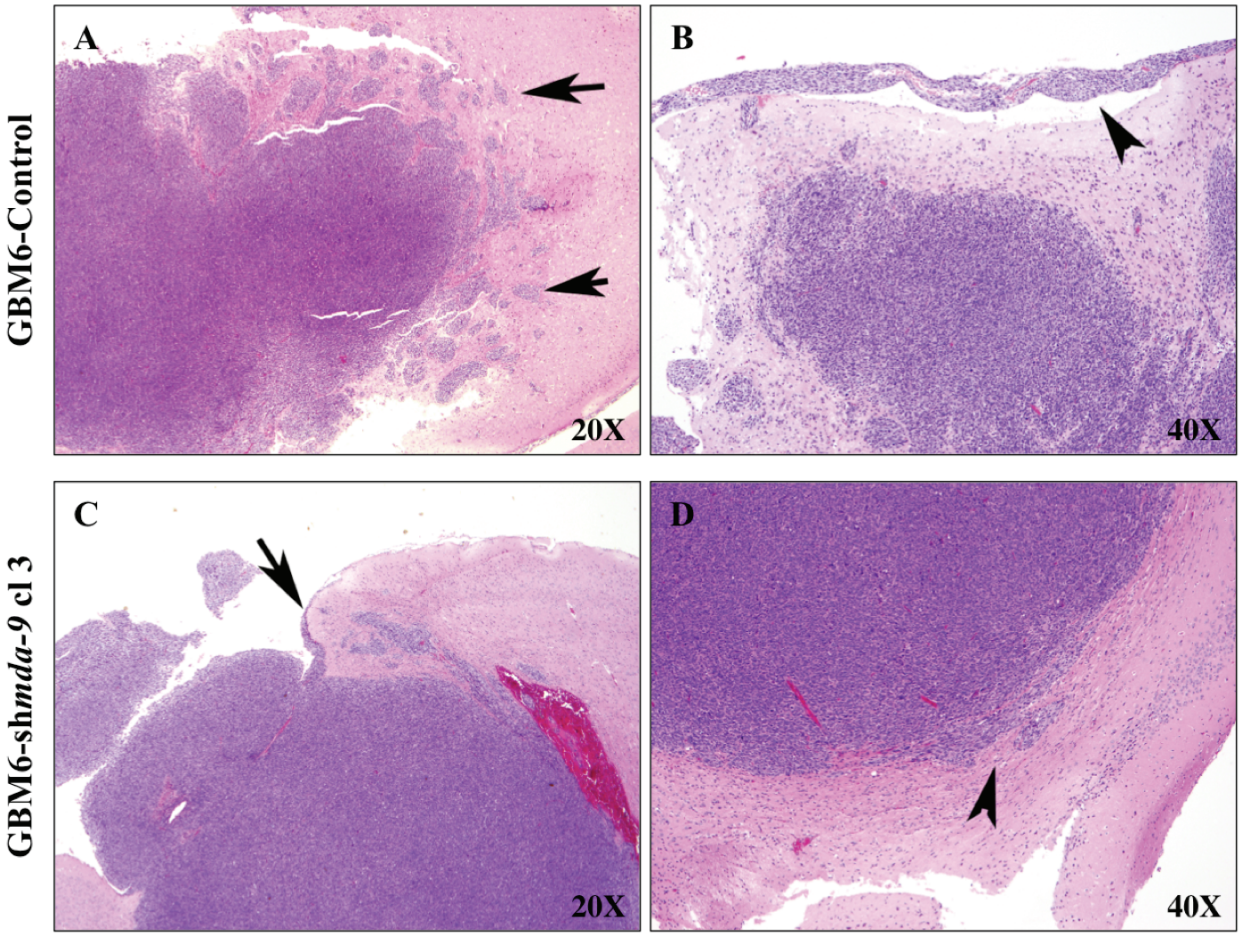


Figure 3.19 MDA-9/Syntenin knockdown reduces hallmarks of invasion. H & E staining from tissue collected from GBM6-control and GBM-sh*mda-9* tumors. **A,** GBM6-con - Note tumor invasion surrounding brain parenchyma, with finger-like projections and tumor nests apart from the main tumor mass showing infiltration along Virchow-Robin spaces (arrows). **B**, GBM6-con - Note leptomeningeal invasion (arrowhead). **C,** GBM6-sh*mda-9* – Very focal leptomeningeal invasion compared to control (arrow). **D,** GBM6-sh*mda-9* – minimal focal tumor invasion into surrounding brain parenchyma (arrowhead) with a largely demarcated margin.

IV. Discussion

We provide definitive evidence for a role of MDA-9/Syntenin in mediating astrocytoma invasion and pathogenesis. Earlier studies implicated MDA-9/Syntenin in numerous cellular functions and found its expression to be particularly relevant in cancer metastasis. While systemic metastasis of astrocytoma is highly uncommon, the propensity for local invasion of the brain parenchyma leads to challenges in treatment and inevitable tumor recurrence (257). We found MDA-9/Syntenin to be overexpressed in astrocytoma and have a correlation with tumor grade (Figure 3.1, Figure 3.2), as grade IV tumors (GBM) had the highest level of MDA-9/Syntenin expression. Uncovering new targets for treatment in these tumors will be particularly important, as median survival remains around 15 months despite aggressive therapy (257). Our invasion studies indicated that MDA-9/Syntenin was a significant factor in determining invasiveness and migratory capacity of astrocytoma cells as both forced overexpression and knockdown of MDA-9/Syntenin led to alterations in invasive ability. Given the involvement of MDA-9/Syntenin in crucial early stages of development in the central nervous system, this suggests that MDA-9/Syntenin may be an important genetic component in less differentiated precursor cells, with which glioma cells share remarkable similarities, notably the ability of single cells to migrate throughout the brain (469).

An essential aspect of tumor migration and invasion is the interaction of single cells with the extracellular matrix (ECM). Vital contributors in these interactions include one of the most important groups of adhesion molecules in glioma migration and invasion, the integrins, which act as signal mediators and anchors to the ECM (470). Specifically, high expression of the integrin β1 subunit correlates with invasive behavior in glioma (471). Immunoprecipitation of

β1 integrin in melanoma and breast cancer revealed fibronectin interactions increased FAK-c-Src-MDA-9/Syntenin signaling complexes, shown to be important regulators of the migration and invasion machinery (37, 472). We document that in astrocytoma, MDA-9/Syntenin expression leads to c-Src activation and downstream effects on the P38MAPK pathway, resulting in enhanced invasion. Furthermore, MDA-9/Syntenin has been linked to a key mediator of integrin and PI3K signaling, ILK (37). This serine/threonine kinase acts as a molecular tether point, regulates protein-protein interactions, and coordinates the organization of the actin cytoskeleton (473). Conspicuously, PTEN, inactivated in a large percentage of high-grade astrocytomas (175) can inhibit the activity of ILK in addition to PI3K (474, 475).

NF-κB is activated through overexpression of MDA-9/Syntenin and, conversely, it can be inhibited through knockdown of MDA-9/Syntenin. Syndecan-1, a member of a major group of cell surface proteoglycans, is overexpressed in GBM samples and may be regulated by NF-κB (476). This serves as another possible link between MDA-9/Syntenin and glioma as one of the earlier observations of MDA-9/Syntenin was its identification as binding the cytoplasmic domain of syndecans (14). Additionally, pharmacological inhibition of downstream c-Src effectors abrogates the invasion gains accrued through MDA-9/Syntenin overexpression. Upstream, c-Src has been linked to an important and common alteration found in GBM, the amplification or mutation of epidermal growth factor receptors (EGFR), giving credence to the possibility of MDA-9/Syntenin playing a role in this important signaling pathway(172, 477-479). Moreover, in a recent study a physical interaction and colocalization of MDA-9/Syntenin and EGFR was evident in human urothelial cell carcinoma cell lines and primary tumors (49).

While anti-angiogenic therapy currently exists for high-grade astrocytoma, these tumors have been shown to evade cell death through hypoxia-induced autophagy, adapting to therapy

through HIF-1α induction(480). Notably, MDA-9/Syntenin induction leads to HIF-1α expression in human melanoma, which results in increased IGFBP-2 secretion (59). IL-8 has proven to be a particularly important cytokine in glioma biology. Mutant EGFR signaling acts through NF-κB to induce secretion of IL-8, leading to an enhanced xenograft neovascularization (150). Additionally, IL-8 is a mediator of invasion in gliomas, also acting through the NF-κB pathway(481). The angiogenic effect of MDA-9/Syntenin in glioma deserves further exploration as the syndecan-MDA-9/Syntenin-ALIX axis is implicated in the biogenesis of exosomes, which can be secreted from tumor cells and contain mRNA and proteins (21). In GBM, exosomes have been shown to contain translatable mRNA and can contribute to growth and angiogenesis. In addition to the clinical importance of MDA-9/Syntenin in patient survival demonstrated in Figure 3.1, mining the NCI REMBRANDT database corroborated this finding in human glioma patients. In GBM patients with *mda*-9/syntenin up-regulated > 2.0X, survival was significantly shorter ($p = 0.0016$). Furthermore, in all glioma patients with *mda*-9/syntenin up-regulated > 2.0X, survival was again significantly reduced ($p = 6.0 \times 10^{-5}$) (482).

Through our *in vivo* studies, we demonstrate that MDA-9/Syntenin is a major contributor to the deadliest aspect of GBM pathology, invasion. Combined with our analysis of patient samples and *in vitro* data, we propose that MDA-9/Syntenin could prove to be a valuable target for the treatment of high-grade glioma.

Chapter 4 – Targeting MDA-9/Syntenin Complements Glioma Radiotherapy

I. Introduction

Despite advances in surgical, pharmacological, and radiation approaches, GBM remains a particularly aggressive and ultimately intractable tumor with median survival less than 15 months and 5-year survival at 5% (457, 483). The current standard of care includes maximal surgical resection followed by radiation and temozolomide chemotherapy. However, inevitable recurrence occurs near resection margins and within the high-dose radiation field, implying that intrinsic invasiveness and radioresistance contributes greatly to relapse (484, 485). Radiation produces a variety of damage in DNA, though double strand breaks (DSBs) appear to drive the majority of radiation-induced apoptosis by activating DNA damage response (DDR) pathways (486). While highly successful in inducing cytotoxicity in a majority of tumor cells, sub-lethal radiation has repeatedly been shown to induce invasion and migration in surviving tumor cells, enhancing the very property that makes curative treatment so difficult (378, 487, 488). A contributing factor to tumor relapse and recurrence is the ability of tumor cells to break from the primary tumor mass (489), which underscores the importance of developing anti-invasive therapies that complement, and ideally enhance, conventional approaches (490). Therefore, gaining a deeper understanding of the crucial molecular signaling events and molecules will help identify targets for anti-invasive and radiosensitizing approaches.

Studies of MDA-9/Syntenin present such a candidate for targeting, as it has been demonstrated in multiple cancer settings to be involved in invasion and metastatic signaling (25, 49, 58, 491). MDA-9 serves critical roles in signal transduction, as well as in cell-cell, and cell-matrix adhesion (20, 460). In addition to its well-described roles in melanoma metastasis and tumor progression, mda-9 was shown to be highly expressed and involved in breast, gastric, and urothelial cell cancers (49, 460). Our work, discussed in Chapter 3, showed that MDA-9/Syntenin is an important regulator of GBM invasion, angiogenesis, and tumor progression (50), and that inhibiting the expression of MDA-9/Syntenin can decrease GBM invasion (50), and enhance survival. Gene expression analysis of the TCGA database revealed that patients whose tumors express high levels of MDA-9/Syntenin have a poor prognosis and reduced survival compared to low-expressing MDA-9/Syntenin tumors. MDA-9/Syntenin expression correlates positively with astrocytoma grade, as analyzed through tissue samples and gene expression databases, and is most highly expressed in GBM. In both melanoma and glioma, MDA-9/Syntenin is involved in NF-κB activation through a c-Src/p38 MAPK signaling (25, 491). Inhibiting mda-9/syntenin expression can lead to a reduction in NF-κB target gene expression such as MMP2, a critical secreted metalloproteinase involved in GBM invasion.

A vital characteristic of MDA-9/syntenin is the inclusion of two tandem PDZ domains, so named for their discovery in **P**SD95/SAP90, **D**LGA, and **Z**O-1(4). PDZ domains are common to a number of scaffolding proteins, critical for facilitating protein-protein interactions throughout the cell. MDA-9/Syntenin utilizes these motifs to successfully facilitate the interaction of c-Src/FAK kinase complexes, noted for involvement in pro-invasive signaling in cancer (25). While inhibiting the interaction between MDA-9/Syntenin and its targets could be fruitful, to date, no molecular inhibitor of the PDZ domain of mda-9/syntenin has been developed.

Fragment-based lead design or fragment-based drug discovery (FBDD) is an emerging and useful strategy for the development of biologically active compounds. Interrogation of a library of compounds through detection of target-mediated interligand transferred nuclear Overhauser effects (ILOEs) has been used with success to select scaffolds that simultaneously bind several Bcl-2 family proteins, including both Bcl-x_L and Mcl-1 (492), thus overcoming the compensatory gain in Mcl-1 expression as a mechanism of resistance when targeting other Bcl-2 family members. Here, we demonstrate the efficacy of supplementing radiotherapy by targeting MDA-9/Syntenin in GBM. By counteracting gains in Src, FAK, and EphA2 signaling, mda-9/syntenin inhibition can reduce radiation-induced invasion, as well as radiosensitize GBM cells, highly beneficial properties to complement radiation treatment.

II. Materials and Methods

Construction of plasmids, adenoviruses and stable cell lines

Small hairpin RNA for *mda*-9/*syntenin* (sh*mda-9*) was constructed with pSilencerTM hygro expression vectors according to the manufacturer's protocol (Ambion Inc. TX) as previously described (50). Plasmids pAd.5/3-sh*mda-9* or pAd.5/3-shNTC were constructed as described (50), and the resultant plasmids were cleaved with PacI to release the recombinant adenovirus genomes and then transfected to HEK-293 cells to rescue the corresponding Ad.5/3-based vectors. The rescued viruses were upscaled using HEK-293 cells and purified by cesium chloride double ultracentrifugation using standard protocol and the titers of infectious viral particles were determined by plaque assay using HEK-293 cells as described(463).

Cell lines, cell culture, and treatments

Human malignant glioma cells U87, U251, and T98G as well as grade III astrocytoma lines Sw1088 and Sw1783 were purchased from the American Type Culture Collection (Manassas, VA) Human primary GBM6 cells were described previously and were provided by Dr. C. David James (University of California, San Francisco, CA) (152). These and U1242 (493) were cultured in DMEM + F12 supplemented with 10% FCS in a 37°C incubator supplemented with 5% CO_2. U87-EGFRvIII cells were a kind gift from Dr. John Laterra and Dr. Bachchu Lal and maintained as described previously(494). Primary human fetal astrocytes (PHFA) were obtained from pre-term abortions as previously described with IRB approval, and h-TERT-immortalized primary human fetal astrocytes (IM-PHFA) were produced and cultured as described (464). All cells were routinely screened for mycoplasmal contamination. Cells were treated with PDZ1i at the indicated concentrations 2 hours prior to irradiation. Irradiations were done using an MDS Nordion Gammacell 40 research irradiator with a 137-Cs source delivering a dose rate of 0.896 Gy/min. Viral infection conditions and protocols were performed as delineated previously (15).

Antibodies and reagents

Antibodies against FAK, phospho-FAK (Tyr 576/577), IGF-1R, phospho-IGF-1R (1135/36), p38MAPK, phospho-p38MAPK (Thr180/Tyr182), p65, and phospho-p65(S536), Src, and phospho-Src (Tyr 416), were obtained from Cell Signaling (Beverly, MA). β-actin and α-Tubulin were obtained from Abcam (Cambridge, MA), and MDA-9/Syntenin from Abnova (Taipei, Taiwan).

Preparation of whole-cell lysates and Western blotting analysis

Preparation of whole-cell lysates and Western blotting analysis was performed as described (465). For densitometric evaluation, X-ray films were scanned and analyzed with ImageJ software (NIH).

Extraction of Total RNA and Real-Time PCR

Total RNA was extracted from cells using the QIAGEN miRNAeasy Mini Kit (QIAGEN, Hilden, Germany). cDNA preparation was done using ABI cDNA synthesis kit (Applied Biosystems, Foster City, CA). Real-time polymerase chain reaction (RT-PCR) was performed using an ABI ViiA7 fast real-time PCR system and Taqman gene expression assays according to the manufacturer's protocol (Applied Biosystems, Foster City, CA).

Immunohistochemistry

A portion of the frozen tumor specimen was fixed in phosphate-buffered formalin and preserved as paraffin sections following standard procedure for the maintenance of histological structure. Paraffin-embedded sections were dewaxed and rehydrated through incubations in xylene and a gradient series of alcohol. Antigen retrieval was processed in 10 mM citric acid (pH 6.0) with microwave treatment for 20 min. Endogenous hydrogen peroxidase was quenched with 3% H_2O_2 for 20 min. After blocking non-specific binding sites with 5% normal sera, the sections were incubated overnight with antibody. The sections were incubated with appropriate biotinylated secondary antibody and subsequently with ABC-peroxidase (Vector Elite, Vector laboratories, Burlingame CA). Colorimetric reactions were developed by incubation in DAB substrate (0.02% DAB, 0.005% hydrogen peroxide), and counterstained with 10% Harris' hematoxylin. Hematoxylin & eosin staining was conducted following a standard protocol (15).

The images were analyzed under the Olympus BX41 microscope system equipped with DP25 digital camera and software.

Invasion and migration assays

Invasion was measured using 24-well BioCoat cell culture inserts (BD Biosciences, Bedford, MA) with an 8-μm-porosity polyethylene terephthalate membrane coated with Matrigel Basement Membrane Matrix (100 μg/cm^2). Briefly, the Matrigel was allowed to rehydrate for 2 h at room temperature by adding warm, serum-free DMEM. The wells of the lower chamber were filled with medium containing 10% fetal bovine serum. Cells (5 x 10^4) were seeded in the upper compartment (6.25-mm membrane size) in serum-free medium. The invasion assay was done at 37°C in a 5% CO_2 humidified incubator for 18 h. At the end of the invasion assay, filters were removed, fixed, and stained with the Diff-Quick Staining kit (IMEB, San Marcos, CA). Cells remaining on the upper surface of the filters were removed by wiping with a cotton swab, and invasion was determined by counting the cells that migrated to the lower side of the filter using at least 5 fields per insert at 100x magnification.

Survival and viability analysis

Clonogenic radiosurvival experiments were carried out as described previously (493, 495). Briefly, cells were diluted, seeded on 6-cm dishes, and after the cells attached, treated with DMSO or PDZ1i (12.5, 25, or 50 μM) in the medium for 2 hours before irradiation. Cells were incubated overnight, and the medium changed to nondrug containing medium 16 hours postirradiation. Cells were incubated for further 14 days, stained with 2% Giemsa solution, and colonies consisting of 50 or more were counted. Cell viability was assessed through MTT analysis as described previously (496).

Conditioned media (CM) isolation and analysis

CM was harvested from an overnight culture of plated cells in serum-free DMEM + F12 media, filtered with 0.2 μM filters and further concentrated 10-fold on an Amicon Ultra centrifugal filter – 3K (Millipore, Billerica, MA). Protein levels were analyzed via the Proteome Profiler Human Protease Array Kit (R&D Systems, Minneapolis, MN). Relative quantification was performed by densitometric evaluation. X-ray films were scanned and analyzed with ImageJ software (NIH).

Intracranial implant of cells in nude mice

Athymic female NCr-nu/nu mice (NCI-Fredrick) weighing ~25 g were used for this study. Mice were maintained under pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U.S. Department of Agriculture, Washington, DC, the U.S. Department of Health and Human Services, Washington, DC, and the NIH, Bethesda, MD. At least 5 mice per group were utilized. Mice were anesthetized through i.p. administration of ketamine (40 mg/kg) and xylazine (3 mg/kg), and immobilized in a stereotactic frame (Stoelting, Wood Dale, IL). A 24-gauge needle attached to a Hamilton syringe was inserted into the right basal ganglia to a depth of 3.5-mm and then withdrawn 0.5-mm to make space for tumor cell accumulation. The entry point at the skull was 2-mm lateral and 1-mm dorsal to the bregma. Intracerebral injections of 1.5×10^4 cells in 2 μL per mouse were completed over 10 min using an automated injector (Stoelting, Wood Dale, IL). The skull opening was enclosed with sterile bone wax and the skin incision was closed using sterile surgical staples. Post-sacrifice, the tumors were resected, weighed, and preserved for IHC staining.

Database Mining

The REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) (http://rembrandt.nci.nih.gov) database was accessed and mined for all Astrocytoma and GBM patients (482). These were filtered for those with no previous history of radiation therapy, but had record of undergoing subsequent radiation. This population was stratified for *SDCBP* (*mda-9/syntenin*) expression. The survival data for the resulting groups was downloaded and analyzed via GraphPad Prism.

Statistical analysis

The data are reported as the mean ± S.D. of the values from three independent determinations and significance analysis was performed using the Student's t test in comparison with corresponding controls. Probability values < 0.05 were considered statistically significant. Survival curves were analyzed using Cox Proportional Hazards Survival Regression using GraphPad Prism.

III. Results

MDA-9/Syntenin inhibition leads to radiosensitization and inhibition of radiation – induced invasion

Our previous results indicated that MDA-9/Syntenin is an important mediator of glioma progression. Specifically, MDA-9/Syntenin was shown to be a valuable target in addressing one of the deadliest aspects of GBM, its propensity to invade (50). Since radiation therapy has been demonstrated to induce invasion in GBM cells, targeting MDA-9/Syntenin could be a worthwhile approach to complement conventional treatment. Gliomas with higher expression of MDA-9/Syntenin are more likely to be high-grade, and patients whose tumors had high levels of MDA-9/Syntenin had a worse prognosis (50). With this in mind, we looked for possible connections between MDA-9/Syntenin expression and radiotherapy in glioma. We turned to the REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) database, a publicly available dataset with information on tumor gene expression, treatment history, and survival (482). In patients with no history of radiation who then underwent subsequent radiotherapy, we asked if survival time correlated with MDA-9/Syntenin expression. We found that patients whose tumors had higher expression of MDA-9/Syntenin had significantly shorter survival (Figure 4.1 $p<0.01$). Median survival was reduced nearly threefold in patients with tumors expressing high levels of MDA-9/Syntenin (15.2 months) compared to others (43.6 months).

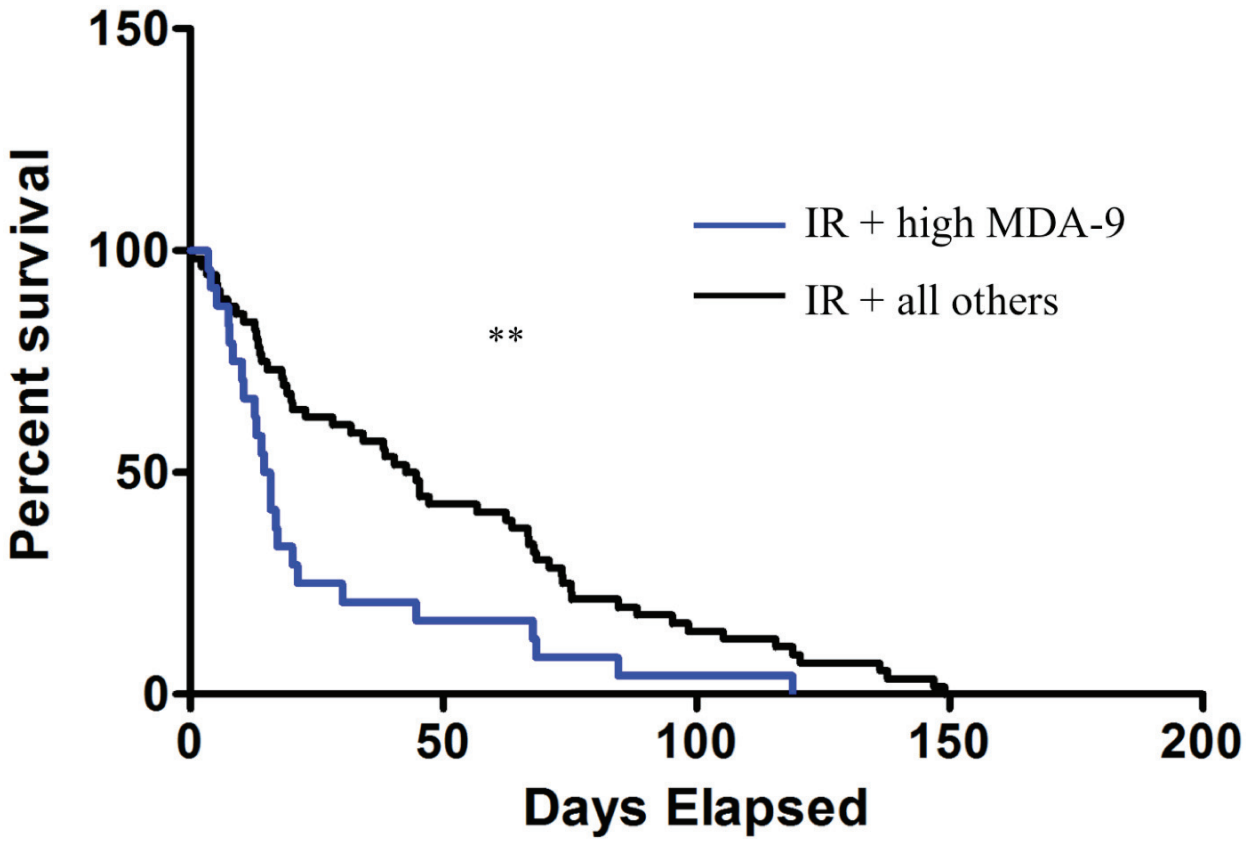


Figure 4.1 High MDA-9/Syntenin expression correlates with poor response to radiotherapy. A. The REMBRANDT database was mined for patients with no previous radiation treatment who then underwent radiation therapy. These were stratified by *SDCBP* (*mda-9/syntenin*) expression (High was set >1.5 fold overexpression). High tumor MDA-9/Syntenin led to a worse prognosis in those undergoing radiotherapy. ** $p < 0.01$.

We then asked if inhibiting the expression of MDA-9/Syntenin would result in changes in radiosensitivity in vitro. Using GBM cells expressing a control shRNA, U1242-shcon, or shRNA targeting MDA-9/Syntenin, U1242-shMDA-9/Syntenin, we found that low levels of MDA-9/Syntenin radiosensitized GBM in a colony formation assay (Figure 4.2). Furthermore, proliferation was inhibited in cells transiently reduced in MDA-9/Syntenin expression via adenoviral vector Ad.5/3 mda-9si (Figure 4.2). This indicates MDA-9/Syntenin may have an important role in cell survival following radiation exposure.

Radiation has consistently been shown to increase motility and invasion in GBM cells (378, 487, 497, 498). We exposed four cell lines to radiation after treatment with control adenovirus, Ad.5/3 NTCsi, or Ad.5/3 mda-9si. Each of the cell lines was exposed to a range of radiation doses, and the dose that demonstrated maximal radiation gains was used for MDA-9/Syntenin knockdown studies. As expected, knockdown of MDA-9/Syntenin levels reduced invasion with no radiation exposure. After irradiation, each GBM cell line exhibited at least a twofold invasion gain, while MDA-9/Syntenin knockdown significantly curtailed these gains (Figure 4.3). GBM invasion following radiation was reduced with MDA-9/Syntenin knockdown to about 33% of levels observed in controls. This represents a meaningful reduction to an undesirable side effect displayed upon exposure to conventional radiotherapy.

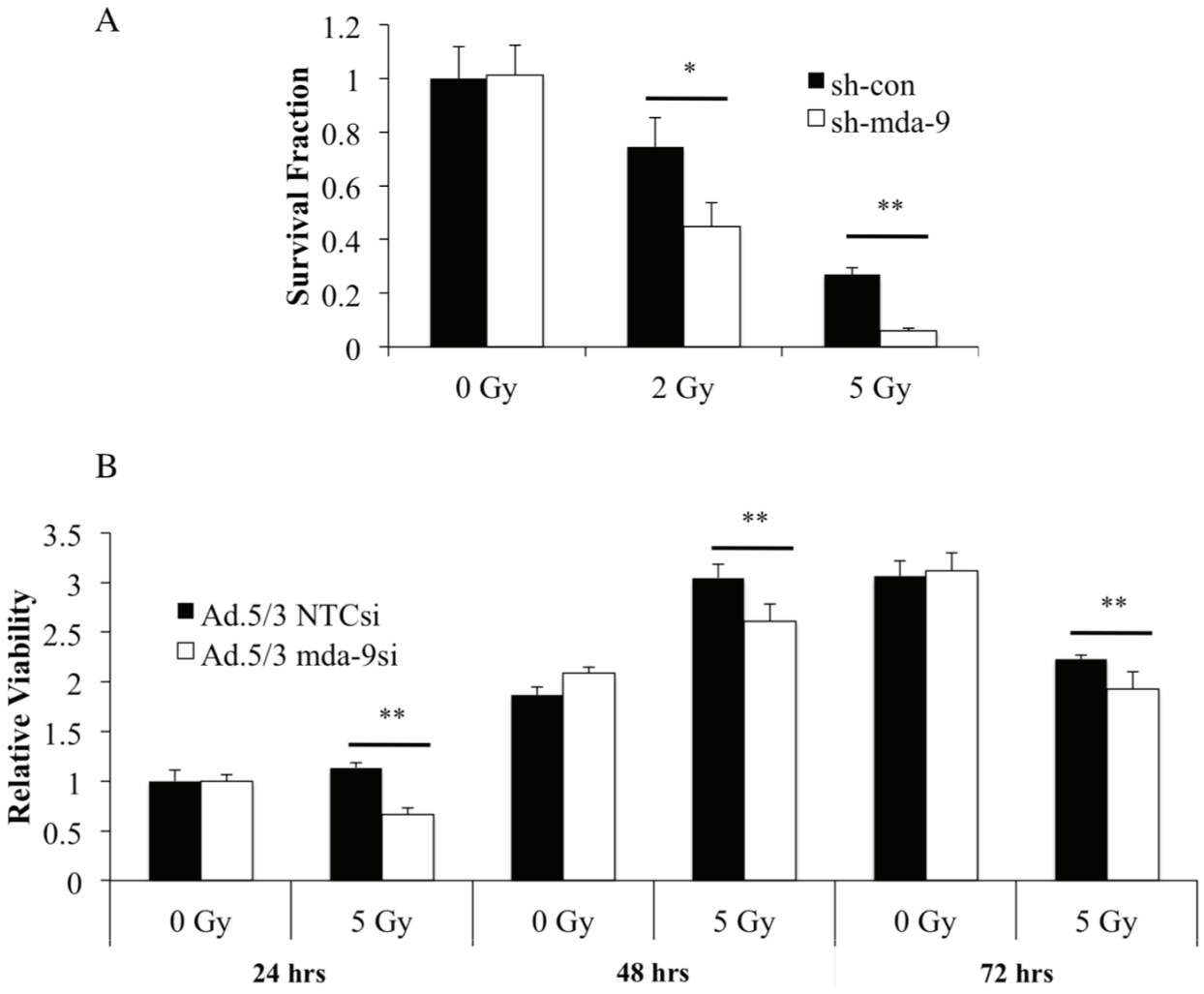
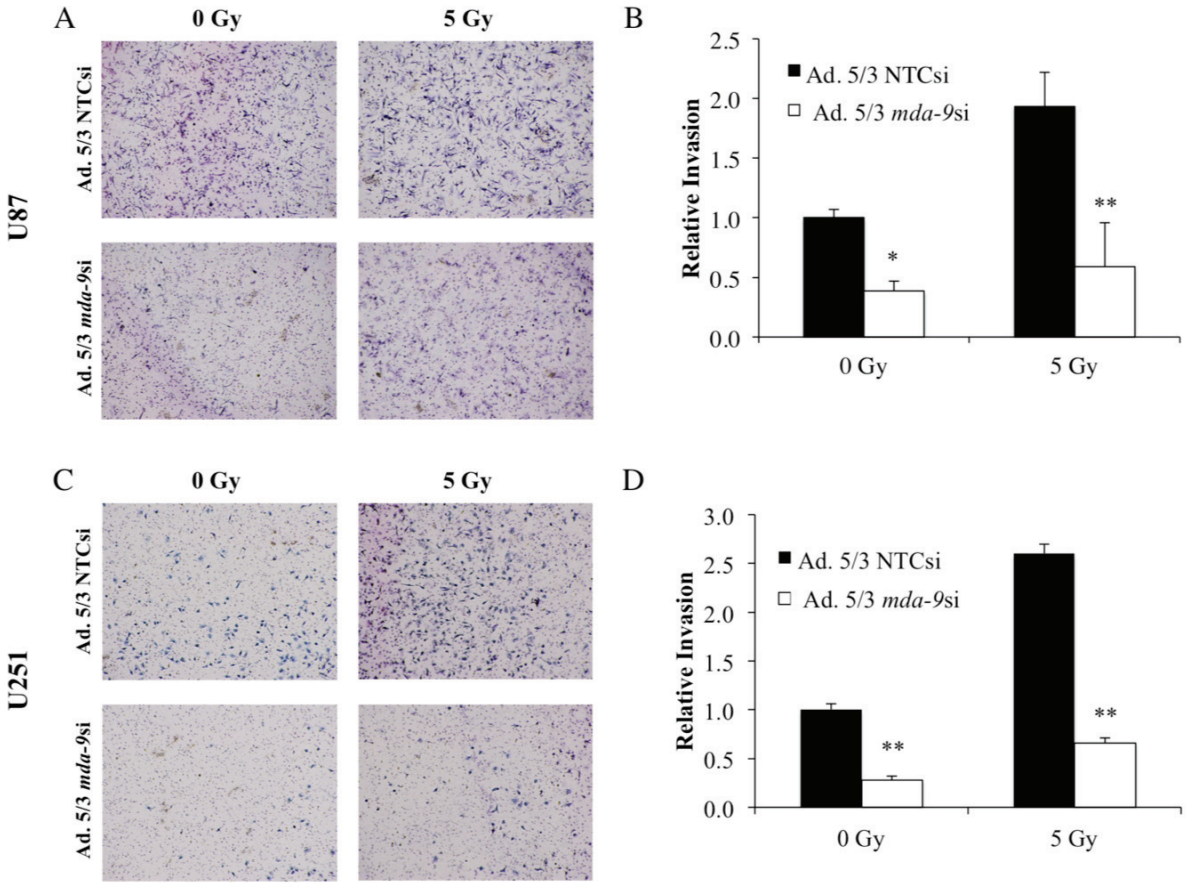


Figure 4.2 Knockdown of MDA-9/Syntenin radiosensitizes GBM. A, U1242-shcon and U1242-sh*mda-9* cells were analyzed for colony formation 14 days post radiation treatment. **B,** U1242 cells treated with either Ad.5/3 NTCsi or Ad.5/3 *mda-9*si were analyzed for viability at the indicated time points via MTT assay. * $p < 0.05$, ** $p < 0.01$.


A
0 Gy
5 Gy
U87
Ad. 5/3 NTCsi
Ad. 5/3 mda-9si
B
Relative Invasion
Ad. 5/3 NTCsi
Ad. 5/3 mda-9si
0 Gy
5 Gy
C
U251
D

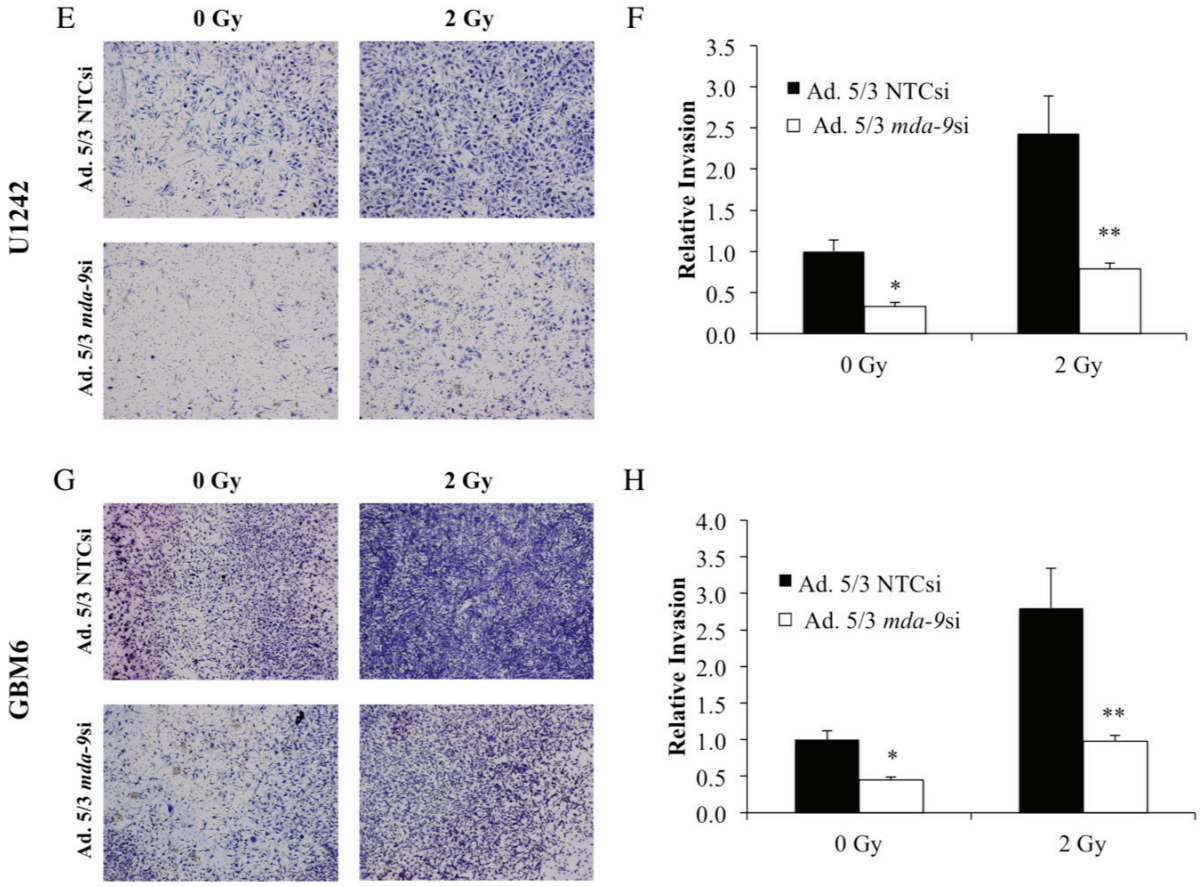


Figure 4.3 MDA-9/Syntenin knockdown inhibits radiation-induced invasion. U87, U251, U1242, and GBM6 cells were treated with either Ad.5/3 NTCsi or Ad.5/3 *mda-9*si and radiated 48 hours later. These cells were then seeded in a trans-well Matrigel invasion assay and stained after 24h. Representative images are shown in **A, C, E, G,** and quantified respectively from 5 random fields in **B, D, F, H**. Error bars = ± s.d. * $p < 0.05$, ** $p < 0.01$.

Knockdown of mda-9/syntenin inhibits Src and EphA2 activation post-radiation

MDA-9/Syntenin has been shown to amplify Src and NF-κB signaling in melanoma and glioma (15, 25, 57, 461). Src is a recognized enhancer of invasive signals in cancer settings and has demonstrated involvement in post-radiation signaling (497). While a modest changes in MDA-9/Syntenin levels 24 h post-radiation was observed, we detected a significant increase in levels of activated Src. MDA-9/Syntenin knockdown reduced those activation levels in each of the GBM cell lines tested (Figure 4.4). EphA2 is a member of the Eph-receptor family, the largest subfamily of receptor tyrosine kinases (RTKs), and are involved in numerous cellular processes including angiogenesis and motility (499). EphA2 overexpression correlates with poor prognosis and increased metastasis, is implicated in the pathogenesis of numerous tumors, including those of the brain (500). Important to our findings, EphA2 has been shown to interact with Src in invasion signaling (97), is upregulated following radiation, and enhances the malignant phenotype in melanoma (501). We found total and activated EphA2 levels to be increased after radiation exposure, yet MDA-9/Syntenin inhibition reduced these gains effectively in each case (Figure 4.4). Since MDA-9/Syntenin inhibition negated the gains in Src and EphA2 signaling post-radiation, it may be an effective target in controlling radiation-induced pathogenesis.

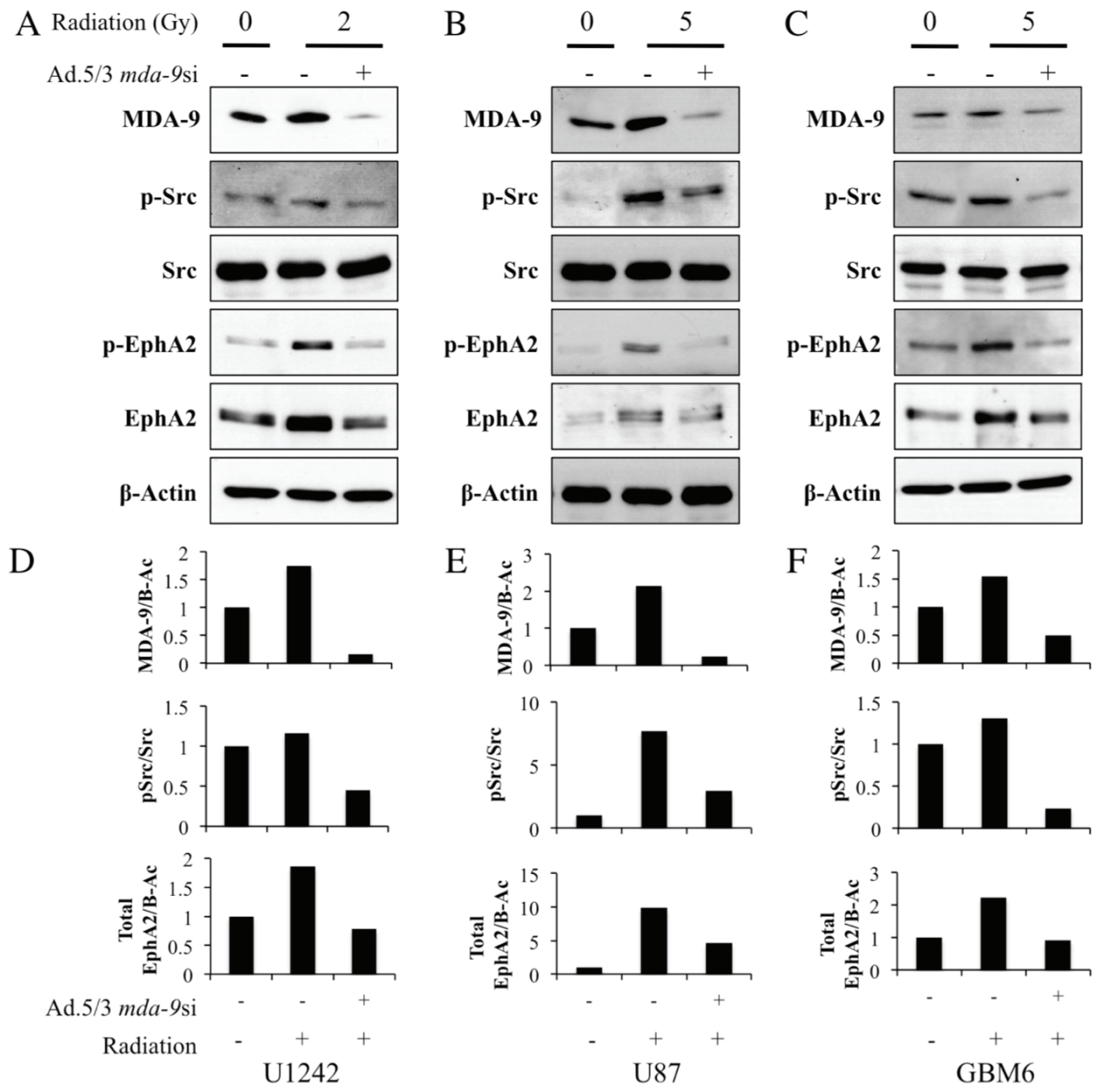


Figure 4.4 Inhibition of MDA-9/Syntenin impairs Src-EphA2 signaling. A-C. Immunoblot analysis of GBM cells treated with either Ad.5/3 NTCsi or Ad.5/3 *mda-9*si and radiated 48 hours later. Cell lysates were collected 24h post-radiation. Changes in protein levels were quantified in **D-F**. β-actin used as protein loading control.

A novel small molecule targeting MDA-9/Syntenin inhibits invasion and radiosensitizes GBM

To develop a deliverable agent that could effectively inhibit the action of MDA-9/Syntenin, we collaborated with the lab of Maurizio Pellecchia of the Sanford Burnham Medical Research Institute, who used a Fragment Based Drug Discovery (FBDD) approach. FBDD is an important technique used with success in recent efforts to develop small molecule inhibitors of the anti-apoptotic Bcl-2 family proteins(502). A library design method based on subsequent detection of target-mediated ligand-ligand transferred nuclear Overhauser effects (ILOEs) was used in this process(492). After collecting a set of initial hits from a 5,000 compound library, traditional medicinal chemistry and structure activity relationships (SAR) enhanced the affinity of first site ligands from millimolar to high micromolar ranges. PDZ1i (113B7) represents the initial, high-affinity bi-dentate compound that resulted from these steps when targeting the PDZ1 domain of MDA-9/Syntenin.

Initial studies with PDZ1i revealed that it effectively inhibited MDA-9/Syntenin – induced invasion in ImPHFA cells following MDA-9/Syntenin overexpression (Figure 4.5A). Moreover, PDZ1i treatment reduced invasion in T98G and U87 cells (Figure 4.5B).

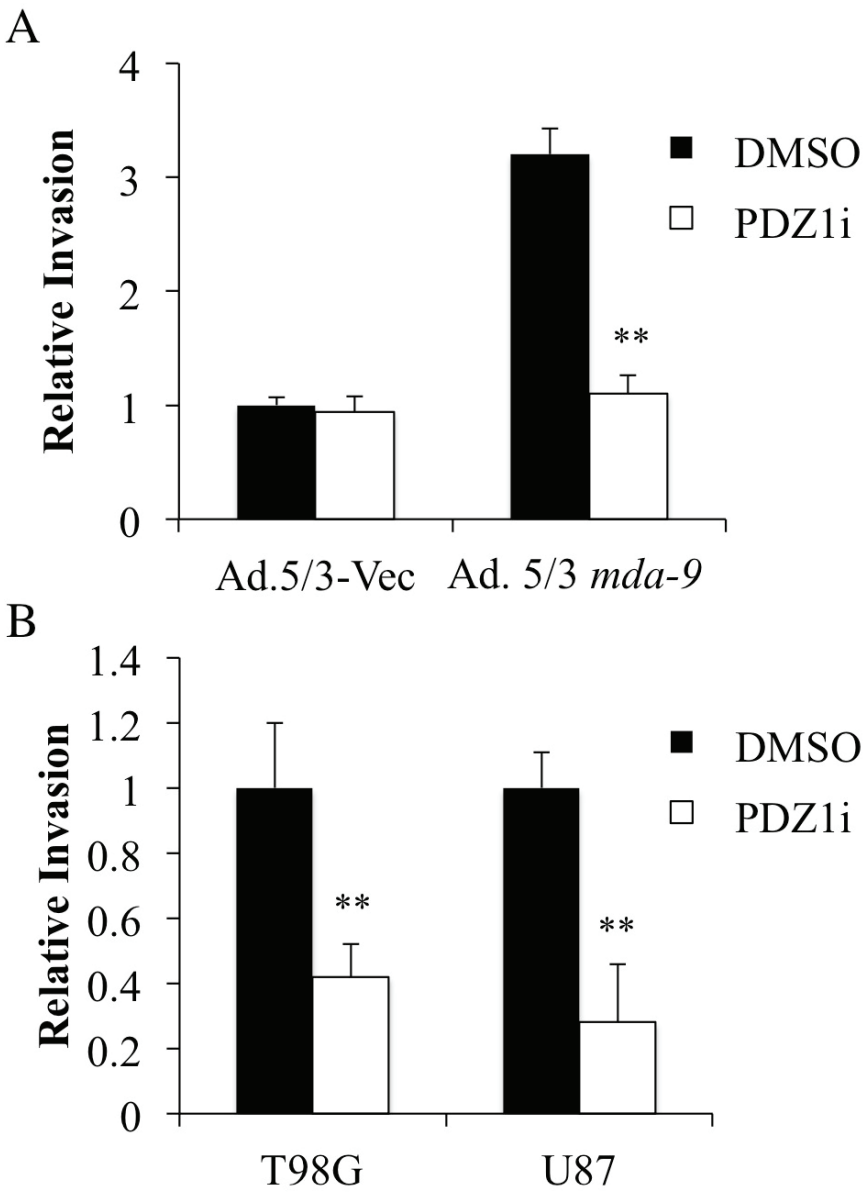


Figure 4.5 PDZ1i inhibits mda-9/syntenin-mediated invasion. Im-PHFA, primary immortal human fetal astrocyte cells were infected with an mda-9/syntenin expression plasmid carrying adenovirus. 48 h post-infection both the control and mda-9/syntenin overexpressing cells were treated with DMSO or 50μM PDZ1i followed by seeding in a trans-well Matrigel invasion assay and stained after 24h. T98G and U87 were treated with DMSO or 50μM PDZ1i and seeding in a trans-well Matrigel invasion assay and stained after 24h. Invasion was quantified from 5 random fields. ** $p < 0.01$.

We then tested if PDZ1i treatment would radiosensitize glioma cells. We exposed immortalized astrocytes, ImPHFA, and GBM cell line U87 to 0, 2, and 5Gy radiation after a two hour pretreatment with PDZ1i. As expected, astrocytes showed significant radiosensitivity following radiation exposure, yet PDZ1i treatment did not further radiosensitize these cells (Figure 4.6A). However, U87 cells showed markedly more radiosensitivity when combined with PDZ1i treatment. Proliferation following radiation was also significantly decreased in U87 cells that combined radiation and PDZ1i compared to radiation with control DMSO treatment (Figure 4.6B). Finally, we exposed U87 cells to 2 and 5 Gy of radiation, with or without PDZ1i pretreatment. Radiation exposure increased the ability of these cells to invade, while PDZ1i pretreatment negated these invasion gains (Figure 4.7).

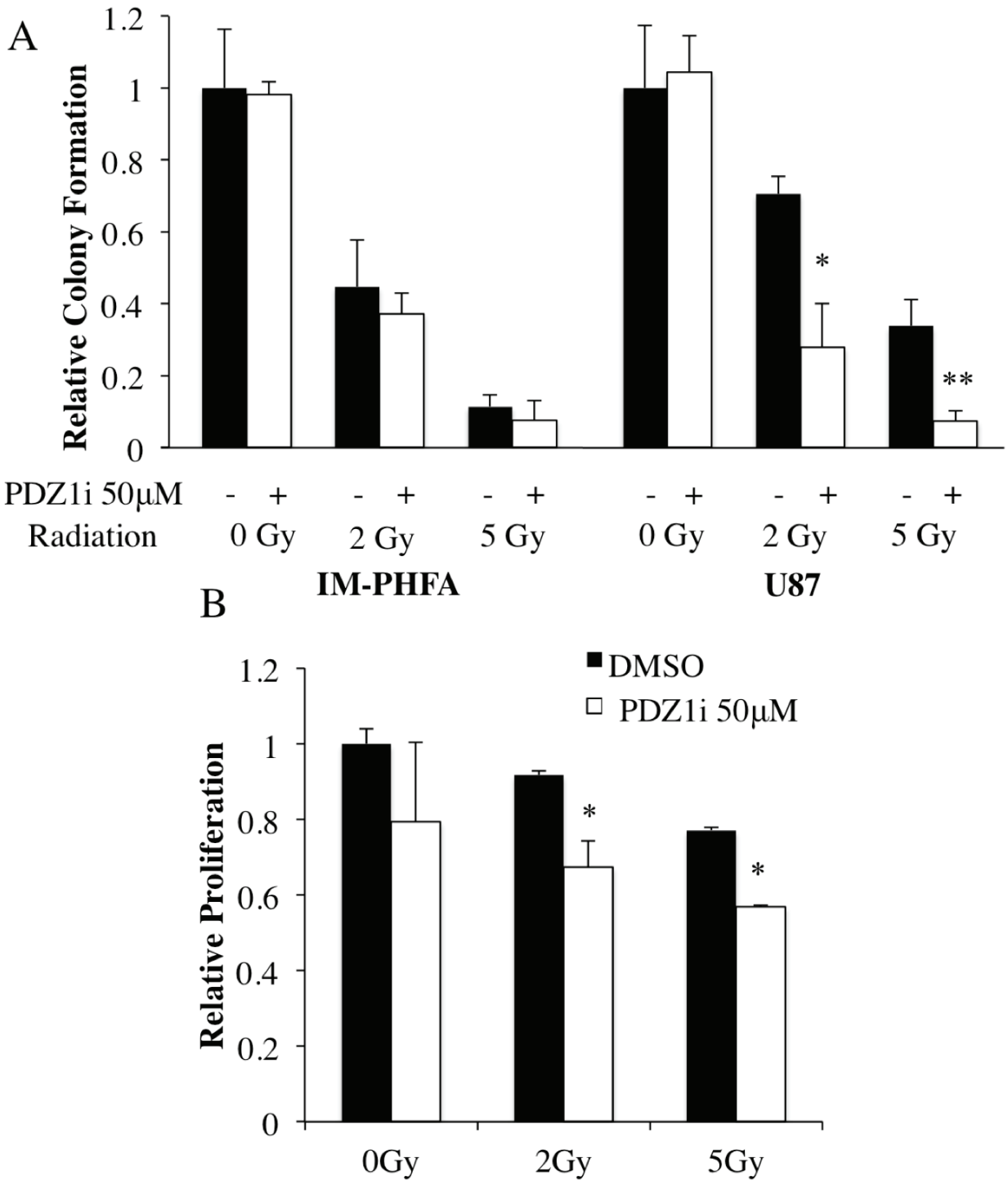


Figure 4.6 PDZ1i treatment inhibits colony formation and proliferation post-radiation. A, ImPHFA and U87 cells were treated with 50μM PDZ1i 2 hours prior to radiation and analyzed for colony formation after 14 days. **B,** U87 cells were treated with 50μM PDZ1i 2 hours prior to radiation and analyzed via MTT assay after 24 hours. * $p < 0.05$.

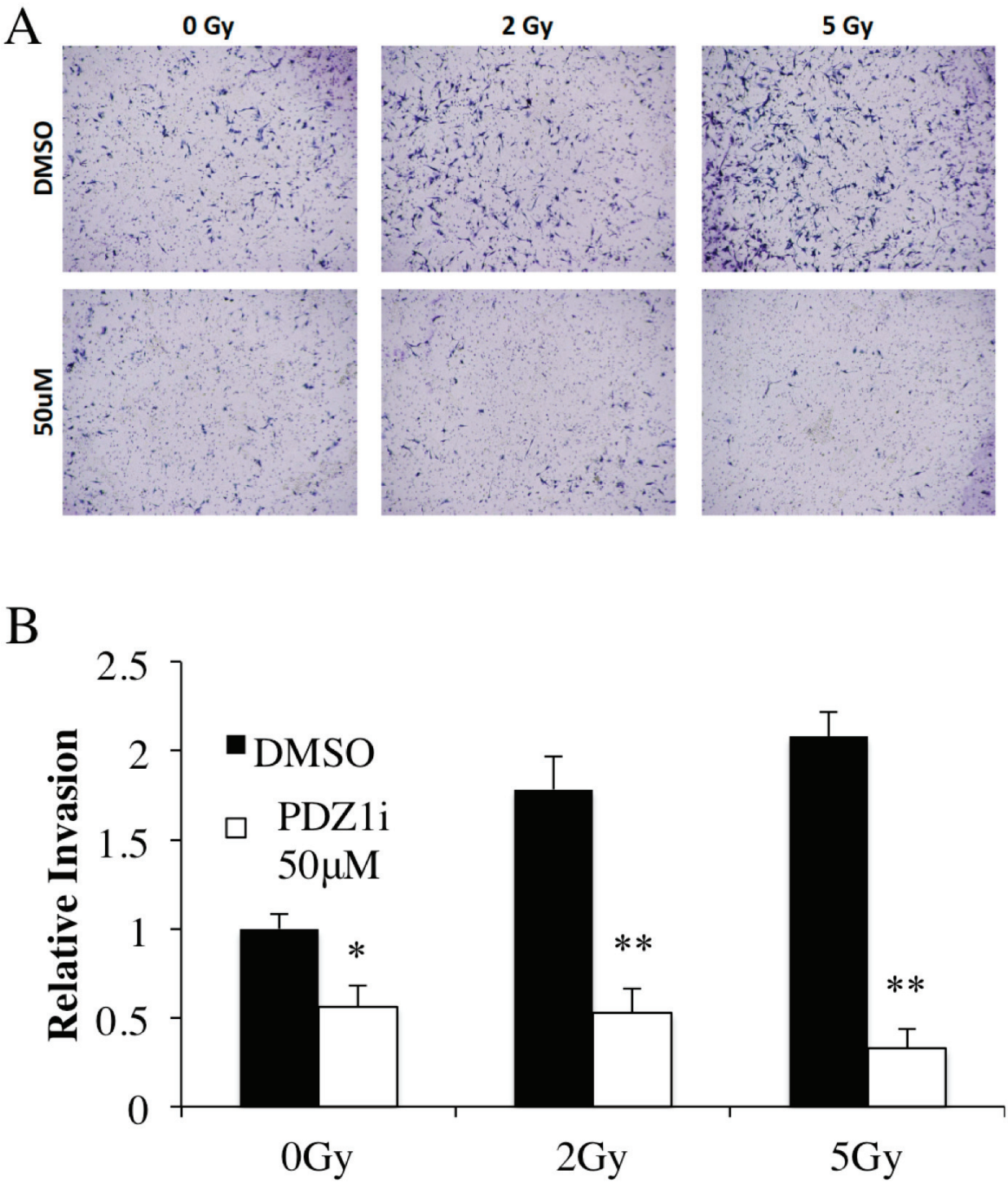


Figure 4.7 PDZ1i inhibits radiation-induced invasion. A, U87 cells were pretreated with 50μM PDZ1i 2 hours prior to radiation and subsequently seeded in a trans-well Matrigel invasion assay and stained after 24h. **B,** Invasion was quantified from 5 random fields in. * $p < 0.05$, ** $p < 0.01$.

PDZ1i inhibits EGFRvIII-driven signaling in GBM and reduces MMP secretion

EGFR amplification and mutation is both a common and important alteration in GBM(120, 503). A particular mutation of this receptor, EGFRvIII, is often co-expressed along with EGFR amplification in GBM. Recent data has demonstrated that EGFRvIII and FAK interact as part of a complex to mediate EGFRvIII-mediated MAPK activation(504, 505). Given the demonstrated role of MDA-9/Syntenin in enhancing downstream signaling of the FAK/Src complex in melanoma (25), we asked if PDZ1i could inhibit EGFRvIII signaling in GBM. We used U87 cells stably expressing EGFRvIII, U87-EGFRvIII (Figure 4.8A), and treated them with PDZ1i prior to radiation. In both radiated and non-radiated cells, phospho-EGFR was significantly reduced in the presence of PDZ1i. FAK activation was enhanced after radiation, and this activation was negated by PDZ1i treatment (Figure 4.8). Downstream, we observe that NF-κB activation, enhanced following radiation, is reduced in the presence of PDZ1i. From these findings, we can gather PDZ1i disrupts EGFRvIII-FAK signaling, ultimately reducing observed post-radiation gains in NF-κB activation.

Secreted factors can have a significant impact on cancer cell invasion, and MDA-9/Syntenin can affect the secretion of important factors such as MMP2 and VEGF in glioma (50). Radiation can enhance the release of important enzymes, including other members of the MMP family(497). We asked if PDZ1i could affect the secretion of invasion-related proteins following radiation therapy. U1242 cells were treated for 2 h prior to radiation therapy, and their media collected after 48 hours. Several MMP family members showed a significant increase in expression following radiation therapy, including MMP2 and MMP9, both accepted as promoters of GBM-related invasion. PDZ1i treatment reduced the levels of these enzymes

following radiation therapy (Figure 4.9), an important aspect of reducing radiation-induced invasion gains.

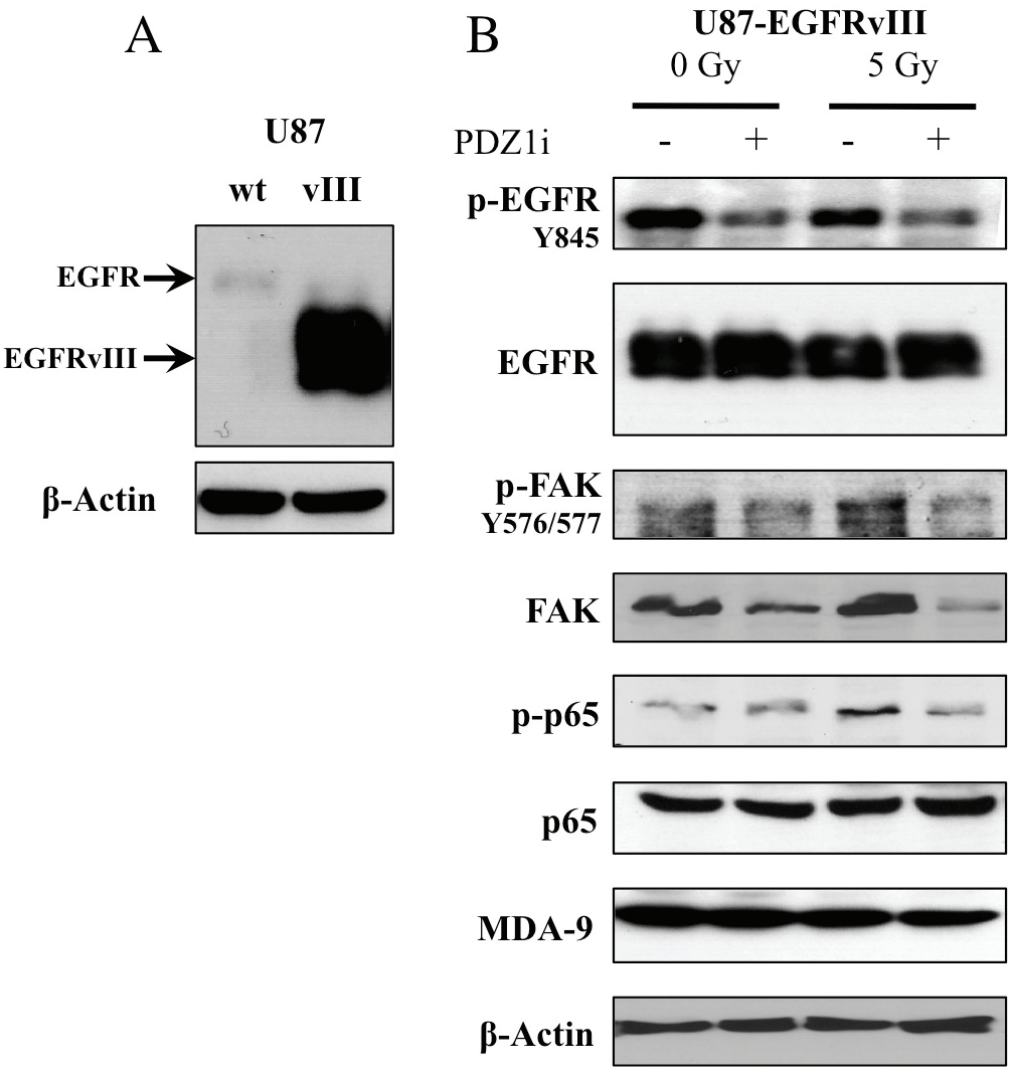


Figure 4.8 PDZ1i inhibits EGFRvIII signaling. A. Total EGFR antibody demonstrates the presence of EGFRvIII in U87-EGFRvIII cells. **B.** U87-EGFRvIII cells were treated with PDZ1i 2 h prior to radiation. Cell lysates were collected 24 h post radiation and analyzed for protein expression. Total EGFR antibody was again used to detect EGFRvIII in these cells. β-actin was used as protein loading control.

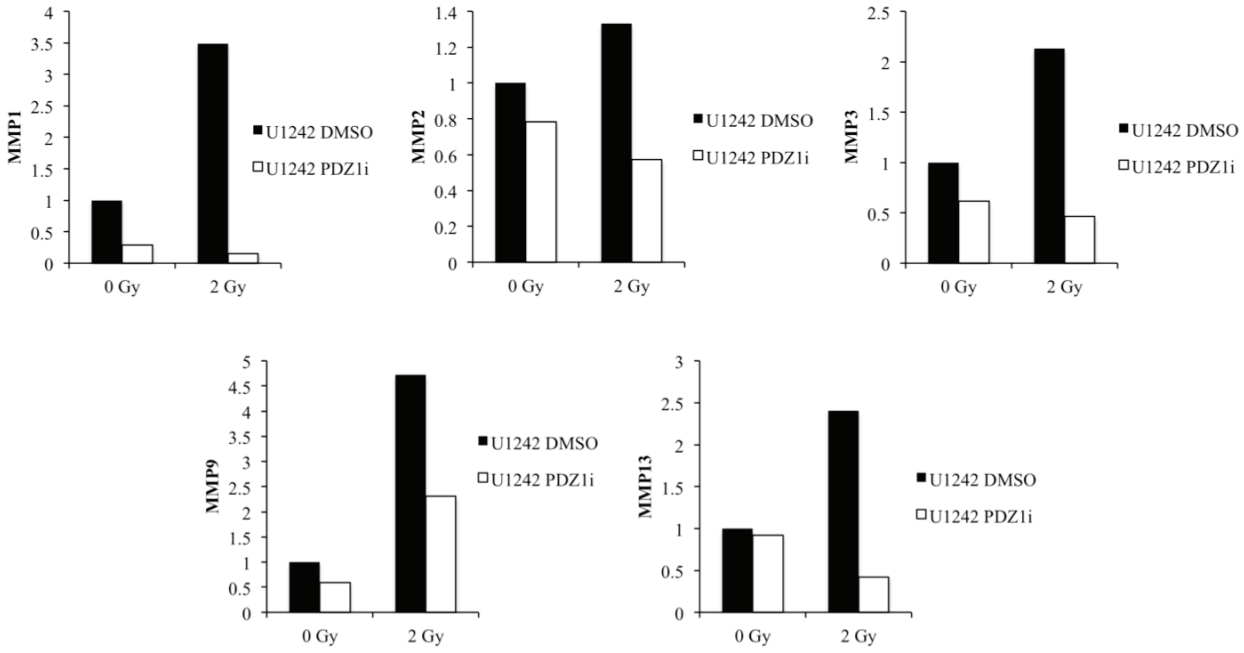


Figure 4.9 PDZ1i reverses radiation-induced expression of key MMP-family members. U1242 cells were treated with either DMSO or 50μM PDZ1i 2 hours prior to radiation in serum-free media. After 48 hours, media was collected analyzed via the Proteome Profiler Human Protease Array Kit (R&D Systems). Relative protein amounts were quantified via ImageJ.

PDZ1i crosses the blood-brain barrier to inhibit invasion

A critical property of a potential therapeutic agent when treating glioma pharmaceutically is the ability for a compound to cross the blood brain barrier. We first tested this property in an *in vitro* model of the BBB by seeding human brain microvascular endothelial cells (HBMEC) in the upper chamber of transwell inserts placed on top of wells containing GBM6 cells. After 24 hours, we assessed the invasive ability of the treated GBM6 cells compared to pretreated controls. PDZ1i effectively crossed the HBMEC barrier to inhibit invasion in GBM6 cells comparably to the pretreated control with no barrier (Figure 4.10). Taken together, these results suggest PDZ1i is a potent inhibitor of invasion with radiosensitizing effects and favorable properties for CNS treatment. Thus, we moved our assessment of PDZ1i as a potential therapeutic agent into *in vivo* models of GBM.

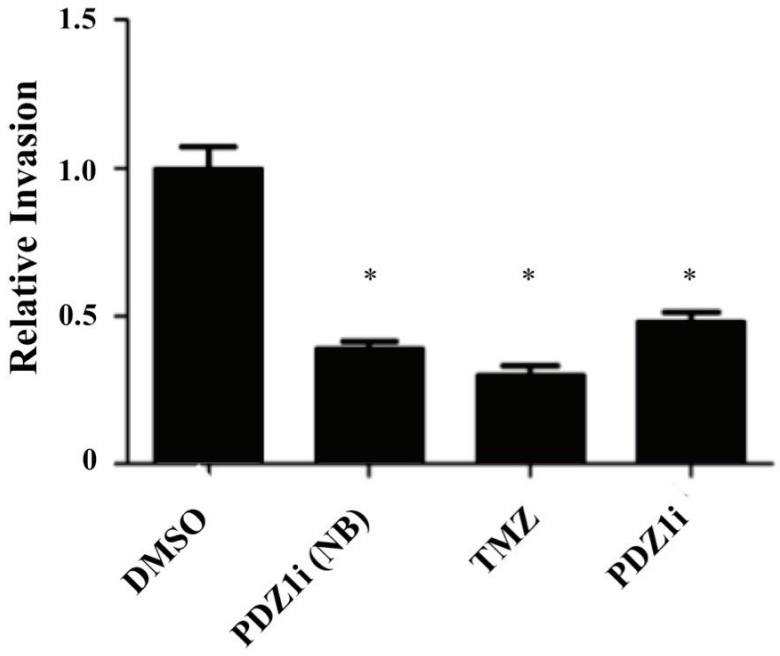


Figure 4.10 PDZ1i penetrates endothelial barrier *in vitro*. Primary human malignant glioma cells (GBM6) were seeded on the bottom well of a transwell chamber. In the top chamber, a monolayer of HBMEC cells separated GBM6 cells from media containing DMSO, 50 μM PDZ1i or Temozolomide (500 μM). After 24h incubation in these conditions, invasion of GBM6 cells were analyzed by seeding in a trans-well Matrigel invasion assay and stained after 24h. NB: PDZ1i treatment and invasion assay without HBMEC barrier. * $p < 0.05$.

To test the efficacy of PDZ1i, we first used an orthotopic xenograft model with GBM6 cells that were pretreated for 2 h with either DMSO or PDZ1i and injected intracranially to form tumors. 7 days post injection, tumor and brain tissue was isolated for sectioning. Tumor cells treated with PDZ1i developed noticeably smaller and more demarcated neoplasms at this time point compared to controls (Figure 4.11A). Separately, untreated cells were injected and tumors established, then mice were treated I.P. with either DMSO or PDZ1i (30mg/kg) three times per week for two weeks. Survival was significantly increased in treated mice compared to controls (Figure 4.11C), and tumors were well circumscribed and less infiltrative on analysis than those of control treated mice **(**Figure 4.11B). Similar to our previous studies knocking down MDA-9/Syntenin expression in an *in vivo* model (50), PDZ1i is effective in reducing tumor invasion and extending survival in an animal model of GBM.

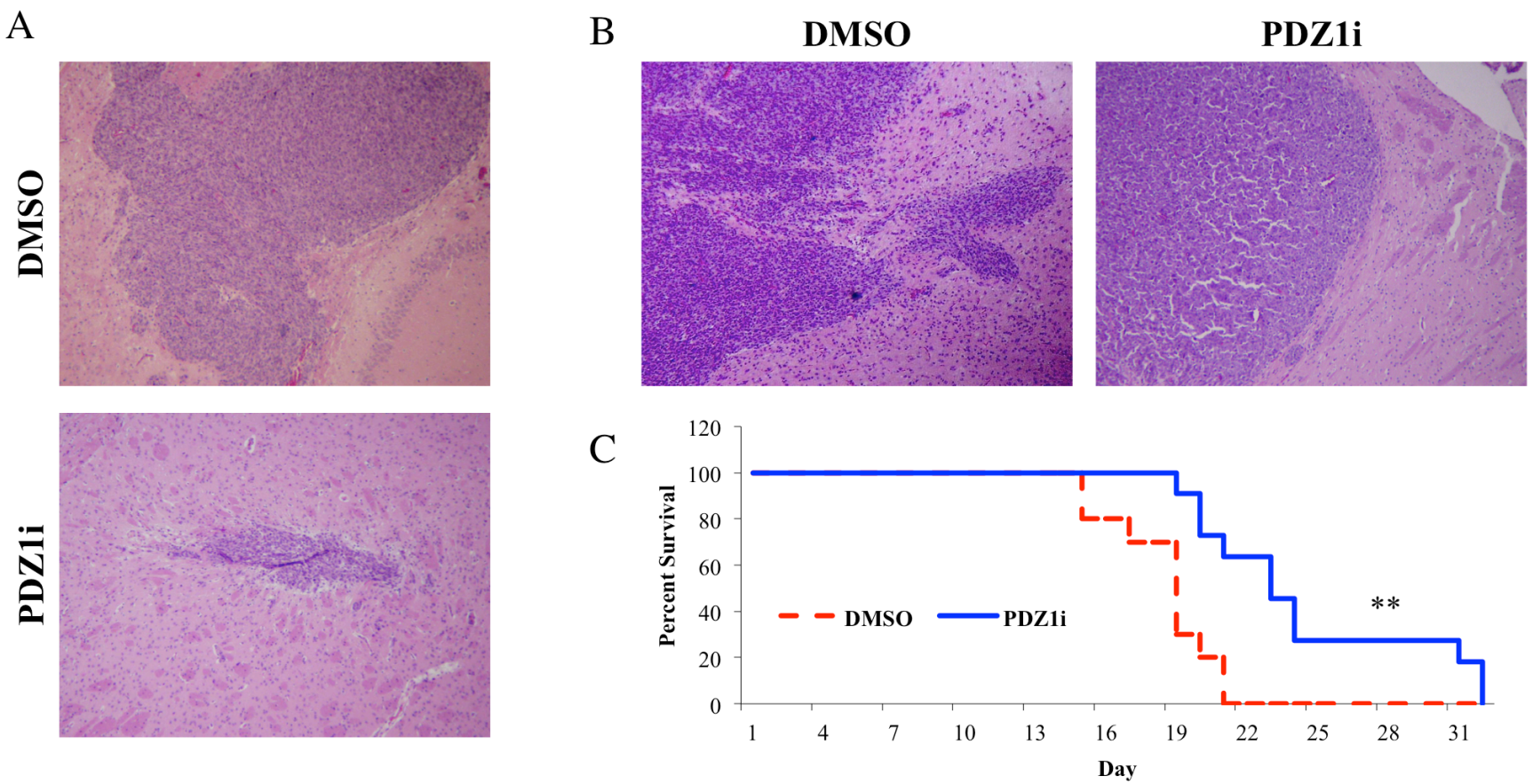


Figure 4.11 Effect of PDZ1i on survival in an *in vivo* model of glioma. A. GBM6 cells were pretreated for 2 hours prior to intracranial injection. After 7 days, brain tissue was isolated and sectioned at injection site. **B.** 7 days after tumor implantation, mice received either vehicle or PDZ1i (30 mg/kg) three times per week for 3 weeks. Brain tissue was isolated and analyzed via H&E stain. **C.** Kaplan-Myer curves of these groups based on animal survival. ** $p < 0.01$.

PDZ1i combines with radiation to extend survival and reduce GBM invasion in vivo

We next investigated the combination of PDZ1i with radiotherapy *in vivo*. U1242-luc cells were injected intracranially under anesthesia and formed tumors, confirmed by imaging at 7 days post injection. At this point, mice were randomized to 4 groups: DMSO treatment, PDZ1i treatment (30mg/kg), DMSO + IR, and PDZ1i + IR. Treatment began on day 11 post injection and mice receiving radiation were treated with 2.5Gy for 4 consecutive days (Figure 4.12A). PDZ1i was administered 2 hours prior to radiation treatment on each of the 4 treatment days (Figure 4.12A). Control treated mice had an average survival of 41.3 days while PDZ1i treatment extended that to 54.7 days. Radiation alone extended survival to 62.8 days while PDZ1i treatment + IR therapy led to survival of 78.8 days (Figure 4.12B). Combination therapy was significantly better than control or PDZ1i alone ($p = 0.01$), and though not statistically significant in this trial, a clear trend emerges in extending survival when combining PDZ1i with radiotherapy. Finally, we asked if there were changes in tumor morphology and invasion pattern between treatment groups. To investigate this, brain tissue sections were collected and H&E stains were analyzed. Indeed, U1242 cells developed diffusely infiltrating tumors in control groups. Tumor margins were slightly more demarcated in groups treated with PDZ1i (Figure 4.13). Radiation treatment led to generally smaller, yet still diffuse tumor patterns, which frequently crossed the midline of the brain with invasive outgrowths. The combination of PDZ1i with radiation led to tumors with markedly more circumscribed margins, less invasive outgrowths, and less spread to the leptomeninges (Figure 4.13). This data leads us to conclude that targeting MDA-9/Syntenin is a promising approach to enhancing conventional radiation treatment.

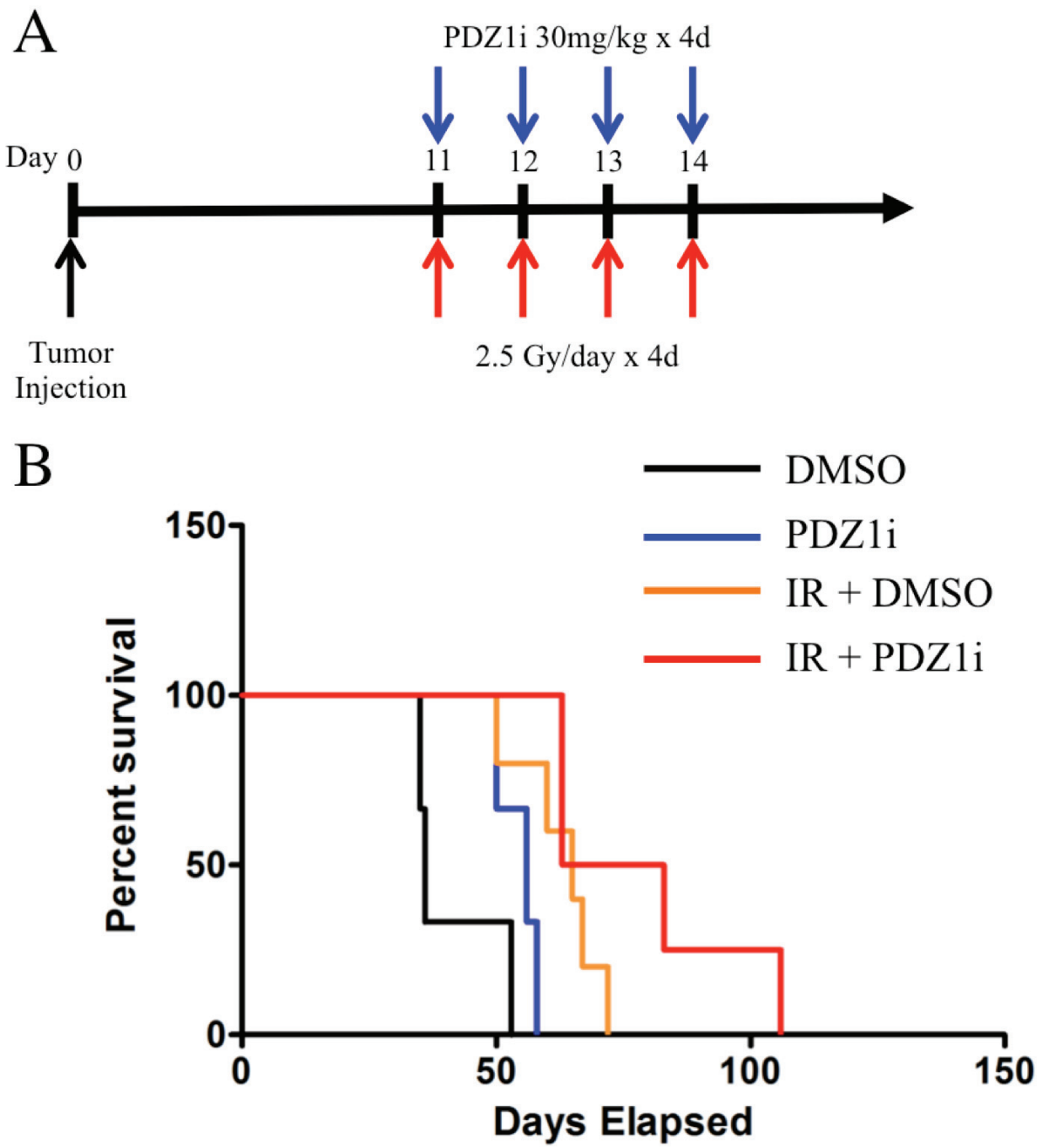


Figure 4.12 PDZ1i treatment combined with radiation in an *in vivo* model of GBM. A. U1242 cells were injected intracranially into nude mice. After 7 days mice were randomized to 4 groups, and mice receiving therapy were treated on days 11-14 as pictured. **B.** Kaplan-Meier survival curves for each treatment group.

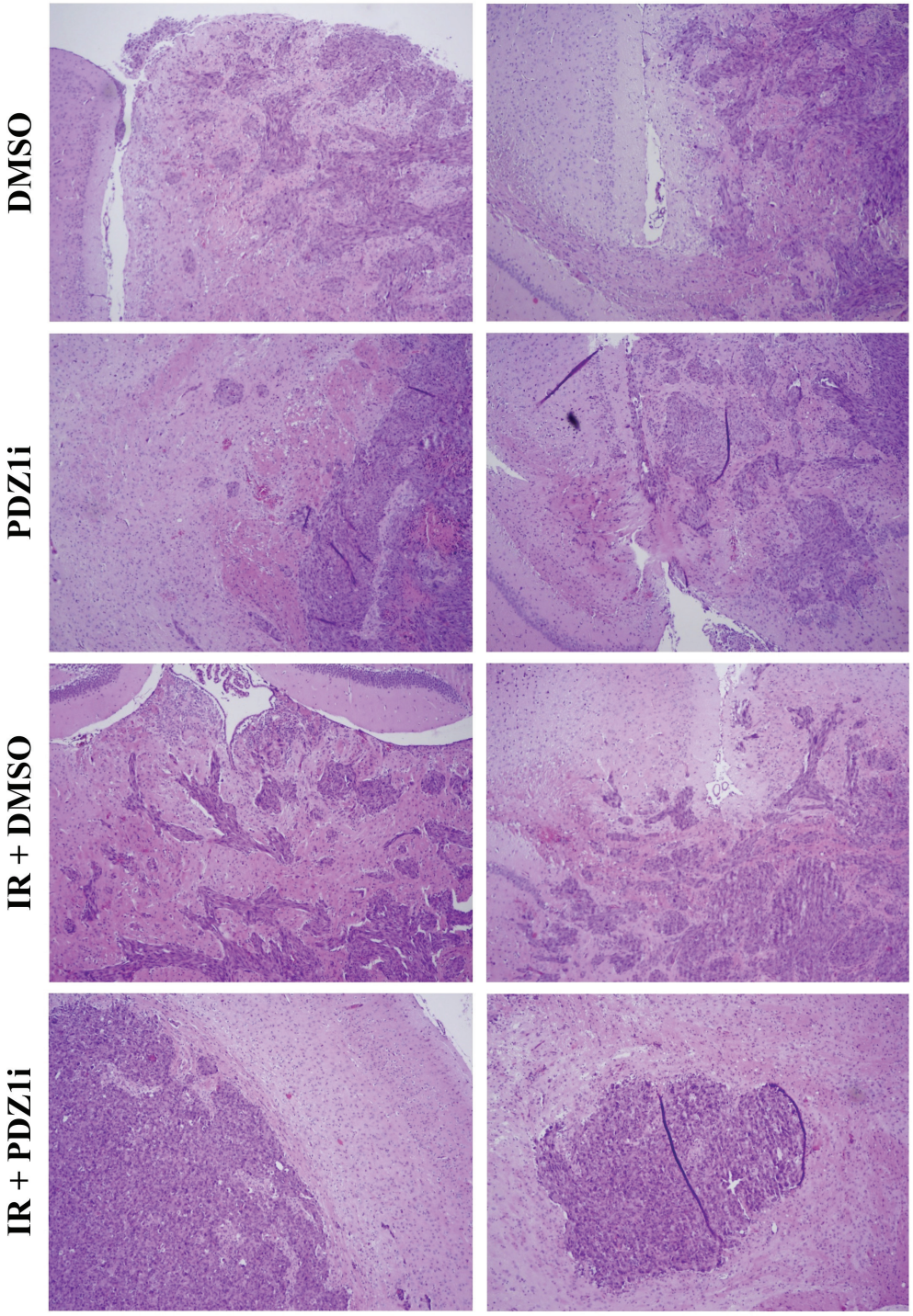


Figure 4.13 PDZ1i combined with radiation *in vivo*. U1242 cells were injected in R Hemisphere and treated with PDZ1i, radiation, both, or neither. Above, H&E sections of brain tissue collected from each group. Note the changes in tumor margins and invasive fronts in each.

IV. Discussion

We demonstrate that MDA-9/Syntenin is an important mediator of the post-radiation signaling process, negating the invasion induced by radiation. Our database analysis of REMBRANDT clinical samples support the idea that high MDA-9/Syntenin expression can lead to a more radioresistant phenotype compared to tumors with lower MDA-9/Syntenin expression. Interesting links in glioma to MDA-9/Syntenin related signaling have emerged in recent reports. A study of database expression profiles comparing long term survivors (LTS) of GBM (>48 months) to short term survivors (<12 months) revealed a 40% decrease in IGFBP2, a noted downstream product of MDA-9/Syntenin signaling (59), in LTS tumors (506). Additionally, identification of a radiosensitive gene signature via expression data from the NCI-60 cancer cell panel revealed that known MDA-9/Syntenin binding partner integrin linked kinase (ILK) (79) was significantly downregulated in radiosensitive cells (507). An ideal complement to radiotherapy would sensitize tumor cells to the cytotoxic effects of radiation while negating the unwanted pro-invasive effects. We establish MDA-9/Syntenin as a promising target by showing that knockdown potentiates radiosensitivity, and abrogates radiation-induced invasion.

MDA-9/Syntenin functions to activate multiple signaling pathways, including enhancing Src signaling. Src is known to be involved in numerous cancer-related processes, including survival, motility, and invasion(508). Especially relevant for GBM, Src interacts with EGFR, including wild-type and mutant versions, and can activate EGFR through phosphorylation(508, 509). Notably, radiation induces Src-dependent activation of EGFR in GBM, leading to MMP2 expression and invasion(497). Additionally, signaling through VEGFR2 activates downstream kinases, including Src and FAK, including in response to post-radiation increases in VEGF levels, enhancing glioma cell motility(510, 511). Since numerous previous studies reported that

MDA-9/Syntenin inhibition results in a reduction of Src signaling (25, 50), we hypothesized we could inhibit radiation induced invasion signaling by targeting MDA-9/Syntenin, thus attenuating related pathways. While extensive preclinical data support the targeting of Src directly for cancer therapy, the results from clinical trials have overall been disappointing(508). Low or no response rates as a single agent or in combination have been reported in breast cancer and GBM(508), and GBM patients who have progressed while on Bevacizumab also did not respond on Src inhibitors(512). A proposed method of escape and resistance to Src inhibitors is shown through upregulation of IGFBP2/FAK signaling(513). High levels of IGFBP2 correlated with resistance, and exogenous IGFBP2 rendered cells less sensitive to dasatinib. Additionally, FAK activation has been linked to radioresistance in several studies(513-516). Targeting MDA-9/Syntenin is known to reduce FAK phosphorylation as well as IGFBP2 production in addition to Src activation. Therefore, our approach of inhibiting the action of MDA-9/Syntenin may prove to preempt methods of resistance stemming from Src inhibition.

Recent efforts have focused on revealing novel mechanisms by which cells evade the cytotoxic effects of radiation therapy. Exosome signaling has emerged as a potential pathway by which cancer cells survive under the stress of radiation damage (517, 518). Amongst exosome cargo are full-length protein receptors, ligands, RNA, and DNA, including oncogenes (518). Radiation therapy can induce greater exosome release from both GBM cells and normal astrocytes. These exosomes contain much higher levels of IGFBP2 and enhance migration in recipient cells. Furthermore, radiation increases cellular uptake of exosomes (519), and uptake following radiation promotes FAK and Src activation in target cells (518). This is especially relevant to our work since MDA-9/Syntenin gain enhances exosome yield, while MDA-9/Syntenin knockdown reduces exosome release (21). We showed that MDA-9/Syntenin

targeting is an effective inhibitor of Src activation in glioma and reduced release of MMP family proteins. Thus, PDZ1i could prove to be useful in reducing both the efficiency of exosomes release, as well as their effect on the ECM and in recipient cells. Further studies focusing on the exosome-related aspect of MDA-9/Syntenin targeting are warranted.

The combination of PDZ1i and radiotherapy in a model of GBM showed evidence of improvement over each method alone. *In vivo*, important interactions occur within the tumor microenvironment, including extracellular communication between tumor cells, but also between tumor cells and normal cells, such as endothelial cells. Recent work points towards a mechanism of radioresistance through endothelial cell FAK - NF-κB signaling (520). In normal endothelial cells within the tumor vasculature, DNA damaging therapy induced FAK activation, NF-κB translocation and activation, leading to secretion of protective cytokines and the development of a sheltering perivascular niche following radiation (520). Therefore, our PDZ1i has the potential to have positive therapeutic effects on both tumor and normal cells within the tumor microenvironment.

Overall, we show that MDA-9/Syntenin targeting is a viable approach for combating radiation-induced invasion and can radiosensitize GBM. Development of a targeted inhibitor leads to a reduction in phenotypes enhanced by MDA-9/Syntenin, and can effectively be combined with conventional radiotherapy *in vivo*. As MDA-9/Syntenin is involved in many important cancer-related cellular processes and signaling events, further investigation into the extent of action for PDZ1i will be enlightening.

Chapter 5 - Future Perspectives

Our studies demonstrate that MDA-9/Syntenin has vital roles in high grade glioma, and inhibiting MDA-9/Syntenin can reduce invasion, angiogenesis, and complement radiation therapy, adding to the growing literature that support its potential as a target for cancer treatment. Novel proteins able to be targeted pharmaceutically will be highly valuable in the continuing effort to combat GBM progression. This class of tumor presents particularly difficult obstacles for future research, but targeting MDA-9/Syntenin has the potential to aid in overcoming many of them.

The view of GBM as a heterogeneous disease with subpopulations of cells that comprise tumors emphasizes the difficulty in treating this neoplasm. While similar processes undoubtedly occur in other cancer types, the nature of GBM presents additional hurdles. A propensity for invasion, the invaluable nature of the surrounding tissue to a patient's quality of life, and the pitfalls of delivering treatment across the BBB are a few of the challenges unique to GBM. Looking ahead, we must create a tangible connection from retrospective, population based genomic characterization to the planning of treatment in individual patients by using profiling of clinical samples (521, 522).

Ample opportunities exist to improve upon the already targeted treatments developed to combat GBM. To date, EGFR and PDGFRα inhibitors have failed to show significant clinical benefit to the outcomes in patient trials (523). Although considerable effort has been put forth to

produce targeted agents that negate the oncogenic effect of these signaling pathways, the high rates of proliferation and mutation within GBM tumors lead to subpopulations that evade such treatments. Additional signaling cascades may cooperate and compensate to maintain a minimum signaling threshold that is not adequately attenuated by inhibition of a single RTK (164, 165). RTK signaling can often lead to the production of MMPs and enhanced invasive ability for GBM cells. However, even MMP inhibition through marimastat treatment did not show efficacy in a clinical trial setting. Much more work remains to investigate possible compensatory methods with which GBM cells evade current treatments. The combination of non-redundant treatment options that address multiple pathways involved in invasion and angiogenesis will be necessary.

A number of recent studies have been aimed at defining the importance of cells of origin in gliomas, and the role of MDA-9/Syntenin in regulating cancer stem cell populations in the tumor environment requires investigation. Targeting this population is the focus of numerous current efforts, as these cells are often responsible for recurrence, invasion, or in other cancer types, metastatic events. Further investigation into the plasticity of tumor cells, or their ability to de-differentiate, and how MDA-9/Syntenin may play an influential role could yield another aspect of this protein that makes it an attractive focus for drug development. In what is a recurring trend for this tumor, a heterogeneous population appears to be responsible. As we progress towards a more complete understanding of developmental gliomagenesis, comprehensive genetic characterization and deep sequencing efforts will provide insight into the underlying programs that govern the most aggressive GBM phenotypes. The ability to take inventory of differences in epigenetic signatures and transcriptional expression profiles will result in the identification of novel targets for therapy. This next generation of therapeutics will

have to attack the capability of cells to maintain multipotentiality, self-renewal, and de-differentiation, which all contribute to the overall aggressive nature of the tumor.

Initial screens for novel compounds that may be active in inhibiting multiple signaling pathways will be aided by the ability to perform more high-throughput type analysis *in vivo*. The development of glioma models in *Drosophila* and zebrafish will aid in achieving these goals. Ultimately, screens of compound libraries in whole organism settings could be used to analyze potential targets for GBM treatment. In zebrafish, such a method for performing high-throughput screening for anti-angiogenic compounds has already been described, identifying and validating the involvement of phosphorylase kinase subunit G1 (PhKG1) (524). Further, this target was shown to be upregulated and copy number alterations demonstrated in human tumor samples. In *Drosophila*, global *in vivo* RNAi screening has been defined in the context of heart function regulators. The critical function of CCR4-Not components was discovered and subsequently validated in a *not3* heterozygous knockout mouse (525). Similar approaches utilizing organisms conducive to high throughput analysis, such as *Drosophila* and zebrafish, could be highly valuable in the screening for novel genetic targets and compound library analysis for therapeutic development in GBM. Follow-up studies investigating prospective genes or novel molecules in mouse models could lead to higher rates of success in developing candidate therapies for clinical trials.

As we develop animal models to more closely reflect the disease progression observed in humans, the possibility of combining advanced techniques with cutting edge imaging techniques will be particularly useful. As described previously, a xenograft model utilizing resection techniques recapitulates the surgical intervention commonly incorporated into management of human GBM (451). One could envision the combination of this approach in a transgenic mouse

model, especially when employing viral gene transfer. While traditional transgenic mouse models often show multifocal tumor development throughout the brain, through the use of viral gene transfer, the likelihood of more focused tumorigenesis is achieved. Monitoring of tumor development through imaging techniques could be coupled with a resection protocol, ultimately leading to the development of a model that imitates the local recurrence after surgical resection observed in humans. Novel approaches to reduce this phenomenon could lead to methods to combat the most lethal aspect of GBM.

Expanding the utilization of veterinary clinical studies opens another avenue to gain insight into novel treatments and protocols for human patients. A closer evolutionary relationship exists between the canine genome and humans than the mouse, and dogs have four times the rate of spontaneous intracranial neoplasia (15-20 per 100,000 canines per year) when compared to humans (526-529). Additionally, the typically advanced age of 8.6 years at the time of astrocytoma diagnosis mirrors the generally older patient typical in human GBM (530). GSCs have been identified (531) and overexpression of EGFR, PDGFRα, and IGFBP2 were demonstrated (532) in spontaneous canine GBM. No gold standard of care is established for canines undergoing treatment for GBM, which allows for the opportunity to introduce earlier testing of novel treatments in veterinary clinical trials (533). As with any veterinary clinical trial, the number of subjects available for testing would be dependent on pet owners' consent. The relatively low numbers of subjects balanced by the flexibility of the treatment options puts the use of spontaneous cases of canine GBM in a niche between mouse modeling and human clinical trials. While no animal model completely replicates all the characteristics of human disease, each are useful for both investigating glioma pathogenesis and preclinical screening of novel therapeutic strategies.

Outside of GBM and melanoma, MDA-9/Syntenin has become a protein of interest in a variety of areas within the cancer field and in other concentrations due to its diverse subcellular functions. Early studies of MDA-9/Syntenin focused on its interaction partners, mainly through its PDZ domains. Recent efforts have shed light on the structure and function of its N- and C-terminal domains, and the roles they play in regulating conformation and binding patterns. Of particular interest is a mechanism describing how MDA-9/Syntenin dimers can bind ubiquitinated proteins, pointing towards a process by which MDA-9/Syntenin can facilitate the interaction of proteins for which it does not have direct binding sites, but can interact with the Ub that the target displays. These studies, and others elucidating the role of N- and C- terminal phosphorylation, provide much needed information on how MDA-9/Syntenin is regulated post-transcriptionally. However, the details of transcriptional regulation have not yet been elucidated. It has been observed that *mda-9/syntenin* is not often amplified, nor does hypomethylation of its promoter appear to be a factor (49). Nonetheless, it is abundantly clear through numerous studies that the expression of MDA-9/Syntenin increases with tumor grade in a variety of cancer types. Investigation into possible promoter regulation will be warranted for future work. Additionally, the possibility of microRNA regulation should be explored. To date, only one publication mentions regulation by a miRNA, in this case miRNA-155, targeting *mda-9/syntenin* and resulting the breakdown of the BBB during neuroinflammation (534). However, this regulation is not characterized with the veracity of many miRNA-based regulatory processes. Exploring candidate miRNAs could be constructive in uncovering additional layers of regulation. Continued work into these areas will be worthwhile as we learn more about this versatile cellular adapter.

An enhanced focus of the cancer field on invasion research is evident in the numerous studies that pursue the underlying mechanisms of cellular pathways commandeered by tumors. This effort will have a significant impact in extending progression free survival and preventing relapse, as treatment resistance and invasion lead to the deadliest sequelae of cancer: damage of nearby normal tissue and the formation of distant metastases. Since MDA-9/Syntenin is now recognized to be a crucially involved in invasive signaling, it will play a prominent role in future treatments targeting invasion. As more is uncovered about the mechanics of tumor invasion, the more these pathways overlap with developmental programs, which are normally under tight regulation in stem and progenitor cells. Cancer cells successful in invasion have circumvented the regulatory pathways preventing this process, and utilize relevant molecules to break away from the primary tumor. The involvement of MDA-9/Syntenin as a facilitator of key developmental processes in a range of tissues supports its role observed in invasive cancer cells.

To gain a more complete understanding of MDA-9/Syntenin's functions and relationships within different cellular contexts, the development and evaluation of transgenic and knockout mice will prove highly useful. Crossing these mice with spontaneous models of tumor formation could prove particularly beneficial for analyzing the role of MDA-9/Syntenin in early tumorigenesis. Even combination with syngeneic models could be valuable as this could isolate effects on tumor growth and pathogenesis inside a tumor microenvironment lacking or overexpressing MDA-9/Syntenin.

Notably, the recent observations of a role of MDA-9/Syntenin in exosome biology could be an area of focus in the near future. Tumor cells have been shown to induce invasion through secreted factors, and some cancer therapies, including radiation, can enhance the release of exosomes. This can be through targeting both adjacent tumor cell populations, or influencing the

behavior of the surrounding microenvironment. Uncovering related roles of MDA-9/Syntenin in the context of different cancers as well as how it behaves in normal cells that make up the tumor milieu, will be useful in understanding the full extent of this protein's influence on invasion.

Ultimately, MDA-9/Syntenin could prove to be a valuable target for inhibiting invasion and metastasis in a wide variety of cancer indications. Results in a range of tumors utilizing genetic inhibition of MDA-9/Syntenin have thus far supported this view. Nonetheless, pharmacological inhibition of MDA-9/Syntenin's invasion-promoting attributes would be ideal. We describe the effects of a small molecule targeting MDA-9/Syntenin, PDZ1i, which has shown promising results in reducing invasion in GBM, as well as other cancers not detailed here. This molecule was designed through a FBDD approach coupled with NMR analysis (492, 535-540). In the future, this and other methods that enable probing of large libraries of potential molecules, such as mRNA display (541, 542), will aid in the quest to design superior molecules that specifically inhibit difficult structures like MDA-9's PDZ domain. Of course, a challenge during this process will be the relatively slower pace of *in vitro* and *in vivo* validation of these candidates compared to timeline of their development. Another significant hurdle will be identifying molecules that avoid rapid clearance from the body, are sufficiently distributed to tumor cells, and have minimal observed toxicities.

As is evident by the results of years of cancer drug discovery and development, there is unlikely to be a single "magic bullet" that can completely eliminate aggressive tumors. Nonetheless, targeting MDA-9/Syntenin could serve as an ideal complement to many conventional and newer therapy strategies. While inhibiting MDA-9/Syntenin can reduce the proliferation rate of some cancer types, the level to which it slows growth is not nearly as

dramatic as true cytotoxic therapies. Therefore, MDA-9/Syntenin targeting is promising as part of a combination approach that utilizes chemotherapy or, as shown here, radiotherapy. It could be particularly well suited to this task in light of the observation that some current treatments, such as radiation and bevacizumab, can actually enhance the invasive potential of surviving cells. In both cases, key MDA-9/Syntenin interacting partners, such as c-Src and FAK, are activated in these invasive cells.

Due to the overlapping nature of angiogenesis and invasion, it is not surprising that MDA-9/Syntenin can be involved in this crucial tumor phenotype as well. Furthermore, microvascular proliferation is defining feature of GBM as it develops strong angiogenic signaling within the tumor microenvironment. Targeting angiogenic signaling has yielded some success with the use of Bevacizumab in GBM. However, evidence showing that angiogenic inhibitors can promote invasive growth patterns could explain the highly refractive nature of residual tumor cells that survive Bevacizumab treatment (291, 292). Further investigation of MDA-9/Syntenin's role in upregulating pro-angiogenic factors, and its influence on tumor endothelial cells and vasculature could prove valuable in this respect.

Overall, MDA-9/Syntenin is an emerging protein in the world of cancer biology. It has a diverse network of involvement in facilitating both intracellular and extracellular effectors of invasion and angiogenesis. This, coupled with its expression and function in a variety of cancer types, makes it an exciting potential therapeutic target that warrants further in-depth study. The use of anti-invasive agents combined with cytotoxic therapies may provide a novel approach for effectively treating both primary tumors and metastases. In these contexts, inhibitors of MDA-9/Syntenin and its critical downstream pathways may usher in new approaches for successfully treating and potentially preventing tumor spread and metastasis.

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Vita

Timothy Patrick Kegelman was born on April 25, 1985 in St. Louis, Missouri and is an American citizen. He graduated from Tabb High School, Yorktown, Virginia in 2003. In 2007, he earned a Bachelor of Science in Chemical Engineering from the University of Notre Dame, Notre Dame, Indiana. Subsequently, he enrolled in the M.D., Ph.D. program at the Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia in 2007