Necl-5 Promotes Glioblastoma Pathogenesis by Enhancing MMP-2 Production and Protecting Cells from Fas-mediated Apoptosis

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ABSTRACT

This thesis describes functions of the cell surface adhesion molecule Nectin-like molecule 5 (Necl-5) and its role in the pathogenesis of the deadly brain tumor glioblastoma (GBM). This thesis presents data showing that Necl-5 has two functions that have not been previously described. First, it promotes MMP-2 production, which is important for the invasive phenotype of GBM, and that MMP-2 production correlates with Necl-5 dependent Akt activation. Akt is known to promote MMP-2 expression in GBM, but this is the first link between Necl-5, Akt, and MMP-2 to be described. Second, Necl-5 protects GBM cells from receptor-mediated apoptosis, but not from chemotherapy-induced apoptosis. Furthermore, Necl-5 participates with CD44 to afford GBM cells this protection. It was previously shown that CD44 binds Necl-5, but the relevance of this interaction was heretofore unknown. As these processes are central to GBM pathogenesis, they support the idea that inhibition of Necl-5 function could inhibit tumor growth and survival.

Acknowledgements

I have many people to thank for this thesis. First and foremost to Dan Jay, my advisor, who launched this project with an audacious goal: to probe the cell surface proteome and find out what a cancer cell needed in order to invade. This in turn could tell us how to stop invasion. This project discovered 3 major targets, which led to several excellent publications (including one in Nature Cell Biology) and a number of theses (including this one). The screen gave us the first evidence of extracellular Hsp-90 function. It also uncovered a role for Neuropilin-1 and Necl-5 (then called PVR or CD155) in glioblastoma invasion. The latter target formed the basis for my project. I am grateful to Dan for his mentorship throughout this project. His guidance was particularly helpful while writing the paper, poster, and thesis that resulted from this work. I also thank my thesis committee: Ira Herman and Brent Cochran, who served as chair. I thank all my co-workers in the Jay Lab: Jean Stewart, Rob Hauptschein, Jessi Sims, Tugba Bagci, Jessica McCready, and Jason Cain. Others from M&V 7 and beyond: Ayana Hinton, Naiomi Sayre, Sarah Bond, Jen Durham, and Maureen Sherry. I'd like to thank my fellow MD-PhD class of 2003 for their support: Chudi Ezeokonkwo, Jane Rosen, and Richard Wong. Thanks to everyone for a great grad school experience. Finally thanks to my wife and son, April and Alex, for love and support. I thank them equally even though Alex was only around for the last year or so.

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CHAPTER 1: INTRODUCTION

CHAPTER 1

This chapter introduces glioblastoma (GBM) and its pathological hallmarks. Two important hallmarks, invasion and apoptosis are given special emphasis. Nectins and nectin-like molecules are also discussed, with emphasis on their known roles in normal cell physiology and cancer pathogenesis.

GLIOBLASTOMA

Glioblastoma (GBM) is the world's most common and aggressive malignant brain tumor. It is a tumor of astrocytic origin, and is the highest grade of malignant glioma in the World Health Organization's definition of astrocytic gliomas [1]. It is a "diffuse" glioma, meaning that it exhibits early, extensive invasion of tumor cells into the brain parenchyma [2]. This leads to an indistinct tumor margin, and little chance for complete tumor resection [3]. Residual tumor cells can establish a recurrent GBM in greater than 90% of cases [3]. In addition to this dispersive growth, GBM is characterized by high proliferation rates, efficient induction of angiogenesis, and resistance to apoptosis [2]. The propensity for dispersal, as well as resistance to chemotherapeutic agents that lead to apoptosis, results in the uniformly poor prognosis of GBM patients. The role of Necl-5 in these two important areas of GBM pathology will be explored in this thesis.

The incidence of malignant CNS tumors is approximately 5 in 100,000, and GBM accounts for over 60% of these [4]. Despite the low incidence of GBM, malignant brain tumors nearly always fatal, and malignant gliomas are responsible for 2% of all cancer deaths in the US [2]. Currently, median survival of GBM patients is approximately one year, and 5-year survival is less than 3% [1]. GBM affects more males than females, and Caucasians more than any other ethnic group [5, 6]. Certain heritable genetic syndromes, such as Li Fraumeni syndrome and Turcot's syndrome (mutations in TP53 and APC, respectively) increase a patient's chances of developing GBM [7]. Also, first-degree relatives have a slightly higher risk for GBM than unrelated people. This suggests a genetic component to GBM predisposition, but the mode of inheritance is unknown [7].

Currently, the only environmental risk factor strongly associated with GBM development is ionizing radiation [8].

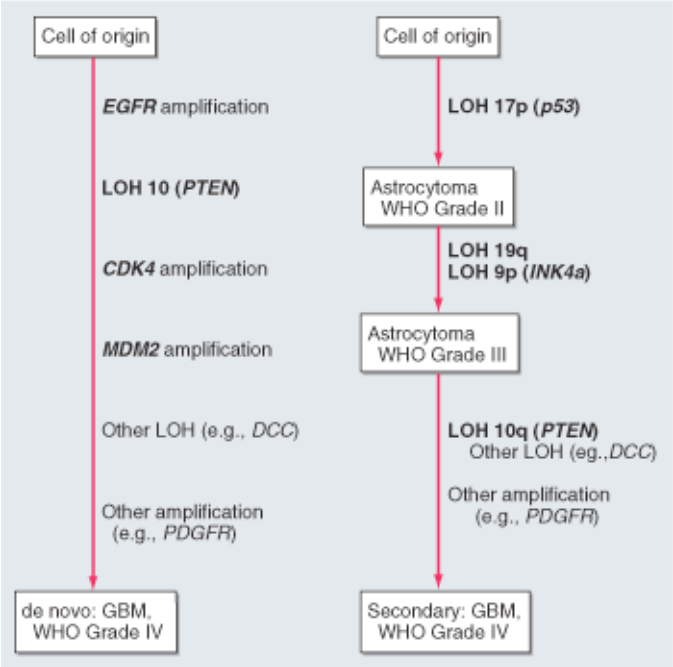

Figure 1. Genetic alterations leading to GBM. Primary GBM is characterized by EGFR amplification, and secondary GBM by loss of p53 function. From Harrison's Principles of Internal Medicine 17th edition

Glioblastoma can arise either de novo (primary GBM) or through evolution from lower grade gliomas (secondary GBM) [2]. Primary GBM is characterized by EGFR amplification, PTEN loss, CDKN2A (p16) deletion, and, less often MDM2 amplification (Fig. 1) [9]. Secondary GBMs usually have TP53 and PTEN mutations [9]. Interestingly, EGFR and p53 mutations rarely coexist. It's thought that one mutation precludes the other, however the mechanism for this is unknown [10]. Although both forms of GBM arise through different mechanisms, they are clinically indistinct and share all the common hallmarks of GBM [2]. Diagnosis is confirmed by tumor biopsy, which shows characteristic areas of necrosis, neovascularization, frequent mitoses, and pseudopallasade formation [11].

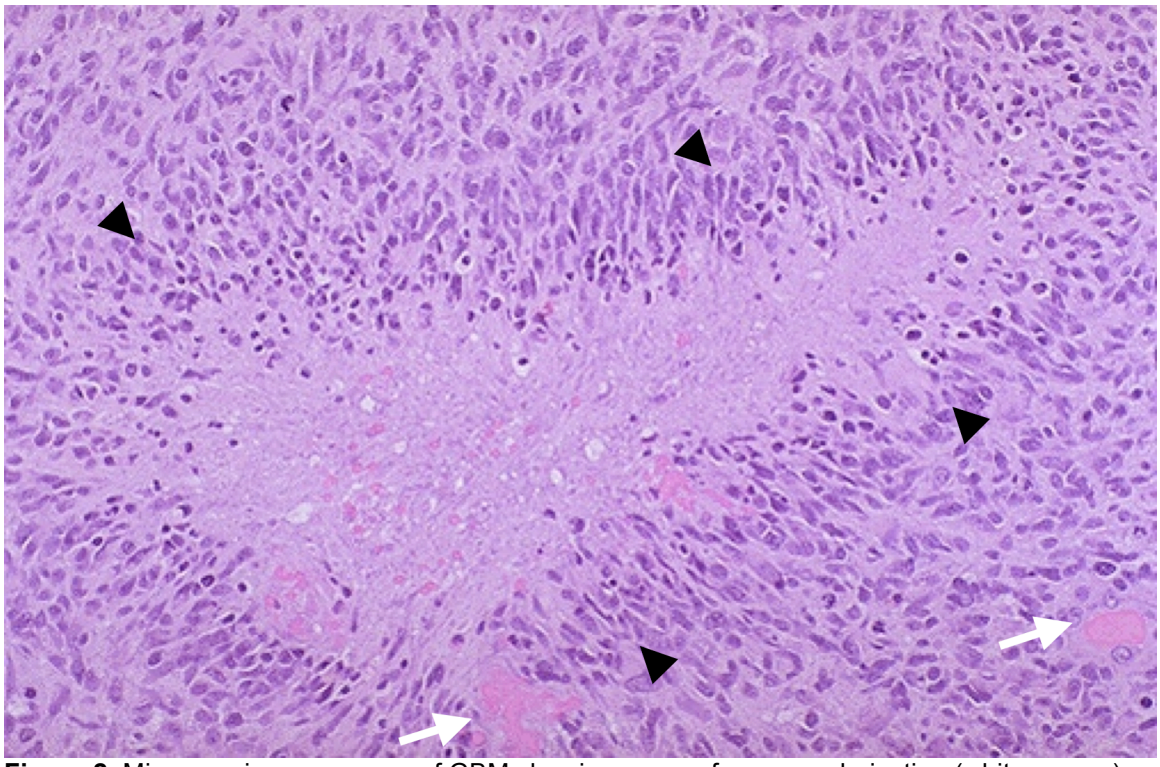

Figure 2. Microscopic appearance of GBM showing areas of neovascularization (white arrows) and pseudopallasading necrosis (black arrowheads). From WebPath http://library.med.utah.edu/WebPath/CNSHTML/CNS139.html

Expression of the marker OLIG2 is restricted to diffuse astrocytomas, and can differentiate GBM and other astrocytomas from metastatic tumors or tumors of other CNS lineages [12].

Despite vigorous research into the pathogenesis of GBM, survival has increased only moderately, and, as described below, only in certain patients [6]. The only strong prognostic indicator of survival is age at the time of diagnosis, with those diagnosed at less than 45 years of age having greater survival [6]. Treatment for GBM consists of surgery, radiation, and chemotherapy. Surgery is performed to debulk the tumor and to relieve the patient of symptoms associated with increased cranial pressure [4]. Radiation therapy increases survival time to 12 months and 2-year survival to 10% [2, 13]. In 2005, the oral alkylating agent temozolomide (TMZ) was shown to increase survival to 15 months and increase 2-year survival to 26% when given after radiation [14]. The benefit

from TMZ was only apparent in patients whose tumor did not express the DNA repair gene MGMT, indicating the utility of genetic analysis and personalized medicine in the treatment of GBM [15]. Other alkylating agents, like carmustine (BCNU) are sometimes used. BCNU is impregnated upon implantable wafers to allow delivery of the drug directly to the tumor resection cavity after surgery [16].

One major pathophysiological feature of GBM is the tumor cells ability to invade the surrounding parenchyma [17]. Invasion involves destruction of an ECM barrier followed by migration into or through the new space. It is the aggregate behavior of invading cells at the tumor margins that gives GBM its diffuse appearance. Invasion is not random, but occurs along preferred routes: perivasular basement membrane areas and white matter tracts [18]. This likely reflects the GBM cells ability to recognize extracellular matrix (ECM) proteins in this area, or the ability to degrade ECM components in these areas [17]. GBM cells near the tumor border express several proteins that permit migration and invasion. These include integrins that allow adhesion to certain substrates, such as fibronectin, laminin, or vitronectin [19]. Several integrins are expressed preferentially in GBM, such as $\alpha v\beta 3$ and $\alpha v\beta 5$ [20, 21]. Also, $\beta 1$ integrin is expressed, and its expression correlates with increased invasion [21]. Furthermore, GBM cells can secrete matrix components, such as vitronectin, in order to stimulate integrin dependent invasion [22].

Matrix metalloproteinase expression is also important to GBM pathogenesis and the ability to invade. Major proteases secreted by GBM include the matrix metalloproteinases MMP-2, MMP-9, and MT-MMP-1. A recent review on glioblastoma invasion gives four reasons why MMP activity is important in GBM invasion: First,

MMPs -2, -9, and MT-MMP-1 can cooperatively degrade nearly every ECM substrate found in the brain. Second, these proteases are activated in tumors and adjacent areas rather than normal brain. Third, activation and expression of MMPs correlates with tumor cell invasion and poor clinical outcome. Lastly, invasion can be suppressed *in vitro* by inhibiting these MMPs with siRNA [17].

A second pathological hallmark of GBM is resistance to apoptosis. This is due to several reasons, including: activation of the PI3K/Akt signaling pathway by receptor tyrosine kinases activity and loss of the PI3K/Akt antagonist PTEN [2], expression of anti-apoptotic proteins like Bcl-2 and Bcl-XL [23, 24], and expression of non-functional decoy death receptors and caspase inhibitors like DcR3 and c-FLIP [25, 26]. Apoptotic resistance is important in GBM, because it may underlie the inherent resistance to radiation and chemotherapy. In fact, the relative expression of pro- and anti-apoptotic proteins is a predictor of radiosensitivity [27]. Also, GBM cells often express Fas and Fas ligand (FasL), and are partially sensitive to FasL treatment *ex vivo* [28] [29]. Therapeutic sensitization of GBM cells to mediators of extrinsic apoptosis may allow selective destruction of tumor cells using exogenous death receptor ligands such as Fas or TRAIL [30].

DYSREGULATION OF SIGNALING PATHWAYS IN GLIOBLASTOMA

Epidermal growth factor receptor (EGFR) and platelet derived growth factor receptor (PDGFR) activities are active in glioma cells and their activities can promote invasion [2]. EGFR gene amplification is common in primary GBM occurring in about 40% of GBM, and amplification is associated with gene rearrangement [1]. Overexpression of EGFR is associated with constitutive signaling, and affects the cell by upregulation of

genes associated with survival, motility, apoptotic resistance and angiogenesis [31]. Also, a constitutively active mutant of EGFR, called EGFRvIII, is common, especially in those gliomas that already show EGFR amplification [32]. This mutant has deletions in exons 2 through 7, is constitutively active, and promotes tumorigenesis by promoting proliferation and preventing apoptosis [33-35]. Unlike EGFR, PDGFR is rarely overexpressed or constitutively active due to mutation in GBM [36]. Rather, the tumor cell expresses both receptor (PDGFRα) and ligand (PDGF-A or -B) and activates signaling using an autocrine or paracrine mechanism [37] [38].

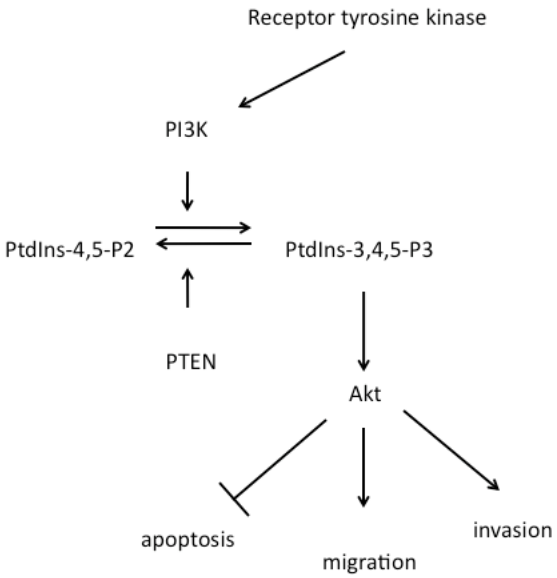

Figure 3. Activation of Akt in glioblastoma

Glioblastoma cells often show elevated activation of the PI3K/Akt system [39], and activation of this pathway correlates with increasing tumor grade, reduced apoptosis, and reduced survival [40]. EGFR and PDGFR activity can lead to activation of the PI3K/Akt pathway. Akt activation has been reviewed extensively [41, 42], and works as follows: Phosphatidylinositol 3-kinse (PI3K) is activated by receptor tyrosine kinase activity, and catalyzes the conversion of phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P2) to phosphatidylinositol-3,4,5-triphosphate (PtdIns-3,4,5-P3). This occurs at the inner leaflet of the plasma membrane and recruits several proteins to bind via a pleckstrin homology domain. PI3K activity is antagonized by the phosphatase PTEN, which catalyzes the reverse reaction of PI3K, resulting in an attenuation of the signal [43]. PTEN is inactivated in up to 50% of gliomas, and this results in constitutive PI3K signaling and PtdIns-3,4,5-P3 formation [43, 44]. PtdIns-3,4,5-P3 forms a docking site that recruits proteins that contain a pleckstrin homology domain, the most important of which is Akt. Upon recruitment, Akt can be phosphorylated by PDK1 or TORC2 at two critical residues: serine 473 and threonine 308 [41, 42]. Constitutive receptor activation coupled with PTEN mutation leads to constitutive Akt activation in GBM, and up to 85% of primary GBM samples have activated Akt [39]. Cellular proliferation and resistance to apoptosis are two

important functions that are regulated by Akt [2, 3]. Recently, several groups have shown a correlation between Akt activation and MMP-2 production in gliomas, suggesting that Akt could have a role in invasion [45-49]. For example, Park et al have shown that GBM cells respond to ionizing radiation (IR) by activating Akt and increasing MMP-2 production and promoting invasion. MMP-2 promoter activity was increased following IR, and could be inhibited by expression of dominant negative (DN) Akt, DN PI3K p85, or wild-type PTEN [47]. Lee et al showed that treatment of GBM cells with the NSAID sulindac resulted in the decrease of Akt activation and MMP-2 expression, and this could be restored by expression of constitutively active Akt [46]. In an interesting synthesis of apoptosis and migration, Joy et al have shown that migrating glioma cells show elevated levels of Akt activation, and that these migrating cells are more resistant to apoptosis [50]. PI3K activation is linked to this effect, because treatment with a PI3K inhibitor sensitized migrating cells to apoptotic induction by either the intrinsic or extrinsic pathway [50]. The central importance of the Akt pathway in GBM can be seen in EGFR inhibitor studies, in which clinical response to gefitinib or erlotinib, inhibiors of EGFR activity, are dependent on the presence of PTEN and concomitant reduction in Akt activity [51]. Those tumors with intact PTEN and low activated Akt levels showed more favorable response to inhibition of EGFR or EGFRvIII than did tumors with deleted PTEN and high Akt levels [51].

In summary, Akt activtion is common in GBM due to characteristic mutations in receptors, ligands, and antagonists. This enhanced Akt activation gives the tumor a selective advantage by enhancing invasion and apoptotic resistance. Methods to reduce

Akt activation could improve patient outcome by preventing a dispersive phenotype, and sensitizing GBM cells to chemotherapeutics or radiation.

INVASION

Invasion is central to the pathogenesis of GBM. It is the propensity for GBM cells to invade into the brain parenchyma that causes its gross diffuse appearance [2]. Also, the cells that invade from the tumor to surrounding areas can be left behind after surgical resection, and give rise to recurrent GBM [3]. Understanding invasion, and how cells acquire that phenotype, is important for understanding how to inhibit recurrence, which is the major cause of death from GBM [3].

Invasion requires the cell to coordinate several biological functions. As reviewed by Nakada et al [17], the cell must first detach from a substratum and other surrounding cells. Next, the cell must adhere to the surrounding ECM by altering its adhesion receptor repertoire, then degrade ECM components and create a space to migrate into. This is done with proteases secreted by the tumor cell. The cell then extends membrane protrusions, like lamellipodia and filopodia, in the direction of migration, which is due to actin-based cytoskeletal rearrangements coordinated by small GTPases like Rac and Cdc42. Finally, adhesion to these new sites is followed by cytoskeletal contraction and propulsion of the cell forward.

Glioblastoma invasion doesn't proceed randomly, but instead follows white matter tracts and perivascular regions in the brain [18, 52]. These areas contain extracellular matrix (ECM) components that promote invasion, such as fibronectin, laminin, collagen, vitronectin, and tenascin [18, 52]. Glioblastoma cells can also secrete

ECM components, such as vitronectin, which has been shown not only to increase invasion, but protects the cell from apoptosis [22].

INTEGRINS

Glioblastoma adhesion to the ECM is mediated by a variety of transmembrane proteins, such as integrins, cell surface glycoproteins, and members of the immunoglobulin superfamily, which includes nectins and nectin-like receptors [17]. Integrins are the most important group of adhesion molecules in GBM, and they consist of heterodimers of 14 α subunits and 8 β subunits. Together they make at least 24 α-β combinations that have specificity for several ECM components [17, 19, 53]. Integrins bind ECM proteins via an RGD motif, and binding is a result of a switch from a low-affinity form to a high-affinity form [19]. Integrins tether cells to the ECM, and the cytoplasmic domain of the β subunit can interact with the cytoskeleton [19]. These interactions can lead to cytoskeletal rearrangements that promote motility [3].

Glioma cells express a different integrin repertoire than normal astrocytes, and these integrins promote migration and invasion rather than adhesion. GBM enriched integrins include α3β1, αvβ1, αvβ3, and αvβ5, and each of these contributes to glioma cell migration [20, 21, 54-56]. Also, upregulation of β1 or β5 containing integrins promote migration [57]. Expression of β1 integrin in rat C6 glioma cells promotes diffuse invasion *in vivo* [58], and neutralizing antibodies against β1 integrin inhibits migration [59]. Vitronectin binds αvβ3 and αvβ5, and this interaction induces anti-apoptotic Bcl-2 and Bcl-XL expression, indicating that the tumor cells are able to promote both their invasion and survival [22]. This survival phenotype is best illustrated by the actions of the αv antagonist EMD121974. Cells treated with this small RGD-containing peptide

cannot adhere to vitronectin or tenascin via their αvβ3 and αvβ5 integrin receptors and subsequently undergo apoptosis, further linking the effects of migration and apoptotic resistance [60]. Integrins can also initiate signaling upon binding to the ECM. Integrins have no intrinsic kinase activity, but they can recruit signaling molecules such as FAK to adhesion sites [55]. During migration, clustering of integrins leads to FAK activation, and recruitment and activation of the signaling moleules Src and paxillin, which could initiate or enhance a motile phenotype [61].

CD44

CD44 is a highly glycosylated transmembrane adhesion protein that is expressed in glioblastoma, and is involved in adhesion, migration, and resistance to apoptosis [62]. The main ligand for CD44 is hyaluronic acid, which is a large component of brain ECM [52, 62]. Binding of CD44 to hyaluronin prevents migration, but the ligand-binding domain of CD44 can be cleaved by ADAM (a disintigrin and metalloproteinase) -10 and -17, which liberates the cell from the ECM and promotes migration [63]. Inhibition of CD44 cleavage inhibited migration from a hyaluronin matrix [63]. Furthermore, treating cells with monoclonal antibodies directed at CD44 prevented invasion *in vitro* and *in vivo* [64, 65], indicating that this protein has a role in enhancing migration, perhaps by generation of pro-migratory fragments upon binding [66].

MIGRATION: ROLE OF SMALL GTPASES

Actin-based membrane dynamics during migration are controlled by the Rho family of small GTPases: Rac, Rho, and Cdc42. These proteins cycle between active GTP bound states and inactive GDP bound states, and are controlled by guanine nucleotide exchange factors, GTPase activating proteins, and guanine nucleotide dissociation inhibitors [53].

Rac activation leads to lamellipodia formation and focal adhesion disassembly, which promotes migration [67, 68]. Cdc42 activation promotes filopodia formation, another type of membrane extension seen in migrating cells [69]. Rho activation leads to contractile stress fiber formation, and is primarily responsible for migration [53]. In cancer, the small GTPases are involved in transformation and invasion, and can contribute to these phenotypes by activating the PI3K pathway [70, 71]. In gliomas, siRNA mediated depletion of Rac1 decreases cell migration and invasion [72], and expression of a dominant negative Rac1 induces apoptotic death in gliomas, but not normal astrocytes [73].

MATRIX METALLOPROTEINASES

Cells express proteases at the leading edge in order to degrade ECM components that impede directional migration. Gliomas express several types of proteases, such as the urokinase-type plasminogen activator and cathepsins, but matrix metalloproteinases (MMPs) are believed to be most important. MMPs are zinc-dependent endopeptidases that regulate ECM turnover in both physiological and disease processes [74]. MMPs are important in glioblastoma invasion because there is a good correlation between the types and levels of MMPs expressed and the invasive behavior of GBM cells [17, 74]. Although several MMPs exist, and almost every cancer type expresses MMPs, there is a core of MMPs that are particularly important for GBM pathogenesis. This core consists of MMP-2, MMP-9, and the membrane-type MMP, MT-MMP-1 (MMP-14) [17].

GBM cells often show elevated expression of MMP-2 as compared to lower grade gliomas [75, 76], and its expression correlates with invasive behavior in GBM patient samples [77, 78]. MMP-2 inhibitors block glioma invasion *in vitro*, indicating its

importance as a possible target for GBM therapy [79-81]. Regulation of MMP-2 expression is not similar to regulation of other MMPs like MMP-1 and MMP-9, which contain an AP1 element in their promoters [74]. MMP-2 expression can be activated by the transcription factor Snail, which promotes epithelial to mesenchymal transition, and the gain of an invasive phenotype [82, 83]. MMP-2 is secreted as an inactive zymogen, and activation of MMP-2 occurs at the cell surface, and involves the actions of MT1-MMP and TIMP-2 [84]. MMP-2 and can be localized to the cell's leading edge by binding to $\alpha v\beta 3$ integrin, which is though to direct its proteolytic activity to the direction of migration [85]. MMP-9 is a 92kDa gelatinase, whose expression correlates with glioma severity in several model systems [74], and is often seen upregulted with MMP-2 [78] [48]. MT1-MMP is an MMP that is tethered to the plasma membrane by a transmembrane domain. It has a unique role in glioma invasion, in that it helps to activate secreted pro-MMPs [86, 87]. Like the ADAM proteins, MT1-MMP can cleave CD44 at the cell surface, untethering the cell from its surroundings and generating pro-migratory fragments. [66]

The link between MMP expression and invasion is not simply limited to matrix degradation. For example, MMPs -2 and -9 can promote angiogenesis [88, 89]. When glioma cells were cocultured with endothelial cells, tube formation (a measure of angiogenesis) was promoted, and this could be inhibited by blocking antibodies to MMP-9, or by blocking MMP-9 expression [88]. Also, tumor angiogenesis is inhibited in MMP-2 deficient mice [89].

APOPTOSIS

Apoptosis is a normal physiological process resulting in non-inflammatory cell death [90]. It is important in development, tissue remodeling, and removal of abnormal cells, and promotes the development and maintenance of normal tissues [90]. Cancer arises from cells that have undergone mutation and have dysregulated signaling [91]. This dysregulation and genetic instability usually results in apoptotic induction. Cells that have the ability to avoid apoptosis are spared from negative selection and grow into tumors [92].

Apoptosis is characterized by chromatin condensation and fragmentation, nuclear fragmentation, cell shrinkage, and nuclear blebbing. Cells eventually break into pieces, and the debris is cleared by phagocytes [90]. Despite the involvement of phagocytes, apoptosis does not induce inflammation. Rather, inflammation is the hallmark necrosis, a mode of cell death from external stimuli such as mechanical trauma [11].

Apoptosis can be triggered by radiation, such as ultraviolet light or X-rays, or by DNA damaging agents, chemotherapeutic drugs, or growth factor withdrawal [91, 93]. Apoptosis can be achieved by stimulation of either the intrinsic or extrinsic pathways. The extrinsic pathway involves cell surface receptors that, when engaged with ligand, signal for the activation of the apoptosis initiator caspase-8, which then activates an apoptotic cascade [94]. The intrinsic pathway for apoptosis involves the loss of mitochondrial membrane integrity, leakage of cytochrome c into the cytosol, and the activation of caspase-9 as the initiator caspase and subsequent induction of apoptosis [94]. These two pathways can intersect, with activation of the extrinsic pathway leading to mitochondrial membrane permeability, and activation of both pathways [95].

THE INTRINSIC PATHWAY OF APOPTOSIS

The intrinsic pathway of apoptosis is initiated by an increase in mitochondrial permeability, and is mediated by caspase-9, members of the Bcl-2 family of proteins, and other cytoplasmic apoptosis modulators [96]. This leads to cytochrome c release into the cytoplasm, which serves as a signal for apoptosis induction by cytoplasmic modulators [97]. Mitochondrial permeability is enhanced by the pro apoptotic repertoire of proteins that includes Bax and Bak [98]. They insert into the outer mitochondrial membrane and promote cytochrome c release into the cytosol [99]. Cytochrome c quickly binds the adapter protein APAF-1, and recruits multiple copies of caspase-9 into a nascent apoptosome [97, 100]. Caspase-9 becomes activated by cleavage and then activates the executioner caspase-3, which cleaves various cytoplasmic targets and commits the cell to apoptosis [97]. Mitochondrial permeability is prevented by the anti-apoptotic Bcl-2 members, such as Bcl-2 and Bcl-XL, which inhibit Bax and Bak directly [101, 102]. Glioblastoma cells often show elevated expression of Bcl-2 and Bcl-XL, and downregulation of the pro-apoptotic Bax, especially in recurrent GBM after treatment [23]. Overexpression of Bcl-XL and Bcl-2 can not only protect from apoptosis, but also enhance motility, which may include upregulation of MMPs [103]. Bcl-XL expression is promoted by EGFRvIII expression [24, 34]. EGFR activity also activates the PI3K/Akt pathway, which promotes survival by phosphorylating and inhibiting Bad and caspase-9 [104, 105]. The loss of PTEN in many GBM cells also contributes to the anti-apoptotic phenotype seen in this tumor type by enhancing the Akt signal [106].

The extrinsic and intrinsic pathways can be linked by the pro-apoptotic protein Bid [95, 107]. Active caspase-8 can cleave Bid, and this cleavage promotes its association with Bax and Bak, and their subsequent insertion into the outer mitochondrial

membrane. Certain cells, called Type II cells, require this "mitochondrial kick" in order to fully activate the apoptotic program.

THE EXTRINSIC PATHWAY OF APOPTOSIS: DEATH RECEPTORS

The three major systems for extrinsic apoptotic induction are the Fas/FasL system, the TNFα system, and TRAIL. These are commonly called death receptors. Regardless of the system involved, the downstream signaling events are the same [108]. Interaction of Fas with its ligand, FasL, initiates the extrinsic pathway. Initiation involves the trimerization of the receptor, and recruitment of Fas-associated death domain (FADD) protein to the receptor [108]. This induces inactive caspase-8 to bind the complex and this complete complex is called the death-inducing signaling complex (DISC). Caspase-8 is then cleaved, and this active caspase-8 then proteolytically cleaves and activates the executioner caspases -3 and -7 [108]. These caspases then cleave several cellular targets that result in the irreversible commitment to apoptotic death.

Glioblastomas express both Fas and FasL, and expression correlates with tumor grade [28]. Most human glioma cell lines are at least partially sensitive to Fas mediated apoptosis [29], and expression of Fas or FasL from adenovirus vectors enhances apoptosis of gliomas [109, 110]. Even though apoptosis can be initiated by FasL treatment in gliomas, sensitivity is usually weak [29]. This may indicate the presence of protective systems in gliomas. One such system involves the production of non-functional decoy receptors that should attenuate a FasL signal. Soluble decoy receptor 3 (DcR3) is expressed preferentially on high-grade malignant glioma cells, and may serve to protect the cells from immune-mediated clearance [25]. Also, Fas signaling can be opposed by the cFLIP proteins. This family is homologous to caspase-8, but cannot be

processed into an active protease upon DISC formation [111]. This prevents caspase-8 processing and inhibits apoptosis. The cFLIPs can bind and prevent signaling from all death receptors.

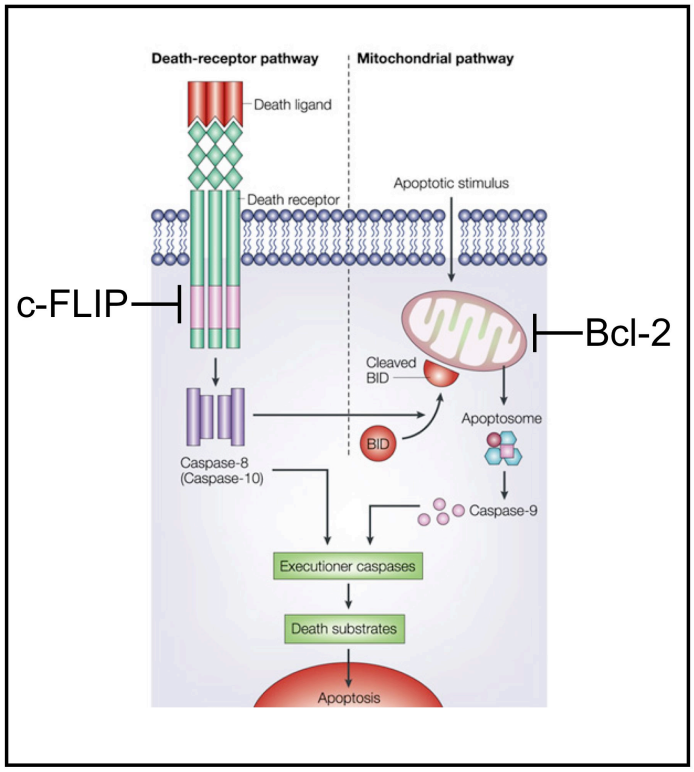

Figure 4. Apoptosis. Diagram of the extrinsic and intrinsic pathways of apoptosis. These two pathways are connected by caspase-8 cleavage of Bid. Adapted from: Igney F.H. and Krammer P.H. Death and anti-death: tumour resistance to apoptosis. Nat Rev Cancer **2**,277-288 (2002).

Expression of CD44 is also thought to protect cells from Fas-mediated apoptosis. Cross-linking of CD44 on synovial cells from patients with rheumatoid arthritis made these cells more susceptible to Fas-mediated apoptosis [112]. Another report showed that CD44 knockdown by siRNA sensitized HT1080 fibrosarcoma cells to Fas-mediated apoptosis and that the presence of CD44 protected cells from FasL by inhibiting DISC formation [113]. CD44 has several splice variants, which are determined by expression of one or more variant exons [62]. The standard CD44, CD44s, expresses none of these

exons. CD44v is the designation for isoforms that contain one or more of the varient exons. Experiments have shown that two isoforms, which express exons v6 and v9, can protect immune cells from Fas-mediated apoptosis by direct interaction with Fas [114].

NECTINS AND NECTIN-LIKE MOLECULES

Nectins, and their related family members, the Nectin-like molecules (Necls), are calcium-independent cell adhesion molecules, but they also have other biological functions, including proliferation and movement [115]. There are 4 nectins [116] and 5 Necls [115], and although they differ in their functions and binding affinities, they share a common structure [115]. Each member has an extracellular domain with 3 immunoglobulin (Ig) like loops, a single transmembrane domain, and a short cytoplasmic domain [115]. Nectins and Necls can bind other members in *cis* or *trans*, as well as bind other classes of molecules such as growth factor receptors and integrins [115, 117, 118].

ROLE OF NECTINS IN CELL JUNCTION FORMATION

Nectins primarily mediate cell-cell contacts in epithelial tissues, and promote formation of adherens junctions and tight junctions [116]. Nectins initiate adherens junction formation between epithelial cells before, and independently of, cadherin recruitment to these junctions [115]. Nectins achieve this by interacting with each other *in trans* on opposing cells, and one important interaction is between nectins -1 and -3 [116]. This interaction activates the tyrosine kinase Src. Activated Src then lead to the activation of the small G protein Rap1, and the subsequent activation of FRG, which is the guanine nucleotide exchange factor for Cdc42 [119, 120]. Activation of Cdc42 leads to the formation of filopodia by actin rearrangement, and the subsequent activation of Rac, which promotes lamellipodia formation [121]. These membrane protrusions are

thought to increase the surface area between opposing cells, and lead to increased contact and strength of interaction between them. Nectins interact with signaling molecules as well as other nectins. For example, Src activation is due to the recruitment of integrin αvβ3 to new regions of cell-cell contact, and the presence of this integrin is indespensible for Src activation [122]. Nectin-3, a strong *trans* binding partner of nectin-1, has been shown to bind the PDGF receptor, which activates the Akt pathway, and subsequently protects cells from apoptosis [123]. Nectins bind the f-actin binding protein afadin, and this recruits actin to adherens junctions [116], which strengthens interactions and helps give epithelial cells their shape.

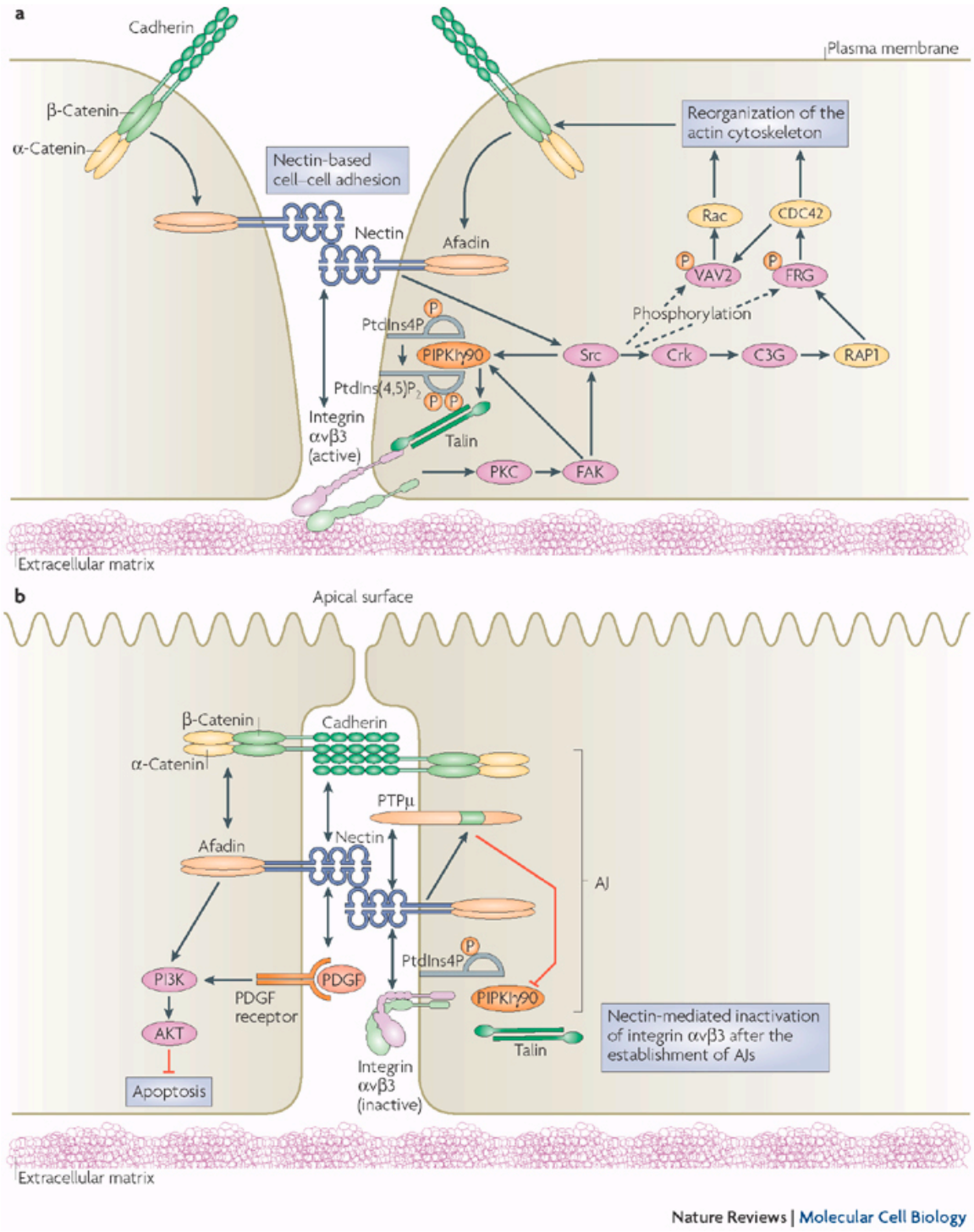

Figure 5. a. Formation of and **b.** maintenance of adherens junctions by *trans* nectin interactions. From: Yoshimi Takai, Jun Miyoshi, Wataru Ikeda & Hisakazu Ogita. Nature Reviews Molecular Cell Biology 9, 603-615 (August 2008)

Necls have a more varied functional repertoire than nectins. Necls differ from nectins in that they do not bind afadin, but they share a similar structure to nectins, including the 3 extracellular Ig loops and short cytoplasmic tail [115]. Necls -1 and -4 are involved in axon myelination by promoting the interaction between neurons and Schwann cells [124, 125]. Necl-2 is involved in mast cell degranulation [126]. Necl-3 is localized to the oligodendrocytes of myelinated axons, where it is thought to promote myelination [127].

NECTIN-LIKE MOLECULE 5

Necl-5 was originally described as the poliovirus receptor [128], and is expressed in Peyer's patches in the lymphoid system of the intestine. Phylogenetic analysis indicates that Necl-5 is more closely related to the nectins than Necls, and may represent a unique member of a third family, distinct from the nectins and other Necls [129]. Necl-5 is unique in that it is not widely expressed in the adult, but is expressed during CNS development, where it promotes axon guidance [130]. Necl-5 is expressed by hepatocytes after mechanical or chemical injury, and expression promotes wound healing [131]. A recent study focused on the repertoire of nectin and Necl molecules upregulated after axotomy in peripheral motoneurons. In situ hybridization was used to probe for Necl-5 mRNA, and upregulation was found in peripheral and spinal motoneurons with the former in close proximity to Schwann cells, indicating that Necl-5 may promote myelination by selectively directing Schwann cell migration toward axons [132].

The two physiological functions of Necl-5 are the promotion of migration and the initiation of mesenchymal to epithelial transition (MET) during development, which results in contact inhibition of cell movement and proliferation [133]. Re-expression of

Necl-5 in cancer may be a hallmark of the epithelial to mesenchymal transition (EMT) where cells take on a migratory phenotype that promotes invasion and metastasis [133]. One role of Necl-5 in MET is the inhibition of cell movement due to cell-cell contacts [134]. Necl-5, like nectin-1, can bind nectin-3 *in trans* [115], and this is thought to be one of the first interactions between moving cells. This interaction activates Cdc42 and Rac by a mechanism similar to nectin-1-nectin-3 interaction, causing an increase in cell contact sites by lamellipodia formation [120]. Necl-5 is eventually internalized by clathrin-mediated endocytosis, where it can no longer integrate signals from αvβ3 integrin and growth factor receptors, thus inhibiting migration [135]. Nectin-3 is not endocytosed, however, but remains at areas of cell-cell contacts and interacts in *trans* with nectin-1 [116]. This promotes the formation of adherens junctions between the cells, as described above. When cells overexpress Necl-5, such as cancer cells or transformed cells carrying a dominant Ras mutation, Necl-5 is not downregulated after cell-cell contact, and the formatin of adherens junctions does not occur [136]. Furthermore, overexpression of Necl-5 negatively regulates the function of sprouty2, which is a negative regulator of mitogenic Ras signaling [137]. Under normal conditions, Necl-5 binds sprouty2, and the endocytotic recycling of Necl-5 during cell-cell contact releases sprouty2, where it can be phosphorylated by Src, and inhibits PDGF-induced activation of Ras [138]. In this manner, contact inhibition of movement can also lead to inhibition of mitogenic signals and inhibition of the cell cycle. When Necl-5 is overexpressed, sprouty is constitutively sequestered, and cannot be phosphorylated by Src, and cannot exert its function [138]. In this way, overexpression of Necl-5 can promote growth factor induced mitogenic activity.

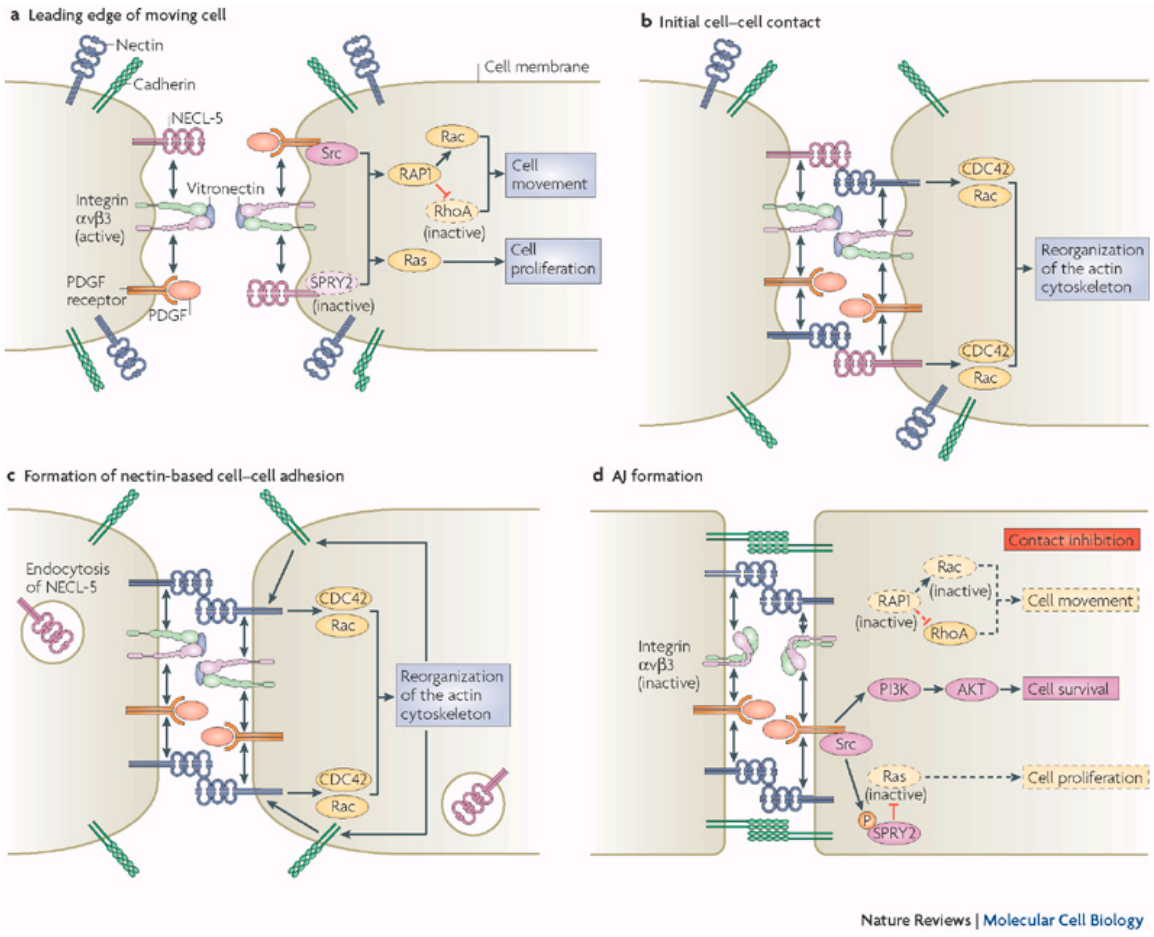

Figure 6. Necl-5 promotes cell adhesion and contact inhibition of cell division following migration. From: Yoshimi Takai, Jun Miyoshi, Wataru Ikeda & Hisakazu Ogita. Nature Reviews Molecular Cell Biology 9, 603-615 (August 2008)

Necl-5 can interact in *trans* with nectin-3, as previously mentioned, but does not interact with itself. Two other transmembrane proteins that Necl-5 is known to bind are CD96/Tactile and CD226/DNAM-1, both of which are important in immune cell function. CD96/Tactile, like Necl-5, is a member of the Ig superfamily, and promotes binding of T-cells to cells expressing Necl-5, and subsequent T-cell activation [139]. CD226/DNAM-1 is also expressed in T-cells, where it enhances T-cell proliferation. This interaction is thought to be important in cancer, as colon cancer cells that express Necl-5 are adherent to platelets via platelet expression of CD226/DNAM-1. This interaction results in enhanced delivery of cancer cells to the lungs [140].

One important function of Necl-5 during migration is to coordinate directional cell movement in response to growth factors at the leading edge. Necl-5 localizes to the leading edge during migraion, and it colocalizes with both αvβ3 integrin and the PDGF receptor [118]. Necl-5 physically interacts with both of these molecules, as determined by co-immunoprecipitation experiments [117]. By virtue of its binding abilities, Necl-5 localizes these proteins to the leading edge, and thus polarizes the cell in preparation for directional movement [117]. Leading edge structures, such as lamellipodia and filopodia, are created by actin rearrangements due to the activation of Rac, Rho, and Cdc42, which themselves are activated by PDGFR or integrin activity. These structures are converted into focal complexes, then focal adhesions as migration progresses [133]. Focal complexes, which contain only Necl-5 and αvβ3 integrin are formed by the activity of Rac and Cdc42. Focal complexes are transformed into focal adhesions, which are more stable structures that contain only αvβ3 integrin, by the activity of Rho, and the inactivation of Cdc42 and Rac [133]. *In vitro* experiments have shown how Necl-5 enhances cell migration by binding to PDGFR and αvβ3 integrin. Upon PDGF addition, these proteins cluster in the direction of stimulation, which defines the leading edge [141]. The cytoplasmic proteins Crk and C3G are then recruited to the areas of the cluster. Crk binds C3G and phosphorylated PDGF receptor, and C3G is a GEF that activates the small G protein Rap1 [141]. In this way, Rap1 is activated, and exerts three important functions. First, it activates Rac at the leading edge by activating its GEF, Vav2 [141]. Rac activation leads to the formation of peripheral ruffles and focal complexes. Second, Rap1 inactivates RhoA, which inhibits the conversion of focal complexes to focal adhesions. Rap1 achieves this by binding ARAP-1, which promotes

the GTPase activity of RhoA [133]. Finally, Rap1 promotes the activity of Spa1, a GAP for Rap1, which promotes its inactivation. This leads to the promotion of RhoA at the leading edge, as the Rap1 inhibition is removed [133]. Also, as PDGFR is recycled by binding to PDGF, the Rap1 signal is attenuated, leading to further RhoA activation and the conversion of focal contacts to focal adhesions [133]. This is the cycle of Necl-5 during a single round of migration.

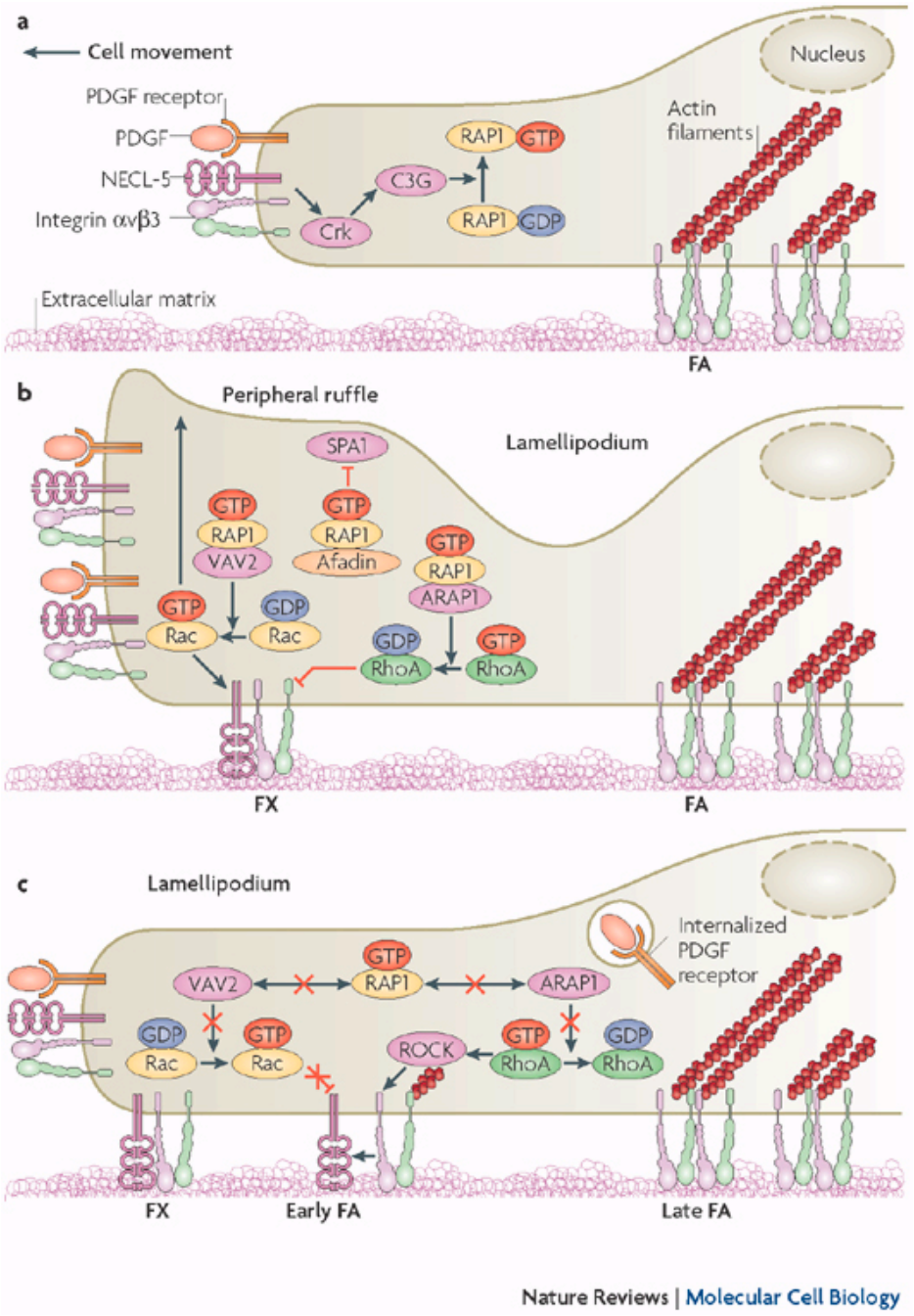

Figure 7. Necl-5 promotes migration by coordinating the activities of small GTPases. From: Yoshimi Takai, Jun Miyoshi, Wataru Ikeda & Hisakazu Ogita. Nature Reviews Molecular Cell Biology 9, 603-615 (August 2008).

Several cancers, including gastric cancer [142], colon cancer [143], and hepatocellular carcinoma [131] express Necl-5. In keeping with its role in cancer, the rat homolog, Tage4, was identified as an epitope expressed in rat colon cancer and

hepatocellular carcinoma [144, 145]. The role of Necl-5 in colon cancer tumorigenesis has recently been described. In this study, Necl-5 knockout mice were generated, and treated with dimethylhydrazine and/or dextran sodium sulphate to initiate experimental colitis and initiate neoplasia. Necl-5 knockout mice and heterozygotes showed significantly fewer tumors compared to wild-type mice after treatment. Furthermore, non-neoplastic cell proliferation was increased in wild type compared to Necl-5 knockout and heterozygotes, leading the authors to conclude that Necl-5 promoted colonic cell proliferation following treatment [146].

Our lab identified Necl-5 as a positive mediator of fibrosarcoma cell invasion using a FALI-based functional proteomic screen [147]. Necl-5 is expressed in glioblastoma cells, and knockdown by siRNA reduced migration of these cells [147]. Necl-5 colocalized with αv integrin at the leading edge of migrating cells [147], and further studies showed that Necl-5 promoted Src and FAK signaling in response to vitronectin, implicating an integrin-mediated mechanism [61]. Necl-5 is not downregulated upon cell contact in cancer, and can promote continuous migration and prevent EMT.

ROLE OF NECL-5 IN INVASION AND APOPTOTIC RESISTANCE IN GLIOBLASTOMA

This thesis describes two new functions of Necl-5. The first, outlined in Chapter 2, shows that MMP-2 expression is promoted by Necl-5. Mechanistically, Necl-5 promotes Akt activation, and this pathway partially controls MMP-2 production. Thus, MMP-2 production is regulated by Necl-5 via Akt activation. Given that MMP-2 is

strongly linked to glioma pathogenesis, Necl-5 may represent a target for glioma therapy, the inhibition of which may prevent invasion and dispersal.

Chapter 3 shows that Necl-5 protects the glioma cell from Fas-mediated, but not chemotherapy-induced, apoptosis. Furthermore, Necl-5 functions in the same pathway with CD44, as knockdown of both proteins by siRNA has no additive effect than knockdown of CD44 singly. The physical connection between CD44 and Necl-5 has long been known, but the consequences of this interaction were heretofore unknown. This thesis provides evidence to support the hypothesis that CD44 and Necl-5 interact to prevent Fas activation in the presence of ligand, and thus to prevent receptor-mediated apoptosis.

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Chapter 2: Necl-5 (CD155/PVR) promotes glioblastoma dispersal by increasing MMP-2 expression via phosphatidylinositol 3-kinase/Akt activation

CHAPTER 2

Data presented in this chapter demonstrates the requirement for Necl-5 in GBM cell invasion using a 3D matrix model. It then shows that Necl-5 promotes MMP-2 expression at the transcriptional level, and loss of Necl-5 reduces total active MMP-2 secretion. MMP-2 is induced by GBM cells when they are plated on vitronectin, and this induction coincides with Akt activation. Activation of Akt is inhibited upon depletion of Necl-5, suggesting that Necl-5 promotes MMP-2 expression by promoting Akt activation. Finally, integrin-linked kinase is responsible for phosphorylating Akt in response to vitronectin and for MMP-2 production.

Necl-5 (CD155/PVR) promotes glioblastoma dispersal by increasing MMP-2 expression via phosphatidylinositol 3-kinase/Akt activation

Brian M. Enloe and Daniel G. Jay

ABSTRACT

Patients afflicted with glioblastoma (GBM) have poor survival due to dispersive invasion throughout the brain. Necl-5, a cell surface receptor for vitronectin, is expressed in GBM but not normal brain. In several GBM cell lines Necl-5 promotes migration and invasion but the mechanism is poorly understood. In this study, we show that knockdown of Necl-5 by RNAi results in markedly decreased invasion of A172 GBM cells in a 3-dimensional matrix. There is a concomitant decrease in the expression and activity of matrix metalloproteinase-2 (MMP-2), a known factor in GBM invasion and disease severity. Knockdown of Necl-5 diminishes basal activation of Akt, an established mediator of MMP-2 expression, thereby limiting the maximal Akt activation in response to vitronectin, which is due to Integrin-linked kinase (ILK). During migration, Necl-5, Akt and ILK co-localize at focal contacts at the leading edge of the plasma membrane, suggesting that these molecules may act to integrate Akt signaling at the leading edge. By virtue of its restricted expression in GBM and its role in invasion, Necl-5 may be an attractive target for limiting MMP-2 production in glioblastoma, and therefore limiting dispersal.

INTRODUCTION

Glioblastoma (GBM) is the most common and aggressive brain tumor in adults. Patients with GBM have a median survival of only 12 months, even with aggressive treatments such as surgical resection, chemotherapy and radiation (1). Despite recent advances in understanding the biology of glioblastoma, survival has not increased (1). GBM is called a "diffuse" glioma because it readily disperses beyond the tumor margins and into the brain parenchyma (1). Dispersal results in indistinct tumor margins, making complete surgical resection impossible. Tumor recurrence is due to dispersal, and usually occurs within 2 cm of the surgical margin (2). The majority of deaths are caused by recurrence, so migration and invasion, which are necessary for dispersal, are important in the pathogenesis of GBM. Understanding the molecular mechanisms of these processes would aid in developing therapies to limit GBM dispersal.

We identified Nectin-like protein-5 (Necl-5, also called CD155 or the poliovirus receptor) as a positive mediator of invasion in HT1080 fibrosarcoma cells using a functional proteomic screen (3). Necl-5 was subsequently found to promote GBM cell migration by promoting focal adhesion disassembly (4). Necl-5 is a member of the nectin-like family of proteins, and is a single-pass transmembrane protein with 3 extracellular immunoglobulin-like loops and a short cytoplasmic domain (5). Necl-5 is not expressed in the adult CNS, but is expressed in GBM (3, 6) as well as other cancers (7-9). When expressed in fibroblasts, Necl-5 physically interacts with the PDGF receptor and $\alpha_v\beta_3$ integrin at the leading edge of migration, and this interaction is believed to initiate integrin clustering and the formation of focal contacts (10). Both Necl-5 and $\alpha_v\beta_3$ integrin bind the extracellular matrix protein vitronectin, which is found along routes that glioblastoma cells travel during dispersal, such as perivascular areas of the brain (11).

Vitronectin is also secreted by GBM cells at the tumor-brain interface, where it confers protection from apoptosis (12). Vitronectin can induce matrix metalloproteinase 2 (MMP-2) expression in invasive cancers such as melanoma (13). MMP-2 is a secreted endopeptidase that can degrade ECM components such as collagen IV, and GBM cells often show elevated expression of this protease (14). MMP-2 expression positively correlates with glioblastoma invasion *in vivo* (15, 16), and MMP-2 inhibitors block glioma invasion *in vitro* (17-19). MMPs are thought to function in invasion by degrading ECM proteins in the vicinity of the tumor cell and allowing unimpeded movement, but may have other functions affecting proliferation, apoptosis, and angiogenesis (20).

In this study we addressed the role of Necl-5 in GBM dispersal, implicating it in an Akt-dependent increase in MMP-2 production. We show that Necl-5 promotes GBM cell dispersal in a 3D matrix assay. Loss of Necl-5 in the GBM cell lines A172 and U251MG reduces MMP-2 secretion, which correlates with lowered phosphorylation of Akt. Upon binding vitronectin, Akt phosphorylation and MMP-2 production is increased, and these activities were attenuated by RNAi mediated loss of Necl-5 or by pharmacological inhibition of phosphatidylinositol 3-kinase (PI3K). Integrin-linked kinase (ILK) is required for Akt phosphorylation in response to vitronectin and for MMP-2 production. Necl-5 is highly localized to the leading edge in migrating cells where it colocalizes with ILK and Akt, suggesting their potential interaction in a signaling complex. We propose that these proteins localize to the leading edge in order to integrate signals from the ECM and coordinate Akt activation and MMP production during cell migration.

RESULTS

Necl-5 promotes glioblastoma cell dispersal in a 3-dimensional matrix. It was previously shown that Necl-5 promotes glioma migration (3). Both gain and loss of Necl-5 expression showed significant effects on transwell migration but the magnitude of these effects were modest. In contrast, gain of Necl-5 expression caused marked dispersal in an *ex vivo* assay on brain slice that more closely mimics infiltration *in vivo* (4). To address this difference and assess the role of Necl-5 in GBM dispersal we used a 3-dimensional collagen matrix spheroid assay. In this assay cells must detach from a spheroid and migrate into a semi-solid matrix, which may be a better model for dispersal (21). A172 cells were implanted into a collagen I matrix and photographed immediately after collagen solidification, and then 24 hours later. The cells that moved from the spheroid into the matrix were then counted. Control cells exhibited robust dispersal from the spheroid as compared to Necl-5 depleted cells (Fig. 1A). Fig. 1B shows a detail of both control and Necl-5 depleted spheroids after 24 hours. In the Necl-5 depleted spheroids, dispersal is mostly limited to the edge of the spheroid, whereas control cells were better able to fully detach from the spheroid and move into the matrix. Figure 1C shows the quantification of dispersal, and the marked effect that Necl-5 has on this process (in control spheroids, 198 ± 36 cells/sphere disperse, whereas 52 ± 5 cells/sphere disperse in Necl-5 depleted spheres (Student's t-test; $p < 0.005$)). Not only did more control cells disperse, but they also dispersed farther than Necl-5 depleted cells. Control cells were found beyond a 1.8 mm radius from the spheroid, while the Necl-5 depleted cells did not migrate farther than 1.3 mm (Fig. 1D). From this experiment we conclude that Necl-5 is necessary for glioblastoma tumor cell dispersal because its loss markedly

decreases both numbers of cells and distance dispersed. It further suggests that Necl-5 may affect processes required for dispersal beyond what is tested in transwell migration.

Necl-5 knockdown by RNAi decreases MMP-2 production. Why might Necl-5 have a greater effect on dispersal than it does on migration? Dispersal is a complex process that must coordinate many cellular functions in addition to migration, such as matrix remodeling. One protein required for matrix remodeling is matrix metalloproteinase 2 (MMP-2). MMP-2 is well characterized in glioblastoma pathogenesis. Its expression is linked to disease severity (14, 15, 22, 23), and is correlated with increased glioma cell invasion in another 3D collagen matrix model (24). We therefore tested whether Necl-5 expression affects MMP-2 production using standard 2D cell culture conditions. We assessed the levels of MMP-2 secreted from A172 and U251MG glioblastoma cells treated with either control or Necl-5 specific siRNA. The loss of Necl-5 resulted in a 69% decrease in MMP-2 protein in conditioned medium compared to control RNAi treated A172 cells (Fig. 2A). This effect was greater for A172 than for U251MG (44%), perhaps due to less efficient Necl-5 knockdown in U251MG, so further characterization of Necl-5 effects on MMP-2 was done in A172. MMP-2 is secreted in an inactive form, so we tested if the change in MMP-2 levels correlated with enzymatic activity by using a flurogenic gelatin assay. Conditioned medium from cells lacking Necl-5 had significantly less gelatinase activity than did medium from control cells. MMP-2 activity in conditioned medium from Necl-5 depleted cells was 40% less than that from control cells after a 42-hour incubation with the flurogenic gelatin (Student's t-test; $p < 0.005$; Fig. 2B). Quantitative RT-PCR analysis shows a 40% reduction in MMP-2 mRNA upon

Necl-5 knockdown as compared to controls (Student's t-test; $p < 0.05$; Fig. 2C), suggesting that Necl-5 affects MMP-2 transcription. Together, these data show that catalytically active MMP-2 is diminished in cells that lack Necl-5, and that the reduction is in part due to transcriptional regulation.

Necl-5 enhances Akt phosphorylation and MMP-2 production in GBM cells. Akt activation promotes cellular growth and survival, and control of gelatinase activity in gliomas has been linked to activation of the PI3K/Akt pathway (25-29). Given this, we hypothesized that Necl-5 may facilitate PI3K/Akt activation in these cells. To confirm that the PI3K/Akt pathway affects MMP-2 production in gliomas, we treated A172 cells with the PI3K inhibitor LY294002 and assessed phosphorylation of Akt and MMP-2 production. The IC_{50} of LY294002 for PI3K is 1.4 μM, and inhibition is specific for PI3K at concentrations up to 50 μM (30). Phosphorylation of Akt at serine 473 is necessary for its serine/threonine kinase activity, and also correlates with metastatic properties of several cancer cell lines (31). Treatment with 50 μM LY294002 reduced both Akt phosphorylation and secretion of MMP-2 as compared to cells treated with vehicle control. The magnitude of the MMP-2 decrease resulting from LY294002 treatment was about 60% (Fig. 3A), and this was similar to the decrease observed after Necl-5 knockdown. We then tested if PI3K inhibition decreased dispersal from spheroids. Figure 3B shows control and LY294002 treated A172 spheroids. Spheroids treated with 50 μM LY294002 showed a significant 64% reduction in dispersal compared to spheroids treated with DMSO (Student's t-test; $p < 0.0001$; Fig. 3C). These results link MMP-2 production and dispersal in our model to PI3K activity. We next tested if Necl-5

knockdown affected Akt phosphorylation, which is a consequence of PI3K activity. A172 and U87MG cells depleted of Necl-5 had 47% and 62% less phosphorylated Akt, respectively, as compared to controls. Total Akt levels were unaffected (Fig. 3D). This demonstrates that Necl-5 promotes basal Akt phosphorylation in GBM cells.

Vitronectin, a ligand for Necl-5 and integrins, can induce MMP-2 expression in melanoma (13), and is a major component of GBM infiltration pathways in the brain (11). We then tested if Akt phosphorylation is enhanced by plating cells on vitronectin as compared to plating on collagen I, which does not ligate Necl-5. Control A172 cells had a 3-fold increase in Akt phosphorylation when plated on vitronectin for 60 minutes as compared to cells plated on collagen. Cells depleted of Necl-5 also showed a 3-fold increase in Akt phosphorylation when plated on vitronectin, but had an overall reduced phosphorylation by about 50% compared to control (Fig. 3E). The vitronectin-induced increase in Akt phosphorylation requires PI3K activity, as it can be completely abolished in the presence of LY294002 (Fig. 3F). These data implicate the PI3K/Akt pathway in MMP-2 production in these cells. They further suggest that Necl-5 promotes a high cellular steady-state Akt phosphorylation level, but does not affect the induced phosphorylation of Akt in response to vitronectin. Finally, we tested if vitronectin could induce MMP-2 production, and if the induction required Necl-5. Plating A172 cells on vitronectin for 24 hours increased MMP-2 secretion by 50% as compared to cells plated on collagen (Fig. 3G) and this effect was eliminated upon Necl-5 knockdown. Analysis of cell lysates from this experiment showed continued elevation of phosphorylated Akt in response to vitronectin after 24 hours, indicating a sustained signaling effect. This effect was diminished in Necl-5 depleted cells. These data demonstrate that loss of Necl-5

results in diminished Akt phosphorylation, and an inability to induce MMP-2, even in response to vitronectin.

ILK is required for Akt phosphorylation in response to vitronectin and for MMP-2 production in A172 cells. The integrin linked kinase (ILK) is expressed in several cancer types, and its expression often correlates with advancing tumor grade or disease severity (32). ILK promotes glioblastoma growth and invasion (27, 33, 34), associates with β integrins, and phosphorylates Akt at serine 473 in a PI3K-dependent manner (35). We hypothesized that ILK is responsible for the vitronectin-dependent Akt phosphorylation effect. To test this, we examined the effect of ILK knockdown on Akt phosphorylation of A172 cells plated on either collagen or vitronectin. Akt phosphorylation in response to vitronectin was completely inhibited when cells were treated with ILK specific siRNA (Fig. 4A). Basal levels of phosphorylated Akt were reduced as compared to both control and Necl-5 siRNA treated cells. ILK has been linked to MMP-2 expression and invasion in several systems (36, 37), including gliomas (27). To determine if ILK expression correlates with MMP-2 production, conditioned media from A172 control, Necl-5, or ILK knockdown cells was analyzed for MMP-2 content. Compared to control or Necl-5 depleted cells, the ILK depleted cells showed less MMP-2 secretion into conditioned media (Fig. 4B). From these experiments we conclude that ILK is necessary for Akt phosphorylation in response to vitronectin, and controls MMP-2 production in these cells.

Necl-5 associates with Akt and ILK at the plasma membrane during migration. Activation of PI3K leads to phosphatidylinositol (3,4,5) triphosphate (PIP3) production. This recruits Akt and ILK to the plasma membrane via PIP3 interaction with their pleckstrin homology domains (38). We hypothesized that activation of cells with

vitronectin would lead to the recruitment of Akt and ILK at the membrane in the vicinity of Necl-5. To test this, we plated A172 cells onto chamber slides coated with vitronectin. We then used a 20-gauge needle to create a wound in the monolayer, and allowed the cells to recover for 2.5 hours. Cells were then fixed and immunostained for Necl-5 and Akt or ILK. Images were acquired on a Leica TCS SP2 AOBS confocal microscope. Cells next to the wound could be seen extending filopodia into the wound space as migration began. Early focal contacts at the ends of these filopodia are highly enriched in Necl-5, as previously reported (Fig. 5A-B; (3, 39)). Staining for ILK and Akt also was localized to these focal contacts, and showed overlap with Necl-5 (Fig. 5A-B). These data show that Necl-5, Akt, and ILK colocalize to structures at the leading edge of migration during wound healing in A172 cells. Together with the biochemical data showing an activation of Akt and MMP-2 secretion, this suggests an important role for Necl-5 in Akt activation at the leading edge, which results in MMP-2 production and matrix degradation.

Discussion

The poor prognosis of GBM is due to the tumor's ability to disperse throughout the brain, leading to secondary tumor formation. Preventing dispersal could increase survival in GBM patients, but this process is poorly understood. In this study we show that Necl-5 has a major role in dispersal *in vitro*. We also show that Necl-5 functions to increase MMP-2 production and promote basal Akt phosphorylation in GBM cells. Loss of Necl-5 also attenuates the maximal activation of Akt by ILK in response to vitronectin. Our findings link Necl-5 to MMP-2 and Akt activation during migration, and this may be

an important sense and response apparatus that leads to matrix degradation during dispersal.

MMP-2 is well implicated in GBM pathology. It is preferentially expressed in GBM as compared to lower grade gliomas (15, 22) and its expression correlates with invasive behavior in GBM patient samples (23). Necl-5 is expressed in up to 60% of GBM (3, 6) and may contribute to increased MMP-2 expression. In GBM cell lines, MMP-2 expression correlates with *in vitro* invasion (24, 40) and its inhibition reduces invasion (19, 41). Vitronectin is produced by GBM cells (12), can stimulate GBM migration (42), and is found along preferred routes of GBM dispersal, such as perivascular areas (11). MMP-2 expression is increased in response to vitronectin, and this is implicated in the pathogenesis of other cancers such as multiple myeloma (43), and melanoma (13). Our findings provide a potential mechanism for increased GBM dispersal along vitronectin-rich routes in the brain: Necl-5-mediated increases in MMP-2 may enhance infiltration along these routes.

Our findings implicate Akt in Necl-5 mediated MMP-2 production in GBM. Akt is activated in a PI3K-dependent manner by phosphorylation at serine 473, which is antagonized by PTEN (phosphatase and tensin homologue deleted from chromosome 10) (44). The loss of PTEN is a common and important deficiency of GBM, and one consequence of this deficiency is MMP-2 transcriptional upregulation (45). Akt phosphorylation increases in migrating glioma cells (46), and increased Akt phosphorylation correlates with MMP-2 expression in gliomas *in vivo* (28). In our work, Akt phosphorylation was inhibited by loss of Necl-5 and the extent of inhibition was independent of vitronectin suggesting that Necl-5 acts in basal activation of Akt. When

we inhibited PI3K with LY294002, we observed a decrease of MMP-2 that was similar in magnitude to Necl-5 depletion. This is consistent with other reports that link Akt activation with MMP-2 transcription in GBM (26) and lung cancer (47). We also observed a dispersal defect in A172 spheroids due to LY294002, which links dispersal to PI3K/Akt activation. While Akt co-localizes with Necl-5 in the presence of vitronectin, we did not observe direct physical interaction between them. We hypothesized that integrin linked kinase (ILK) activates Akt in response to vitronectin. ILK phosphorylates Akt at serine 473 in a PI3K dependent manner and binds β integrins (35). Necl-5 localizes with $\alpha_v\beta_3$ integrin in GBM (4) and these proteins physically interact when expressed in fibroblasts (10). ILK activity is linked to MMP-2 expression and invasion in other systems (36, 37). We found that RNAi mediated depletion of ILK leads to a reduction of basal Akt phosphorylation, Akt phosphorylation in response to vitronectin, and MMP-2 secretion in A172 cells. We also found that ILK and Akt colocalize with Necl-5 at focal contacts during migration, suggesting that these proteins could act together at focal contacts. We believe a direct physical interaction is unlikely, because co-immunoprecipitation experiments did not demonstrate Necl-5 binding to either Akt or ILK (data not shown).

These findings suggest that Necl-5 serves to enhance PI3K/Akt signaling at the leading edge during migration. This signaling leads to MMP-2 production, which can facilitate invasion. Although Necl-5 is a vitronectin receptor (48), our data show that ILK rather than Necl-5 is responsible for Akt activation in response to vitronectin. ILK is a cytoplasmic protein and is not itself a receptor. We speculate that integrins with which ILK associates, such as $\alpha_v\beta_3$ or $\alpha_v\beta_5$, are the receptors in GBM cells responsible for

vitronectin mediated increases in MMP-2 and dispersal that we have observed. Necl-5 serves to enhance integrin clustering for maximal Akt activation. As Akt participates in several aspects of cellular growth and survival, this may be the selective pressure that leads to Necl-5 expression in cancer cells. In the absence of Necl-5, migration, invasion, and dispersal are inhibited. Basal levels of Akt phosphorylation are also diminished. This basal reduction prevents maximal activation of Akt in response to vitronectin, which itself is likely mediated by integrin and ILK. Therefore, we suggest that a major role of Necl-5 is to increase the basal levels of Akt activation, thereby permitting maximal Akt activation in response to vitronectin-integrin signaling via ILK, leading to MMP-2 production.

How does Necl-5 affect phospho-Akt levels? One possibility is that Necl-5 facilitates signaling from growth factor receptors such as PDGF receptor or EGF receptor, both of which are important in GBM (1). However, we found that Necl-5 depletion does not inhibit Akt activation in response to PDGF or EGF (data not shown). Another possibility is that when overexpressed, Necl-5, by virtue of its ability to bind integrins, can stimulate low-level integrin clustering and ILK-dependant Akt activation even in the absence of ligand. In the absence of Necl-5, integrin clustering is less efficient, even in the presence of vitronectin, and Akt activation is attenuated (Fig. 6). Previous observations that Necl-5 colocalizes with $\alpha_v\beta_3$ integrin (3, 10) and enhances its clustering and focal contact formation (39), and our finding that basal Akt phosphorylation is even further reduced by ILK knockdown are consistent with this interpretation.

This study has implicated Necl-5 in GBM dispersal. Its specific expression in GBM rather than adult brain makes it a candidate drug target. MMP inhibitors are ineffective as therapeutics (49). One major deficiency is that they act systemically and inhibit MMPs secreted by non-tumor cells, which may have physiological activities. Necl-5 inhibition could reduce the total amount of MMP-2 produced by a tumor, without inhibiting physiological expression of MMP-2 at other sites in the body. This is especially important in CNS tumors, where MMP-2 expression is restricted to the tumor and not found in normal brain (14, 15). Necl-5 regulates multiple functions, such as MMP production, focal adhesion turnover, and possibly other Akt-dependent processes, and as such Necl-5 inhibition may be more effective that targeting any process singly. Necl-5, therefore, represents a promising target for glioblastoma therapy, because it serves as a focal point for orchestrating various processes in dispersal.

MATERIALS AND METHODS

Cell Culture

A172 and U251MG human glioma cells were obtained from American Type Culture Collection and cultured in DMEM supplemented with 10% FBS and 100 units/mL penicillin/streptomycin. Cells were incubated at 37°C with humidified air containing 5% CO_2.

Reagents

Purified vitronectin was obtained from Promega (Madison, WI); purified collagen I was obtained from BD Biosciences (San Jose, CA); bovine serum albumin (BSA) and LY294002 were from Sigma (St. Louis, MO). Anti-Necl-5/PVR (clone D171), used for immunocytochemistry, was obtained from Lab Vision (Fremont, CA); anti-Necl-5 used for immunoblotting was a kind gift from Dr. Eckard Wimmer; AlexaFluor 594 and AlexaFluor 488 goat anti-mouse and anti-rabbit IgG secondary antibodies were from Molecular Probes (Eugene, OR); anti-total Akt, anti-ILK, and anti-phospho-Akt (Ser473) were obtained from Cell Signaling Technology (Beverly, MA). Anti-β-actin (clone AC-15) was from Sigma; anti-MMP-2 (clone 101724) was from R&D systems (Minneapolis, MN). Peroxidase-conjugated anti-mouse and anti-rabbit IgG secondary antibodies were obtained from Cell Signaling Technology.

RNAi experiments

A double-stranded siRNA oligonucleotide targeting Necl-5 (5'-CAA-CUU-UAA-UCU-GCA-ACG-UdTdT-3') was chemically synthesized (Dharmacon, Lafayette, CO) and transfected into A172 or U251MG cells using OligofectAMINE following the manufacturer's instructions using 75 nmol/L siRNA per 35 mm dish. Cells were incubated with siRNA in OptiMEM for 6 hours after which time normal growth medium was added. Cells were then incubated for 72 hours to achieve >80% knockdown of Necl-5. Control cells were transfected with a Smartpool siRNA oligonucleotide (Dharmacon) at matching concentrations. The ILK oligonucleotide was purchased from Cell Signaling Technology (Beverly, MA) and used according to the manufacturer’s instructions.

Gelatinase activity assay

Gelatinase activity was measured in concentrated serum-free A172 conditioned media using the EnzChek gelatinase/collagenase assay kit (Invitrogen) according to the manufacturer's instructions. For preparation of conditioned media, cells were incubated with serum-free DMEM for 24 hours. For RNAi experiments, conditioned media was obtained 72 to 96 hours post transfection. Media was collected and concentrated using an Amicon Ultra dialysis spin tube with a 10 kDa MWCO (Millipore). Equal amounts of total protein (approximately 100μg) were used from both control and RNAi treated cells, and incubations were done in a Costar 96 well assay plate. Fluorescence data were obtained using a Spectrafluor Plus plate reader (Tecan) at 488 nm.

Quantitative RT-PCR

Total RNA was extracted from A172 cells using an RNeasy Micro kit according to the manufacturer's instructions (Qiagen). Quantitative PCR was done using the Brilliant II SYBR green 1-step QRT-PCR Master Mix (Stratagene) according to the manufacturer's instructions, a Stratagene Mx4000 cycler, and primers for human MMP-2 (forward: 5'-CTT-CCA-AGT-CTG-GAG-CGA-TGT-3'; reverse: 5'-TAC-CGT-CAA-AGG-GGT-ATC-CAT-3') and human β-actin (forward: 5'-CAT-GTA-CGT-TGC-TAT-CCA-GGC-3'; reverse: 5'-CTC-CTT-AAT-GTC-ACG-CAC-GAT-3').

Adhesion signaling assay and immunoblotting

Control, Necl-5, or ILK siRNA-transfected cells were grown for 48 hours in 35 mm tissue culture dishes, at which time the growth media was replaced with serum-free DMEM and incubated for an additional 24 hours. Cells were detached with Versene, resuspended in serum-free DMEM and 1 x 10^5 cells were plated onto 35 mm culture

dishes coated with vitronectin (2 μg/mL) or collagen I (50 μg/mL), then blocked for 60 minutes with 1% BSA in PBS. Cells were allowed to adhere at 37°C for 60 minutes and then washed with room temperature PBS and incubated in NP40 lysis buffer [1% NP40, 20 mmol/L HEPES (pH 7.4), 2mM EDTA, 150 mmol/L NaCl] supplemented with protease inhibitors (Roche, Nutley, NJ) and phosphatase inhibitors (Sigma) at 4°C. Lysates were collected by scraping, rotated for 20 minutes, cleared by centrifugation for 15 minutes at 10,000 x g and quantified using the DC Protein Assay (Bio-Rad, Hercules, CA). Lysate (20 μg) was separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Membranes were blocked in 5% nonfat dry milk in PBS and probed with primary antibody diluted in 5% BSA in PBS overnight. Antibody binding was detected with peroxidase-conjugated secondary antibodies and visualized using enhanced chemiluminescence substrate (Perkin-Elmer, Boston, MA).

Wound healing and immunocytochemistry

Cells were deposited in 8-chamber culture slides that had been coated with 2 μg/mL vitronectin and blocked with 1% BSA in PBS for 60 minutes. Monolayers were incubated in serum-free DMEM overnight, then scratched with a 20-gauge needle and allowed to recover for 2.5 hours at 37°C. Wounds were then fixed with 2% paraformaldehyde in PBS, and blocked with 1% BSA in PBS. Antibodies were diluted in PBS/10% FBS/0.1% saponin and incubated with cells for 60 minutes at room temperature, then washed three times in PBS/1% BSA. Appropriate species-specific secondary antibodies conjugated to AlexaFluor 488 or 594 were used to visualize antibody staining. Images were acquired on a Leica TCS SP2 AOBS confocal microscope and analyzed with Openlab (Improvision) or ImageJ software (National Institutes of Health, Bethesda MD).

Preparation of conditioned media

Cells were incubated in serum-free DMEM for 24 hours at 37°C. Media was collected and concentrated using an Amicon Ultra dialysis spin tube with a 10 kDa MWCO (Millipore) following the manufacturer's instructions. Concentrated media containing 20 μg of protein was subjected to SDS-PAGE and immunoblotting.

3D collagen matrix invasion assay

This assay is a modification of the method developed by Del Duca et al (21). Briefly, cells were suspended in growth media at a concentration of $5x10^5$ cells per mL. 20 μL was placed on the inside surface of a 10 cm culture dish and incubated over 10 mL DMEM for 72 hours at 37°C. Resulting spheroids were collected and placed into 1.25 mg/mL denatured collagen I. The collagen was allowed to solidify at 37°C and covered with an equal volume of DMEM. Images were acquired by Zeiss inverted microscope immediately after collagen solidification, and then after 24 hours. Images were compiled and analyzed using OpenLab software (Improvision). Cells were counted manually.

FIGURE LEGENDS

FIGURE 1. Necl-5 promotes glioblastoma cell dispersal in a three dimensional matrix. **A.** A172 cell spheroids were implanted into denatured collagen I (1.25 mg/mL) and photographed after gel solidification and then again 24 hours later. Control cells dispersed into the surrounding matrix, but cells lacking Necl-5 did not. Scale bar indicates 500 μm. Arrows indicate dispersing cells. Images are representative of 3 independent experiments. Immunoblots demonstrate the levels of Necl-5 and β-actin

expressed in spheroids. **B.** Closer view of control and Necl-5 depleted spheroids after 24 hours. Scale bars indicate 500 μm. Arrows indicate dispersing cells. **C.** Quantification of cell dispersal, showing an average of 198 ± 36 control cells disperse, whereas 52 ± 5 Necl-5 depleted cells disperse (* $p < 0.005$; Student's t-test). **D.** Number of cells dispersed graphed as a function of distance, showing that a greater number of control cells migrated farther than Necl-5 depleted cells.

FIGURE 2. Necl-5 promotes MMP-2 expression in A172 and U251 glioblastoma cells. **A.** Cells were treated with control or Necl-5 specific siRNA (75 nM) and serum-free conditioned media was collected. Twenty micrograms of protein was loaded in each lane. Immunoblots were probed with an MMP-2 specific monoclonal antibody. The decrease in MMP-2 levels were estimated by band densitometry, which is shown above the blot. Quantification of conditioned media is relative to intensity of the control band. Twenty micrograms of total cell lysate was also run and probed for Necl-5 and β-actin. **B.** Gelatinase activity assay. 100 μg of total protein from conditioned media was incubated with quenched fluorogenic gelatin for 42 hours at 37C. Gelatinase activity was measured by fluorescence intensity (* $p < 0.005$; Student's t-test). Results presented are typical of three independent experiments. **C.** Quantitative real time PCR was used to measure the relative amount of MMP-2 mRNA produced by control compared to Necl-5 depleted cells. Control mRNA levels were set to 100%, and relative level in the Necl-5 depleted cells was quantified using the ΔΔCt method (50) (* $p < 0.05$; Student's t-test). Results are an average of three replicates.

FIGURE 3. Necl-5 expression promotes Akt phosphorylation and MMP-2 expression in GBM cells. **A.** MMP-2 secretion is diminished by PI3 kinase inhibition in A172.

Treatment of cells with the PI3K inhibitor LY294002 (50μM) inhibited both Akt phosphorylation and MMP-2 secretion into the conditioned media. Control cells were treated with an equal volume of DMSO vehicle. Quantification is estimated by band densitometry, and values are shown above the blots. Values are relative to intensity of control. **B.** PI3K inhibition reduces dispersal from A172 cell spheroids. Spheroids were prepared as previously described and incubated in the presence of either 50 μM LY294002 or DMSO vehicle for 24 hours. The drug treated spheroids showed fewer invading cells. Scale bars represent 130 μM. **C.** Quantification of dispersal relative to control. Cells from control or drug treated spheroids were counted and graphed. Drug treated spheroids showed greater than 60% reduction in the number of invading cells compared to vehicle treated spheroids (* $p < 0.0001$; Student's t-test). **D.** A172 and U251MG (U251) cell lysates were subjected to SDS-PAGE and immunoblot using the designated antibodies. Necl-5 depletion leads to a reduction of phosphorylated Akt, but not total Akt. The decrease in phosphorylation is estimated by densitometry, and shown above the blot. Here, the ratio of pAkt to total Akt is used, and control is set to 1. **E.** Adhesion signaling assay. A172 cells were allowed to adhere to dishes coated with collagen I (col) or vitronectin (vn). Phosphorylated Akt levels were elevated upon exposure to vitronectin. This effect was attenuated in cells lacking of Necl-5. The change in phosphorylation is estimated by densitometry, taking the ratio of pAkt to total Akt, and shown above the blot. **F.** Phosphorylation of Akt in response to vitronectin depends upon the activity of PI3K. A172 cells were plated onto vitronectin for 60 minutes in the presence of 50 μM LY294002 (+) or an equal volume of DMSO vehicle (-). **G.** A172 cells induce MMP-2 expression in response to vitronectin. A172 control or Necl-5

depleted cells were plated onto dishes coated with either collagen I (col) or vitronectin (vn) for 24 hours. Concentrated conditioned media or cell lysates (20 μg) were subjected to SDS-PAGE and immunoblotting with specified antibodies. Changes in MMP-2 secretion were estimated by band densitometry, which is shown above the blot. Values are relative to intensity of control. All data are representative of at least 3 experiments.

FIGURE 4. ILK is necessary for vitronectin-induced Akt phosphorylation and MMP-2 production. **A.** A172 cells were treated with control, Necl-5, or ILK specific siRNA and plated onto collagen (col) or vitronectin (vn). Akt phosphorylation was assessed by immunoblot. Akt activation in response to vitronectin was absent upon ILK knockdown. **B.** A172 cells were treated with control, Necl-5, or ILK specific siRNA. Serum-free conditioned media was collected and 20 μg subjected to SDS-PAGE and immunoblot with MMP-2 specific antibody. MMP-2 levels were reduced by Necl-5 depletion, and nearly eliminated by ILK depletion.

FIGURE 5. Necl-5 associates with Akt and ILK during migration. A172 cells were plated onto chamber slides coated with vitronectin. A 20-gauge needle was used to create a wound in the monolayer, and cells were allowed to recover for 2.5 hours. Cells were then fixed and immunostained for Necl-5 and Akt (Fig. 5A) or Necl-5 and ILK (Fig. 5B). Cells extended filopodia into the wound space during migration. Early focal contacts at the ends of these filopodia contained Necl-5. Staining for ILK and Akt also was localized to these focal contacts, and showed overlap with Necl-5. Scale bars represent 30 μM for full field views and 15 μM for magnified views.

FIGURE 6. A model for Necl-5 function in GBM. Necl-5 expression leads to basal integrin clustering and activation of Akt via ILK. This is due to Necl-5 binding integrin. In the presence of vitronectin, Necl-5 binds both integrin and vitronectin, and augments integrin activation. This leads to increased ILK and Akt activity and MMP-2 production. In cells that lack Necl-5, low level clustering is absent, and basal Akt phosphorylation and MMP-2 production is reduced. Also, the response to vitronectin is less efficient, leading to decreased Akt activation and MMP-2 production.

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FIGURE 1

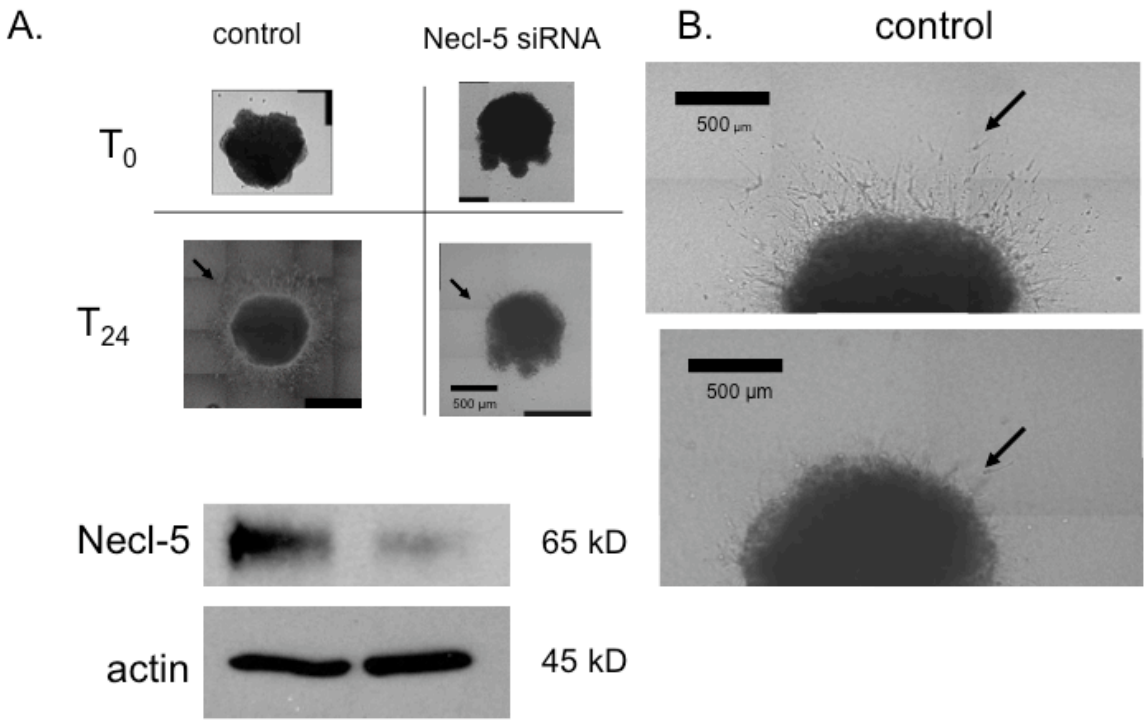
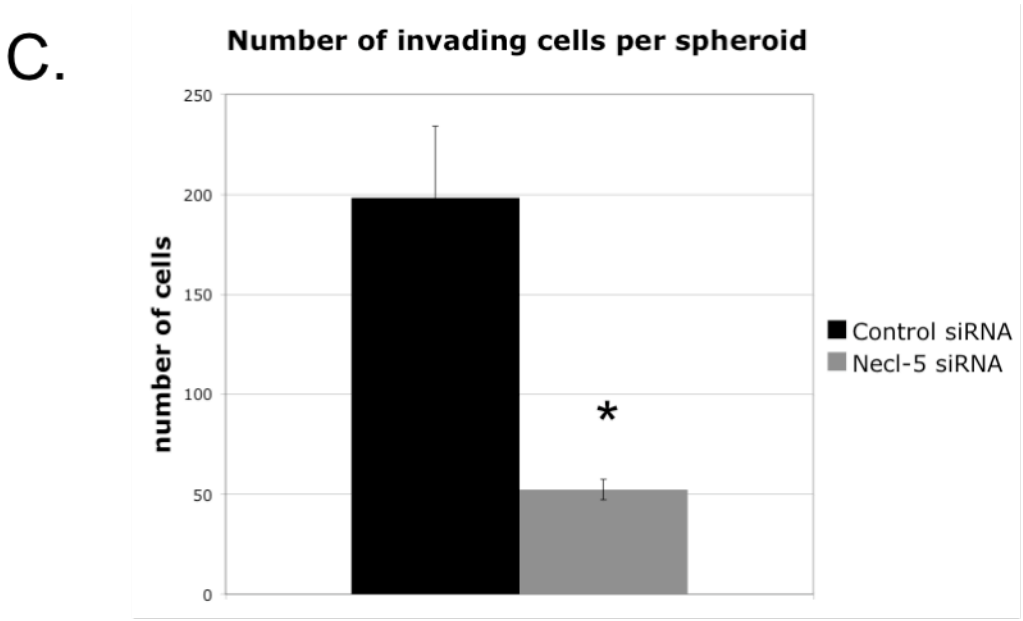


Figure 1. Necl-5 promotes glioblastoma cell dispersal in a three dimensional matrix. A. A172 cell spheroids were implanted into denatured collagen I (1.25 mg/mL) and photographed after gel solidification and then again 24 hours later. Control cells dispersed into the surrounding matrix, but cells lacking Necl-5 did not. Scale bar indicates 500 μm. Arrows indicate dispersing cells. Images are representative of 3 independent experiments. Immunoblots demonstrate the levels of Necl-5 and β-actin expressed in spheroids. B. Closer view of control and Necl-5 depleted spheroids after 24 hours. Scale bars indicate 500 μm. Arrows indicate dispersing cells.

FIGURE 1 continued



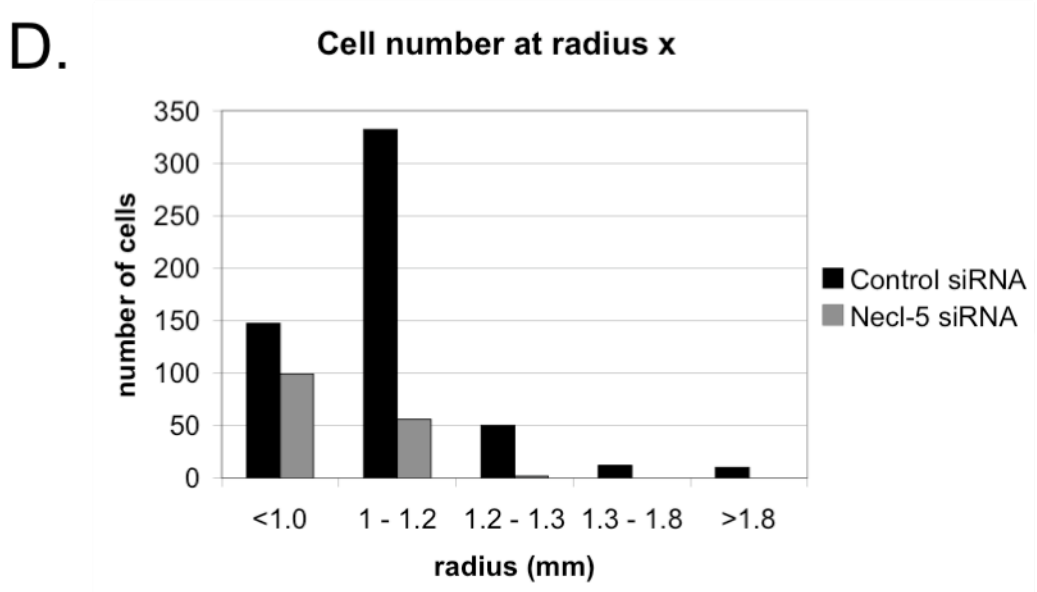


Figure 1 (continued). **C.** Quantification of cell dispersal, showing an average of 198 ± 36 control cells disperse, whereas 52 ± 5 Necl-5 depleted cells disperse (* $p < 0.005$; Student's t-test). **D.** Number of cells dispersed graphed as a function of distance, showing that a greater number of control cells migrated farther than Necl-5 depleted cells.

FIGURE 2

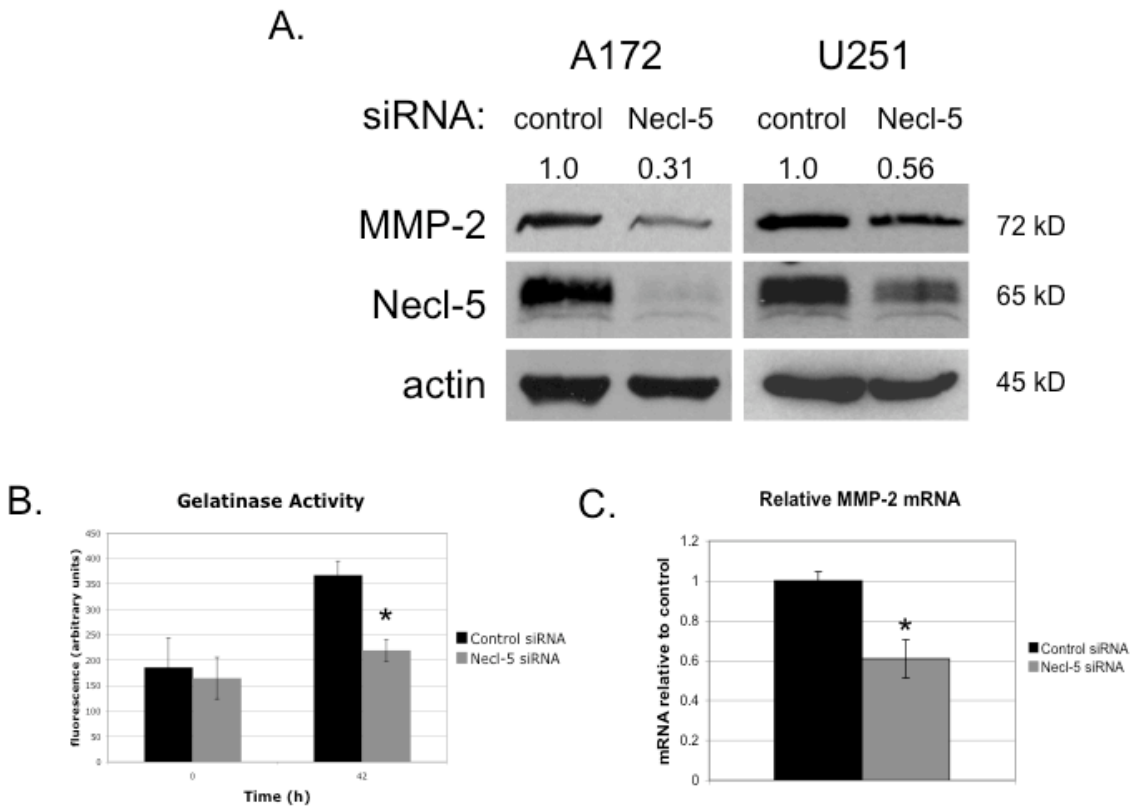


Figure 2. Necl-5 promotes MMP-2 expression in A172 and U251 glioblastoma cells. A. Cells were treated with control or Necl-5 specific siRNA (75 nM) and serum-free conditioned media was collected. Twenty micrograms of protein was loaded in each lane. Immunoblots were probed with an MMP-2 specific monoclonal antibody. The decrease in MMP-2 levels were estimated by band densitometry, which is shown above the blot. Quantification of conditioned media is relative to intensity of the control band. Twenty micrograms of total cell lysate was also run and probed for Necl-5 and β-actin. B. Gelatinase activity assay. 100 μg of total protein from conditioned media was incubated with quenched fluorogenic gelatin for 42 hours at 37C. Gelatinase activity was measured by fluorescence intensity (* $p < 0.005$; Student's t-test). Results presented are typical of three independent experiments. C. Quantitative real time PCR was used to measure the relative amount of MMP-2 mRNA produced by control compared to Necl-5 depleted cells. Control mRNA levels were set to 100%, and relative level in the Necl-5 depleted cells was quantified using the ΔΔCt method (50) (* $p < 0.05$; Student's t-test). Results are an average of three replicates.

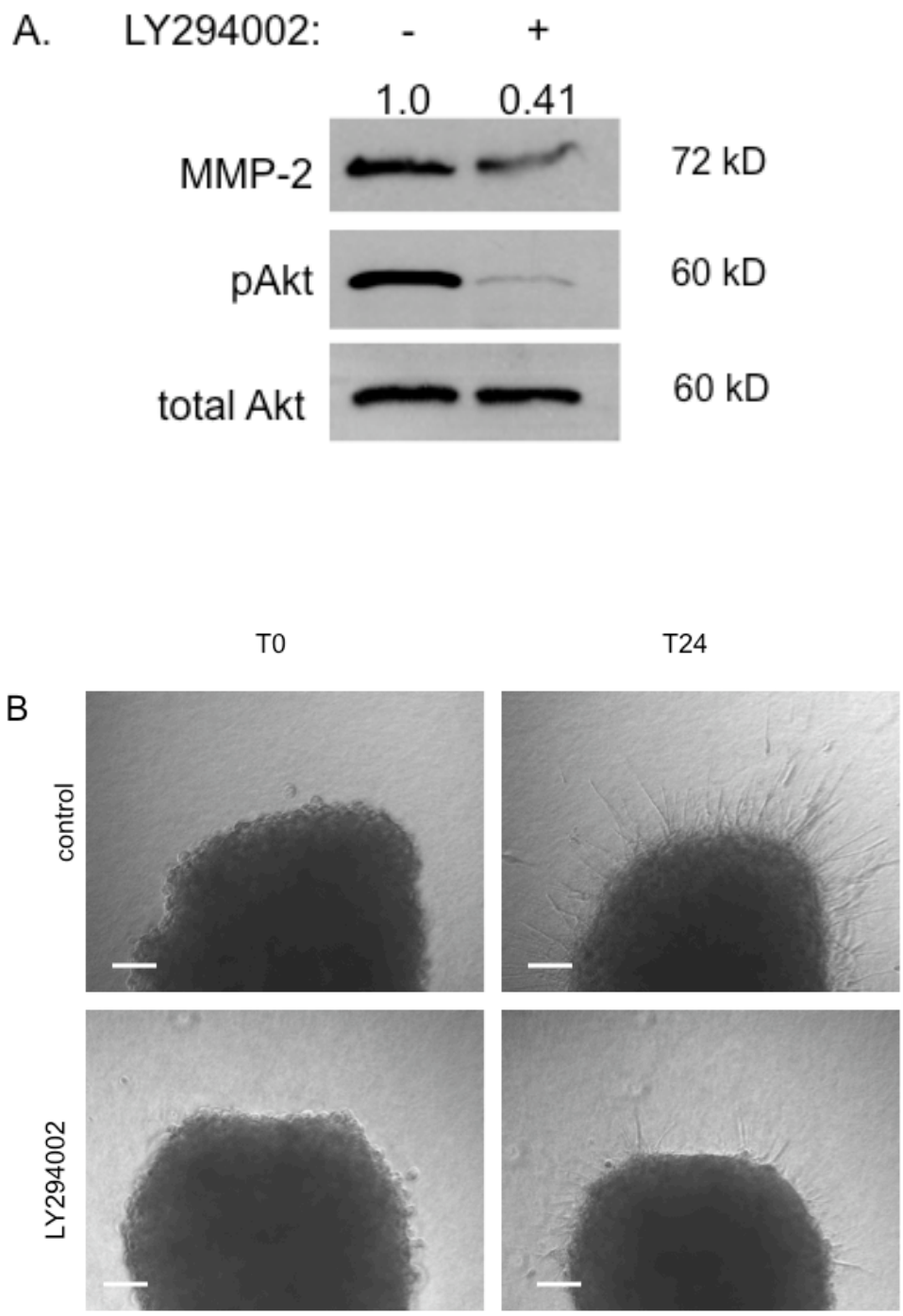


Figure 3. Necl-5 expression promotes Akt phosphorylation and MMP-2 expression in GBM cells. A. MMP-2 secretion is diminished by PI3 kinase inhibition in A172. Treatment of cells with the PI3K inhibitor LY294002 (50μM) inhibited both Akt phosphorylation and MMP-2 secretion into the conditioned media. Control cells were treated with an equal volume of DMSO vehicle. Quantification is estimated by band densitometry, and values are shown above the blots. Values are relative to intensity of control. B. PI3K inhibition reduces dispersal from A172 cell spheroids. Spheroids were prepared as previously described and incubated in the presence of either 50 μM LY294002 or DMSO vehicle for 24 hours. The drug treated spheroids showed fewer invading cells. Scale bars represent 130 μM.

FIGURE 3 continued

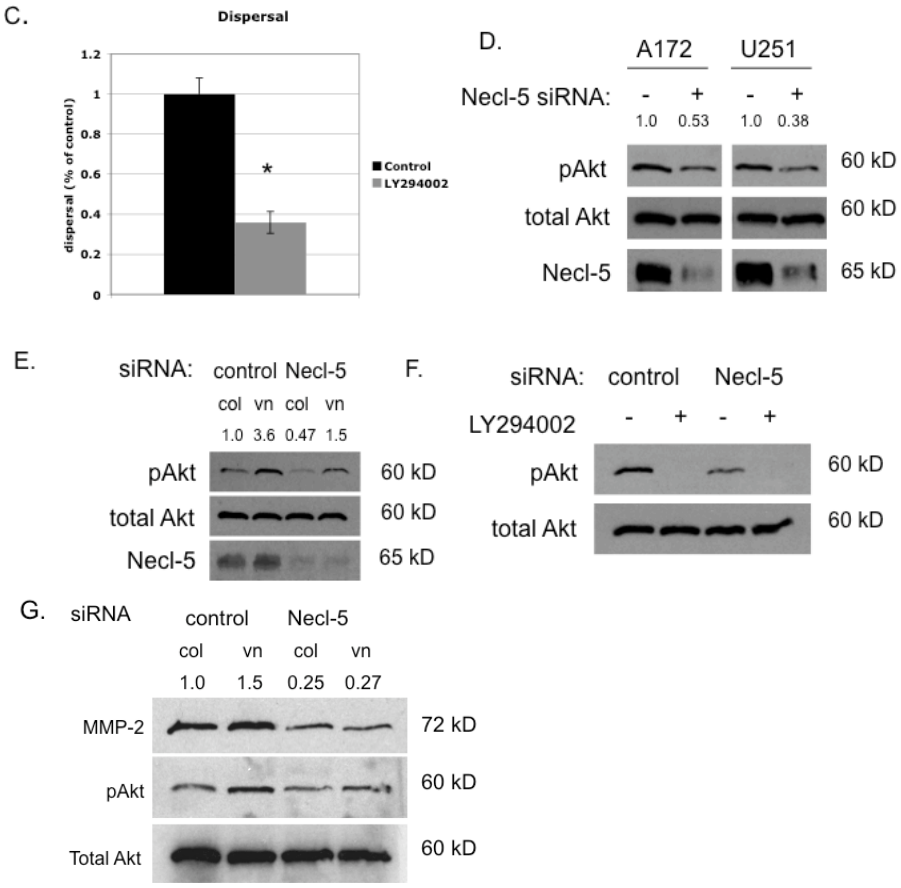


Figure 3. C. Quantification of dispersal relative to control. Cells from control or drug treated spheroids were counted and graphed. Drug treated spheroids showed greater than 60% reduction in the number of invading cells compared to vehicle treated spheroids (* $p < 0.0001$; Student's t-test). D. A172 and U251MG (U251) cell lysates were subjected to SDS-PAGE and immunoblot using the designated antibodies. Necl-5 depletion leads to a reduction of phosphorylated Akt, but not total Akt. The decrease in phosphorylation is estimated by densitometry, and shown above the blot. Here, the ratio of pAkt to total Akt is used, and control is set to 1. E. Adhesion signaling assay. A172 cells were allowed to adhere to dishes coated with collagen I (col) or vitronectin (vn). Phosphorylated Akt levels were elevated upon exposure to vitronectin. This effect was attenuated in cells lacking of Necl-5. The change in phosphorylation is estimated by densitometry, taking the ratio of pAkt to total Akt, and shown above the blot. F. Phosphorylation of Akt in response to vitronectin depends upon the activity of PI3K. A172 cells were plated onto vitronectin for 60 minutes in the presence of 50 μM LY294002 (+) or an equal volume of DMSO vehicle (-). G. A172 cells induce MMP-2 expression in response to vitronectin. A172 control or Necl-5 depleted cells were plated onto dishes coated with either collagen I (col)

or vitronectin (vn) for 24 hours. Concentrated conditioned media or cell lysates (20 μg) were subjected to SDS-PAGE and immunoblotting with specified antibodies. Changes in MMP-2 secretion were estimated by band densitometry, which is shown above the blot. Values are relative to intensity of control. All data are representative of at least 3 experiments

FIGURE 4

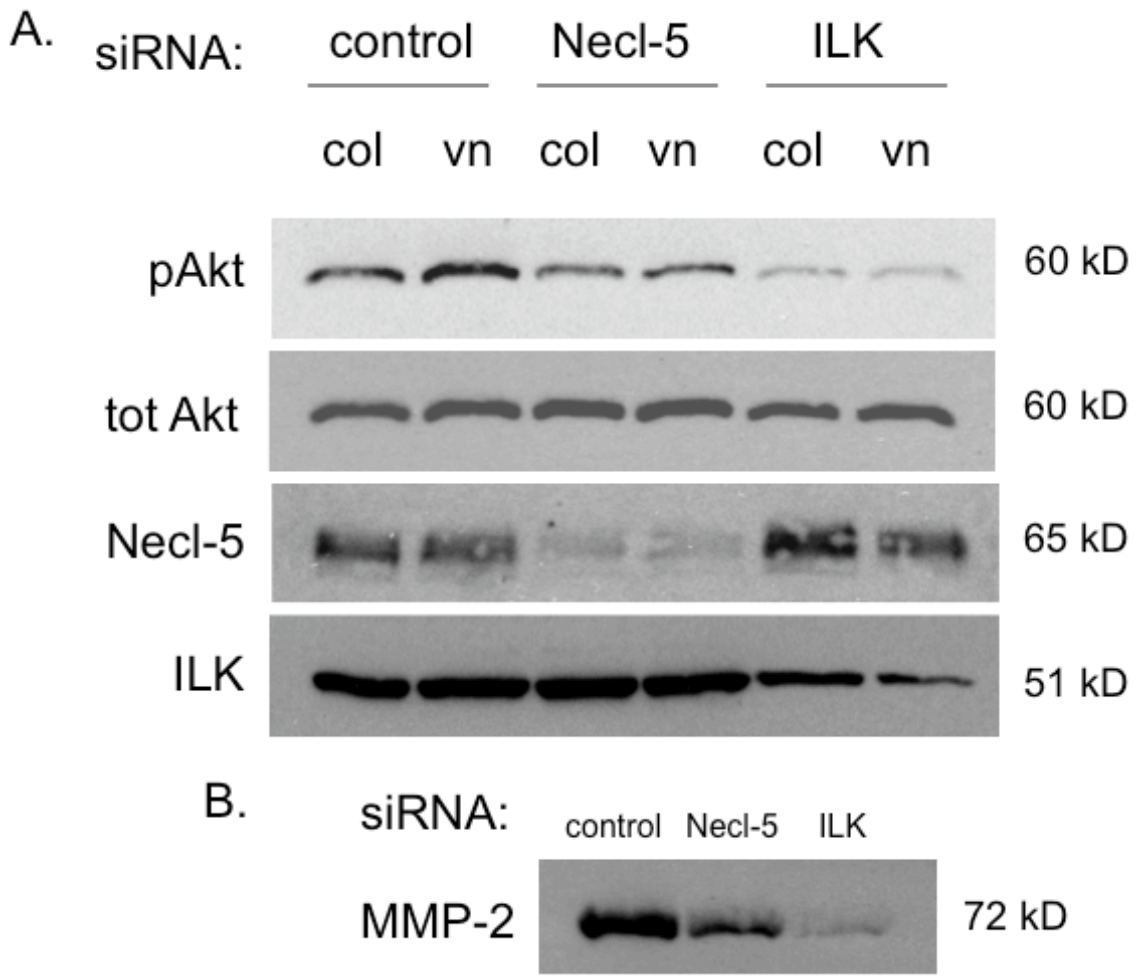


Figure 4 ILK is necessary for vitronectin-induced Akt phosphorylation and MMP-2 production. A. A172 cells were treated with control, Necl-5, or ILK specific siRNA and plated onto collagen (col) or vitronectin (vn). Akt phosphorylation was assessed by immunoblot. Akt activation in response to vitronectin was absent upon ILK knockdown. B. A172 cells were treated with control, Necl-5, or ILK specific siRNA. Serum-free conditioned media was collected and 20 μg subjected to SDS-PAGE and immunoblot with MMP-2 specific antibody. MMP-2 levels were reduced by Necl-5 depletion, and nearly eliminated by ILK depletion.

FIGURE 5

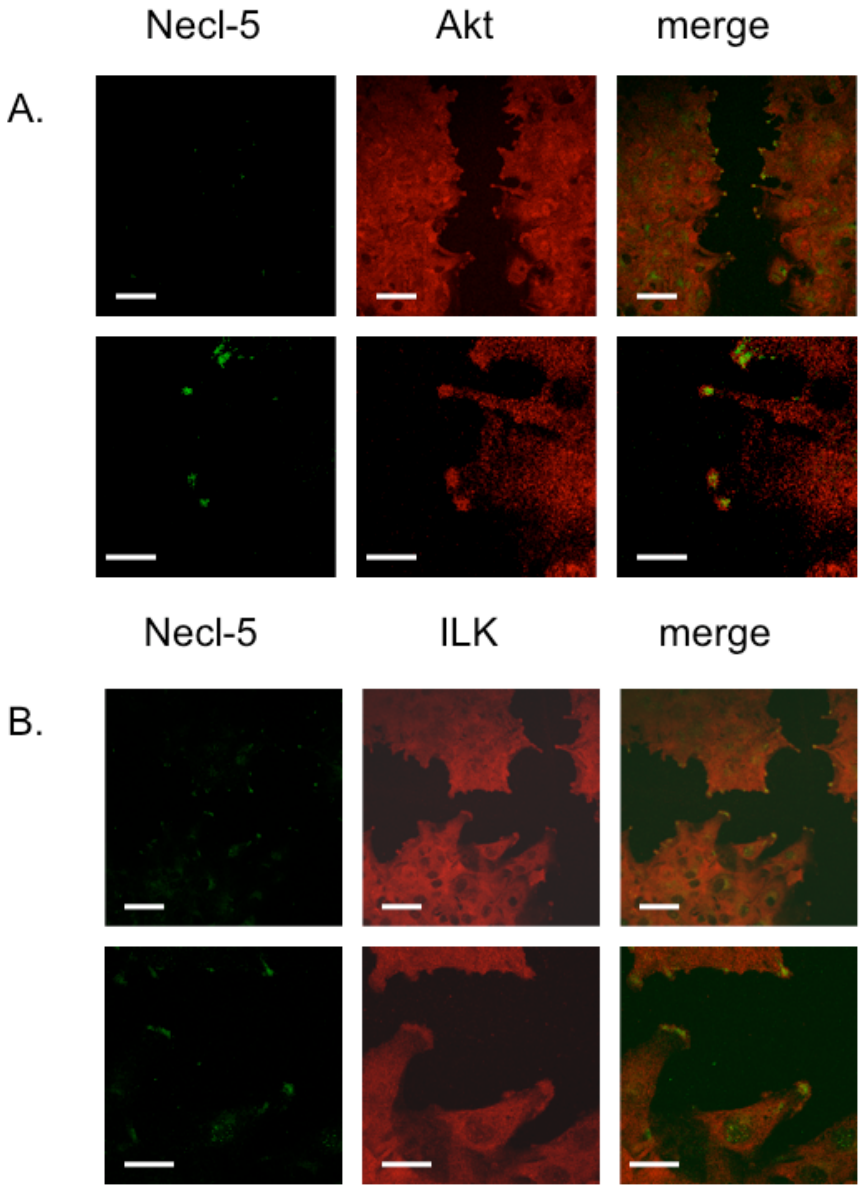


Figure 5. Necl-5 associates with Akt and ILK during migration. A172 cells were plated onto chamber slides coated with vitronectin. A 20-gauge needle was used to create a wound in the monolayer, and cells were allowed to recover for 2.5 hours. Cells were then fixed and immunostained for Necl-5 and Akt (Fig. 5A) or Necl-5 and ILK (Fig. 5B). Cells extended filopodia into the wound space during migration. Early focal contacts at the ends of these filopodia contained Necl-5. Staining for ILK and Akt also was localized to these focal contacts, and showed overlap with Necl-5. Scale bars represent 30 μM for full field views and 15 μM for magnified views.

FIGURE 6

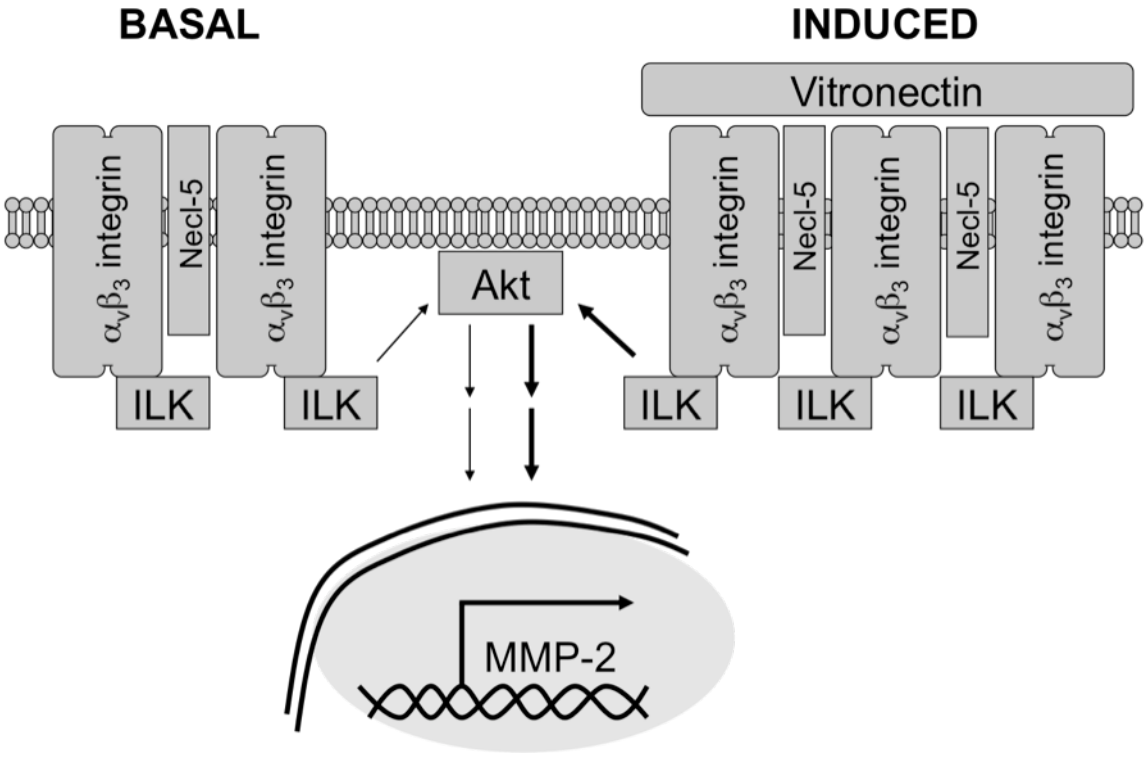


Figure 6. A model for Necl-5 function in GBM. Necl-5 expression leads to basal integrin clustering and activation of Akt via ILK. This is due to Necl-5 binding integrin. In the presence of vitronectin, Necl-5 binds both integrin and vitronectin, and augments integrin activation. This leads to increased ILK and Akt activity and MMP-2 production. In cells that lack Necl-5, low level clustering is absent, and basal Akt phosphorylation and MMP-2 production is reduced. Also, the response to vitronectin is less efficient, leading to decreased Akt activation and MMP-2 production.

Chapter 3: Necl-5 protects A172 glioblastoma cells from Fas-mediated apoptosis by its interaction with CD44

CHAPTER 3

This chapter deals with the novel role of Necl-5 in protection from Fas-mediated apoptosis. Knockdown of Necl-5 sensitized A172 cells to Fas ligand, a mediator of the extrinsic pathway of apoptosis. Necl-5 depletion did not sensitize cells to chemotherapeutic agents, however, demonstrating no role for protection from intrinsic apoptosis. I also show that in knockdown studies, both Necl-5 and CD44 protect from FasL, and there is no additive effect from double knockdown, suggesting these proteins act in the same pathway. The physical association between Necl-5 and CD44 has been known for many years, but its biological relevance has been unknown. Here, evidence is provided that they cooperate to form a complex that inhibits Fas activation in the presence of FasL.

Necl-5 protects A172 glioblastoma cells from Fas-mediated apoptosis by its interaction with CD44

By Brian M. Enloe and Daniel G. Jay

Abstract

Glioblastoma is a severe, incurable brain tumor characterized by invasive growth and apoptotic resistance. GBM cells often express Fas and Fas ligand (FasL), but are resistant to extrinsic apoptotic induction by this system. The mechanism of this resistance is unclear, and adjunct treatments to bypass this resistance could lead to apoptotic activating therapies for GBM. In this study, we show that Necl-5, a cell surface vitronectin receptor, protects GBM cells from Fas-mediated apoptosis. Further, we show that CD44, a protein known to interact with Necl-5, also protects GBM from FasL. Double knockdown experiments of both Necl-5 and CD44 show no additive effect, suggesting that they operate in the same pathway. Immunocytochemistry of Necl-5 and Fas show colocalization in areas outside of focal adhesions. Together, these findings suggest that Necl-5, CD44, and Fas form a complex that inhibits Fas signaling, and that the Necl-5-CD44 interaction is critical for resistance to Fas induced apoptosis.

Introduction

Glioblastoma (GBM) is the most common and severe brain tumor in adults. Median survival for patients afflicted with this tumor is approximately 12 months, even with aggressive treatment including surgery, chemotherapy, and radiation [1]. The severe

lethality of this cancer highlights the importance for the development of therapeutics to treat GBM. Despite intensive research into GBM pathology and the development of new potential therapies, survival times remain low [2]. Current treatments include the chemotherapeutic agents temozolomide and carmustine, which cause DNA methylation or alkylation and lead to apoptotic death [3]. Paclitaxel (taxol), which induces apoptosis by causing irreversible polymerization of microtubule subunits [4], is currently being studied as an adjuvant to alkylating agents [5]. Other therapies in development include immunotherapy using antibodies directed at glioblastoma-specific epitopes [6, 7], use of viruses to induce oncolysis [8], and stimulation of the extrinsic pathway of apoptosis [9-11].

Fas is a transmembrane receptor for Fas ligand (FasL), and ligation induces receptor trimerization, recruitment of adapter proteins and initiator caspases, and activation of caspases -8 and -3 to induce apoptosis [12]. The assembly of adapter proteins and caspases in response to Fas ligand creates the death-induced signaling complex (DISC) [13]. GBM cells express Fas and FasL, but most tumors and cell lines are relatively resistant to apoptotic induction [14]. Fas expression correlates with tumor grade in astrocytomas, with GBM having the highest expression [15, 16]. Fas expression also correlates with a high apoptotic index (AI) [17], but the relevance of this to survival is unclear. Some groups have reported that high AI leads to no increase in survival [18], while others report significant increases in survival of patients whose tumors have a high AI[19]. Investigators have sought to activate Fas signaling in GBM as a method of tumor specific cell killing. Groups have found that virus-mediated overexpression of Fas or Fas ligand increases apoptosis in experimental tumors [11, 20]. Also, although GBM cells

are resistant to Fas mediated apoptosis, the machinery of apoptosis remains intact in these cells.

GBM cells express CD44, and expression is thought to enhance invasion [21-23]. CD44 is a single-pass transmembrane glycoprotein that is highly variable in its structure due to post-translational modification such as glycosylation [24]. Our lab has previously shown that CD44, the hyaluronan receptor, interacts with Fas and prevents DISC assembly [25]. Upon siRNA knockdown of CD44, cells become sensitized to Fas mediated apoptosis. The gene for CD44 contains nine so-called "variant" exons that are not expressed in the standard form, but can be present in alternately spliced forms [24]. Other groups have shown that certain splice variants of CD44, specifically CD44v6 and CD44v9, bind and sequester Fas, thus making the receptor unavailable for DISC formation in the presence of ligand [26].

Necl-5 (nectin-like protein 5) is a 64 kDa single-pass transmembrane glycoprotein that was originally identified as the poliovirus receptor, but subsequent research has shown its involvement in cell migration, invasion, cell-cell junction formation, and proliferation [27, 28]. Our lab identified Necl-5 as a positive mediator of migration, and subsequent studies have partly elucidated its role in GBM pathogenesis [29, 30]. It promotes motility by focal adhesion disassembly, and promotes invasion by MMP-2 production [30](Chapter 2 of this thesis). It also affects steady-state Akt phosphorylation, which has been linked to both motility and apoptotic resistance (Chapter 2 of this thesis).

In this study, we show that Necl-5 protects the GBM cell line A172 from Fas-mediated apoptosis, but not from apoptosis resulting from chemotherapy. This implicates

Necl-5 in the extrinsic, but not intrinsic, pathway of apoptosis. A172 cells are sensitized to Fas ligand upon Necl-5 depletion and cell death from Fas ligand is caspase-dependent, and results in the cleavage of caspase-3. We also found that CD44 depletion in A172 also sensitized cells to Fas, but that double knockdown of Necl-5 and CD44 was not additive, suggesting that these proteins act in the same pathway. This result is strengthened by the fact that we also show that Necl-5 and CD44 are co-regulated and that these proteins colocalize in areas outside of focal adhesions. These results lead us to conclude that Necl-5 participates with CD44 to protect cells from Fas-mediated apoptosis. These finding suggest that combination of Fas tratment and Necl-5 depletion could be an efficacious therapy.

MATERIALS AND METHODS

Cell Culture

A172 human glioma cells were obtained from American Type Culture Collection and cultured in DMEM supplemented with 10% FBS and 100 units/mL penicillin/streptomycin. Cells were incubated at 37°C with humidified air containing 5% CO_2.

Reagents

Anti-Necl-5/PVR (clone D171), used for immunocytochemistry, was obtained from Lab Vision (Fremont, CA); anti-Necl-5 used for immunoblotting was a kind gift from Dr. Eckard Wimmer; AlexaFluor 594 and AlexaFluor 488 goat anti-mouse and anti-rabbit IgG secondary antibodies were from Molecular Probes (Eugene, OR); mouse anti-

caspase-8 (C15, Axxora), mouse anti-caspase-9 (C9, Cell Signaling Technology), rabit anti-caspase-3 (Cell Signaling Technology) rabbit anti-Fas (C20, Santa Cruz) rabbit CD44 (H300, Santa Cruz). Peroxidase-conjugated anti-mouse and anti-rabbit IgG secondary antibodies were obtained from Cell Signaling Technology. Apoptosis was induced with *Super*Fas Ligand (Alexis) at 500 ng/mL. Temozolomide, carmustine (BCNU), and paclitaxel (taxol) were obtained from Sigma.

RNAi experiments

Double-stranded siRNA oligonucleotide targeting Necl-5 (5'-CAA-CUU-UAA-UCU-GCA-ACG-UdTdT-3') or CD44 (5'-GAACGAAUCCUGAAGACAUdTdT-3') was chemically synthesized (Dharmacon, Lafayette, CO) and transfected into A172 cells using OligofectAMINE following the manufacturer's instructions using 75 nmol/L siRNA per 35 mm dish. Cells were incubated with siRNA in OptiMEM for 6 hours after which time normal growth medium was added. Cells were then incubated for 72 hours to achieve >80% knockdown of Necl-5. Control cells were transfected with a Smartpool siRNA oligonucleotide (Dharmacon) at matching concentrations.

Immunocytochemistry

Cells were plates onto glass 8-chamber slides overnight, then fixed with 2% paraformaldehyde in PBS, and blocked with 1% BSA in PBS. Antibodies were diluted in PBS/10% FBS/0.1% saponin and incubated with cells for 60 minutes at room temperature, then washed three times in PBS/1% BSA. Appropriate species-specific secondary antibodies conjugated to AlexaFluor 488 or 594 were used to visualize antibody staining. Images were acquired on a Leica TCS SP2 AOBS confocal

microscope and analyzed with Openlab (Improvision) or ImageJ software (National Institutes of Health, Bethesda MD).

Anoikis assay

35-mm dishes were coated twice with 1 mL of 12mg/mL poly(2-hydroxyethyl methacrylate) (poly-HEMA; Sigma) and allowed to air dry after each coating. Dishes were then washed twice with PBS. 2.5 x 10^5 A172 cells in 2 mL DMEM were plated and allowed to incubate overnight. Cells were then collected, washed once in PBS, and stained with an alexa fluor 488-annexin V conjugate and propidium iodide from the Vybrant Apoptosis Assay Kit #2 (Invitrogen) according to the manufacturer's protocol. Cells were then wet-mounted onto glass slides and observed on a Nikon Diaphot 200 microscope. Cells were scored as indicated in the text.

Cell death assay

Cells were treated with drug, FasL, or vehicle control for 72 hours, then live cells were detected using CellTiter 96 non-radioactive cell proliferation assay (MTS; Promega) following the manufacturer's instructions. Results were quantified using a fluorescent plate reader (SpectraFluor Plus, Tecan). The MTS assay counts live cells, and thus cannot distinguish apoptotic death from reduced proliferation. However, our lab has previously shown that siRNA mediated knockdown of Necl-5 does not alter the growth kinetics of A172 cells [30].

RESULTS

Necl-5 protects cells in suspension from anoikis. Detached cells with unligated integrins can undergo a form of apoptosis termed "anoikis" [31]. This process is important in metastasis, especially when cells are disseminated hematogenously or through the lymphatic system. Cells detach from the main tumor, intravasate through a capillary wall, and then are carried through the circulation in suspension. Cells that are sensitive to anoikis will not disseminate through blood or lymph. Cells that have acquired resistance to anoikis, however, may survive and populate distant organs [32]. We tested whether A172 glioblastoma cells were sensitive to anoikis by growing them in dishes coated with poly-hema, a hydrophobic polymer that does not permit cell attachment. After 24 hours, the cells were collected and stained with Alexa-488 conjugated Annexin-V and propidium iodide (PI). Cells were mounted and observed using a Zeiss axioplan inverted fluorescent microscope. Cells were scored as "live" if no staining was present, "dead" if the nucleus was stained and had normal morphology, and "apoptotic" if the cell membranes were stained with annexin and showed either no nuclear staining (early anoikis) or condensed, pyknotic nuclear staining (late anoikis). Fig.1 shows these data from a total of 248 control cells grown on poly hema and 26 (10.5%) cells were apoptotic, 77 (31%) cells were dead, and 145 (58.5%) cells were alive. Cells depleted of Necl-5 by siRNA were also grown in suspension for 24 hours, stained and counted. In this case, of the 261 cells counted, 73 (28%) cells were apoptotic, 120 (46%) cells were dead, and 68 (26%) cells were alive. Therefore, loss of Necl-5 promoted a 2.5-fold increase in apoptotic cells as compared to control cells. We therefore conclude that Necl-5 protects cells from anoikis.

One possible mechanism that explains this phenomenon is the ability of Necl-5 to cluster integrins and promote their activation, even in the absence of ligand. We have previously demonstrated the ability of Necl-5 to activate Akt in the absence of an integrin ligand. Loss of Necl-5 diminishes steady-state pAkt levels in A172 and U251MG cells, and attenuates Akt phosphorylation by ILK in the presence of vitronectin (chapter 2). This process likely requires integrin (such as αvβ3 or αvβ5) as the vitronectin receptor. We speculated that Necl-5 does this by binding integrin and promoting its clustering and activation, even in the absence of ligand. In the presence of vitronectin, clustering and activation is less efficient, leading to attenuated Akt activation. In an analogous mechanism, we speculate that Necl-5 leads to low level clustering and activation of integrins, and this inhibits an anoikis signal from being generated in suspension.

GBM cells are not metastatic in the conventional sense. They do not usually move from one organ to another. Instead, their major mechanism of pathogenesis is invasion throughout the brain and subsequent secondary tumor formation. For this reason we propose that protection from anoikis may be most important in tumors that express Necl-5 and also spread hematogenously, such as colon adenocarcinomas.

Necl-5 does not protect glioblastoma cells from alkylating agents or microtubule stabilizers. Frontline chemotherapeutic agents used in the treatment of GBM include the alkylating agents carmustine (BCNU) and temozolomide [33]. Paclitaxel (Taxol) is currently being studied for use as an adjuvant for glioblastoma chemotherapy, and works by promoting microtubule assembly, and inhibiting disassembly [34]. Treatment with these drugs may lead to apoptosis in GBM. As Akt activation correlates with protection

from apoptosis, and Necl-5 promotes Akt activation, we hypothesized that Necl-5 may protect GBM cells from chemotherapeutic apoptosis.

To test this, we treated A172 cells with temozolomide (TMZ; 100 μM), carmustine (BCNU; 75 μg/mL) or paclitaxel (taxol; 25 μM) for 72 hours, and then measured remaining live cells using an MTS assay. For TMZ, we found an approximately 20% reduction in cell number; for BCNU, we found an approximately 70% reduction in cell number; for taxol we found an approximately 50% reduction in cell number (Fig. 2A-C). These values did not change significantly in Necl-5 depleted cells, nor did they change in response to plating on vitronectin, which we have previously shown to cause a 3-fold increase in pAkt levels in A172 (Fig. S1). From these data, we conclude that Necl-5 does not protect GBM cells from chemotherapy-induced cell death or apoptosis. As chemotherapeutic agents are thought to promote cell death by activating the intrinsic pathway of apoptosis [35], these results suggest that Necl-5 does not promote resistance to effectors of this pathway.

Necl-5 protects GBM cells from Fas-mediated apoptosis. Many GBM tumors and cell lines express Fas [15], but are weakly sensitive to Fas-induced apoptosis [14, 36]. We tested the role of Necl-5 in protecting cells from Fas-mediated apoptosis by treating either control or Necl-5 depleted A172 cells with either 500 ng/mL recombinant Fas ligand (super-Fas ligand; Alexis) or vehicle (PBS). Necl-5 depleted cells showed greater than 80% cell death by apoptosis over 72 hours, a significant increase (Fig. 3A, $p < 0.0001$;Student's t-test). By comparison, control cells showed no significant cell death after 72 hours exposure to Fas ligand (Fig 3A). U251MG cells showed significantly

more resistance to Fas, even upon Necl-5 knockdown, varying between 0% and 20% cell death between experiments (Fig. S1).

Fas-mediated apoptosis in A172 cells is caspase dependent, but does not result in detectable caspase-8 cleavage. One possible mechanism for Necl-5 protecting the cell from Fas-mediated apoptosis is that Necl-5 may inhibit Fas expression. We tested this by treating control and Necl-5 depleted cells with Fas or vehicle alone. Unstimulated A172 cells express Fas, and treatment with Fas ligand causes upregulation of Fas (Fig. 3C). Cells depleted of Necl-5 also express Fas and upregulate Fas in the presence of Fas ligand. This experiment demonstrates that Necl-5 does not inhibit Fas expression or its upregulation in the presence of Fas ligand.

The first caspase mediated step of receptor-mediated apoptosis occurs after assembly of the DISC and results in the cleavage of caspase 8 from a 55 kDa pro form to generate active 43/41 kDa and 18 kDa fragments [37]. A172 cells express caspase-8, but it is not cleaved in response to Fas (Fig. 4B). In order to determine if cell death from Fas is caspase dependent, we treated A172 cells with Fas in the presence of 50 μM of the pan-caspase inhibitor Z-VAD-FMK. Control cells treated with Fas were resistant to apoptosis, as expected. Control cells treated with the caspase inhibitor were not affected, indicating that the drug was not toxic at this concentration. Cells lacking Necl-5 were sensitive to Fas, and the caspase inhibitor rescued this sensitivity (Fig. 3B). This experiment demonstrates that the cell death in response to Fas is caspase dependent.

With respect to extrinsic apoptosis, cells can be divided into Type I or Type II cells. Type I cells can efficiently transduce a signal from Fas to caspase-3 by activating a

large amount of caspase-8. Type II cells, by contrast, respond to Fas ligand by cleaving a small amount of caspase-8 and then relying on the intrinsic pathway, including caspase-9 activation, to eventually activate caspase-3 [38, 39]. Given that A172 cells do not show detectable caspase-8 cleavage in response to Fas ligand, we tested for caspase-9 cleavage. A western blot for caspase-9 showed a detectable increase in cleavage of this caspase after Fas ligand treatment Fig. 4C. This was measured by densitometry and then by taking the ratio of cleaved caspase-9 to full length. This ratio was set to 1 for control cells treated with vehicle alone, then compared to other conditions. As seen in Fig. 4C, the activation index for caspase-9 increases 6-fold in Necl-5 depleted cells treated with Fas ligand, as compared to untreated controls. Treated controls and untreated Necl-5 knockdown cells show nearly a 4-fold and 3-fold increase respectively, but this is not correlated with an increase in apoptosis as estimated by MTS assay. However, 6-fold increase in Fas ligand treated Necl-5 depleted cells is consistent with the behavior of Type II cells, and suggests that Fas mediated apoptosis in A172 occurs in conjunction with the intrinsic pathway.

Caspase-3 is an executioner caspase, and cleavage of this protein irreversibly commits the cell to apoptosis. We found that caspase-3 was cleaved after Fas treatment only in cells that had been depleted of Necl-5 and treated with Fas ligand (Fig. 4A). This is additional evidence that Fas mediates caspase dependent apoptosis is A172 cells, and that Necl-5 protects the cell from this.

Necl-5 and CD44 act together to inhibit Fas-mediated apoptosis in A172. CD44 is a transmembrane glycoprotein that acts as the hyaluronan receptor. CD44 expression has been linked to diverse cellular functions such as migration, invasion, and drug resistance

[24]. Our lab has previously demonstrated that CD44 protects the cell from Fas mediated apoptosis [25]. CD44 has been shown to colocalize with Necl-5 [40], and treating cells with CD44 antibodies prevents poliovirus infection *in vitro* [41]. CD44 shows no involvement in poliovirus entry or replication [42, 43], suggesting that the interaction of Necl-5 and CD44 may have to do with the cellular function of these proteins, rather than a viral function. To test whether Necl-5 and CD44 work together to prevent Fas-mediated apoptosis, we treated A172 cells with CD44 specific siRNA, Necl-5 specific siRNA, or both. The CD44 siRNA targets exon 5, which is a sequence common to all isoforms. We then treated cells with either 5 or 500 ng/mL Fas ligand, and assayed for cell viability after 72 hours. As seen in Fig. 5A, treatment with 500 ng/mL Fas ligand has no significant effect on cells treated with control siRNA. However, treatment with Necl-5 specific siRNA reduces cell number by about 65% compared to control. Treatment with CD44 specific siRNA reduces cell number by about 75%. Cells lacking both Necl-5 and CD44 also showed about 75% reduction in cell number when exposed to Fas ligand, and the difference was not statistically significant to CD44 knockdown alone (Student's t-test; $p = 0.18$). This indicates that the contributions of Necl-5 and CD44 are not additive, and suggests that these proteins are operating in the same pathway. In order to exclude the possibility that we were reading a maximal apoptotic signal, we repeated the experiment using 5 ng/mL Fas ligand (Fig. 5B). In this case, total cell death was less than that induced by 500 ng/mL Fas ligand. Necl-5 depletion resulted in a 25% reduction in cell number, and either CD44 or CD44-Necl-5 depletion resulted in a 30% reduction in cell number. The difference between CD44 and CD44-Necl-5 was not significant (Student's t-test; $p = 0.27$).

In order to confirm Necl-5 and CD44 knockdown, we performed a western blot on lysates obtained from cells treated with control siRNA, Necl-5 siRNA, or CD44 siRNA. As shown in Fig. 5C, knockdown of each species was achieved. Surprisingly, knockdown of Necl-5 resulted in an increase in CD44 expression, and knockdown of CD44 resulted in increased Necl-5 expression. Treatment with both Necl-5 and CD44 siRNA reduced both species. This can be explained in terms of coregulation. A loss either CD44 or Necl-5 causes transcriptional upregulation from promoters at both loci. Both Necl-5 and CD44 are known to be regulated by an AP-1 binding site in their promoters. Ras activity is also known to stimulate AP-1 dependent transcription of both promoters.

Colocalization of Necl-5 with CD44 and Fas. One model for CD44v modulation of the Fas signal is that CD44 sequesters Fas and prevents DISC assembly [25, 26]. In support of this hypothesis is immunocytochemical data showing colocalization of CD44v6 and Fas [26]. Also, our lab has shown that FALI-treated CD44 results in FAS oligomerization, which is also consistent with a direct interaction with Fas [25]. Given this, we tested if Necl-5 and Fas colocalized in A172 cells. As shown in Fig. 6A, costaining for Necl-5 (green) and Fas (red) showed several areas of overlap. Much of the overlap was in perinuclear areas, possibly indicating a common expression pathway to the surface. Costaining was also apparent in peripheral membrane areas, suggesting areas of functional overlap. We also observed colocalization of Necl-5 and CD44. As shown in Fig 6B, areas of overlap are seen in perinuclear areas and in limited areas around the membrane. There are other areas where no localization occurs. This may reflect different functional compartmentalization, such as Necl-5 but not CD44 localization to focal

adhesions. Also, it may reflect different affinities of Necl-5 for different isoforms of CD44. These data are consistent with the interpretation that Necl-5 and CD44 participate to sequester Fas such that DISC formation is not efficient in the presence of FasL.

DISCUSSION

In this study, we demonstrate the role of Necl-5 in apoptosis. We have shown that it protects A172 glioblastoma cells from both anoikis and Fas mediated apoptosis, but has no effect on chemotherapy-induced apoptosis from alkylating agents or paclitaxel. As resistance to apoptosis is a hallmark of cancer, Necl-5 may be an attractive target for sensitizing tumors to pro-apoptotic stimuli such as Fas, which stimulates the extrinsic apoptotic pathway.

Necl-5 has a well-described relationship with integrins. It is known to bind αvβ3 integrin at the leading edge of migration, and this interaction results in cell polarization and directional movement [44]. Necl-5 also potentiates integrin signaling. Loss of Necl-5 reduces the maximal activation of Akt in response to vitronectin, which involves integrin linked kinase, and presumably either αvβ3 or αvβ5 integrin (Chapter 2). It was this connection between integrins and Necl-5 that led to the hypothesis that Necl-5 could protect cancer cells from anoikis, which is apoptosis resulting from unligated integrins. We found that when grown in suspension, the proportion of apoptotic cells increased from 10% to 28% upon Necl-5 knockdown. Loss of Necl-5 affects several pathways that are involved in survival signaling from integrins, such as Akt, FAK, and p130Cas [30]. Akt could contribute to stabilization of mitochondrial permeability via phosphorylation and inactivation of the pro-apoptotic caspase-9 and Bad [45, 46]. FAK, and ILK are

kinases that reside at focal adhesions. Constitutively active FAK or ILK can protect cells from anoikis [47, 48]. Necl-5 may act to protect cells by providing constitutive integrin activation via ILK or FAK. It may do this by virtue of its ability to bind and cluster integrin, even in the absence of ligand.

Anoikis is not thought to be important in GBM. GBM cells are not distributed hematogenously or via lymphatics, and so it seems unlikely that GBM cells would not be in a position to experience unligated integrins *in vivo*. Colon cancer cells express Necl-5 [49], and this cancer type has a well-documented pathway that leads from cancer initiation, to carcinoma in situ, to metastatic disease [50]. One location that colon cancer often metastasizes to is the liver [51]. It does this by invading through capillary walls and traveling through the portal circulation to the portal vein and is delivered to the liver sinusoids. Cells then attach to ECM proteins in the liver and initiate metastatic disease. As this process requires cells to travel through the circulation, Necl-5 expression may enhance metastatic progression.

Our lab has recently demonstrated a role for Necl-5 in Akt activation (Chapter 2). We found that Necl-5 promotes steady-state Akt phosphorylation, and permitted maximal Akt activation in response to vitronectin. Akt promotes cell survival, and one way it does this is by inhibiting the activity of anti-apoptotic proteins such as those of the Bcl-2 family [45]. Given the role of Necl-5 in Akt activation in GBM, we hypothesized that Necl-5 expression would promote a general resistance to apoptosis, and that this general resistance could manifest as drug resistance. To test this hypothesis, we treated A172 and U251MG cells with three drugs that are either front-line GBM therapeutics (temozolomide, carmustine) or adjuvants under study for efficacy in GBM (paclitaxel)

[33]. Temozolomide (TMZ) and carmustine (BCNU) are alkylating agents that damage DNA and cause mitotic catastrophe, and paclitaxel (taxol) promotes the stable and irreversible interaction of microtubule subunits [4]. In each case, the cell lines were sensitive to the drug, but sensitivity did not increase upon Necl-5 depletion. We have also previously shown that Necl-5 expression can lead to maximal Akt activation in response to vitronectin, a ligand for both Necl-5 and αvβ3 or αvβ5 integrin (Chapter 2). Plating cells on vitronectin as compared to collagen before drug treatment had no significant effect on survival, arguing against the importance of Akt signaling in response to these drugs in GBM.

Fas is an important mediator of extrinsic apoptosis [37]. This type of apoptosis requires a receptor-ligand interaction, and does not necessarily require an increase in mitochondrial permeability [38]. Fas is activated by Fas ligand, and this initiates the formation of the death induced signaling complex [12]. In the presence of ligand, Fas forms a homotrimer, then recruits Fas associated Death Domain (FADD) and caspase-8 or -10. Cleavage of the initiator caspase results in activation of the executioner caspase, caspase-3. Cleavage of caspase-3 activates its proteolytic activity and allows it to cleave downstream targets that commit the cell to apoptosis [12].

It is curious to note that many GBM tumors and cell lines express both Fas and Fas ligand [15]. Immunocytochemical analysis of GBM shows that Fas is expressed in areas near necrotic areas, and that tumors that express Fas highly also have a high apoptotic index [17, 52]. It has also been shown that astrocytoma grade correlates with Fas expression [16]. Recent studies have tried to take advantage of this by engineering

GBM to express Fas or Fas ligand more highly and such an approach may offer a powerful tool for induction of apoptosis and treatment of GBM [11, 20].

We have found that A172 GBM cells are highly resistant to Fas-mediated apoptosis, despite expressing Fas. Cells that are treated with Fas ligand increase expression of Fas, but this does not increase their susceptibility to Fas, even at concentrations up to 500 ng/mL. In contrast, when A172 cells are depleted of Necl-5 using siRNA, they become highly susceptible to apoptosis in response to Fas ligand. We found that A172 cells lacking Necl-5 showed only 20% to 30% survival over 72 hours exposure to 500 ng/mL Fas ligand compared to cells expressing Necl-5. There was no statistical difference between control siRNA cells treated with Fas ligand and control cells exposed to vehicle alone. This result implicates Necl-5 specifically in protecting cells from apoptosis by Fas and Fas ligand.

We have also found that stimulation of A172 cells depleted of Necl-5 with Fas ligand induced apoptosis in a caspase dependent manner, excluding alternate forms of cell death such as autophagy or necrosis. Our finding that Z-VAD-FMK reverses the cell viability phenotype of Necl-5 depleted cells implicates caspases as the cell death effectors. Z-VAD-FMK is a pan-caspase inhibitor, and gives us no information about the type of apoptotic cascade that leads to apoptosis in this experiment. We also examined the cleavage of three major caspases: Caspase-8, -9, and -3. Caspase-8 is the initiator caspase for Fas, TNFα, and TRAIL induced apoptosis. Caspase-9 is an initiator caspase for the intrinsic pathway of apoptosis and is induced by mitochondrial membrane permeability. Both caspases eventually cleave caspase-3, the executioner caspase, which is responsible for cleaving a diverse array of substrates in the cell, such as poly-ADP-

ribose polymerase (PARP) and the δ isoform of protein kinase C. Our finding that caspase-8 cleavage was undetectable following FasL treatment after Necl-5 knockdown is surprising, but could be explained by the presence of FLIP, a caspase homolog that cannot be cleaved and which may promote resistance by inhibition of DISC formation [53]. This would lead to apparent inefficient caspase-8 cleavage, which is consistent with our findings. The finding that caspase-9, which initiates the intrinsic pathway, is cleaved upon treatment with Fas ligand, supports the interpretation that A172 are Type II cells, which require both intrinsic and extrinsic apoptotic pathways [38]. Finally, we provide evidence that caspase-3, the executioner caspase, is cleaved only in A172 cells both depleted of Necl-5 and treated with Fas ligand. Overall, these experiments support the model where Fas ligand stimulation leads to low-level caspase-8 activation, followed by activation of caspases-9 and -3.

How does Necl-5 protect cells from this type of apoptosis? Several recent studies have demonstrated a role for cell-surface receptors in sequestration of Fas, preventing its trimerization, inhibiting DISC formation, and preventing Fas-mediated apoptosis. For example, our lab identified CD44 as having a role in DISC inhibition [25]. Another lab specifically implicated the variant isoforms of CD44, CD44v6 and CD44v9, in this process [26]. There is also evidence that CD44 and Necl-5 physically interact in that a CD44 monoclonal antibody blocked poliovirus infection of Hela cells, even though only Necl-5, and not CD44, acts as the poliovirus receptor [41]. This suggests that an antibody to CD44 inhibited viral infection by steric hinderance, because of close association with Necl-5. Given these facts, we hypothesized that Necl-5 and CD44 act together to prevent Fas activation in the presence of ligand. Double knockdown experiments support this

hypothesis. Single knockdown of either Necl-5 or CD44 sensitizes A172 cells to Fas, leading to 70-80% viability reduction in response to 500 ng/mL Fas ligand. Knockdown of both Necl-5 and CD44 shows no additive effect. This experiment was repeated using 5 ng/mL Fas ligand, and, while overall cell death was less (20-30% reduction in viability), double knockdown still showed no additional effect. These results suggest that these proteins are acting in the same pathway for protection from Fas-mediated apoptosis. Interestingly, these proteins appear to be co-regulated and reciprocally expressed; single knockdown of one protein led to the induction of the other. Although the exact nature of this co-regulation is unknown, the promoters of both Necl-5 and CD44 contain AP-1 binding elements and can respond to a Ras-Raf-MEK-Erk signaling cascade [54, 55]. Co-regulation makes sense teleologically if both proteins are needed for the same biological activity.

From these data we propose the following model for Necl-5 function in protection from Fas-mediated apoptosis in A172 (Fig. 7): GBM cells express Fas, Necl-5 and CD44. CD44 (or a specific isoform) binds Necl-5, and this complex binds Fas, prevents its trimerization in response to Fas ligand, and inhibits DISC formation. In the absence of Necl-5, the stability of the CD44 interaction with Fas is significantly reduced, allowing Fas signaling to operate. Necl-5 has no affinity for Fas, so depletion of either CD44 or Necl-5 has the same effect.

Several unanswered questions still remain. First, does Necl-5 show any preference for any CD44 splice variants? It has been shown that CD44v6 and CD44v9, but not standard form, can protect Jurkat cells from Fas-mediated apoptosis [26]. It would be interesting to see if one or more splice variants would specifically co-immunoprecipitate

with Necl-5. This would demonstrate specificity to the CD44-Necl-5 interaction as it pertains to this biological function. Second, does Necl-5 inhibit DISC formation in response to Fas? Studies from our lab and others have demonstrated the role for CD44 in preventing DISC formation. The double knockdown experiments would suggest that Necl-5 has a similar role, but this remains speculative. Finally, does loss of Necl-5 in glioblastoma sensitize the tumor to therapeutic FasL? Animal studies comparing GBM control and shRNA mediated Necl-5 depleted tumors could answer that, and provide further confirmation of Necl-5's suitability as a drug target.

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FIGURE LEGENDS

Figure 1. Necl-5 protects A172 cells from anoikis. A172 cells were transfected with either control or Necl-5 specific siRNA, then a suspension of $2.5x10^5$ cells were added to a 3.5 cm dish that had previously been coated with 2 mL of 12 mg/mL poly-HEMA in 95% ethanol and left to dry. Cells were allowed to grow in suspension for 24 hours, after which time they were collected and stained with Alexafluor488-annexinV and propidium iodide. Cells were scored as either viable (no membrane annexin staining), apoptotic (prominent annexin staining with or without condensed nuclear staining) or dead (with or without annexin staining, but nuclear staining with a normal morphology). 248 control siRNA treated cells and 261 Necl-5 siRNA treated cells were scored. For control cells, 26 (10.5%) cells were apoptotic, 77 (31.0%) cells were dead, and 145 (58.5%) cells were alive. Cells depleted of Necl-5 by siRNA were also grown in suspension for 24 hours, stained and counted. In this case, of the 261 cells counted, 73 (28.0%) cells were apoptotic, 120 (46.0%) cells were dead, and 68 (26.0%) cells were alive. Loss of Necl-5 promoted a 2.5-fold increase in apoptotic cells as compared to control.

Figure 2. Necl-5 does not protect from chemotherapy-induced cell death. A172 and U251MG GBM cells were treated with **A.** temozolomide (TMZ; 100 μM), **B.** carmustine (BCNU; 75 μg/mL) or **C.** paclitaxel (taxol; 25 μM) for 72 hours, and then assayed for cell number using an MTS. For BCNU, there is an approximately 70% reduction in cell number for A172 and about 40% reduction in cell number for U251MG. For TMZ, there is an approximately 20% reduction in cell number for A172 and about 40% reduction in cell number for U251MG. For taxol there is an approximately 50% reduction in cell number for A172 and 60% reduction in cell number for U251MG. These values did not change significantly in Necl-5 depleted cells. Nor did they change in response to plating on vitronectin, which has been shown to cause a 3-fold increase in pAkt levels in A172 (supplemental figure S1).

Figure 3. Necl-5 protects A172 from Fas mediated cell death. A172 cells were transfected with either control or Necl-5 specific siRNA, then treated with either 500 ng/mL FasL or PBS control for 72 hours. **A.** Necl-5 depletion sensitizes A172 to Fas mediated apoptosis. Treatment with FasL reduced cell viability, as measured by MTS assay, by 84% as compared to control siRNA treated cells ($p < 0.0001$; Student's t-test). **B.** This effect is caspase dependent. Pretreatment of A172 cells with 50 μM of the pan-

caspase inhibitor Z-VAD-FMK completely reverses the cell death effect of FasL on Necl-5 depleted A172 cells. This experiment excludes other forms of cell death, like autophagy or necrosis, from occurring in response to FasL. **C.** Fas is expressed in A172. Cells were treated as indicated and then lysed in NP-40 buffer, subjected to SDS-PAGE and immunoblotted for Fas and actin. A172 cells express Fas, and Fas is upregulated in response to FasL. Necl-5 depletion does not upregulate Fas, although Fas is still upregulated in response to FasL. This excludes dysregulated Fas expression as the mechanism for Necl-5 mediated FasL sensitivity.

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Figure 5. Necl-5 and CD44 protect A172 from Fas mediated apoptosis by using the same pathway. **A.** Cells were treated as indicated for 72 hours, after which cell number was measured with an MTS assay. Knockdown of either Necl-5 or CD44 significantly increased cell susceptibility to FasL. Double knockdown had no additive effect, suggesting that these proteins operate in the same pathway. **B.** This experiment was also performed using 5 ng/mL FasL, with similar results. **C.** Necl-5 and CD44 are coregulated. Cells were treated as indicated, then lysed in NP-40 buffer, 20 μg subjected to SDS-PAGE, and immunoblotted for Necl-5, CD44, and actin. Knockdown of Necl-5 led to an increase in CD44 expression, and knockdown of CD44 led to an increase in Necl-5 expression. Double knockdown reduced expression of both proteins, but this did not lead to any additional sensitivity to FasL (see above).

Figure 6. Colocalization of Necl-5 with CD44 and Fas. **A.** A172 cells were stained with anti Necl-5 (D171) and anti Fas (Santa Cruz), then counterstained with Alexa 488 conjugated anti-mouse or Alexa 594 conjugated anti-rabbit antibody. **B.** A172 cells were stained with anti Necl-5 (D171) and anti CD44 (Santa Cruz), then counterstained with Alexa 488 conjugated anti-mouse or Alexa 594 conjugated anti-rabbit antibody.

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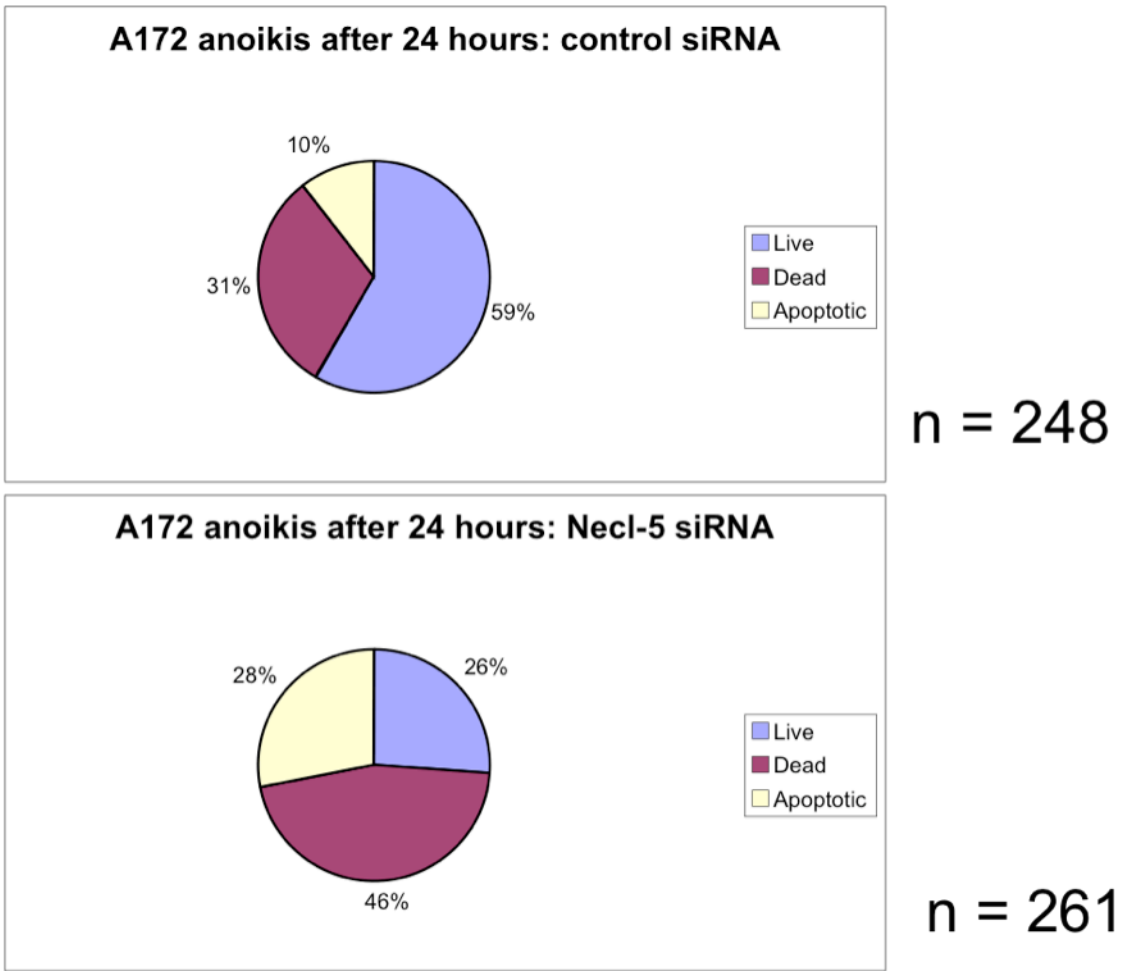
Figure S1. Complete A172 and U251 drug resistance series on collagen I (c), or vitronectin (v). Cells were treated with control (c) or Necl-5 specific siRNA. The first 4 in the series are A172 and the last 4 are U251.

FIGURE 1



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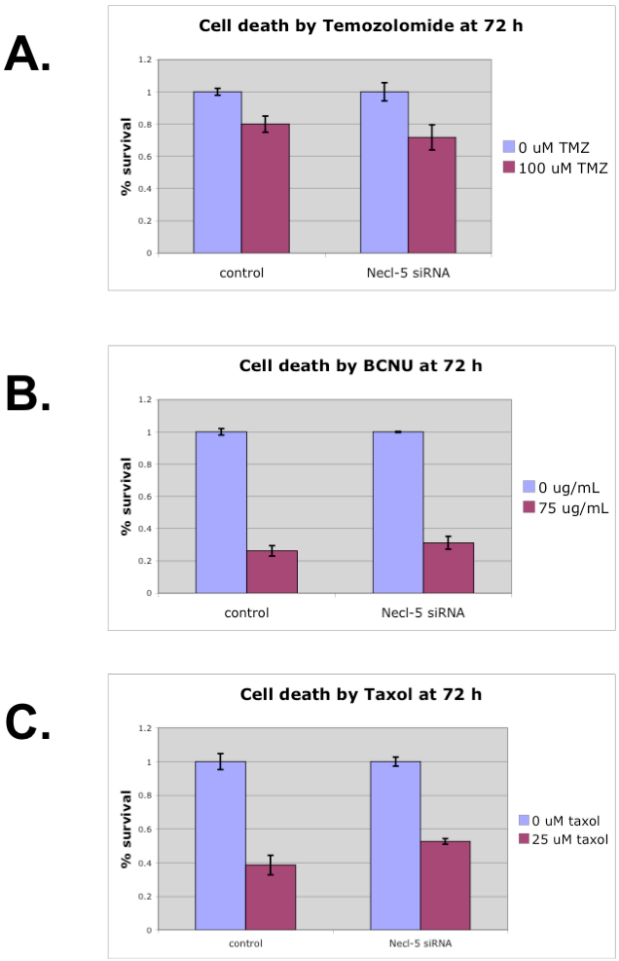

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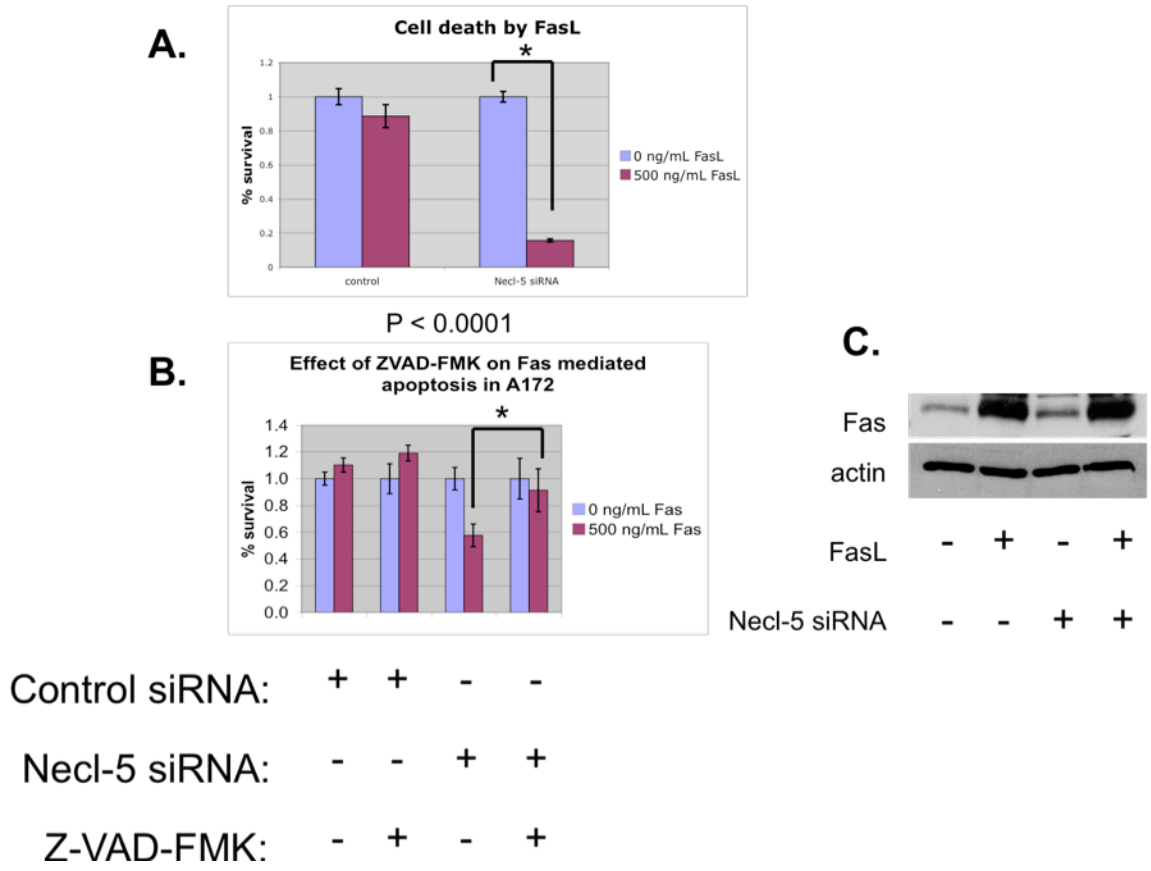

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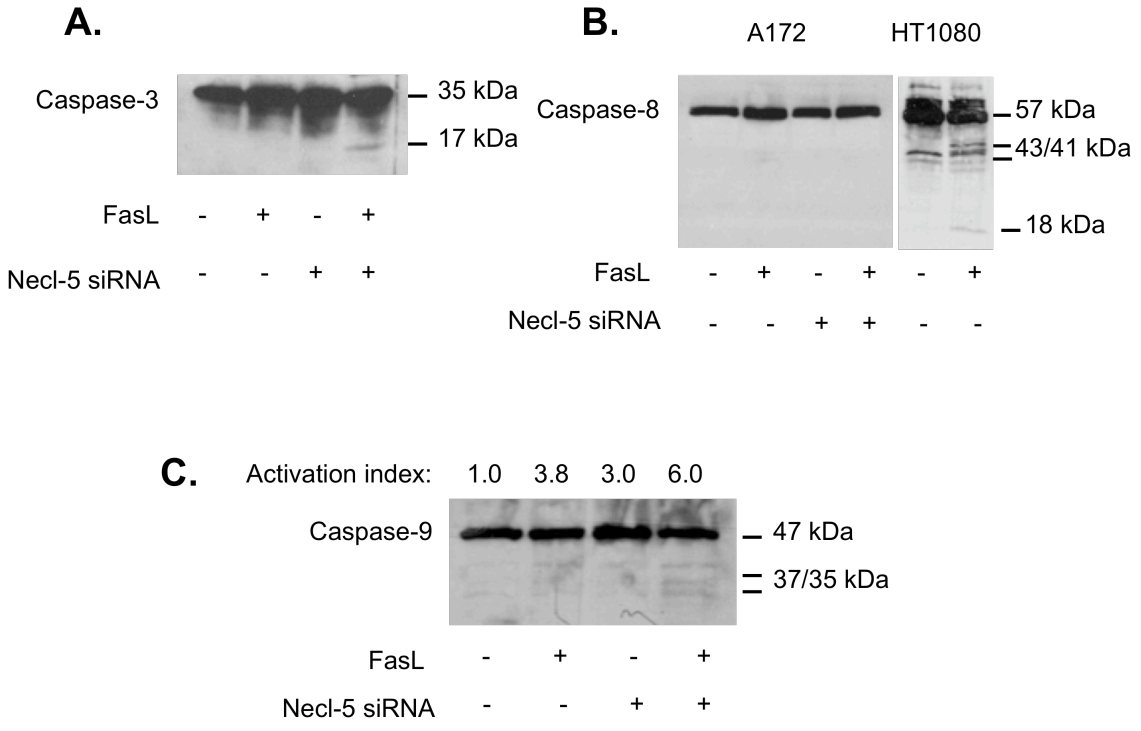

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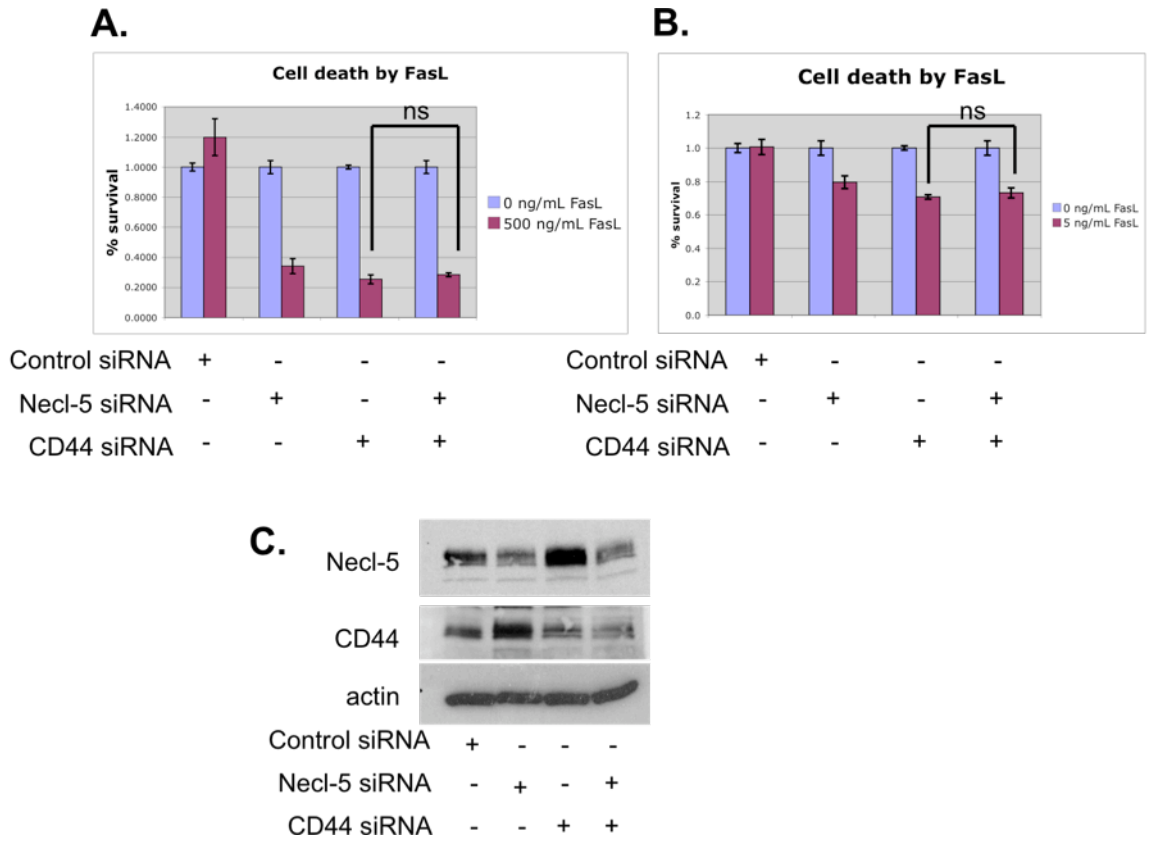

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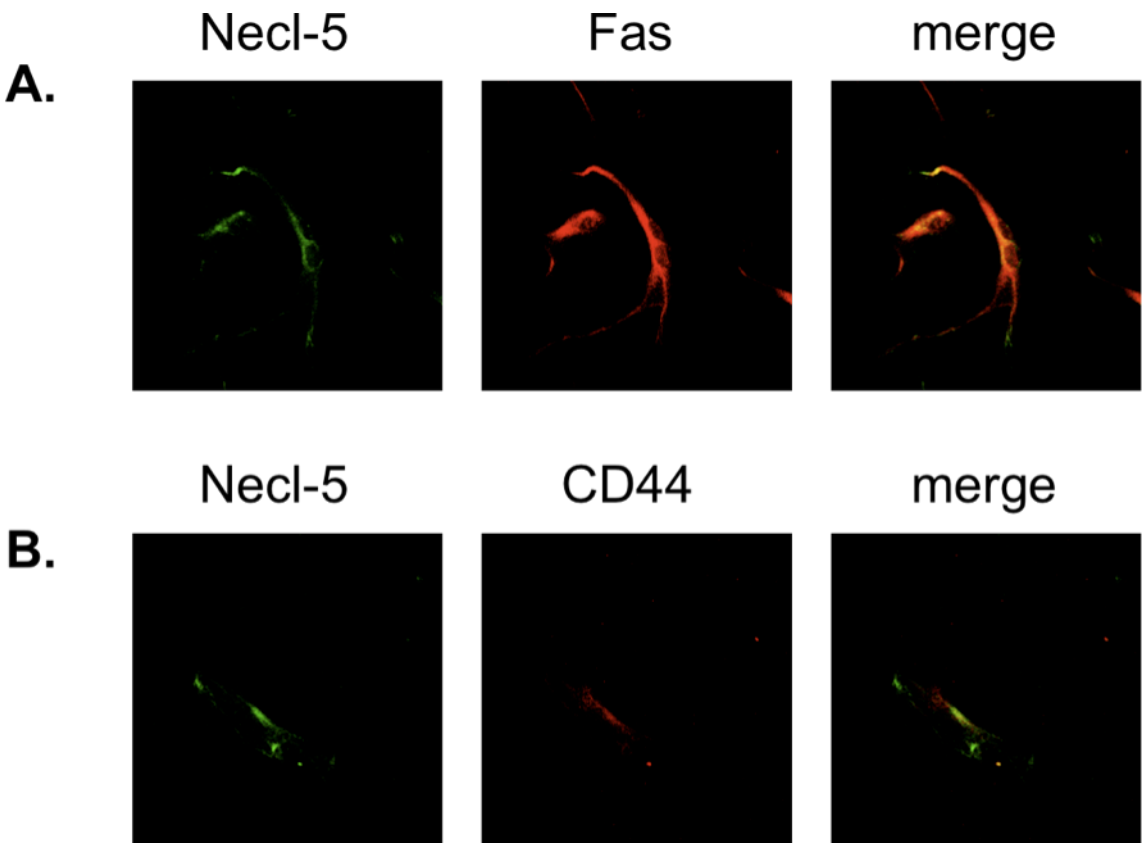
FIGURE 6



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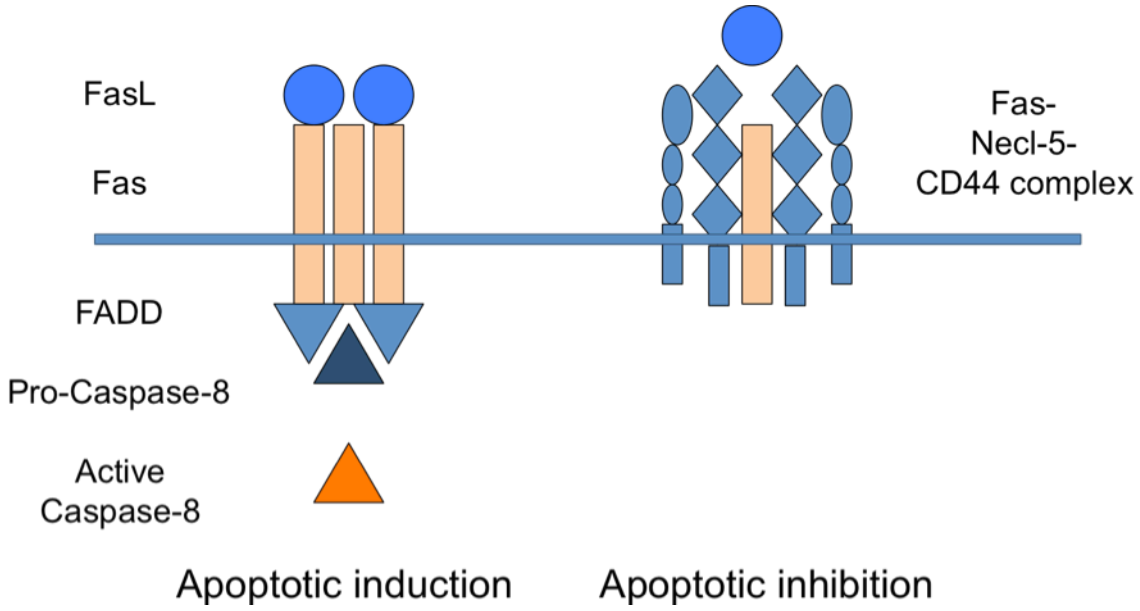

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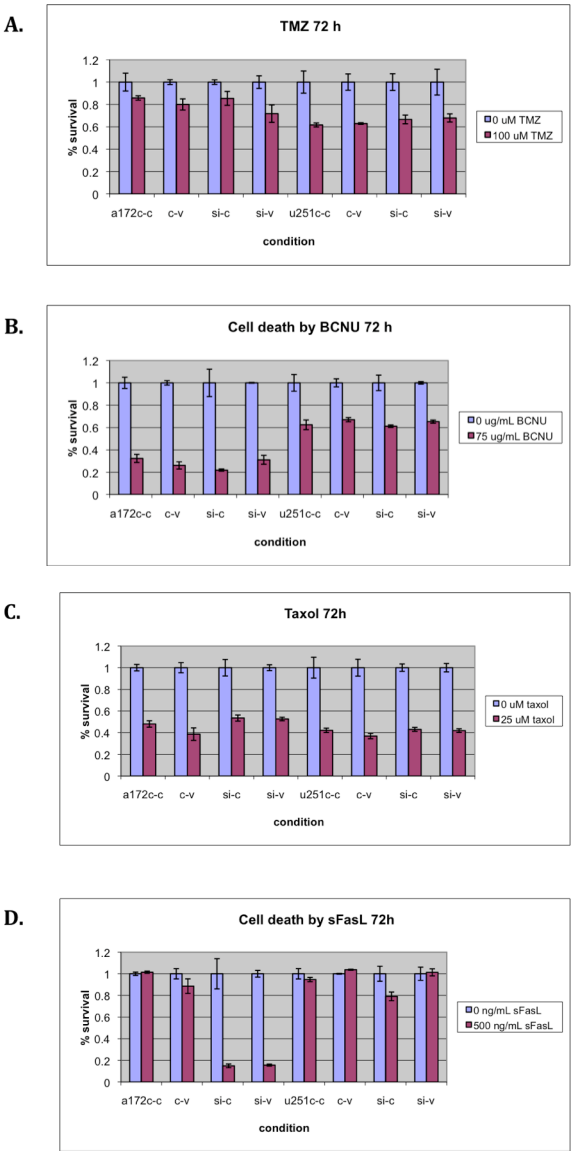

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Chapter 4: Contributions of this Thesis

CHAPTER 4

This chapter reviews and discusses the findings of this thesis, and their relevance to the field of glioblastoma in biology and medicine. Follow-up experiments are proposed to address unanswered questions.

Necl-5 promotes dispersal in a 3-D matrix

Dispersal is a phenotypic hallmark of GBM [1]. Dispersal leads to indistinct tumor margins, incomplete surgical resection, and subsequent tumor recurrence [1]. In order to achieve this phenotype, the GBM cell must possess at least two abilities: invasion and tumor initiation. In order to study invasion, investigators must use relevant models. These models should ideally allow for cell manipulations, such as protein overexpression and knockdown, drug treatments, and cell tracking. Models of cell movement must also allow manipulation of the matrix, or provide a physiologically relevant matrix.

Current models for studying cell migration and invasion, which are necessary for dispersal include transwell or Boyden chamber type models [2], wound healing assays [3], scattering assays on immobilized substrates or physiological tissues [4, 5], directional migration assays using flow chambers [6], and three-dimensional matrix invasion assays [7]. *In vivo* or *ex vivo* invasion models have high physiological relevance, but are difficult to perform and do not allow facile manipulations of the substrate. Each of these assays has its virtues and limitations. For example, transwell assays allow manipulations of the cell, of the matrix the cell must degrade, and the chemoattractant to induce directional cell migration. However, this assay is limited by its reductionism. Transwell migration does not necessarily recapitulate migration or invasion *in vivo*, but rather tests a cell's ability to migrate or invade only under a few set of conditions defined by the experimenter. Conversely, *ex vivo* tissue invasion models have physiological relevance but do not allow the experimenter to easily vary matrix components, limiting its ability to

demonstrate a role for a particular cellular receptor or extracellular matrix component in migration or invasion.

In the second chapter of this thesis, I used a three-dimensional model for glioblastoma cell dispersal. This model involves creating cell spheroids and implanting them in collagen I [7]. Cells must detach and initiate directional migration away from the spheroid. Unlike 2D assays such a transwell migration, both numbers of migrated cells and distance migrated can be assessed. A 3D matrix may provide a more relevant substrate than monolayer migration methods. For example, Sahai and Marshall showed that 3D migration could stimulate either amoeboid movement that required Rho-ROCK function and resulted in rounded cell morphology, or a more elongated mode of motility that requires Cdc42, filopodia formation, and proteolysis [8]. Broad-spectrum protease inhibition could force cells to switch from the elongated mode to the rounder, bleb-like mode. Interestingly, in my results with A172 glioblastoma cells, cell morphology was highly elongated, and involved the formation of filopodia. Recently, Sarkar and Yong used a similar 3D collagen matrix invasion assay and determined that invasion was caused by cytokine-induced MMP-2 and -9 expression in glioma cells [9].

In my work, I found that siRNA mediated knockdown of Necl-5 led to a nearly 75% reduction of the number of cells that had migrated from an A172 spheroid (Ch.2, Fig.1). This was a much larger migratory phenotype than had previously been reported by transwell migration or matrigel invasion assays, which have shown an approximately 25% inhibition. The reason for the discrepancy is unknown, but, as stated earlier, the behavior of cells in monolayer culture and 3D culture may not be equivalent. The 3D microenvironment may promote expression or activate signaling pathways that are not

active in monolayers. It is interesting to note that the results seen on 3D dispersal with Necl-5 manipulation more closely concur with *ex vivo* experiments that show a 5-fold increase in dispersal when Necl-5 is expressed in mouse 9L gliosarcoma cells and allowed to migrate on brain slices [5].

Necl-5 promotes MMP-2 production in GBM

Given that MMP expression is critical for glioblastoma invasion and dispersal, and that several 3D matrix models of glioma invasion have found that MMPs are critical for motility, I assessed the ability of A172 cells to produce MMPs -2 and -9. MMP-2 was readily detected in the culture supernatants, but MMP-9 was not detected. MMP-2 levels produced by A172 were similar to that produced by the invasive HT1080 fibrosarcoma cell line, which is used as a model for in vitro invasiveness. Conditioned media from A172 cells that had been treated with Necl-5 specific siRNA showed a significant reduction in MMP-2 expression and activity (Ch.2, Fig 2 A-C). In A172 cells, RT-PCR analysis showed approximately 40% reduction in MMP-2 mRNA, and nearly 70% reduction in MMP-2 protein in conditioned media. A gelatinase activity assay showed a 40% reduction in activity, which correlates well with reduced mRNA and protein levels, as well as reduced migratory capabilities. The apparent difference in magnitude of MMP-2 mRNA and protein decrease after Necl-5 siRNA treatment may be related to post-transcriptional or post-translational regulation of MMP-2, or a concomitant reduction in MMP-2 activation. A related glioblastoma cell line, U251, which also expresses Necl-5, also showed reduced MMP-2 production, but the magnitude of difference between control and Necl-5 depleted cells in this line is smaller than for A172. U251 cells are also

weakly dispersive in the 3D collagen assay, so linking MMP production and cell movement is difficult in this cell line.

This is the first link identified between Necl-5 and MMP expression in any cell line. MMP-2 is a major factor in promoting metastasis of several cell lines, and has a well-established importance in glioblastoma pathology [10, 11]. For epithelial tumors, such as colon cancer, MMP-2 can digest collagen IV, which is a major component of the basement membrane [12]. The digestion of the basement membrane distinguishes carcinoma *in situ* from cancer, and the disrupted basement membrane permits tumor cell dissemination and metastasis [12].

The link between glioma invasiveness and MMP-2 expression is strong. Several studies have shown that increasing MMP-2 levels correlate with tumor grade in patient samples, with low grade gliomas such as pilocytic astrocytoma expressing less MMP-2, both on a per cell basis and intensity of staining, than anaplastic astrocytoma (grade III) or GBM [13-16]. This implicates MMP-2 as a marker for high-grade glioma, and suggests a role for this protease in tumor progression. Two studies also demonstrated immunohistochemical reactivity against MMP-2 to be localized to glioma cells and tumor-associated vascular cells rather than to normal brain astrocytes [13, 16], indicating that the tumor, not normal brain, is the source of MMP-2.

Gelatinolytic activity of human tumor cells is correlated with grade and MMP-2 expression [17]. Invasive behavior is correlated with MMP-2 expression is cell lines [18, 19], and MMP-2 inhibition using small molecules prevents invasion [20, 21]. This demonstrates that MMP-2 has functional relevance in glioblastoma pathogenesis, and at least one of its roles is to promote invasion. Given these data, the link between Necl-5

and MMP-2 is relevant to GBM pathogenesis. Necl-5 is expressed in 30-60% of GBM, and not in normal brain [2, 22]. MMP inhibition has not shown promise in clinical trials, despite strong connections between expression of this protease and invasive behavior and metastasis [23]. The reasons for this are unclear, but may be related to the inappropriate inhibition of MMPs involved in normal physiological processes. MMPs may be produced by stromal cells, and used for wound healing or angiogenesis at sites away from the tumor [23]. Targeting Necl-5, which is not expressed in normal brain and which shows limited expression in other tissues, may be a way of specifically targeting GBM invasion without adversely effecting MMP activity at other sites. This makes Necl-5 an attractive target for GBM therapy.

Although the link between Necl-5 and MMP-2 expression and activity is clear, it is not certain if the reduced MMP-2 activity in A172 cells completely explains their dispersal defect. One test that would link these two processes is this: express MMP-2 in cells treated with Necl-5 siRNA. The MMP-2 transgene could be expressed from an artificial promoter, such as CMV, so that the regulatory changes that lead to native MMP-2 repression will not apply to the transgene expression. Alternatively, recombinant active MMP-2 could be added to the gels after spheroid implantation. The importance of this link would be supported if MMP-2 activity would restore an invasive phenotype to the cells. However, as Necl-5 is implicated in several biological processes that relate to migration or invasion, such as protection from apoptosis or focal adhesion disassembly, only a partial recovery may be detected.

Distinguishing the MMP effects from the anti-apoptotic effects could be achieved by treating spheroids with the pan-caspase inhibitor Z-VAD-FMK. Caspase inhibition

will prevent apoptosis, and if this treatment reverts Necl-5 depleted cells to an invasive phenotype, then we could conclude that the major defect in invasion in this model is due to apoptosis. Interestingly, apoptosis and MMP-2 production are linked in glioblastoma cells. MMP-2 knockdown using adenoviral shRNA constructs has been shown to induce apoptosis in U87 and U251 glioblastoma cells [24]. Although the mechanism is unknown, MMP-2 knockdown led to activation of caspases -8, -3, and -9, implicating both the intrinsic and extrinsic pathways [24]. One possible mechanism is the ability of αvβ3 to bind MMP-2, and then transmit pro-survival integrin signals. Another possibility is MMP-2 mediated cleavage and inactivation of pro-apoptotic molecules, such as Fas ligand. The study also found that angiogenesis was inhibited *in vivo*, thus linking another hallmark of GBM pathology to MMP-2 [24]. Endothelial cells express αvβ3 integrin [25] and require MMP function during angiogenesis [26], and GBM cells may enhance angiogenesis by providing an additional source of MMP-2.

Another possible role for Necl-5 in GBM dispersal is its role in focal adhesion disassembly. This has only been demonstrated during 2D matrix migration, and not in 3D collagen gels [5]. Focal adhesions connect the cell to ECM components, but neither Necl-5 nor αvβ3 integrin binds to collagen I, thus reducing the liklihood that this mechanism is important in gels. This potential role for Necl-5 could be tested by overexpressing Src or FAK kinases, the activities of which are stimulated by Necl-5-integrin-substrate interactions [5], in a cell depleted of Necl-5. If this mechanism is important, dispersal should be partly recovered.

Role of Akt in MMP-2 production in gliomas

It has been shown that inhibition of PI3K by LY294002 and the resulting loss of Akt phosphorylation gives two phenotypes in A172 cells. First, it inhibits MMP-2 production (Ch.2, Fig. 3A). The degree of inhibition is similar to the MMP-2 defect we see upon Necl-5 depletion, as measured by western blot from conditioned media. This implicates the PI3K/Akt pathway in MMP-2 production in this line. Second, it inhibits invasion from spheroids, with a similar near 75% decline in the number of invading cells, which is comparable to the effect seen after Necl-5 depletion, implicating PI3K/Akt as a promoter of invasion in this model (Ch2. Fig 3B).

The link between PI3K/Akt activation and MMP-2 production is strong in GBM cells. Cells treated with wortmannin or LY294002 show defects in MMP-2 or -9 secretion, and expression of these proteases correlates with Akt phosphorylation in experimental tumors [27]. These are similar to the results shown above for A172 cells, and highlight the importance of this pathway in GBM pathogenesis. Treatment of U87 and D54 glioma cells with rapamycin inhibits MMP-2 production [28]. Rapamycin inhibits the kinase activity of the mTOR/FRAP complex, which is activated by the sequential activation of growth factor receptors (such as PDGFR or EGFR), PI3K, and Akt. The mechanism of action of this complex that leads to MMP induction is not known, but this further implicates the Akt pathway in MMP-2 production in gliomas. A study by Park et al showed that ionizing radiation, such as that used in the initial treatment of GBM, can induce MMP-2 expression [29]. Induction can be inhibited by PI3K, Akt, or mTOR inhibition, mediated either by drug treatment or expression of dominant negative p85 or Akt. Furthermore, Park showed that MMP-2 induction was seen only in cell lines that lacked expression of PTEN, which is often deleted in GBM,

further suggesting that Akt activation promoted this effect [29]. This study was notable for suggesting that radiation treatment, which is front-line therapy for GBM, could induce invasion by PI3K/Akt dependent MMP induction, and suggesting that inhibition of this pathway could prevent invasion. Knockdown of PI3K and Akt by shRNA in both gastric adenocarcinoma and U251 glioma cells also leads to downregulation of MMPs -2 and -9, and an inhibition of invasion [30]. Also, MMP-2 activity can be induced in a PI3K/Akt dependent manner. Kleber et al showed that in GBM cells that are resistant to apoptosis, treating cells with Fas ligand promotes cell invasion by upregulating MMPs -2 and -9, and this requires PI3K/Akt activity [19]. Together, these studies show that the PI3K/Akt pathway can not only control basal or background levels of MMP-2 production, but also that stimulation of this pathway can cause MMP-2 induction.

Certain non-steroidal anti-inflammatory agents can reduce Akt phosphorylation in glioma cells. Sulindac-treated cells show a concomitant decrease in MMP-2 expression and invasiveness, which is reversed by expressing dominant active myristoylated Akt [31]. The same study shows that, conversely, dominant negative Akt inhibits MMP-2 promoter activity [31]. This strongly links Akt activity and MMP-2 transcription.

Akt signaling is enhanced by loss of PTEN in glioblastoma. PTEN is a phosphatase that antagonizes the activity of PI3K. Its loss in glioblastoma is common and promotes constitutive Akt phosphorylation [32]. Ectopic expression of PTEN in the PTEN-null GBM cell lines U87 and U251 resulted in a downregulation of MMP-2 activity as determined by zymography [33, 34]. PTEN gene transfer inhibited U251 and U373 glioma cells invasion on transwell plates and brain slices with concomitant decrease in MMP-2 activity [35].

Given that the PI3K/Akt pathway is linked to MMP-2 production in glioblastomas, and that Necl-5 and MMP-2 expression are linked, I hypothesized that Necl-5 affected Akt activation in glioblastoma cells. I tested this by comparing the ratio of phosphorylated (active) Akt to total Akt in A172 and U251 cells that had been treated with either control or Necl-5 specific siRNA. Western blot and densitometric analysis revealed that A172 and U251 cells showed 47% and 62% reduction in phosphorylated Akt after Necl-5 knockdown as compared to controls (Ch.2 Fig. 3D). This experiment demonstrates a novel link between basal Akt activation and Necl-5 expression. This link is important, as Akt activation in glioblastomas leads to increased cell survival, invasion, and angiogenesis. Activation of these biological processes leads to tumor growth, survival, and spread, each of which are characteristic of glioblastoma pathogenesis [36].

Together, these studies provide a firm link between PI3K/Akt/PTEN/mTOR activity and MMP-2 expression in glioblastoma. Expression of MMP-2 and its function in invasion and protection from apoptosis can be reduced by inhibiting this pathway, and increased by stimulating it. Although the exact mechanism for this transcriptional control of MMP-2 is not known, one plausible model from the studies cited above is as follows (Figure 1): growth factor receptors, such as constitutively active EGFR or autocrine/paracrine stimulation of PDGFR, activates PI3K, which leads to increased production of phosphatidylinositol (3,4,5) triphosphate. This recruits Akt to the inner leaflet of the plasma membrane, where it becomes phosphorylated and activated. This activation of Akt is long-lived, because the antagonistic effects of PTEN are absent. Activation of Akt leads to the sequential activation of the rapamycin-sensitive mTOR/FRAP complex, which can lead to activation of HIF1α, a transcription factor that

is important in VEGF production and subsequent angiogenesis [28]. As MMP-2 is important for angiogenesis, and this protease can be used by endothelial cells to promote angiogenesis, MMP-2 expression could also be controlled by HIF1α, or by a more complex regulation downstream of VEGF. This model would account for regulation of MMP-2 and VEGF in glioblastoma cells, which are both inherently invasive and angiogenic, and the role of constitutive PI3K/Akt signalng in that process. This model only accounts for the PI3K/Akt-dependent contribution to MMP-2 expression, of which Necl-5 is one part. Other inputs may also exist. For example, Stat3 inhibition has been shown to decrease MMP-2 expression and invasion in glioblastoma cells, although in an PTEN-independent fashion [37]. Stimulation of Stat3 by IL-6 in pancreatic carcinoma cells promotes VEGF and MMP-2 expression, and also enhances the cells' ability to invade [38]. Stat3 has been shown to activate both Akt and HIF1 [39]. From this, one can amend Figure 4 to include Stat3, entering at the leval of Akt, activating HIF1, VEGF, and MMP-2, and contributing to the invasive phenotype. The relative contribution of Stat3, or Necl-5, or other potential activators of this pathway can vary between different cell lines and tumors. This is ultimately determined by the series of mutations, tumor environment, and phenotypic selections that occur during tumorigenesis and tumor progression.

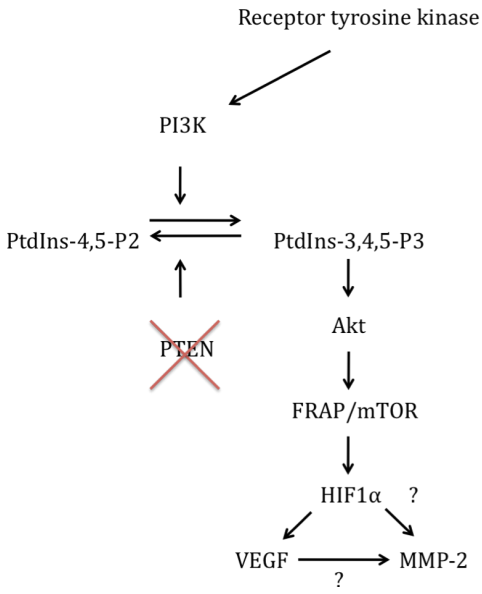


Figure 4: Hypothetical mechanism for MMP-2 induction via Akt activation in glioblastoma cells.

Inhibition of Necl-5, and the subsequent inhibition of Akt activation, could, in principle, reduce invasion, increase apoptosis, and inhibit tumor growth *in vivo*. This leads to the hypothesis that Necl-5 depletion in glioblastomas would inhibit invasion *in vivo*. This can be tested using a mouse model of glioblastoma, and comparing tumor development and invasion between two isogenic tumors, one of which has been depleted of Necl-5. Interestingly, several recently developed mouse models of GBM achieve tumorigenesis by activating Akt. In one study, expression of the constitutively active receptor tyrosine kinase FIG-ROS, which activates Akt, in conjunction with deletion of the Ink4a/Arf locus, leads to GBM formation [40]. In another recent model, co-expression of Akt and H-ras in astrocytes using a lentiviral delivery vector was sufficient to induce GBM tumorigenesis in mice [41]. Akt alone is not sufficient to transform cells [41, 42], suggesting that expression of Akt could contribute to secondary phenotypes that enhance transformed cell survival rather than tumor initiation. Angiogenesis, apoptotic inhibition, or invasion could be affected and contribute to animal survival. As Necl-5 depletion inhibits Akt activation, and Akt does not transform cells, tumor development should not be affected. Instead, one would predict that the resulting tumor would be defective in angiogenesis and invasion. Further, as the Akt-induced protection from apoptosis is absent or reduced, sensitivity to chemotherapeutic agents should be increased.

This leads to the hypothesis that Necl-5 depleted tumors should fail to disperse, and should be more sensitive to chemotherapeutic agents than tumors that express Necl-5. This can be tested by intracranial injection of cell lines into nude or SCID mice, and then assaying for tumor dispersal by histological analysis. Two cell lines should be used,

one expressing Necl-5 and stably transfected with a non-targeting shRNA vector, and the other expressing an shRNA vector targeting Necl-5. One would predict that after tumor establishment and progression, cells expressing Necl-5 would show robust dispersal on necroscopic exam, while the Necl-5 depleted tumor would show reduced dispersal. Furthermore, in a parallel experiment, mice harboring tumors depleted of Necl-5 would show enhanced sensitivity to chemotherapeutic drugs like BCNU or temozolomide, while Necl-5 expressing tumors would be comparatively more resistant. This can be assessed using animal imaging instruments such as the Xenogen Biophotonic Imager, which can measure tumor size by luciferase-mediated light output [43].

Necl-5, along with αvβ3 integrin, can bind vitronectin. This is an ECM protein that is found in the brain, especially along perivasular tracts that serve as routes for GBM cell migration [44]. Vitronectin can induce MMP-2 expression in melanoma cells [45]. Given that Necl-5 is a vitronectin receptor [46], and that its loss causes a reduction in MMP-2 activity, I hypothesized that Necl-5 ligation by vitronectin would increase MMP-2 production. I tested this by plating equal numbers of A172 cells on dishes coated with collagen or vitronectin for 24 hours, and tested for MMP-2 production by western blot of conditioned media. I found a modest 50% increase in MMP-2 production in control cells on vitronectin as compared to control cells on collagen I (Ch.2 Fig. 3G). Cells depleted of Necl-5 showed reduced amounts of MMP-2, and no increase on vitronectin. This leads to the conclusion that Necl-5 permits MMP-2 upregulation in cells plated on vitronectin. Loss of Necl-5 lowers basal levels of MMP-2, and renders the cell less responsive to MMP-2 induction by vitronectin. This may have implications for cell invasion in the brain. As cells move along perivascular tracts that are enriched in

vitronectin, MMP-2 induction is favored, leading to enhanced invasion and protection from apoptosis. Cells lacking Necl-5 may lose the ability to travel along these tracts, leading to reduced invasion.

Given the links between Necl-5, MMP-2, and Akt activation that I had determined, I hypothesized that Necl-5 expression results in Akt activation when exposed to vitronectin. I tested this by plating equal numbers of cells, treated with either control or Necl-5 specific siRNA onto dishes coated with either collagen or vitronectin. I then lysed the cells and performed a western blot for phosphorylated and total Akt. I found that control cells on vitronectin showed a 3-fold increase in active Akt, indicating that vitronectin could induce Akt activity (Ch. 2 Fig. 3E). Total Akt was unaffected. For cells depleted of Necl-5 on collagen, overall activated Akt levels were approximately half that of control cells on collagen. This is similar to what was shown before in adherent A172 and U251 cells. When Necl-5 depleted cells were plated on vitronectin, there was a 3-fold increase in Akt phosphorylation as compared to collagen, but still only half the level of Akt phosphorylation of control cells on vitronectin. This experiment indicates that Necl-5 is not the sole vitronectin receptor, and either that there are multiple vitronectin receptors that increase Akt phosphorylation when exposed to ligand, or that Necl-5 cooperates with another receptor to achieve maximal response to vitronectin. Total levels of Akt are equivalent in all cases, excluding the possibility that reduced pAkt levels result from reduced total Akt in Necl-5 knockdown cells. Other possible vitronectin receptors in the cell include integrins $\alpha v\beta 3$ and $\alpha v\beta 5$. Necl-5 is known to bind $\alpha v\beta 3$ integrin, as determined by co-immunoprecipitation [6, 47]. It is not known, however, if the binding site is at the αv or $\beta 3$ subunit. Necl-5 is known to bind the PDGF

receptor and αvβ3 integrin as part of a tripartite complex at the leading edge of migrating cells, and that Necl-5 enhances clustering of αvβ3 [47], which points to a functional role for the Necl-5-αvβ3 complex during migration. My results suggest that Necl-5 and αvβ3 cooperate in binding vitronectin, and that Akt activity is stimulated by this interaction. In order to test the functional relationship between Necl-5 and αvβ3, one can test the ability of a cell to respond to vitronectin in the absence of either Necl-5, αvβ3, or both. This can be achieved by Necl-5 siRNA and function blocking αvβ3 antibodies, such as LM609. While several studies have shown a link between Necl-5 and αvβ3 integrin, it is not known if this is the only integrin pair that binds Necl-5. For example, if Necl-5 binds the αv subunit, then it could also bind αvβ5, which is also a receptor for vitronectin and is also expressed in A172, and promotes vitronectin-dependent migration in this line [48]. In any event, these data show that Necl-5 depletion severely inhibits vitronectin-induced Akt phosphorylation, and concomitant MMP-2 expression, which is dependent on PI3K/Akt signaling.

ILK activates Akt in response to vitronectin

One important concept in vitronectin-induced Akt activation is the identity of the kinase that activates Akt. We have shown that Akt activation by vitronectin is dependent upon PI3K, because pre-treating cells with LY294002 inhibits Akt phosphorylation in response to plating on vitronectin coated dishes. Also, activation is likely mediated by integrin, because an attenuated activation can still be achieved after knockdown of Necl-5, and Necl-5 is known to associate with integrins that bind vitronectin. It is unlikely that Akt activation results from growth factor receptor activation, as these experiments were done under serum-free conditions, and no exogenous growth factors were added.

Integrin-linked kinase (ILK) is a kinase that functions in a PI3K-dependent manner to phosphorylate Akt upon activation of integrins [49], and binds αvβ3/β5 integrins [50]. ILK is expressed in gliomas, and its activity promotes expression of MMP-9 [51], demonstrating the importance of this kinase to integrin signaling that results in an invasive phenotype. Blocking ILK function in glioblastomas using an antisense knockdown strategy significantly reduced tumor growth in mice [52], suggesting that this kinase could be a therapeutic target. ILK has been linked to MMP-2 production in breast cancers [53], and in the EMT of renal tubular epithelial cells [54] and ovarian epithelial cells [55]. Given this, it was hypothesized that ILK is necessary for Akt activation in response to vitronectin and MMP-2 production in A172 cells. This was tested by treating cells with control or ILK specific siRNA, plating the cells on vitronectin, and assessing the subsequent Akt activation. ILK depleted cells retained the ability to adhere to vitronectin, indicating that ILK activity was not necessary for vitronectin receptor adhesion. Akt activation, as measured by the ratio of phosphorylated to total Akt, was severely inhibited (Ch. 2 Fig. 4A). Both basal levels and vitronectin-induced levels of phophorylation were reduced as compared to control or Necl-5 siRNA treated samples. Basal and vitronectin-induced levels of phosphorylated Akt were equal in the ILK depleted cells, indicating that the vitronectin receptor, while engaged, could transmit no signal to Akt via ILK. Given that basal levels of pAkt were reduced compared to control or Necl-5 depleted sample, this suggests that in the absence of exogenous growth factors, the integrin-ILK axis initiates pAkt signaling in this line. The ability of ILK depleted cells to produce MMP-2 was also assessed. Serum-free culture supernatants of ILK-depleted cells were found to contain very little MMP-2 as compared to control or Necl-5

depleted cells (Ch. 2 Fig. 4B). This indicates that ILK is necessary for basal MMP-2 production in this line.

Given the data presented above, one model for how Necl-5 affects MMP-2 production is as follows: First, Necl-5, by virtue of its ability to bind integrin, can initiate integrin clustering. This is supported by the observation that αvβ3 clustering is enhanced in the presence of Necl-5 [47]. In this study, fibroblasts that overexpress Necl-5 were plated on vitronectin and stimulated with PDGF in a directional manner. Cells were immunostained for αvβ3 and Necl-5, and the membrane ruffles at the leading edge contained punctate areas of overlap between αvβ3 and Necl-5. Cells depleted of Necl-5 showed random distribution of αvβ3, indicating that Necl-5 enhances clustering.

Second, clustering of αvβ3 or αvβ5 by Necl-5 activates ILK. This is supported by the fact that clustering of integrin enhances signaling in the presence or absence of ligand [56]. This study shows that integrin clustering by polyvalent antibodies promoted FAK phosphorylation even without ligand, and that ligand occupancy, in the absence of clustering was insufficient to induce FAK activation. Similarly, as Necl-5 promotes αvβ3 clustering, then ILK activation could follow. This could explain the source of the basal Akt phosphorylation that is dependent upon Necl-5 expression. Necl-5, by virtue of its ability to bind and cluster integrin, promotes integrin signaling. Furthermore, upon loss of Necl-5, the integrin becomes more randomly distributed, and attenuates signaling activity. As ILK depends upon PI3K activity, one might ask about the source of PI3K activity. Necl-5 has also been shown to bind the PDGF receptor (PDGFR), and a tripartite complex of Necl-5, integrin, and PDGFR has been demonstrated [6]. A172 cells express PDGF-BB and PDGFR, and thus have an autocrine activation loop for PDGF receptor

activation [57]. As Necl-5 can cluster PDGFR and integrin, PI3K activity could be stimulated in the vicinity of integrin, and lead to the recruitment and activity of ILK and Akt. Activation of the PDGF receptor is important in the initiation of directional cell movement, and the clustering of PDGFR and integrin ensures efficient transduction of promigratory cues. Activation of ILK and Akt to enhance MMP-2 production could be a part of this program. As cells move and encounter ECM, the clustering of integrin by Necl-5 and ECM leads to enhancement of Akt activation and MMP-2 production, which could have a role in matrix degradation and allowing unimpeded invasion. This would enhance invasion, and promote the dispersive phenotype that is typical of GBM.

Role of Necl-5 in anoikis and chemotherapy-induced apoptosis

Apoptosis is a mechanism of tissue homeostasis that induces cell death as a response to tissue remodeling, infection, or transformation [58]. As opposed to necrosis, the apoptotic program removes damaged cells without evoking inflammatory pathways [58]. Apoptosis is a highly regulated process and occurs in almost all somatic tissues, but most cancer cells have mutations or express proteins that inhibit apoptosis. This resistance to apoptosis is a hallmark of cancer [59]. Apoptosis can occur by an intrinsic pathway, which uses mitochondrial outer membrane permeability to signal for death, an extrinsic pathway, which relies on receptor-mediated initiation of apoptosis, or a combination of both [58]. Glioblastoma cells are highly resistant to apoptosis because of mechanisms such as overexpression of the anti-apoptotic Bcl-2 protein or activation of the PI3K/Akt pathway [58]. Activation of this pathway leads to inactivation of caspases [60] and pro-apoptotic proteins like Bad [61].

Anoikis is apoptosis that is induced by a loss of cell adhesion, or of inappropriate adhesion [62]. Although it has its own classification, it is essentially an apoptotic process [63]. Integrins are responsible for preventing anoikis in non-transformed cells via integrin signaling [64]. Transformed cells are resistant to anoikis, and have mutations and dysregulation of signaling pathways and that prevent this [63]. They do not require anchorage for survival, and can resist apoptosis as they travel through the bloodstream during metastatic dissemination [65]. Two integrin binding proteins, focal adhesion kinase (FAK) and ILK, can suppress anoikis. Overexpression of either protein can independently protect cells from anoikis [66, 67]. The independence of these two proteins in anoikis protection is exemplified by fact that dominant-negative FAK does not promote anoikis in cells that overexpress ILK [67]. Activation of the PI3K/Akt pathway is important to anoikis as well [63, 68].

Integrins are intimately responsible for protection from anoikis, and one strategy that cancer cells use to protect themselves is to alter the pattern of integrin expression. For example, in melanoma, expression of αvβ3 correlates with disease progression, and represents a transition from non-β3 expressing precursors [69]. This integrin is important in melanoma progression, because as the cells migrate into areas rich in collagen, such as the dermis, αvβ3 expression adheres the cells to new substrates and protects the cell from anoikis [70] by upregulating the anti-apoptotic protein Bcl-2 [71].

Given the role of αvβ3 integrin in Necl-5 function [47], and the observation that Akt activation can be influenced by Necl-5 expression (Chapter 2), I hypothesized that Necl-5 expression protects cells from anoikis. I tested this by treating A172 cells with either control or Necl-5 specific siRNA and growing cells in suspension. After 24 hours,

cells depleted of Necl-5 showed a 2.5-fold increase in apoptotic cell number as compared to control cells (Ch.3 Fig. 1). One possible explanation for this phenomenon is that integrin clustering, promoted by Necl-5 expression, led to Akt activation and inhibition of pro-apoptotic signaling. This could be tested by several methods: First, western blot to detect the ratio of pAkt to total Akt would tell if the Necl-5 depleted cells grown in suspension failed to activate Akt signaling. Also, inhibition of Akt by inhibitors like LY294002, or expression of dominant negative Akt should lead to a reduction of viability in cells that express Necl-5. Finally, expression of active Akt in Necl-5 depleted cells should increase viability of cells grown in suspension.

Anoikis is an important way of selecting against cells that have become detached from their surroundings, or to restrict the cell to certain areas based upon ECM-integrin receptor compatibility. Cancerous cells often lose the ability to undergo anoikis, and have a survival advantage when disseminating via blood or lymph during metastasis. Necl-5 may contribute to this survival by activating Akt. However, hematogenous dissemination is not thought to be important in GBM, so this mechanism may be more important in tumors like colon adenocarcinoma.

Necl-5 does not protect cells from chemotherapy-induced apoptosis

Chemotherapy is a part of standard treatment for GBM. Current drugs used to treat GBM are carmustine (BCNU) and temozolomide, both DNA alkylating agents [72]. Paclitaxel (taxol) promotes stable microtubule assembly, and is currently under investigation as an adjuvant to BCNU therapy, and combination therapy of these two drugs causes complete remission of experimental GBM in nude mice [73]. Chemotherapeutic agents are thought to kill cancer cells by induction of apoptosis, because of the apparent death of cells

without inflammation [58]. Glioma cell death from chemotherapeutic agents involves the mitochondrial pathway rather than the death receptor pathway, and is caspase dependent [74, 75]. Along with overexpression of anti-apoptotic proteins like Bcl-2, the resistance of GBM cells to chemotherapeutic agents is promoted by activation of the Akt pathway [76, 77]. Given the importance of Akt in anti-apoptotic survival, and the link between Necl-5 and Akt activation, it was hypothesized that Necl-5 expression, and the concomitant Akt activation, would protect cells from chemotherapy-induced apoptosis. Furthermore, by plating the cells on vitronectin, which increases pAkt levels 3-fold over baseline, cells on vitronectin would be more resistant than cells on collagen. This was tested by plating A172 and U251 cells on these matrices, then adding media containing temozolomide, carmustine, or paclitaxel for 72 hours, and then measuring cell number using an MTS assay (Ch.3 Figs. 2, S1). While all three drugs markedly decreased GBM cell viability, these values did not change significantly in Necl-5 depleted cells, nor did they change in response to plating on vitronectin. Necl-5 does not protect GBM cells from chemotherapy-induced cell death or apoptosis. As these drugs are thought to affect the intrinsic pathway, we may generally conclude that Necl-5 has no role in protection from apoptosis induced by mitochondrial permeability. It is interesting to note that the Akt pathway is thought to be centrally involved in resistance to apoptosis, but plating cells on a substrate that increases pAkt levels does not lead to increased resistance. One possible explanation is that the high levels of these drugs overwhelm the system and are toxic even in the presence of high pAkt levels. One way to optimize these results would be to titrate down the concentration of the drug and observe if cells on vitronectin become statistically more resistant at lower concentrations. An alternate hypothesis is that

chemotherapeutic drugs induce cell death by modes other than intrinsic apoptosis. This can be tested by treating the cells with ZVAD-FMK, the caspase inhibitor, and testing the response to the drugs. Apoptosis is caspase-dependent, and if the cells were dying from apoptosis then inhibiting the caspases would inhibit death from the drug. If the cells continued to die, then this would be evidence that other forms of cell death, like autophagy or necrotic death were prevalent.

Necl-5 protects A172 GBM cells from Fas-mediated apoptosis

The extrinsic pathway of apoptosis is a receptor-mediated event, and is often used by the immune system to eliminate infected or pre-malignant cells [78, 79]. One important input into this pathway is the Fas/Fas ligand (FasL) system. Interaction of Fas with FasL initiates apoptosis by activating the initiator caspase-8, followed by the executioner caspase-3 [80]. Fas activation may also allow activation of the intrinsic apoptotic program. Active Caspase-8 can cleave Bid, a pro-apoptotic member of the Bcl-2 family, which then causes mitochondrial outer membrane permeability, cytochrome c release, and activation of caspase-9 [81].

Glioblastoma cells often express Fas and FasL [82], but are resistant, or only weakly sensitive to Fas-mediated apoptosis [83, 84]. Sensitivity can be enhanced by pretreating glioma cells with chemotherapeutic agents [84-86] or by overexpression of Fas [87]. Since apoptosis can be achieved by FasL treatment, this implies that the apoptotic machinery is intact within the gliobastoma cell, but that the cell is blocking its activation. Several lines of evidence demonstrate that GBM cells resist apoptosis by expression of anti-apoptotic proteins like Bcl-2 and Bcl-XL [88], or by expression of a non-functional decoy receptor [89]. Glioblastomas may also express FLIP, which

inhibits caspase-8 activation at the DISC in response to death receptor agonists [90]. Downregulation of FLIP has been shown to sensitize GBM cells to death receptor agonists [91], indicating that removing the blocks to death-receptor mediated apoptosis can promote therapeutic use of death receptor agonists in GBM.

The response of A172 and U251 to high levels of recombinant, soluble FasL was tested, and both lines are resistant (Ch.3 Figs. 3A, S1). Treatment with 500 ng/mL FasL for 72 h had negligible effects on viability of these lines, based upon MTT assay. In A172 cells depleted of Necl-5, however, treatment with FasL gave a robust apoptotic response, showing about 85% reduction in cell number. No such reduction was seen with Necl-5 depleted U251 cells upon FasL treatment (Ch.3 Fig. S1). This may be due the presence of more stringent anti-apoptotic protein expression in this line, or due to the non-functional p53 status of U251. Transfection of functional p53 into U251 has been shown to induce apoptosis following activation of the Fas pathway [92]. The A172 line expresses functional p53, which has been linked to sensitivity to chemotherapy-induced apoptosis [29].

Fas-mediated apoptosis occurs through a well-defined series of steps, including receptor trimerization in the presence of ligand, recruitment of adapter proteins such as FADD and caspase-8 to the activated receptor, forming the DISC, and subsequent proteolytic cleavage and activation of caspase-8 [58]. Extrinsic pathway activation was measured in FasL exposed, Necl-5 depleted A172 cells by looking for evidence of caspase-8 cleavage. Surprisingly, a western blot for caspase-8 in lysates of treated cells showed no cleavage of this initiator caspase (Ch.3 Fig. 4B). The antibody used in this experiment can detect cleaved caspase-8, as shown by the presence of low molecular

weight fragments of caspase-8 in treated HT1080 cells. This result indicates that either FasL can induce apoptosis in a caspase-8 independent manner, or that caspase-8 cleavage is inefficient and undetectable. In order to test if Fas-mediated cell death in Necl-5 depleted A172 cells is caspase-dependent, cells were treated with both FasL and the pan-caspase inhibitor Z-VAD-FMK. If FasL induces the apoptotic machinery in A172 cells, then treatment with a caspase inhibitor should prevent cell death in response to FasL. The alternate outcome would be that FasL induces cell death in a caspase independent manner, suggesting another mode of cell death such as autophagy or necrotic death. Treating cells with Z-VAD-FMK prevented cell death in response to FasL, which implicates an apoptotic mechanism (Ch.3 Fig. 3B).

The final effecter in the caspase cascade for intrinsic and extrinsic apoptosis is caspase-3. When activated, this caspase irreversibly commits the cell to apoptotic death by cleaving several intracellular substrates. Given that FasL treatment induced caspase-dependent cell death in Necl-5 depleted A172 cells, caspase-3 cleavage was measured. Western blot analysis revealed that caspase-3 cleavage could be detected only in Necl-5 depleted cells treated with FasL (Ch.3 Fig. 4A). This shows that Necl-5 depletion sensitizes the cells to apoptotic induction by Fas. Interestingly, only a small fraction of total caspase-3 was cleaved, as evidenced by the faint band at 17kDa. These cells had been treated with high doses of FasL for 48 hours, and the hallmarks of apoptosis, caspase cleavage, show very little activity, as if FasL is inducing apoptosis very slowly. One explanation for this observation is that Necl-5 is not the only anti-apoptotic protein expressed by A172 cells. The presence of soluble decoy receptor DcR3 could prevent receptor activation by effectively lowering the concentration of FasL available to bind the

true receptor [89]. Expression of FLIP, which binds activated Fas and prevents DISC assembly by inhibiting caspase-8 activation, could also lead to this attenuation of death receptor signaling. FLIP is highly expressed in A172, and its expression correlates with resistance to death-receptor mediated apoptosis [93]. Either of these possibilities would explain the weak reaction of these cells to FasL, and the difficulty in detecting cleaved caspase-8.

Other downstream players in the apoptotic signaling pathway may also be responsible for the blunted response to FasL. Certain types of cells, called Type I cells, can transduce a signal from caspase-8 to caspase-3 and promote apoptosis directly. Type II cells, by contrast, require the mitochondrial pathway to fully activate apoptosis [94, 95]. The intrinsic and extrinsic pathways are linked by the expression of Bid, a pro-apoptotic member that, when cleaved by caspase-8, promotes mitochondrial permeability [81]. Given this, the activation of caspase-9, the initiator of mitochondrial-induced apoptosis, was measured. Western blot analysis showed a small increase in cleaved caspase-9 in Necl-5 depleted FasL treated A172 cells as compared to controls (Ch.3 Fig. 4C). The modest activation may be due to the presence of anti-apoptotic proteins, such as Bcl-2, that inhibit mitochondrial permeability. Type II cells can be protected from FasL by expression of the anti-apoptotic proteins Bcl-2 or Bcl-XL [94]. A172 cells are known to express Bcl-2 [77], and death receptor activity can be abrogated by expression of Bcl-2 in Type II cells [96]. The presence of Bcl-2 may provide resistance to the mitochondrial apoptotic mechanism, and inhibit overall apoptotic response to FasL.

Necl-5 and CD44 function together to prevent apoptosis in response to Fas

CD44 is a receptor for hyaluronic acid [97]. It is a single-pass transmembrane protein, and the expression pattern of its different isoforms is highly variant [97]. CD44 can be expressed as a standard form, or as a variant form, with the presence of one or more of 9 variant exons [97]. The pattern of variant exon expression correlates with tissue type. For example, epithelial CD44, which is expressed in the normal epithelium of many tissues including skin, bladder, and cervix, contains variant exons 8-10 [97, 98]. It is also expressed on tumors that arise from these tissues. The standard CD44, CD44s, contains no variant exons and is found in mesenchymal cells and hematopoietic cells [98]. Cancer cells often express CD44s and variants containing exons 6 and 9 [99].

CD44 can either enhance or prevent extrinsic apoptosis. Studies using a chimeric CD44-Fas protein showed that activation by hyaluronic acid or crosslinking antibodies induces apoptosis [100]. However, in another study using lung cancer cells, ligation of CD44 with hyaluronan decreased Fas expression and reduced apoptosis [101]. In a third study using synovial cells from rheumatoid arthritis patients, crosslinking CD44 or treatment with hyaluronan fragments led to an upregulation of Fas and increased apoptosis after Fas-agonizing antibody treatment [102]. In our lab, downregulation of CD44 by siRNA was found to inhibit Fas-mediated apoptosis by inhibiting DISC formation [103]. Similarly, in a study of Fas-mediated apoptosis of T-cells, it was shown that CD44 isoforms that contained the v6 or v9 variant exons protected the cells from Fas-mediated death, whereas the standard CD44 did not [104]. This paper further shows that inhibition of v6 led to sensitization to Fas, and that v6 and v9 expressing isoforms colocalized with Fas [104]. A later report by this group demonstrates that CD44s

promotes apoptosis, thereby proposing a difference in CD44s and CD44v functions [105].

CD44 has also been shown to bind Necl-5 [106]. Poliovirus entry can be blocked by a CD44 antibody, but CD44 is not a poliovirus receptor [107], nor is CD44 required for poliovirus replication [108, 109]. The relevance of the CD44-Necl-5 interaction is unknown. Given the role of Necl-5 and CD44 in Fas-mediated apoptosis, and their physical interaction, a functional interaction between them was explored. If a functional interaction exists, then knockdown of both proteins by siRNA would show no additive effects as compared to single knockdown. Thi was tested by treating A172 cells with control, Necl-5 specific siRNA, CD44 specific siRNA, or both Necl-5 and CD44 siRNA. The cells were then treated with FasL, and assayed for cell number after 72 hours (Ch.3 Fig. 5A). Necl-5 depleted cells treated with 500 ng/mL FasL showed about 70% reduction in cell number. CD44 depleted cells treated with 500 ng/mL sFasL showed about 80% reduction in cell number. Cells treated with control siRNA were unaffected by FasL. Cells depleted of both proteins also showed 80% reduction in cell number, showing that a Necl-5 depletion does not add to the apoptotic sensitivity of CD44 depletion. To ensure that an additive effect could be detected, and that an 80% decrease didn't represent the maximum number of cells that were sensitive to Fas, The experiment was repeated with 5 ng/mL FasL (Ch.3 Fig. 5B). Treatment for 72 hours at this concentration led to significant decreases in cell number for Necl-5 depleted (20% reduction) and CD44 depleted (30% reduction). Again, loss of both Necl-5 and CD44 had no additive effect, with a 30% reduction. If these two proteins were operating in different pathways, one would predict that the effects would be additive, and each protein

would contribute by its own mechanism. The observation that there are no additive affects suggests that they are operating in the same pathway.

It has been proposed that CD44v binds Fas, sequesters it, and prevents its activation by FasL and inhibits DISC formation [104]. This leads to the hypothesis that if CD44 and Necl-5 operate by the same mechanism, then a Necl-5-CD44-Fas inhibition complex may exist, and Necl-5 should colocalize with Fas. When cells were immunostained with Necl-5 and Fas antibodies, a colocalization in areas outside of focal contacts could be observed (Ch.3 Fig. 6A). Colocalization of Necl-5 and CD44 was also observed, but not at all areas (Ch.3 Fig. 6B). This may be due to the fact that only certain isoforms of CD44 will interact with Necl-5, and this is the basis for their functional interaction in preventing Fas-mediated apoptosis. This could be resolved by the use of isoform-specific antibodies in immunocytochemistry or immunoprecipitation.

A physical interaction between Necl-5 and CD44 may occur at the plasma membrane surface. These proteins, when together, together bind Fas and prevent its trimerization in the presence of FasL. This interaction prevents DISC formation, caspase-8 activation, and subsequent induction of apoptosis (Ch.3 Fig. 7). Neither CD44 nor Necl-5 individually can bind Fas, and knockdown of either prevents complex formation, and allows susceptibility to FasL. The apoptotic induction that results from either CD44 or Necl-5 knockdown is relatively weak, requiring high levels of FasL and long incubation time. This suggests the presence of multiple levels of apoptotic inhibition, such as DcR3, FLIP, or Bcl-2 expression. It is known that A172 cells express the protective Bcl-2 protein and FLIP, but it is not known if these cells express DcR3. The

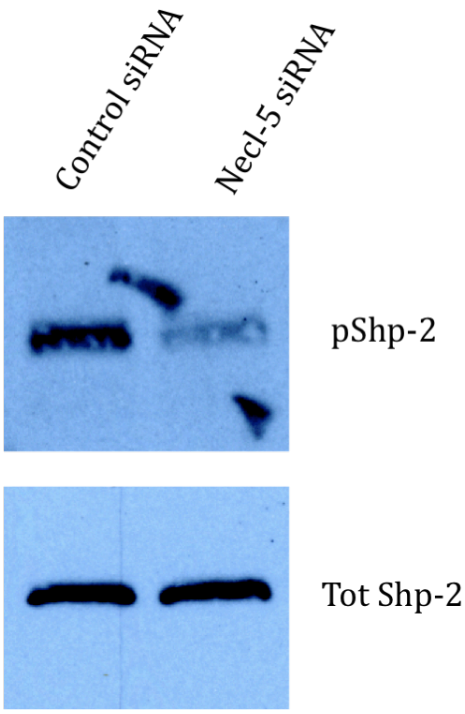
presence of these proteins would explain the inefficiency of caspase-8 activation, and can be tested by western blot.

Contributions of this thesis

This thesis describes the role of Necl-5 in two important hallmarks of cancer: invasion and resistance to apoptosis. Necl-5 has a well-established role in migration, and its role in GBM invasion has recently been elucidated. One mechanistic explanation for how Necl-5 could be promoting invasion in GBM cells is by MMP-2 expression. MMP-2 levels decrease in GBM cells depleted of Necl-5, which may affect the ability of these cells to disperse in a 3D matrix. Furthermore, lack of Necl-5 correlates with reduced activation of Akt, which is often necessary for MMP-2 production in gliomas. Necl-5, therefore, reduces Akt activation, which leads to less MMP-2 production. As MMP-2 is important in the pathogenesis of GBM, Necl-5 may be a candidate drug target to reduce MMP-2 expression and prevent the invasive phenotype that leads to GBM dispersal an eventual recurrence. As the recurrence of GBM leads to death, preventing this phenomenon may offer therapeutic benefit.

Further work on this project may give insight into how Necl-5 affects Akt activation. Necl-5 may promote Akt activation by enhancing integrin clustering, which is one of the known functions of Necl-5. Clustering enhances integrin signaling, leading to Akt activation. In others' work on Necl-5, it has been shown that Necl-5 interacts with several proteins by its extracellular domain, in particular PDGFR and $\alpha v\beta 3$ integrin. It was also shown to bind sprouty, a negative regulator or Ras signaling [110], and Shp-2, a tyrosine phosphatase [111], by its intracellular domain. Necl-5 promotion of migration in fibroblasts requires both the extracellular and intracellular domains, suggesting that its

extracellular and intracellular binding functions may be coupled to promote migration. The model proposed here, however, postulates that only the integrin binding function is required to promote integrin clustering and Akt activation. It would be interesting to test if a mutation or deletion of the Necl-5 intracellular domain would still allow Akt activation. This would strengthen the integrin-clustering model. It is possible that Necl-5 transmits a signal via its intracellular domain that affects Akt activation. A recent report shows that gain-of-function SHP-2 mutants with enhanced phosphatase activity promotes migration of fibroblasts, with concomitant enhanced Akt activation that is dependent upon β1 integrin outside-in signaling [112]. This may suggest a role for Necl-5 in delivering Shp-2 to areas of integrin crosslinking and enhancement of Akt activation. Shp-2 activation is defective in Necl-5 depleted A172 cells, which is consistent with this idea.



It also remains to be determined how activated Akt transmits a signal that results in MMP-2 induction at the transcriptional level. MMP-2 mRNA levels decrease significantly upon Necl-5 knockdown, suggesting transcriptional regulation. It is known that HIF1α is a transcription factor that is activated by Akt, and functions to enhance transcription of VEGF in response to hypoxia [28]. It has also been shown that HIF1α expression can be induced in the absence of hypoxia by serum or EGF stimulation in an Akt-dependent manner in prostate cancer cells [113], and

siRNA knockdown of HIF1α results in reduced MMP-2 activity in gliomas [114]. This leads to the hypothesis that HIF1α promotes MMP-2 transcription in an Akt-dependent, hypoxia-independent manner in A172. This could be tested by chromatin IP of HIF1α, followed by quantitative PCR of the MMP-2 promoter, which would show that HIF1α binds the MMP-2 promoter. Additionally, binding should decrease in the presence of LY294002 or Necl-5 depletion, and increase in the presence of EGF, vitronectin, or hypoxic conditions. If true, this would link Necl-5 to MMP-2 expression by Akt and HIF1α.

In order to definitively establish the role of Necl-5 in GBM dispersal, an animal study could be performed. Cells that express Necl-5, such as A172, U251, or cells from primary brain tumors could be stably infected with vectors expressing either non-targeting or Necl-5 specific shRNA and injected stereotactically into NOD-SCID mice. If tumors develop, slides can be made and assessment of invasion can be made by H&E staining and microscopic exam. One would predict that tumors expressing Necl-5 would show dispersal in areas near the tumor-normal brain boundary, while cells depleted of Necl-5 may show more limited dispersal. Further, immunohistochemisty of phospho-Akt should be stronger in control tumors as compared to Necl-5 depleted tumors.

The third chapter of my thesis describes the role of Necl-5 in protecting GBM cells from Fas-induced apoptosis, a function that was previously unknown for Necl-5. GBM cells express both Fas and FasL, and sensitizing cells to agents that induce extrinsic apoptosis may offer a way to treat GBM medically. Furthermore, Necl-5 and CD44 cooperate to provide this resistance. A physical link between CD44 and Necl-5 had previously been described, but its relevance was unknown.

Recent work has shown that CD44 has a role in protecting cells from Fas-mediated apoptosis. In work from our lab, Rob Hauptschein has shown that knockdown of CD44 by siRNA sensitized HT1080 fibrosarcoma cells to apoptotic induction by FasL, and proposed that CD44 sequesters Fas, making it unavailable for DISC formation. Necl-5 may operate in the same pathway with CD44, and this would predict that loss of Necl-5 would inhibit DISC formation in the presence of Fas as well. This can be tested by using a Type I cell that expresses both Necl-5 and CD44, and which becomed sensitive to apoptosis upon knockdown of either. Control and Necl-5 depleted cells can be treated with FasL, followed by immunoprecipitation of Fas. Efficiency of DISC formation can be estimated by the amount of FADD that co-immunoprecipitates, as FADD is recruited to the DISC in response to FasL. Furthermore, Hauptschein et al showed that cells respond similarly to FasL, TRAIL and TNFα, all mediators of extrinsic apoptosis, after CD44 ablation by FALI. This raises the possibility that Necl-5 depletion could sensitize cells to these other inducers as well. TRAIL has recently been touted as a possible therapeutic apoptosis inducer in GBM, due to its unique safety profile. Necl-5 ablation followed by TRAIL treatment could be a viable therapeutic option for GBM treatment.

Other work has shown that specific CD44 isoforms, CD44v6 and CD44v9, are responsible for binding to and sequestering Fas and preventing Fas activation in response to ligand. This raises the possibility that Necl-5 binds specifically to one or more isoforms, and not the entire CD44 population. It would be worthwhile to test if immunoprecipitation of Necl-5 shows co-immunoprecipitation of CD44v6 and/or v9, which would support the role of Necl-5 in preventing apoptosis by forming a complex with known Fas inhibitors.

This thesis identifies two new roles for Necl-5 in the pathogenesis of glioblastoma. As this disease is very deadly, and few effective treatments are available, these represent significant contributions to the field of glioblastoma biology, which may be exploited for therapeutic advances. Invasion and resistance to apoptosis are two hallmarks of cancer where Necl-5 has a major role. Attenuation of these biological processes reduces a cancer cell's fitness for survival, and could promote tumor regression.

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