Evaluation of Brain Cancer Cell Migration Using Three-dimension Brain Mimetic Hydrogels

Yixiao Cui[1], Jessica O. Winter[1,2]

[1]Department of Biomedical Engineering, [2]William G. Lowrie Department of Chemical and Biomolecular Engineering, The Ohio State University

Abstract

Approximately 22,500 new cases of brain cancer are diagnosed in the United State every year. Glioblastoma (GB) is the most lethal form of brain cancer with a minimal patient survival time~15 months. Unlike other types of cancer, GB rarely metastasizes to other organs but instead diffuses to tissues surrounding the tumor site making it hard to define the tumor region. This invasive nature of GB has caused great challenges in clinical treatments. The current standard of care for GB patients is surgical removal of the tumor mass followed by radiation and/or chemotherapy. However, it's almost impossible to remove the tumor completely due to the unclear tumor edge. GB cells have also demonstrated resistance to radio- and chemotherapies—in some cases, radiation treatments may even increase the migration ability of the tumor cells. Therefore, strategies targeting cell migration has been tested in clinical trials as an addition to the standard but few success has been made. The purpose of this study was to evaluate GB cell migration in a true three-dimension (3D) environment which mimics the brain tissue and better captures cell behavior than conventional 2D cultures. We particularly studied the effects of different inhibition methods on GB migration which offered future directions in brain cancer treatment.

1. Introduction

Brain cancer and brain metastases yield grim prognoses, yet are particularly difficult to study because of anatomical and functional limitations. For example, glioblastoma (GB), an astrocytic brain cancer that represents 80% of primary malignant adult brain tumors [1], has a dismal two year survival rate of only 27% [1]. Despite treatment, including surgery and chemo-radiation [2], patients develop resistance to therapy leading to tumor recurrence and eventually death. GB is highly aggressive, with recurrence in 95% of cases 2-3 cm from the initial surgical site [3], often with significantly more infiltrative phenotypes [4]. Thus, GB patients face the twin challenges of development of therapeutic resistance and the infiltrative nature of the disease.

Two migrating phenotypes with potential clinical significance have been identified in GB: mesenchymal and amoeboid [5]. It is generally believed that GB cells migrate via mesenchymal mechanisms [6], which have been observed in 2D culture, 3D collagen gels [7-10] and *in vivo* mouse models [8]. Mesenchymal migration is characterized by 'crawling' motions, elongated cell morphology, strong cell-extracellular matrix (ECM) interactions [11, 12] mediated by integrins (i.e., particularly subunits β1, β3, and α5), formation of actin stress fibers driven by Rac/Cdc42 signaling [13, 14], use of matrix metalloproteases (MMPs) to degrade the surrounding ECM [15], and slow migration resulting from slow focal adhesion turnover [16]. Of clinical importance, Rac/Cdc42 signaling is associated with poorer progression free survival [17], integrins are upregulated in GB tumors [18], and levels of certain MMPs (e.g., MMP1, MMP2, and MMP9) are correlated with tumor grade [19].

Emerging evidence suggests amoeboid migration may constitute one such salvage strategy, evidenced in reaction to GB tumor microenvironment (TME) changes. Amoeboid migration is characterized by 'gliding' movement, rounded or ellipsoid cell shape, weak cell-matrix interactions with little integrin dependence, low integrin (especially β_1) expression [20-23],

membrane bleb formation [24] through cortical actin regulation via Rho/ROCK signaling [25, 26], low proteolysis, minimal substrate remodeling [25, 26], and higher migration speed than mesenchymal cells (up to 10 fold) because of weak matrix interactions [16, 27]. Of clinical importance, low integrin and MMP expression limits the potential efficacy of integrin- and MMP-targeting therapies to this phenotype.

The observation of two distinct migratory signaling pathways suggests the possibility of a biologically significant mesenchymal to amoeboid transition (MAT) [25], similar to the epithelial to mesenchymal transition (EMT) believed to take place during metastasis of primary tumors [28]. For example, there are correlations between amoeboid phenotypes and resistance to therapy. GB cells exhibit enhanced migration following sub-lethal photon irradiation (IR) [29-31], which is linked to Rho/ROCK signaling [32]. Suppression of Rac signaling associated with mesenchymal cells, which might be expected to force cells into amoeboid modes, enhances migration further [33]. However, the molecular underpinnings of these phenomena are poorly understood [34]. Without this knowledge, interventions that target both migrating phenotypes cannot be developed [16].

Here, we constructed a hydrogel composite model with two components, collagen and hyaluronic acid (HA). With an increasing concentration of HA in collagen gels, we discovered an increased number of rounded GB cells with a lower migration speed. When we added ROCK inhibitor Y27632 to the high HA concentration gels, the population of elongated cells was increased by ~100%. However, no difference was detected in cell migration speed with or without Y27632. It's believe that our model is suitable for studying MAT and AMT of GBM in vitro and can be potentially used for drug screening regarding this purpose.

2. Materials and Methods

2.1 Cell culture

U87MG human glioma cells (ATCC) were cultured in DMEM/F-12 (Gibco) supplemented with 10% fetal bovine serum (FBS) (VWR), 1% penicillin-streptomycin (Corning), and 1x MycoZap (Lonza) and passaged when reached ~80% confluency. Cells with low passage numbers (<15 passages) were used in this study.

2.2 Preparation of collagen-HA hydrogel composites

Hydrogel composites were prepared as previously described[10]. Briefly, hydrogel composites were prepared by mixing DMEM/F-12 with collagen (PureCol, Advanced Biomatrix) and HA (Glycosil, ESI BIO). For all conditions, the concentration of collagen was kept as a constant 1 mg/ml and the concentration of HA was 1 mg/ml or 2 mg/ml for the col-0.1HA or col-0.2HA condition, repectively. To facilitate gel solidification, 10 mg/ml polyethylene glycol diacrylate (Extralink, ESI BIO) was added to the composites at a volume ratio of 1:4 to HA. In a 96-well plate, a layer of gel was first laid down and allowed for solidification. Then, another layer of gel with cells suspended inside was added on top of the first layer to reach a true 3D environment. After full solidification of the gel, cell culture media was added to the wells and changed every 12hr. To inhibit ROCK, small molecule inhibitor Y27632 (Selleck S1049, dissolved in DMSO) was added both to the hydrogels and cell culture media at a concentration of 10 uM.

2.3 Fluorescent and Time-lapse Imaging

For fluorescent imaging, cells were labeled with CellTracker Red CMTPX Dye or CellTracker Green CMFDA Dye (Thermo Fisher) before encapsulated. To capture single cell morphology and migration, CellTracker Green labeled cells were incubated in the hydrogel for overnight and imaged with 10x objective at a time interval of 15 min for 9 h on a fluorescent microscope

(IX81, Nikon). For nucleus staining, Hoechst 33342 (Thermo Fisher, dissolved in 1x PBS) was added at the end point and still images of cells were taken after 10 min incubation. Migration speed of cells was measured then by the 'MTrackJ' plugin in ImageJ (NIH). For high resolution imaging, cells were labeled with CellTracker Red. Samples were prepared using glass-bottom chamber slides (Nunc™ Lab-Tek®) and incubated for overnight before imaging. To detect cell-matrix interactions, samples were observed under a confocal fluorescent and reflectance microscope (Nikon A1 plus). Reflectance signal from collagen fibers were collected simultaneously with fluorescent signal from the cells. All images were processed and analyzed by ImageJ.

2.4 Morphology Analysis

Morphology of GB cells in hydrogels was measured by aspect ratio (AR). Briefly, still images were taken at random location inside of the hydrogels and manually thresheld to remove the background and clustered cells. AR was then analyzed using the 'Analyze Particle' command in ImageJ. Cells with AR higher than 2 were described as elongated, between 1.5-2 were described as intermediate, and less than 1.5 was described as rounded. ARs of cells from different groups were compared statistically.

2.5 Live Actin Staining

Live cells were stained for actin using CellLight Actin-GFP, BacMam 2.0 (Life Technologies) according to manufacturer's manual. In brief, cells were cultured in 24-well plate at a density of 100,000 cells/well for 18hr to reach ~70% confluency. 30 ul of CellLight particles were then added with fresh medium to the cells and incubated for 24hr before used.

2.6 Statistics and Plotting

All data were compared using nonparametric all pairs methods in JMP and plotted in SigmaPlot.

3. Results and Discussion

3.1 Mesenchymal and amoeboid migration behaviors

We detected two distinct type of migration behaviors of U87 cells cultured in col-HA hydrogels through time-lapse imaging. Cells cultured in pure collagen mostly showed an elongated cell shape and strong collagen-binding (Fig.1A). Time-lapse imaging indicated that those cells moved with mesenchymal features as they formed a leading edge which anchored to the ECM fibers and the cell body later followed (Fig. 1C). Cells cultured in 0.1col-HA showed a different morphology as they didn't elongate as much and instead adopted a rounded shape with only small membrane blebs formed (Fig. 1B) and the rounded morphology was remained during the movement (Fig. 1D).

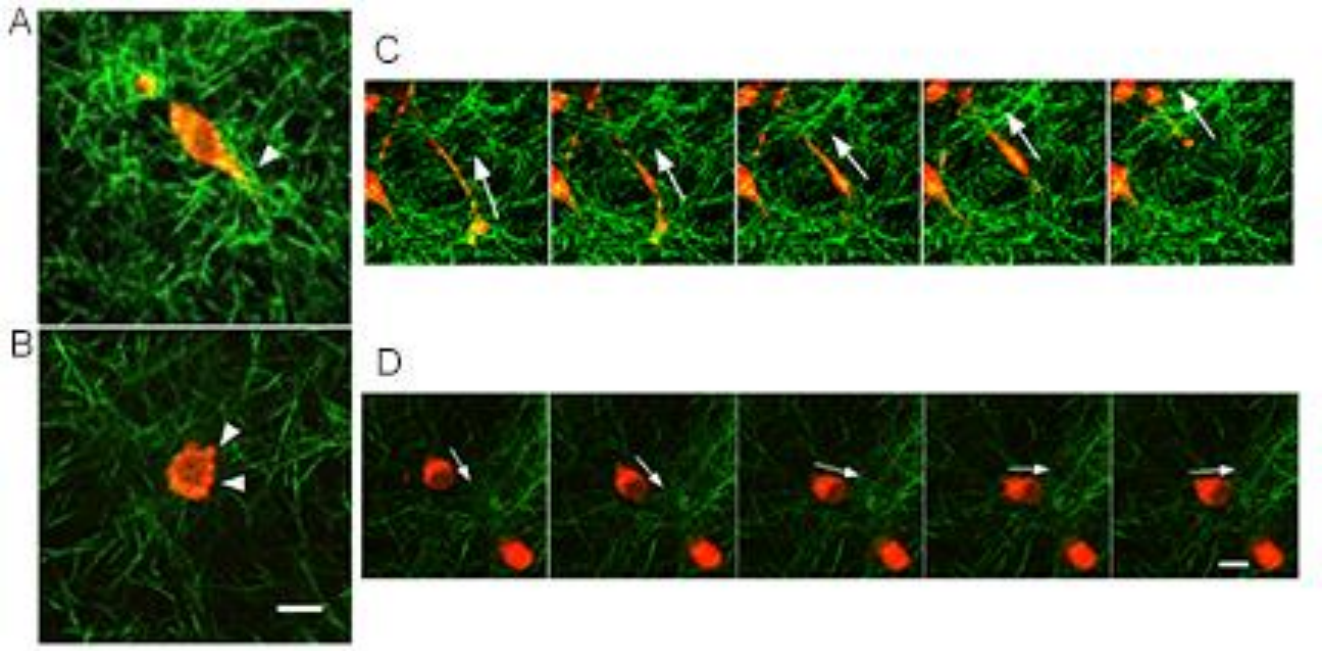

Figure 1 Mesenchymal-like (A,C) and amoeboid-like (B, D) migration behaviors of U87 cells (red) in collagen (green)-HA composites. A, B. Representative image of mesenchymal cells in pure collagen and amoeboid cells in col-0.1HA hydrogels. Bar=10um. A. Majority of cells adopted elongated shape and showed strong interaction with collagen fibers at the cell poles (indicated with arrow head) when cultured in pure collagen. B. Majority of cells remained a rounded shape and formed small blebs on the membrane (indicated with arrow heads). C, D. Still frames from time-lapse imaging depicting cell movement in pure collagen and col-0.1HA hydrogels. Bar=10u. C. Majority of cells in pure collagen migrated in a 'crawling' way. Specifically, cells formed a leading edge, which overlapped with collagen fiber clustering, and then pulled the cell body forward in the same direction. Cell movement direction indicated by the arrow. D. Cells cultured in col-0.1HA gels were less likely to move. The majority of moved cells maintained a rounded cell shape while moving through the gels in a 'gliding' way and minimal cell-fiber interaction was discovered.

3.2 Actin and nuclear deformation

Actin is the main cytoskeleton of cells and accounts for force generation during cell migration. Mesenchymal and amoeboid cells are believed to have different actin distribution, as the former form actin bundles mainly at the leading edge but the latter have actin evenly distributed on the membrane. To confirm that, we did live cell actin staining and compared the actin distribution of the cells with different morphologies. Indeed, strong signals of actin was detected at the leading edge, which was also the major binding site of the cell to the matrix, of the elongated cells (Fig. 2A). For rounded cells, small clusters of actin were discovered on the cell membrane of the rounded cells. However, actin signal still showed different intensities at different locations on the

cell membrane. This is suggesting that for amoeboid cells, there were also smaller actin bundles formed at adhesions sites on the membrane (Fig. 2B).

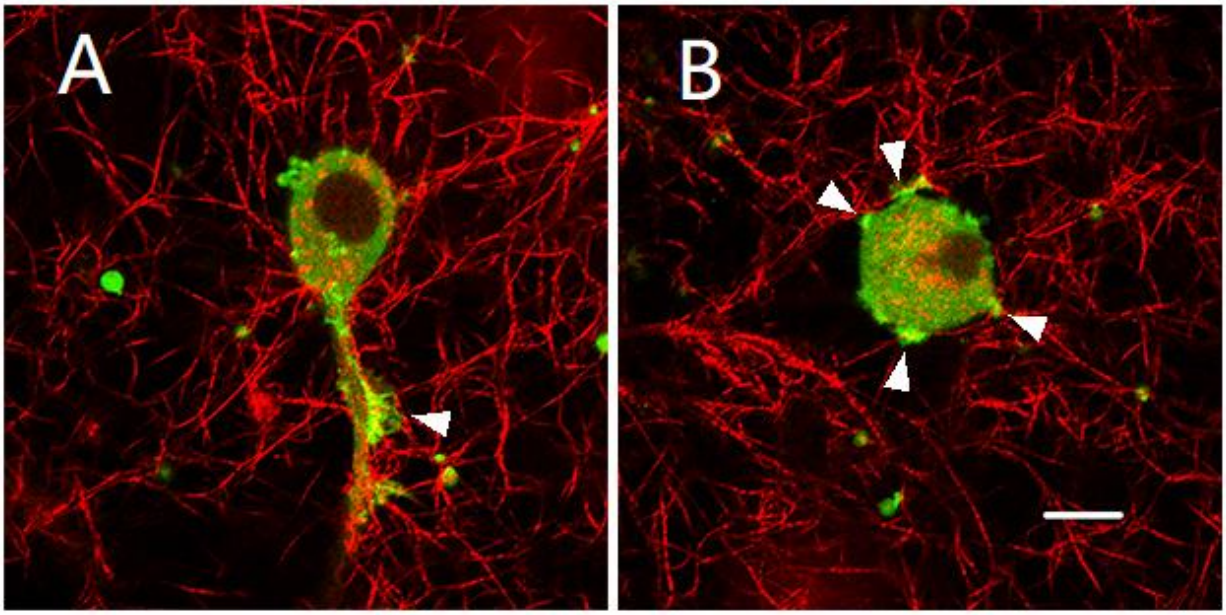

Figure 2. Live actin staining (green) of U87 cultured in collagen (red) hydrogels. A. Actin distribution of an elongated cells. Arrow head indicates high intensity of fluorescent signal coming from actin. B. Actin distribution of a rounded cell. Arrow heads indicate small bundles of actin. Bar=10um.

Because the mesenchymal and amoeboid cells show different degrees of elongation in cell morphology, it's interesting to explore whether that would also cause a difference in nuclear deformation. Therefore, we stained the nuclei of U87 cells cultured in collagen hydrogels with a Hoechst stain to detect the difference of nuclei in elongated and rounded cells. Our results showed that the morphology of the nuclei agrees with the cell shape as the elongate cells had elongated nuclei and the rounded cells had rounded nuclei (Fig. 3).

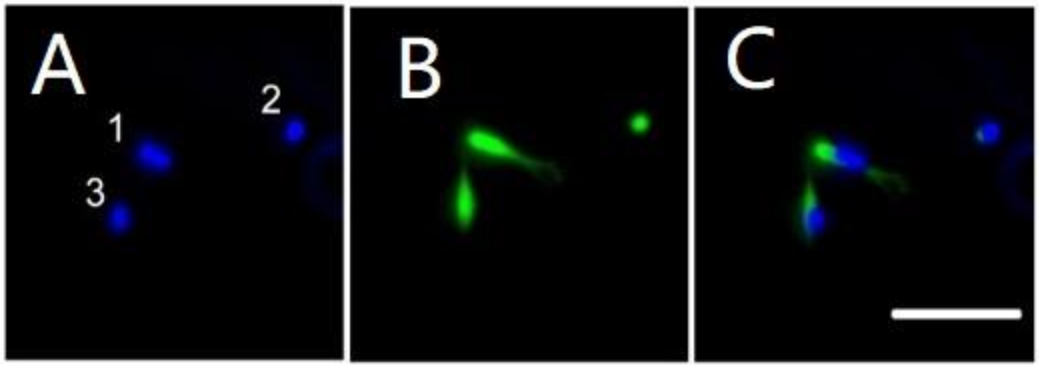

Figure 3. Nuclear staining of U87 in collagen hydrogels. A. Channel of the Hoechst staining of nuclei (blue). B. Channel of the CellTracker green staining of the cell membrane (green). C. Combined imaged of the two channels. Bar=100um.

3.3 Mesenchymal-amoeboid transition and amoeboid-mesenchymal transition

We have analyzed the population of cells with different morphologies by measuring the aspect ratio (AR) of the cell shape. We detected no different of the col-0.1HA condition but an increased number of rounded cells in the col-0.2HA condition as compared to the pure collagen condition. Assuming the cell rounding is related with MAT, we also applied a ROCK inhibitor, Y27632, to the col-0.2HA condition and significantly more elongated cells were discovered as compare to the non-treated col-0.2HA condition. Taking together, we proofed that HA can potentially drive MAT in U87 cells and this transition can be reversed by blocking the ROCK pathway which mediates amoeboid migration (Fig. 4).

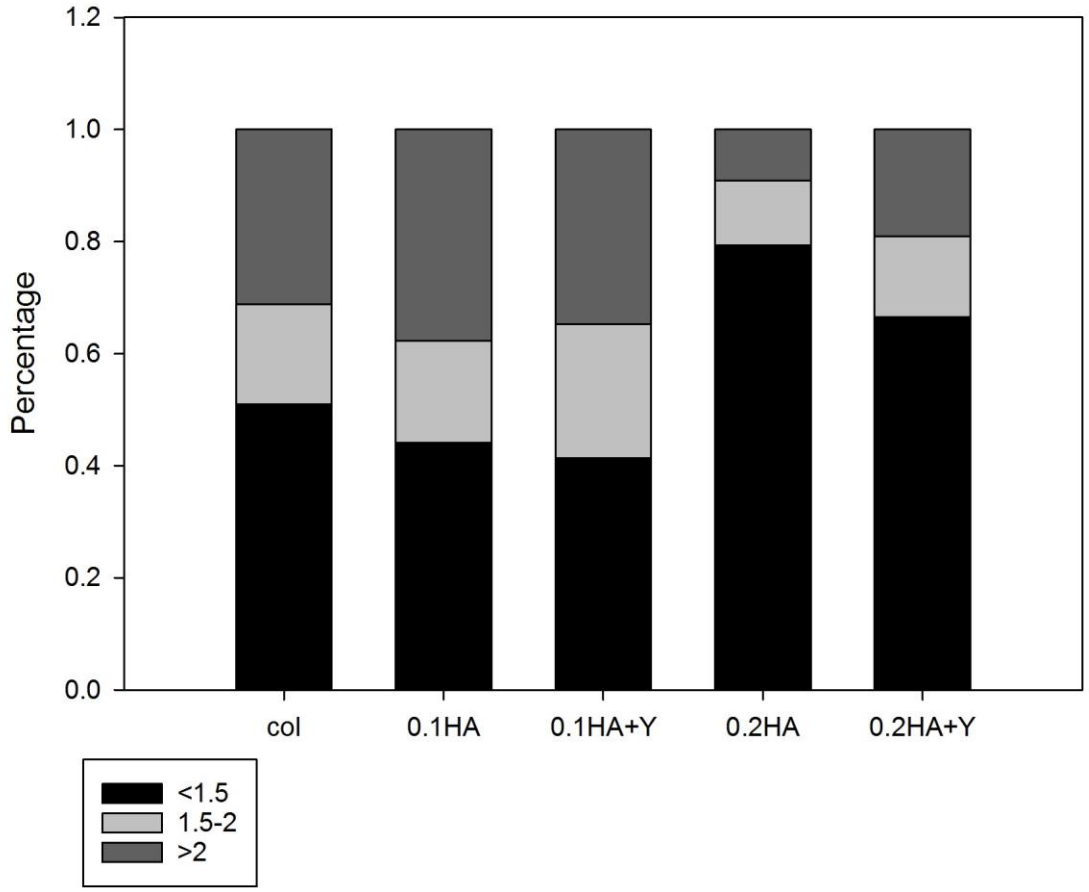

Figure 4. Morphology distribution of U87 cells in col-HA hydrogel composites analyzed by aspect ratio. Y: Y27632.

4. Conclusions

We have characterized two migration modes, mesenchymal and amoeboid, of U87 cells in col-HA hydrogel composites. We found that the mesenchymal cells in general show an elongated cell shape, strong interaction with collagen fibers, elongated actin distribution and nuclear deformation while the amoeboid cells are the opposite. In the col-0.2HA hydrogels, we detected there to be more amoeboid cells as more cells with rounded morphology was detected. By blocking the ROCK pathway with Y27632, this phenomenon was reversed and a significantly higher number of elongated cells was discovered as compared to the untreated gels. We believe

this has potentially indicated the plasticity of glioma cells to undergo different migration mechanisms in reaction to different ECM environment. Further studies will be done to better understand this process.

Acknowledgements

We thank Dr. Sara Cole at the Campus Microscopy and Imaging Facility at The Ohio State University for her help with high resolution confocal imaging. I want to give specially thanks to my grandma who couldn't understand a thing about my study but was so happy for me winning the prize.

References

1. Omuro, A. and L.M. DeAngelis, *Glioblastoma and Other Malignant Gliomas A Clinical Review.* Jama-Journal of the American Medical Association, 2013. **310**(17): p. 1842-1850.
2. Palanichamy, K. and A. Chakravarti, *Combining drugs and radiotherapy: from the bench to the bedside.* Current Opinion in Neurology, 2009. **22**(6): p. 625-632.
3. Salhia, B., et al., *Molecular pathways triggering glioma cell invasion.* Expert Review of Molecular Diagnostics, 2006. **6**(4): p. 613-26.
4. de Groot, J.F., et al., *Tumor invasion after treatment of glioblastoma with bevacizumab: radiographic and pathologic correlation in humans and mice.* Neuro Oncol, 2010. **12**(3): p. 233-42.
5. Sanz-Moreno, V., et al., *Rac activation and inactivation control plasticity of tumor cell movement.* Cell, 2008. **135**(3): p. 510-23.
6. Friedl, P. and K. Wolf, *Tumour-cell invasion and migration: diversity and escape mechanisms.* Nat Rev Cancer, 2003. **3**(5): p. 362-74.
7. Beadle, C., et al., *The Role of Myosin II in Glioma Invasion of the Brain.* Molecular Biology of the Cell, 2008. **19**(8): p. 3357-3368.
8. Caspani, E.M., et al., *Live imaging of glioblastoma cells in brain tissue shows requirement of actin bundles for migration.* Neuron Glia Biology, 2006. **2**(2): p. 105-114.
9. Ulrich, T.A., et al., *Probing cellular mechanobiology in three-dimensional culture with collagen-agarose matrices.* Biomaterials, 2010. **31**(7): p. 1875-84.
10. Rao, S.S., et al., *Glioblastoma Behaviors in Three-Dimensional Collagen-Hyaluronan Composite Hydrogels.* ACS Applied Materials & Interfaces, 2013. **5**(19): p. 9276-9284.
11. Friedl, P. and K. Wolf, *Tumour-cell invasion and migration: Diversity and escape mechanisms.* Nature Reviews Cancer, 2003. **3**(5): p. 362-374.

12. Pathak, A. and S. Kumar, *Biophysical regulation of tumor cell invasion: moving beyond matrix stiffness.* Integrative Biology, 2011. **3**(4): p. 267-278.
13. Nobes, C.D. and A. Hall, *Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia.* Cell, 1995. **81**(1): p. 53-62.
14. Kaibuchi, K., S. Kuroda, and M. Amano, *Regulation of the Cytoskeleton and Cell Adhesion by the Rho Family GTPases in Mammalian Cells.* Annual Review of Biochemistry, 1999. **68**(1): p. 459-486.
15. Kaufman, L.J., et al., *Glioma Expansion in Collagen I Matrices: Analyzing Collagen Concentration-Dependent Growth and Motility Patterns.* Biophysical Journal, 2005. **89**(1): p. 635-650.
16. Paňková, K., et al., *The molecular mechanisms of transition between mesenchymal and amoeboid invasiveness in tumor cells.* Cellular and Molecular Life Sciences, 2010. **67**(1): p. 63-71.
17. Okura, H., et al., *A role for activated Cdc42 in glioblastoma multiforme invasion.* Oncotarget, 2016. **7**(35): p. 56958.
18. Rooprai, H.K., et al., *The role of integrin receptors in aspects of glioma invasion in vitro.* International Journal of Developmental Neuroscience, 1999. **17**(5–6): p. 613-623.
19. Forsyth, P.A., et al., *Gelatinase-A (MMP-2), gelatinase-B (MMP-9) and membrane type matrix metalloproteinase-1 (MT1-MMP) are involved in different aspects of the pathophysiology of malignant gliomas.* British Journal of Cancer, 1999. **79**(11-12): p. 1828-1835.
20. Jaspars, L.H., et al., *EXTRACELLULAR MATRIX AND β1 INTEGRIN EXPRESSION IN NODAL AND EXTRANODAL T-CELL LYMPHOMAS.* The Journal of Pathology, 1996. **178**(1): p. 36-43.
21. Goldman, R., J. Harvey, and N. Hogg, *VLA-2 is the integrin used as a collagen receptor by leukocytes.* European Journal of Immunology, 1992. **22**(5): p. 1109-1114.
22. Falcioni, R., et al., *Expression of β1, β3, β4, and β5 Integrins by Human Lung Carcinoma Cells of Different Histotypes.* Experimental Cell Research, 1994. **210**(1): p. 113-122.
23. Hegerfeldt, Y., et al., *Collective Cell Movement in Primary Melanoma Explants: Plasticity of Cell-Cell Interaction, β1-Integrin Function, and Migration Strategies.* Cancer Research, 2002. **62**(7): p. 2125-2130.
24. Keller, H. and P. Eggli, *Protrusive activity, cytoplasmic compartmentalization, and restriction rings in locomoting blebbing Walker carcinosarcoma cells are related to detachment of cortical actin from the plasma membrane.* Cell Motility and the Cytoskeleton, 1998. **41**(2): p. 181-193.
25. Sahai, E. and C.J. Marshall, *Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis.* Nature Cell Biology, 2003. **5**(8): p. 711-719.
26. Wyckoff, J.B., et al., *ROCK- and Myosin-Dependent Matrix Deformation Enables Protease-Independent Tumor-Cell Invasion In Vivo.* Current Biology, 2006. **16**(15): p. 1515-1523.
27. Pankova, K., et al., *The molecular mechanisms of transition between mesenchymal and amoeboid invasiveness in tumor cells.* Cellular and Molecular Life Sciences, 2010. **67**(1): p. 63-71.
28. Smith, B.N. and N.A. Bhowmick, *Role of EMT in Metastasis and Therapy Resistance.* Journal of Clinical Medicine, 2016. **5**(2).
29. Wild-Bode, C., et al., *Sublethal irradiation promotes migration and invasiveness of glioma cells.* Cancer research, 2001. **61**(6): p. 2744-2750.
30. Goetze, K., et al., *The impact of conventional and heavy ion irradiation on tumor cell migration in vitro.* International Journal of Radiation Biology, 2007. **83**(11-12): p. 889-896.
31. Zaboronok, A., et al., *Proton beam irradiation stimulates migration and invasion of human U87 malignant glioma cells.* Journal of Radiation Research, 2014. **55**(2): p. 283-287.
32. Zhai, G.G., et al., *Radiation Enhances the Invasive Potential of Primary Glioblastoma Cells via Activation of the Rho Signaling Pathway.* Journal of Neuro-Oncology, 2006. **76**(3): p. 227-237.

33. Hwang, S.-Y., et al., *Dominant-negative Rac increases both inherent and ionizing radiation-induced cell migration in C6 rat glioma cells: Rac is Involved in Radiation-Induced Cell Migration in C6 Glioma Cells.* International Journal of Cancer, 2006. **118**(8): p. 2056-2063.
34. Vehlow, A. and N. Cordes, *Invasion as target for therapy of glioblastoma multiforme.* Biochimica Et Biophysica Acta-Reviews on Cancer, 2013. **1836**(2): p. 236-244.