



Elevated Fluid Shear Stress Decreases Ras Expression and Activation in Glioblastoma

Sarah Benjumea^{1,2}, Grace Aclé³, Alexandra Seas^{4,5}, Masanobu Komatsu⁶, Pavlos Anastasiadis^{5,7,8,9}

HONORS COLLEGE
INTEGRATED
LIFE SCIENCES

¹Integrated Life Sciences Honors Program, University of Maryland Honors College, College Park, MD; ²College of Computer, Mathematical, and Natural Sciences University of Maryland, College Park, MD;

³Department of Chemistry and Biochemistry Loyola University Maryland, Baltimore, MD; ⁴Medical Scientist Training Program, University of Maryland School of Medicine, Baltimore, MD;

⁵Department of Neurosurgery, University of Maryland School of Medicine, Baltimore, MD; ⁶Johns Hopkins All Children's Hospital Research Institute, St. Petersburg, FL;

⁷University of Maryland Marlene and Stewart Greenebaum Comprehensive Cancer Center, Baltimore, MD; ⁸Department of Diagnostic Radiology and Nuclear Medicine, University of Maryland School of Medicine, Baltimore, MD;

⁹Fishell Department of Bioengineering, University of Maryland, College Park, MD



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Background

- R-Ras, a small GTPase and member of the Ras superfamily, is involved in cellular signal transduction, migration, proliferation, and differentiation.
- R-Ras and FLNA (a cytoskeletal protein) form the R-Ras/FLNA complex, which is critical for maintaining the integrity of the endothelial barrier and promoting endothelial homeostasis.

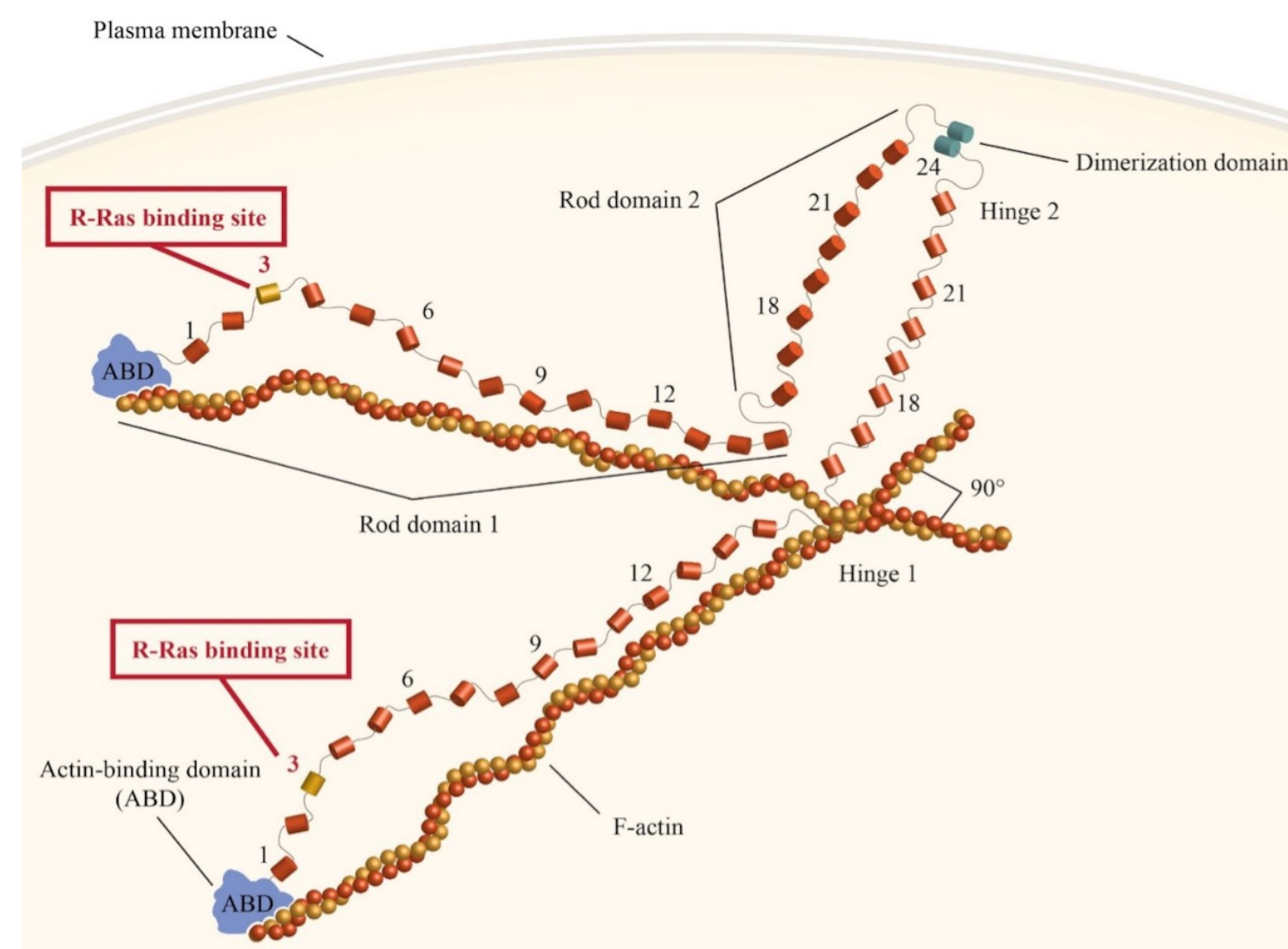


Figure 1. Model of R-Ras and FLNA interaction: R-Ras directly binds to repeat 3 of FLNA. [1]

- The R-Ras/FLNA complex plays a prominent role in the blood-brain barrier (BBB).
- Glioblastoma (GBM), the most lethal primary brain tumor in adults, has a compromised BBB.
- Cells that lack FLNA expression have impaired locomotion function, making them unable to migrate [2,4].

We hypothesize that R-Ras activity affects GBM metabolic activity and migration.

- Ras activity in a cell line can be determined by performing an Active Ras Pull-Down Assay and running Western blots (WB) to probe them with an anti-Ras primary antibody.
- Ras activity monitors all Ras small GTPase activation, not just R-Ras.
- To emulate the fluid shear stress found in the BBB, we utilized cell spinpods at different shear stress levels.

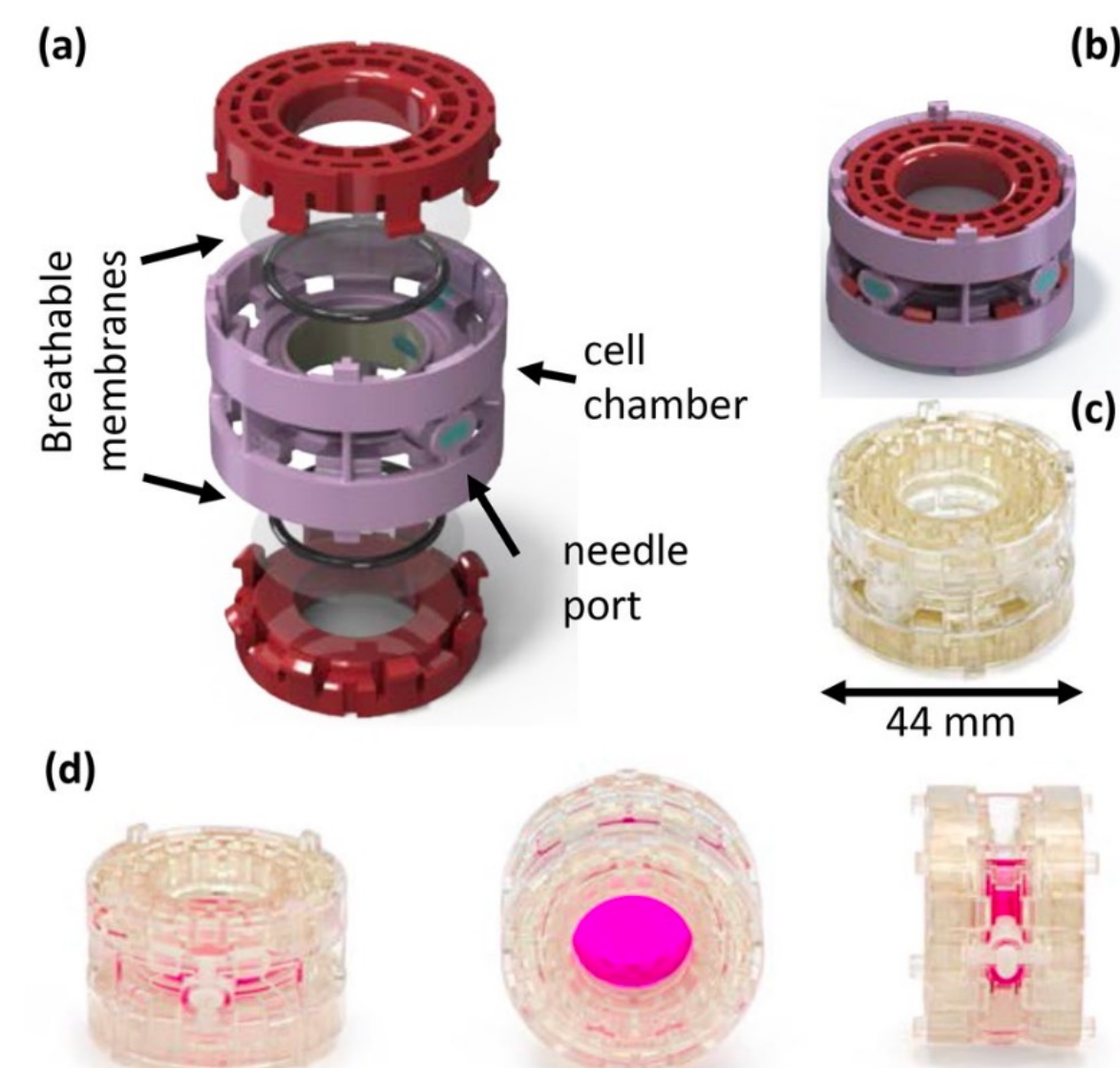


Figure 2. Components and Model of Cell Spinpod: (A-B) CAD renderings of the components of a cell spinpod. (C) Dimension of a cell spinpod. (D) 3 views of a cell spinpod with media inserted. [3]

Results

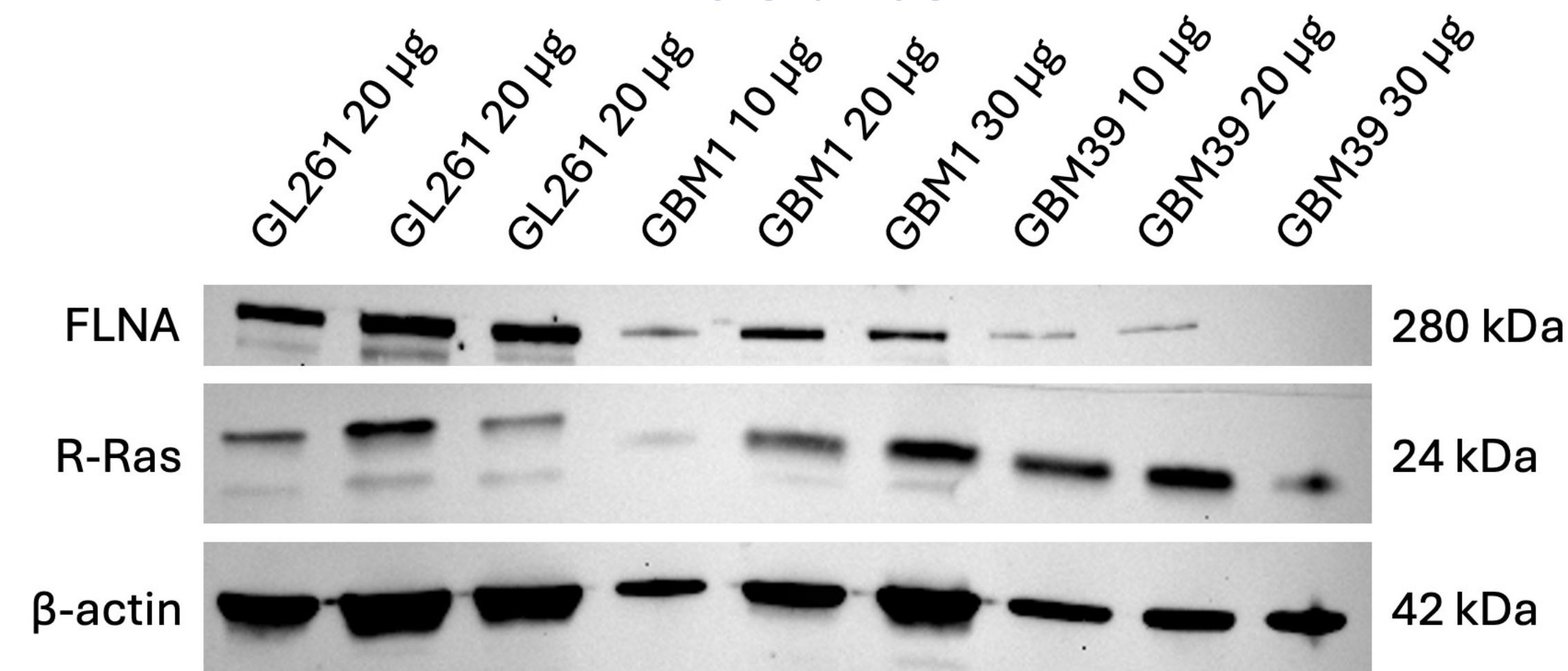
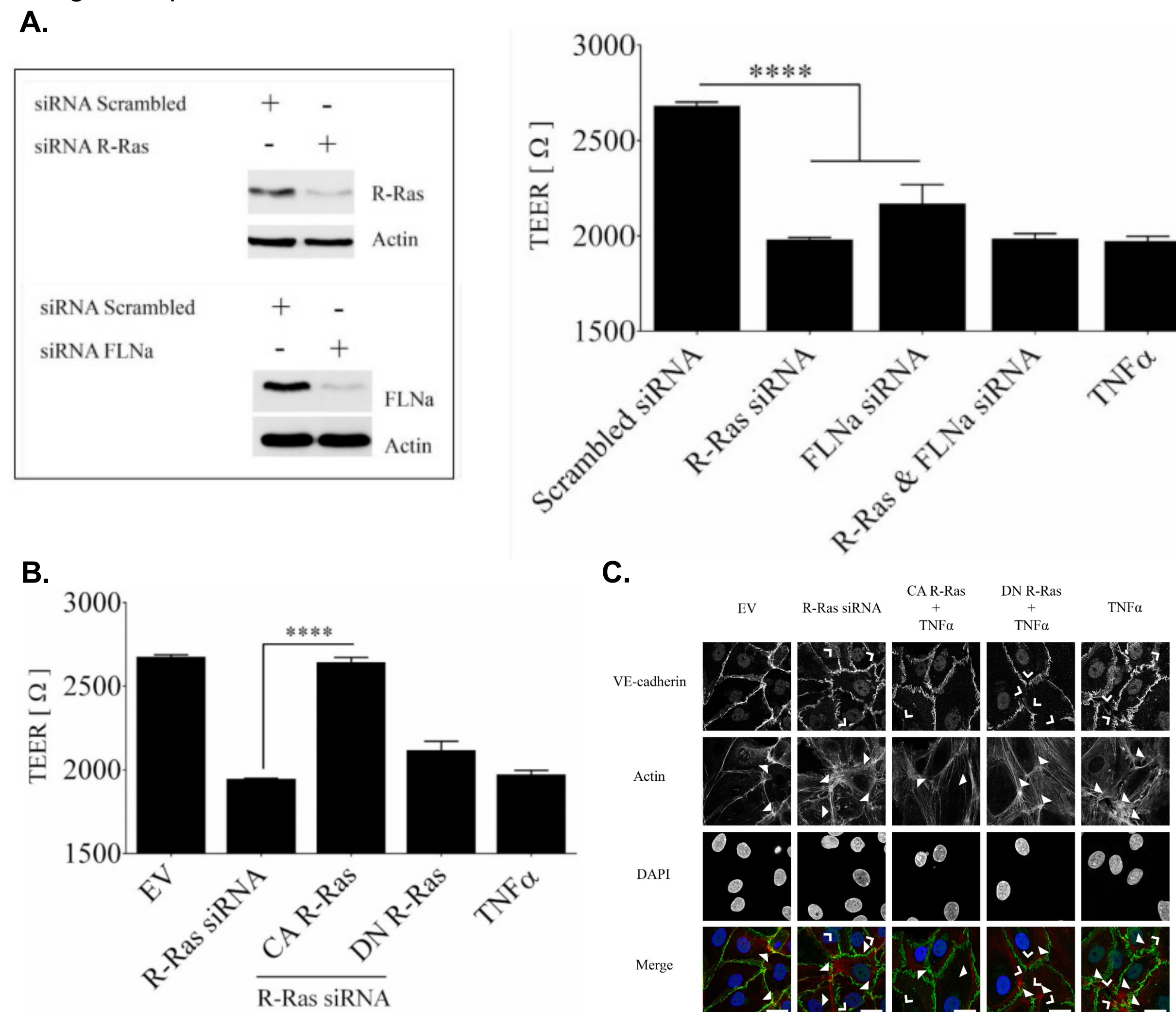
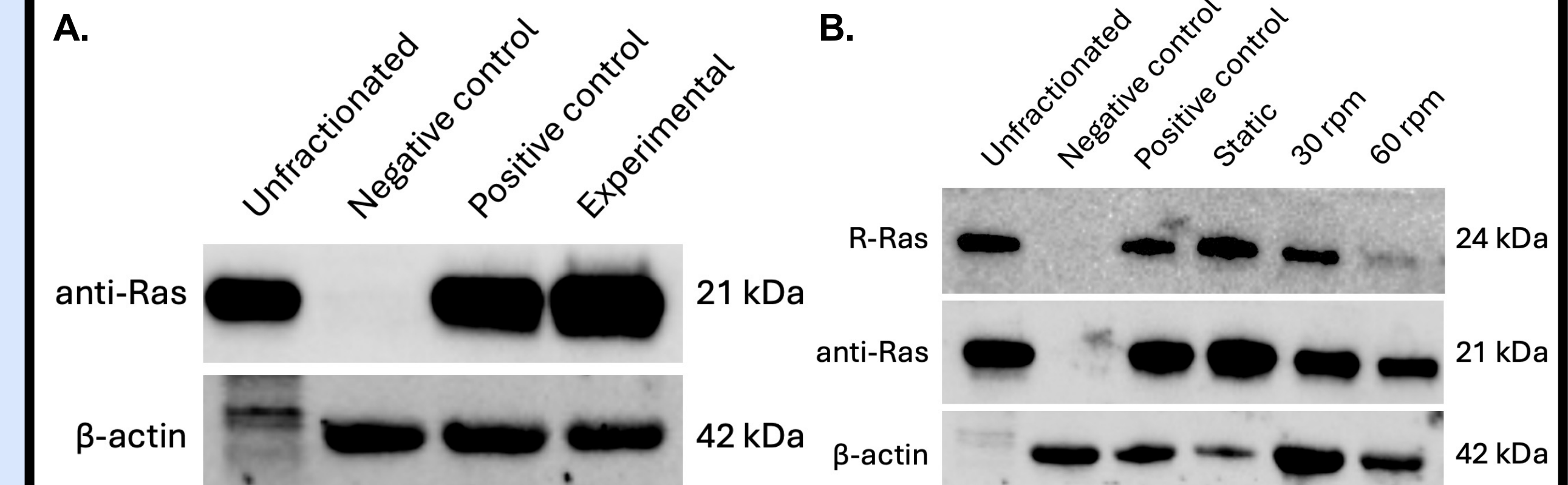


Figure 3. WB of GL261, GBM1, and GBM39 cells: GL261 is a mouse glioma cell line, which has a high expression of R-Ras and FLNA. This expression of R-Ras and FLNA decreases with GBM1 and then again even more with GBM39. Expression levels of R-Ras change with protein concentration of GBM cell lines.



Figures 4A, 4B, and 4C. R-Ras/FLNA activation and knockdowns demonstrate R-Ras/FLNA complex's regulation of endothelial barrier function under shear stress: Endothelial monolayers were subjected to fluid shear stress (FSS) for 15 minutes at 2 dyn/cm² to emulate the FSS found in the BBB. TEER values and vascular permeability are inversely proportional. (A) The loss of R-Ras or FLNA due to knockdown exhibited decreased TEER values (**** p<0.0001). This decreased TEER signifies an increased permeability of the endothelial barrier. (B) Re-expressed constitutively active R-Ras (CA R-Ras + R-Ras siRNA) and re-expressed dominant negative R-Ras (DN R-Ras + R-Ras siRNA) were specifically targeted, due to R-Ras's role in cellular signal transduction. In re-expressed DN R-Ras, endothelial barrier integrity is lost; however, it is restored with re-expressed CA R-Ras (**** p<0.0001). Therefore, re-expressed CA R-Ras can be introduced to decrease the permeability of the endothelial barrier. (C) After 15 minutes of exposure to FSS at 2 dyn/cm², these endothelial monolayers containing specific R-Ras knockdowns were stained with Alexa 594-phalloidin (red), anti-VE-cadherin antibody (green), and counterstained with DAPI (blue) for confocal imaging. The images confirm that cells transfected with CA R-Ras restore permeability back to baseline values. Closed arrowheads indicate stress fiber formation, a signifier of permeability in the endothelial barrier. [1]



Figures 5A and 5B. WB of GBM1 Ras Activity show that increased FSS decreases Ras activation: In an Active Ras Pull-Down Assay, the addition of GST-Raf1-RBD as an experimental sample allows for the quantification of Ras through binding to the GTP-bound state of Ras. The Ras activity bands are shown at the anti-Ras weight, 21 kDa. The Ras small GTPase is activated and inactivated for the positive control (presence of band) and negative control (lack of band), respectively. The unfractionated sample is an untreated GBM1 sample. The difference between the Active Ras Pull-Down WB and the previous R-Ras WB is the Active Ras Pull-Down WB measures the activity level of the Ras small GTPase while the R-Ras WB measures the protein concentration of R-Ras in the sample, even if it is inactive. (A) Static GBM1 cells were cultured and lysed to create samples to probe for Ras activity. Lane 1: unfractionated GBM1; lane 2: negative control, GDP-treated GBM1; lane 3: positive control, GTPγS-treated GBM1; and lane 4: experimental, GST-Raf1-RBD-treated GBM1. (B) Static and FSS GBM1 samples were cultured and lysed to create samples to probe for Ras activity. FSS GBM1 cells are not undergoing shearing as in the BBB, only undergoing shear stress in a protected cell spinpod to emulate FSS. Lane 1: static unfractionated GBM1; lane 2: static negative control, GDP-treated GBM1; lane 3: static positive control, GTPγS-treated GBM1; lane 4: static experimental, GST-Raf1-RBD-treated GBM1; lane 5: FSS 30 rpm (slow) experimental, GST-Raf1-RBD-treated GBM1; and lane 6: FSS 60 rpm (fast) experimental, GST-Raf1-RBD-treated GBM1.

Conclusions & Future Directions

- GBM1 and GBM39 abundantly express R-Ras.
- The R-Ras/FLNA complex is essential for maintaining endothelial homeostasis.
- Ras activity in GBM1 decreases at higher levels of fluid shear stress.
- Next steps:** (1) Using siRNA knockdown for R-Ras under FSS to inhibit GBM cell migration will provide insight into how Ras activity affects GBM1 cells and the BBB. (2) Determining the activation level of patient-derived GBM cells is crucial to understanding the invasive potential of these cells.
- Future research question:** How are R-Ras and FLNA involved in the regulation of the blood-brain barrier in infiltrative gliomas?

References

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Acknowledgements

Part of the research reported in this work is supported by the UMGCCC American Cancer Society Institutional Research Grant DICR INTR-23-1253710-01-DICR INTR to Tonya J. Webb and NIH/National Cancer Institute grants: R25CA186872 to Bret A. Hassel and P30CA134274 to Taofeek Owonikoko; In part of Diversity in Cancer Research Program, University of Maryland Baltimore; This research is also supported by the Bioacoustics and Biophysics Research Laboratory, University of Maryland Baltimore.

