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14. ABSTRACT

This research provided novel insights regarding micro-scale changes in the mechanical properties of acute and chronic demyelinating brain lesions, and how these changes might correlate to remyelination failure in advance stages of MS. We found that changes in matrix stiffness can impact the differentiation of iPSC-derived human glial lineages, and that stiffer matrices promotes astrocyte differentiation over that of oligodendrocytes. Preliminary findings indicate that the subcellular localization of the mechanosensitive transcription factor YAP in oligodendrocytes can be predictive of their ability to differentiative. Specifically, retention of nuclear YAP is negatively correlated with myelin protein expression at the single cell level in myelinating co-cultures. These findings have the potential to help optimize the methods currently used to generate human oligodendrocytes for autologous transplantation using iPSC technology, as well as guide the efforts for the efficient targeting and delivery of these cells to MS patients.

15. SUBJECT TERMS
Multiple sclerosis, mouse models, demyelination, remyelination, tissue stiffness, extracellular matrix, atomic force microscopy

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1. Introduction

Despite the presence of oligodendrocyte progenitor cells (OPC) capable of regenerating myelin after its loss, chronic multiple sclerosis (MS) lesions in the brain and the spinal cord are characterized by remyelination failure. Cell-based therapies involving progenitors derived from autologous stem cells are a promising approach for the treatment of chronic MS[1,2]. However, transplants delivered into CNS areas with extensive damage often result in poor cell engraftment and survival [3-5]. Recent studies have also demonstrated that mechanical cues delivered by the extracellular matrix (ECM) are capable, independently of chemical signals, of directing the differentiation of stem cell populations or promoting the differentiation of mesenchymal stem cells into specific cell types. Similarly, while soft artificial substrates mechanically similar to the healthy brain promote neurogenesis and axonal growth,[6,7], work from our laboratory and others has demonstrated that oligodendrocytes (OL), the myelinating glia of the CNS, are also mechanosensitive[8-12] and that increases in ECM stiffness inhibit their differentiation[12].

The abnormal accumulation of a wide range of ECM proteins in demyelinated lesions has repeatedly been shown to inhibit remyelination[13,14,15]. However, despite the evidence that mechanical signals may play an important role in CNS development and repair, and the fact that changes in ECM deposition resulting from injury or demyelination must change the mechanical properties of the ECM, there has been very little systematic investigation of this problem. More critically, *very little is known about how the ECM changes mechanically during the time course of disease and recovery in the CNS*. Although it is accepted that chronic demyelination ultimately causes remyelination failure[16], and that chronic and acute demyelination are associated with different types of ECM molecules deposition[13,15], there has been no systematic analysis of how demyelinating insults affect the mechanical properties of the ECM in the CNS. Attempts to measure the mechanical properties of the brain parenchyma in animal models of demyelination[17] and human aging[18] using non-invasive methods such as magnetic resonance elastography (MRE) have been limited to a macroscopic spatial resolution. Atomic force microscopy (AFM) provides an alternative that allows the examination of tissue stiffness at scales relevant to cellular mechanotransduction.

To this end we developed a protocol which allowed us to capture both optical images and AFM measurements of the brain tissue at micrometer scales. This information used in combination with immunohistochemistry (IHC) of the same region, made it possible to correlate changes in tissue stiffness with ECM structure and cellular composition of the area being examined. *This innovative approach provided unprecedented level of detail on the mechanical properties of the CNS and the mechanical stimuli experienced by specific cells populations within demyelinated lesions.* We found that acute and chronic demyelination affect the mechanical properties of CNS tissue in distinct ways when examined at cellular rather than macroscopic resolution. Specifically, acute demyelinated lesions are softer than healthy tissue, while chronic demyelinated lesions exhibit increased stiffness, which is associated with elevated ECM deposition. Thus, changes in ECM mechanical properties may be an important contributing factor to the rapid remyelination observed in acute softer lesions and the failure to remyelinate typical of chronic stiffer lesions.

2. Keywords

Multiple sclerosis, mouse models, demyelination, remyelination, tissue stiffness, extracellular matrix, atomic force microscopy.

3. Accomplishments

- **What were the major goals of the project?**
    - Major Task 1: Characterization of the mechanical properties of active and chronic demyelinating MS plaques in human brain tissue in conjunction with analysis of ECM and cellular composition
    - Major Task 2: Characterization of mechanical tissue properties and ECM deposition in a mouse model of demyelination
    - Major Task 3: Establish the ex vivo culture system of cuprizone-demyelinated brain slices and hOPC.
- **What was accomplished under these goals?**

Major Task 1: Characterization of the mechanical properties of active and chronic demyelinating MS plaques in human brain tissue in conjunction with analysis of ECM and cellular composition

Subtask 1: Regulatory Review and approval by the USAMRMC Human Research Protection Office (HRPO).

Completed

Subtask 2: AFM and histological analysis of human MS tissue. **Completed**. A total 12 human MS samples have been analyzed to date (tissue was not mechanically damaged and identifiable lesions were found). Among these six lesions from two samples were adequate for further AFM and histological studies. The results obtained from these studies were published in Scientific Reports.

- **Major Task 1: 100% completed as August 2019**

Major Task 2: Characterization of mechanical tissue properties and ECM deposition in a mouse model of demyelination

Subtask 1: Regulatory Review and approval by the USAMRMC Animal Care and Use Review Office (ACURO. **Completed**

Subtask 2: Setup and maintenance of the mouse cuprizone-induced demyelination cohort. **Completed**

Subtask 3: Assess the mechanical properties of the corpus callosum brains slices generated in subtask 2 via atomic force microscopy (AFM) to generate high resolution force maps of brain tissue. **Completed**

*Subtask 4: Perform IHC of the same regions measured via AFM to determine the extent of demyelination, oligodendrocyte differentiation and survival, monitor gliosis and deposition of extracellular matrix (ECM).***Completed**

- **Major Task 2: 100% Completed as August 2019**

The milestones for major task 1 and 2 are completed. A paper was published in Scientific Reports. Follow-up studies will require obtaining more MS tissue from the tissue bank to further characterize the nature of the cellular and molecular changes driving the changes in stiffness in acute and chronic demyelination. Grant applications were submitted for these studies to both the National Multiple Sclerosis Society, Department of Defense and NIH. On summer 2022, we finally obtained an Investigator-Initiated Research Award of the Department of Defense (W81XWH2211044) that will allows us to continue these studies.

Major Task 3: Establish the ex vivo culture system of cuprizone-demyelinated brain slices and hOPC

Subtask 1: Regulatory Review and approval by the USAMRMC Human Research Protection Office (HRPO).

Completed

Subtask 2: Induction of human OPC (hOPC) from iPSC (See **Fig. 3**). **Completed**

Subtask 3: Pre-conditioning of hOPC prior to co-culture with mouse brain slices. **In progress**. We established that culturing iPSC-derived neurospheres in soft matrices promote OPC differentiation (Olig2+) while stiffer substrates promote astrocyte differentiation (GFAP+) (**Fig. 1**). These data was included on a grant application (GRANT13472904 for MS210138) in response to FY21 Multiple Sclerosis Research Program Investigator-Initiated Research Award of the Department of Defense, that was successfully awarded this year (W81XWH2211044).

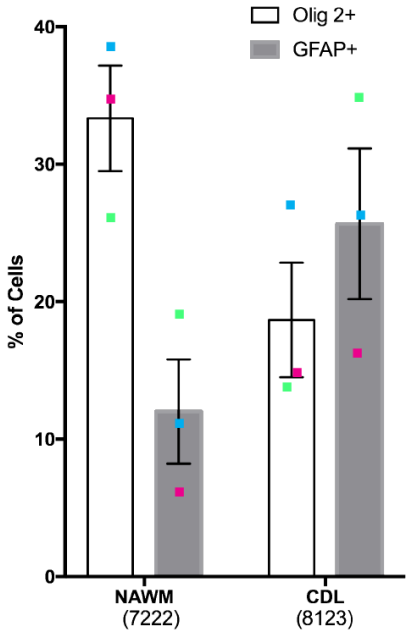

Figure 1. Effect of ECM stiffness on the production of human glia from iPSC lines. Quantification of the percentage of OPC/OL (Olig2+) and astrocytes (GFAP+) derived from 3 iPSC-lines[1,2]. Cultures were kept for 76 (CL121) or 91 days (CL104, CL659) in hybrid hydrogel/fiber matrices mimicking the stiffness of soft NAWM ($E \sim 1.3$ kPa) or stiff CDL ($E \sim 5$ kPa) found in MS tissue in media that promotes OL-differentiation. For all cell lines tested the softer NAWM environment promoted OPC/OL differentiation over astrocytes. In stiffer matrices (CDL) this trend was reversed, i.e., decreased OPC/OL and increased astrocyte production. Bar represents the mean±SD of 3 biological replicates per condition. The total number of cell counted is indicated at the bottom (7222 for NAWM) and (8123 for CDL)

We continue with these studies, specifically on determining how these changes on lineage differentiation are related to the expression and subcellular localization of mechanosensitive transcription factors such YAP/TAZ and Olig1 in both human and rodent OPC. Exciting preliminary data on these studies were recently presented at the Society for Neuroscience Annual Meeting in San Diego, CA. We found that at the single cell level, nuclear retention of YAP is directly correlated with decreased myelin protein expression and myelination (**Fig. 2**). We plan to continue and extend these studies to include other mechanoresponsive transcription factors and epigenetic markers to investigate if OPC/OL establish a mechanical memory of their environment.

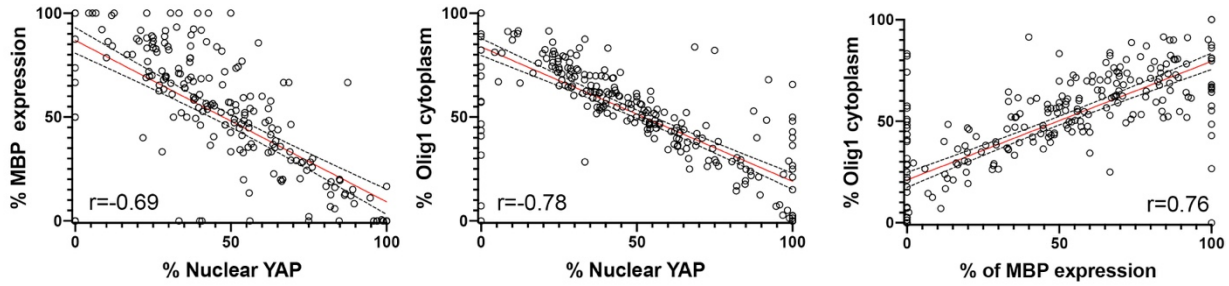

Figure 2. Increased YAP nuclear retention impairs MBP expression and Olig1 cytoplasmic translocation. Data shows the negative correlation at the single cell level between YAP nuclear localization and MBP expression and Olig1. By contrast, a positive correlation was observed between cytoplasmic Olig1 and MBP expression in each cell, 2 cultures per condition, 149 cells counted. (Pearson correlation, $p<0.0001$)

Subtask 4: Setup and maintenance of the mouse cuprizone-induced demyelination cohort. **(Completed as November 2022)**: These studies were initiated on August 2021 with the idea of providing chronically demyelinated tissue for preparation of brain slices (see below).

Subtask 5: Co-culture of hOPC on brain slice cultures. **(Completed as November 2022)**: We tested the viability of adult mouse brain slices (normal and demyelinated) and determined they can be maintained *ex-vivo* for up to a week. However, human iPSC-derived OPC required more time to complete differentiation (6-8 weeks). We changed our approach and decided to use decellularized brain matrices instead of *ex-vivo* slices. We tested various protocols for decellularization of matrices and used them as substrates for human OPC, unfortunately this approach did not work either as the matrices were unable to maintain human cells viable for the required time. Going forward and as part of the new grant we will be pursuing these studies using biomimetics artificial matrices (*in vitro*) and transplantation in rodent brains (*in vivo*).

Subtask 6: IHC analysis of co-cultures and data analysis. **(In progress)** Due to the technical challenges found with of the slice *ex-vivo* system, we started to use cultures of DRG neurons platted in soft vs. stiff hydrogels as an alternative approach to study the role of matrix elasticity on human OPC differentiation and myelination *in vitro* (**Fig 1, 2**). Through a separate collaboration with Dr. Mehmet Kurt at Stevens Institute of Technology (*manuscript in revision*), we were able to measure the stiffness of individual DRG axons in culture and demonstrate that matrix stiffness is transmitted to the axons. We are currently using these cultures to seed human neurospheres and characterize differences in myelination. We have been successful in obtaining differentiation and alignment along DRG axons by human iPSC-derived OPC (**Fig. 3**). These data was included on a grant application (GRANT13472904 for MS210138) in response to FY21 Multiple Sclerosis Research Program Investigator-Initiated Research Award of the Department of Defense, that was successfully awarded on Summer 2022 (W81XWH2211044).

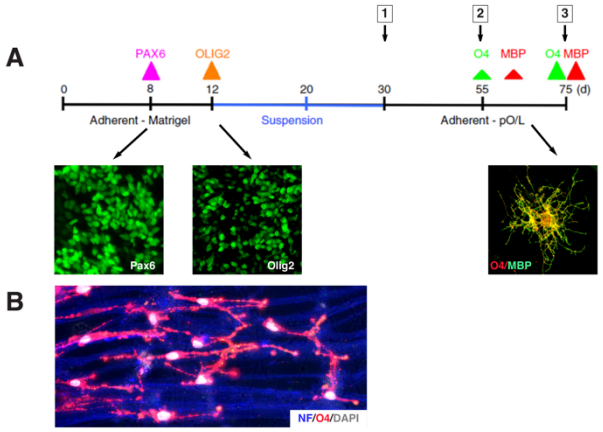

Figure 3. Generation of human iPSC-derived oligodendrocytes progenitors (hOPC) competent for *in vitro* myelination. Frozen iPSC neurospheres containing neuroectoderm (Pax6+) and ventral neural progenitors (Olig2+) were thawed and allowed to differentiate in culture following well-established protocols[1,2]. After 30 days of culture in suspension (1) cells can be transferred to adhesive substrates in media that promotes hOPC differentiation and evaluated for proliferation, survival and/or expression of maturation markers: 04+, 55 days (2) or 04+,MBP+ 93), after 75 days. **B**. Alternatively, hOPC (O4+,red) can be transferred after 30 day in suspension, to dorsal root ganglion (DRG) rodent neurons (neurofilament, blue) and maintained for 6-8 weeks in media promoting differentiation. The image shows that hOPC are able to attach and align their processes along axons. DAPI staining shows hOPC nuclei.

Major task 3 at 70% completion as November 2022: The milestones for major task 3 were mostly completed by the end of the grant period (4 out of 6). A paper is currently under revision, and another in preparation as part of Mr. Ace Alcantara, PhD thesis. Please note that the approach for pre-conditioning hOPC was changed, as the *ex-vivo* slice system did not work (see Subtasks 4,5). The tasks listed as on-going (Subtasks 3,6) are carried out using a modified experimental approach not included in the original application, to grow and pre-condition hOPC. These studies are included as part of the Investigator-Initiated Research Award of the Department of Defense (W81XWH2211044), that will allow us to continue with the characterization of the mechanical responses of human iPSC *in vitro* and *in vivo* for the next 3 years.

- **What opportunities for training and professional development has the project provided?**
    - Dr. Matt Urbanski (Research Assistant) was the leading author of our 2019 publication and continued helping in the with this project until March 2020. Although, we ran out of funding to keep him as a postdoctoral fellow in the lab, he has maintained his professional

ties at Hunter College. He has been working as adjunct teaching faculty and is currently been considered for a full-time tenure track position as doctoral lecturer in our department. Dr. Urbanski has developed an upper-level elective course for undergraduate and graduates students in mechanobiology of the brain. He is also a co-author on a 2023 publication in collaboration with the laboratory of Dr. Brahim Chaqour at SUNY Downstate Medical Center. His contribution to this publication was key as he helped transfer the technology we developed for AFM measure of tissue stiffness from brain to retina.

- Ace Alcantara, a PhD student was trained by former Research Assistant Dr. Matt Urbanski and continues to work on this project as part of his PhD thesis. He has participated in conferences, weekly lab meetings and graduate student progress report seminars. He was a co-author on a 2022 publication in collaboration with the laboratory of Dr. Patrizia Casaccia, a fellow glial scientist at the Advanced Science Research Center of CUNY.
- Joseph Lawrence, a PhD student started his rotation during the summer 2022 and joined the lab this Fall. He will carry his thesis project in our lab and will be working on the continuation of the studies funded by our Investigator-Initiated Research Award (W81XWH2211044)

- **How were the results disseminated to communities of interest?**
  - Nothing to report.
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - Nothing to report

4. Impact

We have provided novel insights regarding micro-scale changes in the mechanical properties of acute and chronic demyelinating MS lesions, and how these changes might correlate to remyelination failure in advance stages of disease. We also found that changes in matrix stiffness can impact the differentiation of human glial lineages, and that stiffer matrices promotes astrocyte differentiation over that of oligodendrocytes. Preliminary findings indicate that the subcellular localization of the mechanosensitive transcription factor YAP in OL can be predictive of their ability to differentiative. Specifically, retention of nuclear YAP is negatively correlated with myelin protein expression at the single cell level in DRG-OPC co-cultures. These findings have the potential to help optimize the methods currently used to generate human OPC for autologous transplantation using iPSC technology, as well as guide the efforts for the efficient targeting and delivery of these cells to MS patients.

- **What was the impact on the development of the principal discipline(s) of the project?**
  - Nothing to Report.
- **What was the impact on other disciplines?**
  - Nothing to Report.
- **What was the impact on technology transfer?**
  - Nothing to Report
- **What was the impact on society beyond science and technology?**
  - Nothing to Report.

5. Changes/Problems

- **Changes in approach and reasons for change**
    - The *ex-vivo* slices approach did not working as anticipated. However we have implemented alternative approaches to grow human iPSC-derived glial cells on substrates of different stiffness. To date the most successful involves the use of hydrogels and DRG neurons in coculture with human iPSC-neurospheres. On separate studies (not covered by this project), we plan to test the delivery of human OPC directly into the corpus callosum of chronically demyelinated mice. These studies are now part of the Investigator-Initiated Research Award of the Department of Defense (W81XWH2211044).
- **Actual or anticipated problems or delays and actions or plans to resolve them**
    - *Not applicable*
- **Changes that had a significant impact on expenditures**
    - *Not applicable.*
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
    - *Not applicable.*
- **Significant changes in use or care of human subjects**
    - *Not applicable.*
- **Significant changes in use or care of vertebrate animals**
    - *Not applicable.*
- **Significant changes in use of biohazards and/or select agents**
    - *Not applicable.*

6. Products

- **Journal publications.**

  Published:
  Chaqour B, Grant MB, Lau LF, Wang B, Urbanski MM, Melendez-Vasquez CV (2023). Atomic Force Microscopy-Based Measurements of Retinal Microvessel Stiffness in Mice with Endothelial-Specific Deletion of CCN1. *Methods Mol Biol*. 2023; 2582:323-334. doi: 10.1007/978-1-0716-2744-0_22. Acknowledgement of federal support: Yes.

  Park HJ, Tsai E, Huang D, Weaver M, Frick L, Alcantara A, Moran JJ, Patzig J, Melendez-Vasquez CV, Crabtree GR, Feltri ML, Svaren J, Casaccia P (2022). ACTL6a coordinates axonal caliber recognition and myelination in the peripheral nerve. *iScience*. 2022 Apr 15;25(4):104132. doi: 10.1016/j.isci.2022.104132. Acknowledgement of federal support: Yes

Urbanski MM, Brendel MB, Melendez-Vasquez CV (2019). Acute and chronic demyelinated CNS lesions exhibit opposite elastic properties. *Sci Rep.* 2019 Jan 30;9(1):999. doi: 10.1038/s41598-018-37745-7. Acknowledgement of federal support: Yes

Other: Chuang, Y; Alcantara, A; Fabris, G; Melendez-Vasquez, CV; Kurt, M. Myelination dictates axonal viscoelasticity . (Manuscript in revision) Acknowledgement of federal support: Yes

- **Other publications, conference papers, and presentations.**

  Wang, X.; Tabassum A, Alcantara**,** A, Melendez-Vasquez, CV. The role of mechanical memory on oligodendrocyte progenitor cells differentiation. *Poster* Society for Neuroscience, San Diego CA, Nov 12-Nov19, 2022

  Chuang, Y; Alcantara, A; Fabris, G; Melendez-Vasquez, CV; Kurt, M. Studying the variation of biomechanics during myelination using an *in vitro* neuron-oligodendrocyte co-ulture model. *Oral Presentation*. SB3C Summer Biomechanics, Bioengineering & Biotransport Conference. June 14 -18, 2021

  Alcantara, A.; Urbanski, M.; Parikh, D.; Wang, H.; Melendez-Vasquez, C. Role of Mechanotransduction in the differentiation of human oligodendrocytes . *Poster*. Glia in Health and Disease 2020 Virtual Poster Session. Cold Spring Harbor Laboratory Meeting July 16-July 19, 2020.

  H. S. Domingues, H. Wang, S. Macedo-Ribeiro, M. M. Urbanski, J. B. Relvas, B. Rubinstein, C. V. Melendez-Vasquez, I. Mendes Pinto. Mechanical plasticity in developing oligodendrocytes. *Oral Presentation*.XIV European Meeting on Glial Cells in Health and Disease (July 10 – 13, 2019. Porto, Portugal).

  M. Urbanski, M. Brendel, C. Melendez-Vasquez. Mechanical Properties of the Injured CNS: Implications for Remyelination and Axonal Repair *Poster*. XIV European Meeting on Glial Cells in Health and Disease (July 10 – 13, 2019. Porto, Portugal).

Invited seminars/lectures by Dr. Melendez-Vasquez

- SUNY Upstate Medical University, Cell and Developmental Biology Department, Syracuse, NY (November 2022)
- Glia Club Seminar at University of Wisconsin-Madison (June 2022)
- Spinal Cord & Brain Injury Research Forum at Indiana University School of Medicine (March 2021)
- Invited Panelist for NYU CoNNExINs: Postdocs: Now and Then (June 2021)
- Mahoney Institute for Neurosciences (MINS) at the University of Pennsylvania (October 2021)
- The ASRC at 5: Showcasing Interdisciplinary Excellence, ASRC of CUNY New York, NY (October 2019)
- International Iberian Nanotechnology, Department of Life Sciences Nanomedicine Group, Porto, Portugal (July 2019)

7. Participants and other collaborating organizations

Name:	*Carmen Melendez-Vasquez*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-8752-8956*
Nearest person month worked:	*1 CM*
Contribution to Project:	*Reviewed data. Manuscript preparation. Prepared report.*

Name:	*Mateusz Urbanski*
Project Role:	*Research Associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12 CM*
Contribution to Project:	Carried out experiments for Major Task 1. Trained a graduate student assisting in the project.

Name:	*Ace Alcantara*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3 CM*
Contribution to Project:	*In charge of experiments in major task 3*
Funding Support:	*NIH-NIGMS RISE Program*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
    - Nothing to Report.
- **What other organizations were involved as partners?**
    - Nothing to Report.

8. Special Reporting Requirements

Not applicable.

9. Appendices

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