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TITLE: Extracellular Vesicles as Potential Drivers of Myelin Health and Myelin Repair in Pregnant MS Patients

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CONTRACTING ORGANIZATION: The Research Foundation of SUNY, Stony Brook, NY

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**14. ABSTRACT**

This research study explores whether Extracellular Vesicles (EVs) that are produced during pregnancy may have beneficial roles in Multiple Sclerosis, in particular, to myelin health and/or repair capacity. We have characterized EVs from both pregnant and control mice, have completed studies to determine the effect of pregnancy EVs on oligodendrocyte progenitor cell proliferation, differentiation, and myelin gene expression. In addition we have sequenced miRNA cargos contained within pregnancy EVs compared to non-pregnant control EVs. We are currently working to establish the cellular and temporal requirements for EV uptake in the brain, as well as for the capacity of pregnancy EVs to alter myelin repair trajectories using both cuprizone-mediated demyelination in the brain, and lysolecithin-mediated demyelination in cerebellar slice culturs.

**15. SUBJECT TERMS**
None listed.

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## INTRODUCTION

While current MS therapies may decrease the frequency and severity of damage that occurs to myelinated axons, no current MS therapy directly stimulates cellular processes in oligodendrocytes (OLs) that lead to improved myelin stability or repair. One reason is that the molecular mechanisms that regulate the ability of an OL to successfully undergo myelin repair remain poorly understood, or, have not proven to be readily "drug-able". Therefore there is urgent need for a better understanding of these molecular mechanisms in order to develop successful oligodendrocyte regenerative and/or myelin repair therapies for MS. Pregnancy provides researchers a powerful clue as to where to look. Indeed hormones that are dramatically altered during pregnancy having been explored for years as potential MS therapies, but unfortunately have not proven to be clinically viable. The current proposal takes a novel approach by examining a completely different physiological response of pregnancy, that of increased extracellular vesicle (EV) circulation. While pregnancy EVs have been explored as immune modulators, we hypothesize that pregnancy-EVs will also have unique properties that may prove beneficial to the OL regenerative capacity in MS. By characterizing these effects and the molecular mechanisms that underlie them we hope to identify new therapeutic strategies for MS. The approach described here has the potential to identify more relevant cargos, and, to identify ways to increase cellular targeting of beneficial EVs. Overall, the experiments of the proposal will generate novel areas for MS drug discovery research.

## KEYWORDS

Myelin repair, extracellular vesicles, oligodendrocyte, cuprizone, Multiple Sclerosis

## ACCOMPLISHMENTS

**3a. What were the major goals of the project**?

The major goals of the project were divided into the three following specific aims (see Figure 1), which were each further subdivided into multiple subtasks, as follows:

**Aim 1: Determine whether treatment with LP-EVs improves OPC/OL health and differentiation in the rodent CNS following cuprizone-induced demyelination.** Late pregnancy-specific plasma factors with immunoregulatory capacity have been suggested to be responsible for pregnancy-mediated disease suppression in EAE, however the ability of LP-EVs to influence OPC/OL health and differentiation dynamics remains unclear. We will therefore investigate the effects of intranasal delivery of LP-EVs or C-EVs in the cuprizone-induced demyelination model, which reliably triggers robust demyelination of the corpus callosum without peripheral immune involvement, allowing us to selectively study OL regeneration and remyelination *independently* from EV effects on the peripheral immune system.

**Aim 2: To investigate whether LP-EVs directly impact OPC/OL health and differentiation.** OPC/OL cultures provide a controlled environment for the study of molecular signals responsible for OPC proliferation, OPC differentiation into mature OLs, and OPC/OL health, all properties that contribute to OL regeneration capacity. We will test whether LP-EVs can directly influence OPC/OL dynamics, both in healthy OPC/OLs as well as in OPC/OLs exposed to conditioned medium obtained from pro-inflammatory microglia, conditions designed to mimic the inflammatory environment during MS.

**Aim 3: Assess the cargo and surface proteins of circulating EVs from late pregnancy and virgin mice.** While immunoregulatory cargos of pregnancy EVs have been described, it remains unknown whether pregnancy EVs contain cargos that influence OPC/OL proliferation/differentiation/health. miRNAs are important regulators of OL development and differentiation, and EV surface proteins (integrins, tetraspanins, & other adhesion proteins) are also critical as they can both act to promote uptake by cells, and/or can help to target EVs to particular cell types. We will therefore determine whether pregnancy alters EV cargos and/or surface proteins, which could serve to influence OL/myelin repair dynamics in MS.

**3b. What was accomplished under these goals?**

Aim 1: During the first 6 months of the grant period, we were in the process of obtaining regulatory approval for mouse work, which involved several revisions to first our IACUC, followed by submission of the ACURO

followed by revisions, then approval. As Aim 1 is largely mouse work, we were not able to proceed with is Aim, however we were still able to test out several key antibody reagents on other tissue and EV samples that we had on hand in the lab, as well as research and optimize experimental strategies, all in preparation for the approved cuprizone work. We were granted ACURO approval in March 2021, but then we hit a snag in that our IACUC due for its 3-year renewal in June 2021. This IACUC tri-renewal was applied for and was granted in June 2021, i.e., on time so that we did not have any gap in IACUC approval on our project, however the tri-annual IACUC renewal precipitated a requirement to submit a brand new ACURO, only 3 months after getting the first one approved. We therefore wrote another ACURO, which was largely the same as the previously approved one, which was reviewed but found to require additional revisions. Those revisions were submitted and approved on August 24, 2022, after which we were finally able to proceed with animal work. Because of this delay, we have only done a few pilot experiments to determine the extent of nasal delivery in anesthetized mice using labeled EVs. Here, we have optimized labeling with Infrared Dye (IR) and detecting labeled IR-EV in the brain following nasal administration (Figure 1). Thus far we observe IR-EV in the rostral areas of the brain but have not detected them in more caudal areas. We are currently optimizing our detection methods.

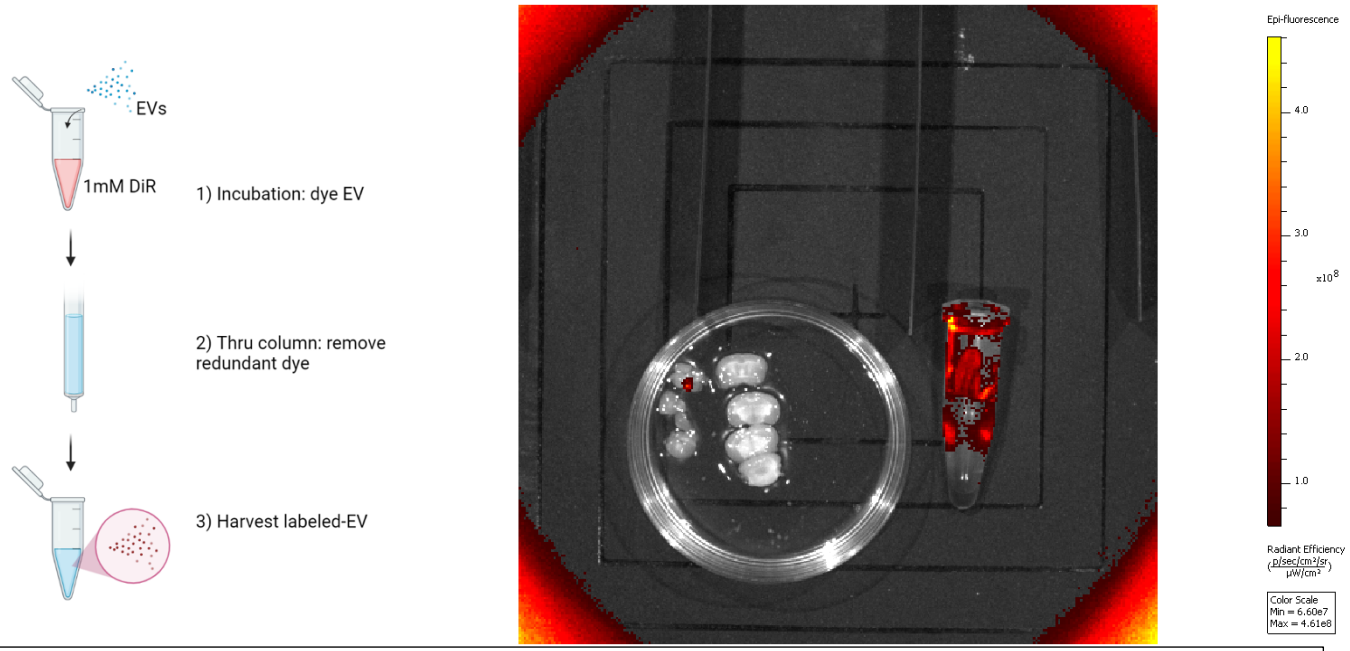



**Figure 1.** Delivery of InfraRed-labeled EVs via nasal delivery, followed by detection using IVIS.

Aim 2: Here, we have made substantial progress. We previously found that delivery of EVs to solo OPC and OL cultures did not alter developmental maturation. We have now pioneered the use of cerebellar slice cultures so that we can monitor OPC and OL outcomes in a cultures system that brings multiple cell types together, as well as allows us to ask whether we can track OPC and OL behaviors under conditions of myelin damage. (Figure 2).

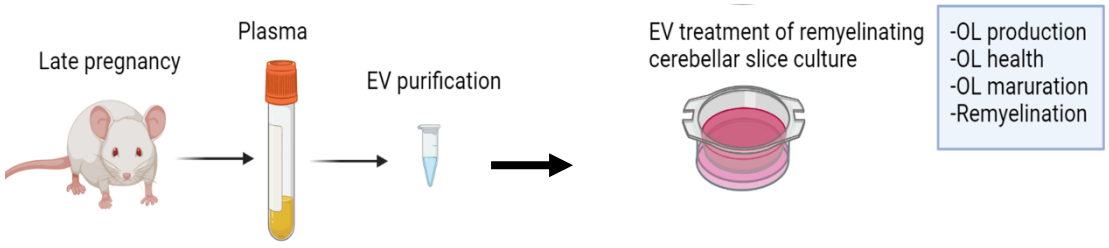



**Figure. 2.** Schematic of cerebellar slice culture experiments to assess OPC and OL dynamics in response to EVs

By modifying the cellular environment of our OPC/OLs, we now have preliminary indication that pregnancy-EVs can alter the number of Olig2+ cells at 2 days post-demyelination (Figure 3). We are currently investigating whether is due to increased OPC proliferation, as well as working to repeat this experiment to enable us to determine if differences are statistically significant.

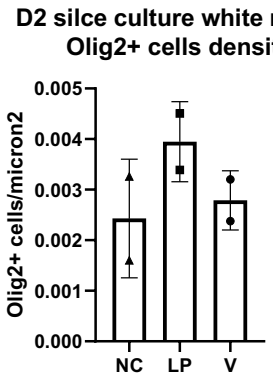
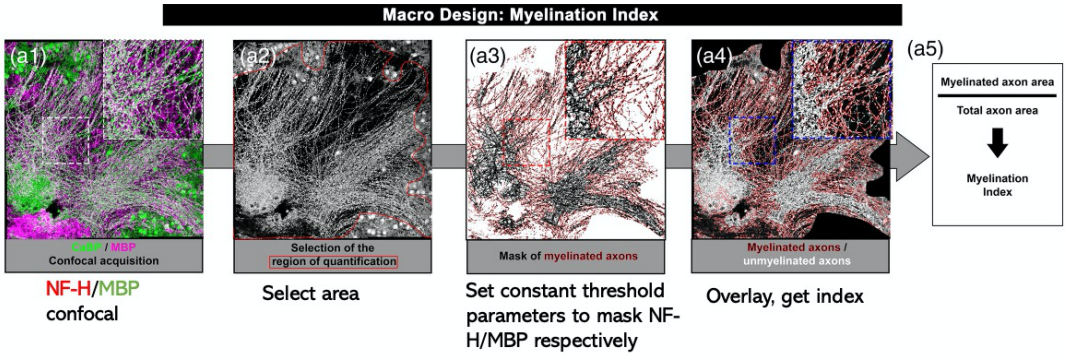
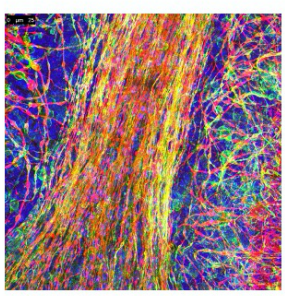
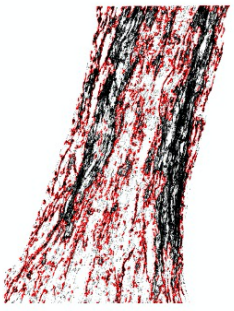



**Figure 3.** The influence of EVs from late pregnancy (LP) or virgin (V) mice on Oligg2+ cell density at day 2 post demyelination in cerebellar slice cultures (n=2).

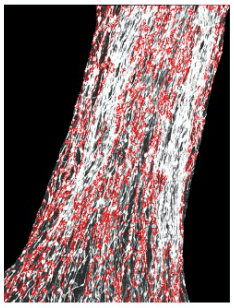
We have also set up a way to assess myelin as an index relative to neuronal axons following demyelination in the cerebellar slice cultures (see Figure 4). Thus far using this index method we have not observed changes in response to EV treatment however we have only performed this on an n of 2 thus far. We are currently working on generating additional sets.





Mask of myelinated axon



Merge of myelinated area

| | Total Area |
|---|---|
| MBP+ NF-H+ | 13605.69 |
| NF-H+ | 28425.31 |

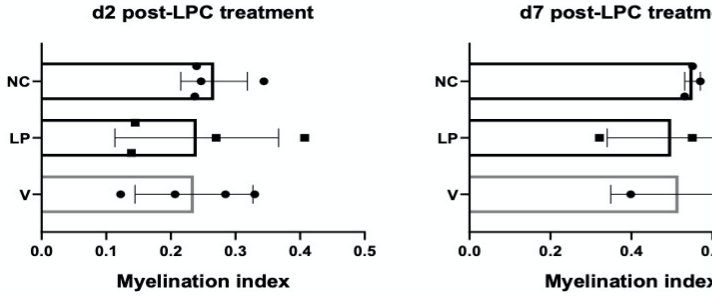



**Figure 4.** Scheme to determine the myelination index in cerebellar slice cultures.

**3c. What opportunities for training and professional development has the project provided?**
There have been extensive training opportunities during the project. The PI and co-PI have been training a graduate student in oligodendrocyte assay techniques, cerebellar slice cultures, immunohistochemistry, confocal image acquisition, EV preparation and analysis. As a result, this graduate student (Mr. Zijian Shao) has been making substantial contributions to the project during the past ~12 months.

**3d. How were the results disseminated to communities of interest?**
We have not yet presented this work at an external conference, however a graduate student who is working on the projected has presented the findings at a graduate student symposium this year.

**3e. What do you plan to do during the next reporting period to accomplish the goals?**
We will continue to design and construct the remaining plasmids for experiments in Aims 2 and 3, and will test out each one, first in cell culture using our newly established and optimized glial culture system, followed by in vivo analysis using nasal electroporation. We will continue to *mdx*$^{3cv}$ phenotypes (stem, progenitor, and oligodendrocyte cellular phenotypes including proliferation, differentiation, and survival, myelination timing, and myelination ultrastructure).

## IMPACT

**4a. What was the impact on the development of the principal discipline(s) of the project?**
There is considerable interest in the Multiple Sclerosis research community in finding mechanisms that may promote oligodendrocyte health, maturation, and capacity to ensheath multiple neuronal axons with myelin (i.e., myelination capacity). Therefore our early findings that LP-EVs may enhance OPC generation suggest that LP-EVs may contain cargos that directly impact OPC biology.

**4b. What was the impact on other disciplines?**
Given the potential effect of LP-EVs on OPC phenotypes, we may be able to expand our findings to other diseases (e.g., Alzheimer's disease) in which myelin loss is believed to be a factor in promoting ongoing neurodegeneration.

**4c. What was the impact on technology transfer?**
Nothing to report.

**4d. What was the impact on society beyond science and technology?**
Nothing to report.

## CHANGES/PROBLEMS:

**5a. Changes in approach and reasons for change**
We have introduced a cerebellar slice culture to supplement our approach in Aim 2. This was to provide a way to assess OPC and OL responses in cell culture in a way that better modeled the environment of the brain in MS.

**5b. Actual or anticipated problems or delays and actions or plans to resolve them.**
Due to the delay in awaiting ACURO approval, we were not able to use funds to support the animal work on this project for a section of the reporting period. However we shifted to work more extensively on other aspects of the problem, and optimized many of the methods needed for Aim 1 such as the best way to detectably label EVs.

**5c. Changes that had a significant impact on expenditures**. Due to the delay in awaiting ACURO approval, we were not able to use funds to support the animal work on this project for a section of the reporting period.

**5d. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**.

Nothing to report.

**5e. Significant changes in use or care of human subjects**.
Not applicable.

**5f. Significant changes in use or care of vertebrate animals.**
Nothing to report.

**5g. Significant changes in use of biohazards and/or select agents**.
Not applicable.

## PRODUCTS:

Not applicable.

## PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

| | |
|---|---|
| Name: | *Holly Colognato* |
| Project Role: | *PI* |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | *0.6* |
| Contribution to Project: | *Dr. Colognato has supervised the project and analyzed data.* |
| Funding Support: | |

| | |
|---|---|
| Name: | *Maricedes Acosta-Martinez* |
| Project Role: | *Co-PI* |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 0.6 |

| | |
|---|---|
| Contribution to Project: | *Dr. Acosta-Martinez has supervised the project and analyzed data.* |
| Funding Support: | |

| | |
|---|---|
| Name: | *Zijian Shao* |
| Project Role: | *Graduate Student* |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | *12* |
| Contribution to Project: | *Mr. Shao has optimized EV purification methods, characterized EVs, assessed the effect of EVs on oligodendrocyte cultures. Mr. Shao has also been working on optimizing methods to assess miRNA and other cargos from isolated EVs, and has optimized using a label to tag EVs for detection in vitro and in vivo. Lastly, he has pioneered the use of cerebellar slice cultures to track OPC and OL responses to myelin injury.* |
| Funding Support: | |

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
Yes; see attached for revised Support document.

**What other organizations were involved as partners?**
Nothing to report.

## SPECIAL REPORTING REQUIREMENTS
Not applicable.

## APPENDICES
Not applicable.