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

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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 purified and characterized EVs from both pregnant and control mice, have performed pilot studies to determine the effect of pregnancy EVs on oligodendrocyte progenitor cell proliferation, differentiation, and myelin gene expression, and have begun our efforts to sequence miRNA cargos contained within pregnancy EVs.

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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 now found to require additional revisions. Currently we are currently in the process of addressing the requested revisions so we can resubmit this.

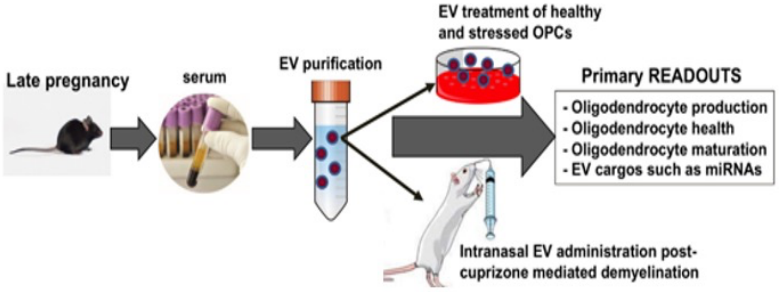

Figure 1. Project overview.

Aim 2: Here we were able to use existing oligodendrocytes cultures prepared for another project (see Subtask 7), to proceed with examining the effect of existing (from our previous pilots) LP-EVs and C-EVs on oligodendrocyte progenitor cell phenotypes. First, we have done substantial work in characterizing our LP- and C-EVs (Figure 2). After optimizing our purification procedures, we analyzed EVs and find that we are routinely obtaining EVs of expected size (Figure 2, 105 - 120 nm diameter), expected yield (ranging from 1 – $3x10^{11}$ particles per mL, not shown), and expected marker expression (e.g., CD9 western blot, **Figure 2**).

While awaiting animal approval we made progress on Aim 2, which involves characterizing the effect of LP- and C-EVs on oligodendrocyte progenitor cells (OPCs) grown in culture conditions. First, we determined a range of EV concentrations to use in culture, ensuring that this range did not induce cell toxicity by tracking cell numbers over time (not shown). Second, we established media conditions that were conducive to EV assessment but still induced sufficient numbers of oligodendrocytes to differentiate from OPCs. Here, our standard differentiation medium (SATO's medium plus 0.5% FBS and T3) was problematic as commercial FBS preparations contain high numbers of EVs from bovine serum. We attempted a culture condition without serum that had been previously used in the lab for short term differentiation assays (SATO plus T3, as well as 0.2 ng/mL PDGF to promote oligodendrocyte survival) however we found that in longer term (e.g., 5 days) differentiation assays this condition only permitted 5-10% of the OPCs to reach the MBP+ state. Therefore we explored different ways to deplete FBS of EVs, then tested whether the EV-depleted FBS could still support high degree of OPC-to-oligodendrocyte differentiation. We were able to identify a centrifugation method that successfully depleted FBS of EVs but still supported OPC differentiation (as judged by CNP+ and MBP+ immunoreactive cells; not shown), then used this for subsequent experiments.

Thus far we have tested 3 different concentrations of EVs on OPC differentiation outcomes: low ($4x10^7$ EV/mL), medium ($8x10^7$ EV/mL), and high ($16x10^7$ EV/mL). We evaluated OPC differentiation at 1, 3, and 5 days in differentiation-promoting medium as optimized above, using antibodies to detect NG2, CNP, and MBP oligodendrocyte lineage stage-specific proteins (see example images of immunocytochemistry in **Figure 3**).

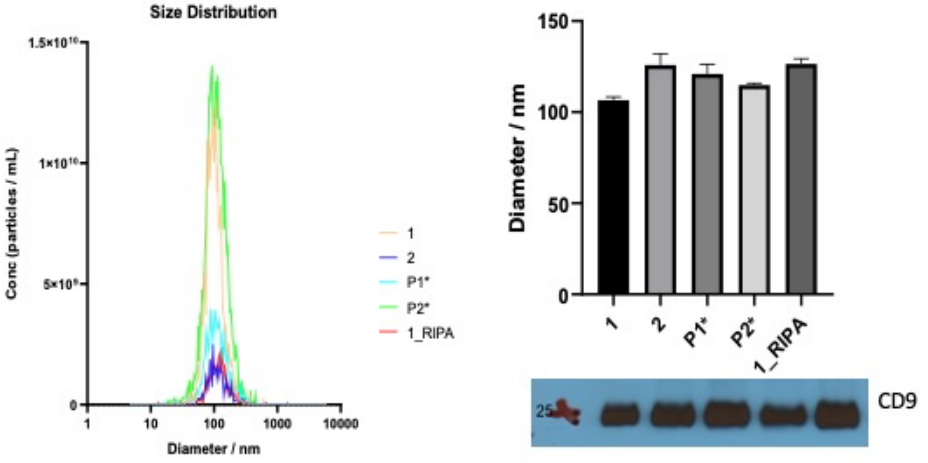

Figure 2. Purification and characterization of extracellular vesicles.

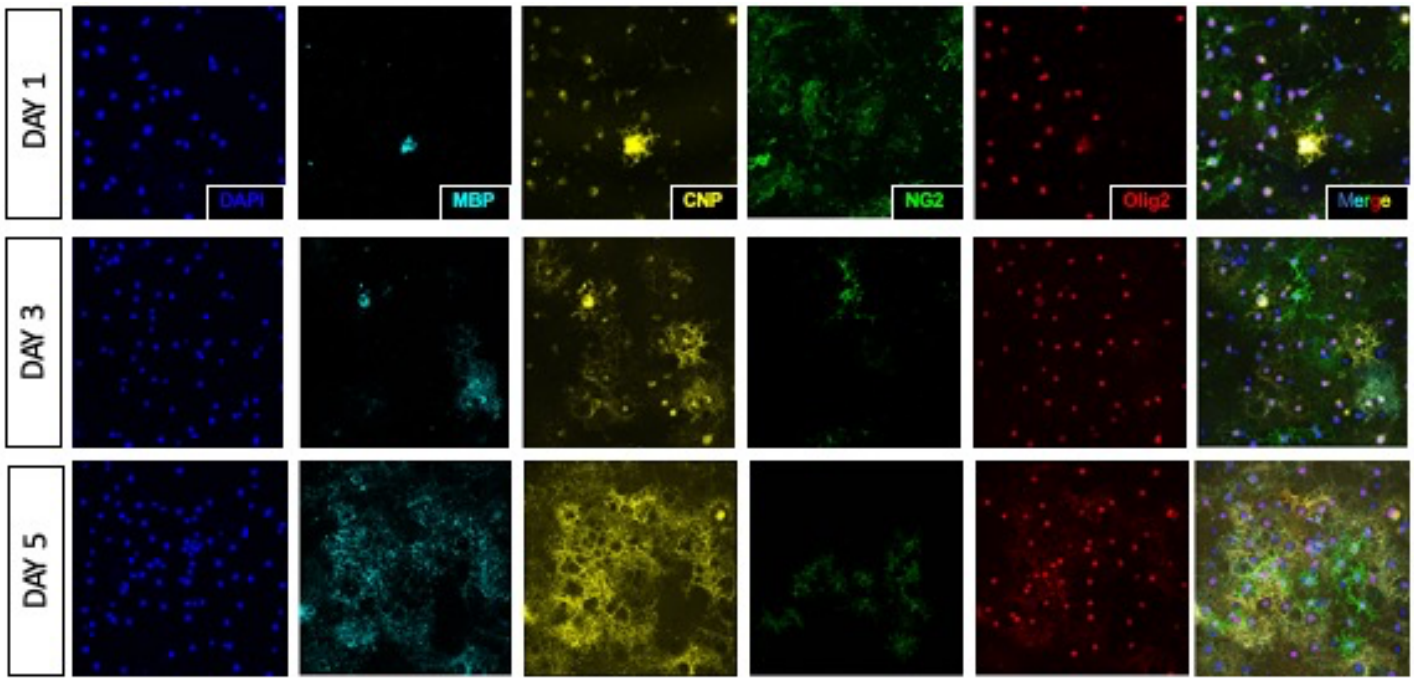

Figure 3. Oligodendrocyte-lineage stage specific proteins are used to identify OPCs (NG2+), newly-born oligodendrocytes (CNP+, MBP-), and mature oligodendrocytes (CNP+, MBP+).

Thus far, we find that both LP- and C-EVs induce some alterations in OPC-to-oligodendrocyte differentiation (**Figure 4)**. However we noticed a significant degree of variation between different EV preparations and are currently characterizing the different preps further to determine whether the EV preparations can be further stratified to better understand the potential for influence on oligodendrocyte lineage cells.

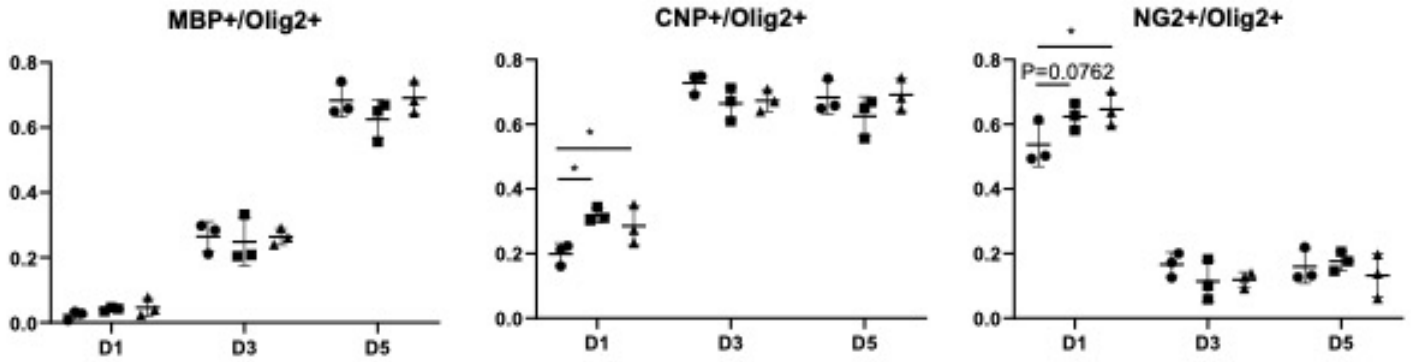

Figure 4. Quantification of oligodendrocyte phenotypes in response to 16x10^7 EV/mL over 5 days. *Circles*, saline treated cultures; *squares*, LP-EV treated cultures; *triangles*, C-EV treated cultures.

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 purifications and cell culture assay techniques, as well as in immunohistochemistry, confocal image acquisition, EV preparation and analysis. As a result this graduate student (Mr. Zijian Shao) has been making substantial contributions to Aim 2 of 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 Mr. Shao has presented his preliminary findings at the Molecular and Cellular Pharmacology Research Symposium in September.

3e. What do you plan to do during the next reporting period to accomplish the goals?
We hope to obtain approval for our revised ACURO application, after which we can begin nasal administration experiments with LP- and C-EVs. In addition, we plan to prepare new, larger batches of EVs in order to embark upon EV proteomic and miRNA analysis.

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 early stages of OPC differentiation into OLs suggest that LP-EVs may contain cargos that directly impact OPC differentiation capacity.

4b. What was the impact on other disciplines?
Given the potential effect of LP-EVs on oligodendrocyte 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 performed some pilot experiments using miRNA PCR panels and found that the amount of miRNA purified from a single mouse plasma preparation is insufficient. We have tried pooling material from 2 or 3 mice, but have not made a significant enough change in miRNA concentration to get reliable PCR data using this approach. In the meantime, we have sent some miRNA preps for RNAseq, in order to determine if we can get sufficient reads on a per mouse basis.

5b. Actual or anticipated problems or delays and actions or plans to resolve them.
At the start of the project, we still had serious delays in access to some campus facilities and support due to partial or complete shutdowns related to COVID. At this point in the project, however, these issues have largely been overcome. However some aspects of the project have been stymied by delays in obtaining animal regulatory approval on ACURO (we has a long delay on our first ACURO but after 3 months we had to reapply since we had our supporting IACUC put through its tri-annual review).

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 much of the first year.

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:	*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. In addition Mr. Shao has been working on optimizing methods to assess miRNA and other cargos from isolated EVs.*
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?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

SPECIAL REPORTING REQUIREMENTS

Not applicable.

APPENDICES

Not applicable.