Human Oligodendrocyte Spheroid Model of Hypoxic-Ischemic Encephalopathy

Background and Significance: White matter injury (WMI), the most prevalent form of brain injury in preterm children (born <37 post-conception weeks (PCW)), sensitizes the infant brain to other injuries and correlates with serious motor, cognitive, and learning disorders later in life. This prevalence is due to perinatal inflammatory and hypoxic-ischemic conditions, resulting from clinical challenges associated with preterm birth (including chorioamnionitis, neonatal sepsis, pulmonary immaturity, and perinatal hemodynamic instability) (1). The World Health Organization estimates that 15 million children (1 in every 10 births) are born prematurely each year worldwide (1,2). Children born 23-32 PCW are at particularly high risk of WMI. During this high-risk period, pre-myelinating oligodendrocytes (pre-OLs) are the predominant cell type in cerebral white matter (1,2,3). Thus, the high-risk period occurs before the onset of myelination (2). Hypoxia-ischemia (HI), often associated with preterm birth, induces oxidative stress (4). Pre-OLs are highly vulnerable to oxidative stress and are the primary target for cell death in white matter lesions (1,2). Perinatal asphyxia, a form of HI, is a lack of gas exchange or blood flow to or from the fetus immediately before, during, or shortly after birth (5). Preterm infants are at high risk of perinatal asphyxia due to lung immaturity, hypotension, and impaired cerebral flow regulation (6). Hypoxic-ischemic encephalopathy (HIE) refers to the neurological sequelae of perinatal asphyxia (5). HIE incidence in “developed” countries is 1.5 per 1000 live births and varies between 2.3-26.5 per 1000 live births in “developing countries”, although there is little information on magnitude, mortality rates, and disability outcomes in some “developing” settings (7). Globally, HIE fatality rates range between 10-60% of cases (7). As HIE can cause long-term neuronal damage and WMI, of surviving patients, 5-10% of infants display persistent motor deficits, and 20-50% display sensory or cognitive challenges that continue into adolescence (8). Furthermore, only 50-60% HIE patients were reported as following a “typical” developmental trajectory (8). Disorders included epilepsy, loss of hearing and vision, language disorders, microcephaly, and muscle spasticity (8). Thus, I propose the model illustrated in Fig 1. Historically, it has been near impossible to study HIE in humans, as it is difficult to directly investigate the human fetal brain, there is a not enough affected human neonatal tissue, and corticogenesis in animal models does not mirror the timing nor complexity of this process in the human fetus (1,6). Therefore, despite extensive research on WMI, the cellular substrates and molecular mechanisms underlying how HI disrupts myelination and the implications for corticogenesis in humans are not well understood (6). As a result, critical time periods for WMI therapeutics are poorly defined, and options for therapeutics are limited (1). Hypothermia is the only licensed treatment for HIE which, although effective in the short-run, is insufficient to prevent all brain injuries and neurological symptoms (9). Therefore, there is great need for additional HIE therapies (9). MicroRNAs (miRNAs), as important epigenetic regulators of brain development, have a critical role in OL maturation, the formation of myelin; and the pathophysiology of HIE (10,11). MiRNAs impacted by HI-induced brain injury are termed “hypoxamiRs”. How HI impacts hypoxamiR expression, contributing to important events in oligodendrogenesis and OL differentiation is unknown. Studying the role of miRNAs in OL development could be an important area of therapeutics targeted towards hypoxiamiRs (11). **MiRNAs:** miRNAs are small, non-coding RNAs, approximately 22 nucleotides long, that bind to target mRNA which causes alterations in gene expression (10,13). Mature miRNAs mediate post-transcriptional regulation of gene expression by degrading target the mRNA and/or suppressing translation (14). A singular miRNA can target multiple mRNAs simultaneously, and a singular mRNA can be regulated by different miRNAs (14). MiRNAs regulate cell fate specification and the differentiation of neurogenic niches, the intricate microenvironment that regulates neural progenitor cells (NPCs), including neural stem cells (NSCs)

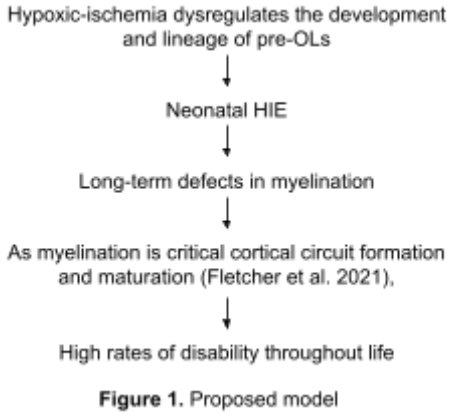

Figure 1. Proposed model

(11,15). **Organoid model:** Organoids are cells with stem cell potential that develop *in vitro* by adhesion, self-organization, and differentiation into cell masses that resemble the tissues of a particular organ (16,17). Cerebral organoids can be created using human pluripotent stem cell (hPSC) lines (16). Organoids are an ideal model for investigating interactions between cultured cells and studying the beginning properties of groups of cells, resembling an organ (18). Organoids can be develop *in vitro* for a long time (over several years) (18). Furthermore, human cerebral organoids share proteomic similarities with fetal brain tissue (19). Thus, it is possible to study HIE in the human cerebral organoid.
Model System: Marton et al (2019) studied human oligodendrogenesis and the interactions between OLs with neurons and astrocytes in human OL spheroids (hOLS) that resembled "typical" human development. They found that the OL-lineage cells followed the transitions through each developmental stage of primary human OLs (Fig 2). Furthermore, the morphology of the OL-lineage cells changes as they mature *in vitro* and begin to myelinate. Therefore, it's possible to create hOLS, and these hOLS, if derived from healthy cells, should imitate the transitions through each developmental stage as seen in primary human OLs. Paşca et al (2019) studied the impact of hypoxic conditions on corticogenesis in 3D brain region-specific organoids. They demonstrated that oxygen (O2) deprivation lead to defects in intermediate progenitor cells and verified their findings using slices of human cortex (6). They suggest future studies combine organoid models which have OLs, astrocytes, and neurons and with their proposed HI induction method to understand defects in myelination (6). Thus, it is possible to apply the methods used in Paşca et al (2019) to hOLS. Marton et al (2019) and Paşca et al (2019) combined offer an opportunity to research how HI impacts myelination. I propose to leverage the hOLS model, put forth by Marton et al (2019) combined with the model of hypoxic-ischemia proposed by Paşca et al. 2019 to investigate how HI impacts the functioning of miRNAs in oligodendrogenesis and OL differentiation, eventually leading to myelination defects and disruption to corticogenesis. I propose to investigate several of the most critical hypoxamiRs, focusing on miR-219, miR-338, and miR-138. I chose these hypoxamiRs based on the review article by Cho et al (2019) which discusses the roles of hypoxamiRs in brain development and perinatal brain injury as well as based on the empirical article by Letzen et al (2010) which profiles miRNA expression of OL differentiation using human embryonic stem cells. **Intellectual merit:** This study is important because it will inform our understanding of how WMI can impact myelination. Specifically, the study illuminates how O2 deprivation impacts myelination in neonates and the implications for corticogenesis. The model I propose is innovative because it combines the human cerebral organoid model, traditionally used to study neuron-neuron interactions, with an organoid model that contains OLs and astrocytes, in addition to neurons. My investigation of the role of miRNAs in OL differentiation and myelination is an important contribution to the field, as studying miRNAs can offer critical insights into hypoxamiR-targeted therapeutics for WMI.

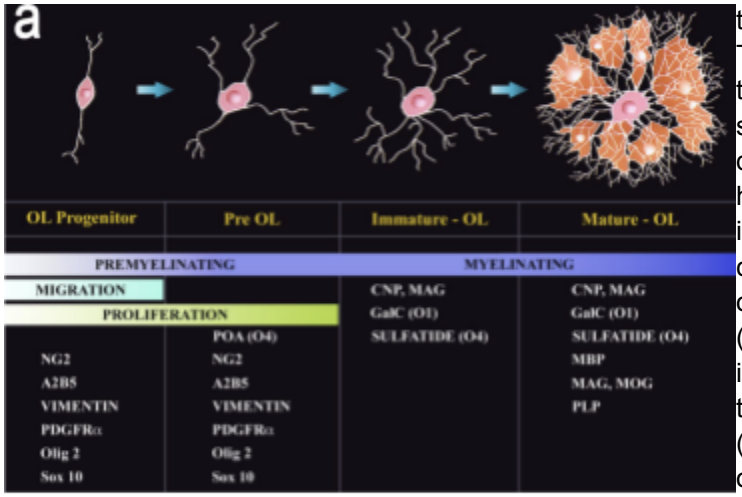

Figure 2. Typical OL maturation and associated genes (2)

Aims overview: I propose to expose hOLS to HI to investigate how O2 deprivation impacts 1) pre-OLs, 2) OL differentiation, 3) number of mature OLs, and 4) myelination defects (Aim 1). For Aim 2, I propose to track and analyze how O2 deprivation changes the behavior of miRNAs critical to the four goals of Aim 1. For Aim 3, which is geared towards hypoxamiR-therapeutics, I propose a loss-of-function experiment on miR-219 to understand its role in OL maturation and myelin production under hypoxic-ischemic

conditions. Although these three aims build upon one another, it is possible to carry out each one independent of the other(s), should one of the aims produce unexpected results. **Aim 1: a) create hOLS** based on the model proposed by Marton et al (2019). Obtain human-induced pluripotent stem cell (hiPSC) lines from healthy subjects. Culture hiPSC. Generate hOLS from hiPSC. Perform cryopreservation. Reference the results from Marton et al (2019) as information on how hOLS would typically behave without the inducement of HI. **b) expose hOLS to HI** using the model proposed by Paşca et al (2019). **Hypothesis 1:** Exposing hOLS to HI will 1) influence the interactions between OLs with astrocytes and neurons to deviate from the transitions through developmental stages seen in primary human OLs, 2) result in a smaller count of mature OLs than reported in Marton et al (2019), and 3) lead to more myelination defects than reported in Marton et al (2019). **Rationale 1:** Marton et al (2019) demonstrated that OL-lineage cells in hOLS transition through similar developmental phases as seen in primary human OLs. However, oxidative stress was found to be associated with changes in both the expression of OL differentiation-regulating genes and in global histone acetylation patterns (3). Thus, the authors conclude that oxidative stress may directly shift the program of OL differentiation and eventually lead to demyelination. Thus, oxidative stress may impact the phase of differentiation (pre-OLs), thus impacting the following stage of myelination. **Approach 1:** *a) HI induction*. Allow hOLS to develop until 10 weeks *in vitro*, as at this time, human cortical spheroids (hCS) transcriptionally resemble the human cerebral cortex at 19-24 PCW, corresponding with extreme prematurity (6). At day 75 of differentiation, expose hOLS to low O2 tension (<1%) for 48 hours using a gas control chamber (6). Follow with re-introduction to 21% O2 (6). Monitor O2 partial pressure changes using a needle-type fiber optic O2 microsensor (6). Perform RNA sequencing at 24 and 48 hours (middle and end of <1% O2 exposure) and after 72 hours of the re-oxygenation period to investigate transcriptional changes (6). Identify differentially expressed genes between the <1% O2-exposed hOLS and 21% O2-exposed hOLS (6). Validate critical genes using qPCR. *b) Tracking OL maturation*. Determine if OL-lineage cells are enriched or not by tracking the gene expression of *OLIG2*, *NKX2–2*, and *MBP* in hOLS using RT-qPCR (20). Determine whether OL progenitor cells (OPCs) are maturing into OLs by immunostaining cryosections obtained from hOLS at 100-160 days of *in vitro* differentiation (20). Determine whether the number of mature OLs are increasing in hOLS over time by quantifying the density of MBP+ cells in whole cryosections between days 50 and 160 of differentiation (20). Fully characterize the OL-lineage cells using single-cell RNA sequencing (20). Further understand the developmental progression of OL-lineage cells using Monocle analysis (20). Determine whether OPCs derived from hOLS migrate in hOLS as they do *in vivo* using temperature-controlled live imaging with *Sox10-MCS5::eGFP* reporter at *in vitro* differentiation stages from day 65 up to day 275 in culture (20). Determine whether OLs are interacting with and myelinating neuronal axons by immunostaining hOLS cryosections at day 115 for MBP and NF-H to observe the interaction of OL processes and nearby axons (20). **Predicted results 1:** Pre-OLs will be vulnerable to oxidative stress and selectively degenerate (2). After the hOLS equivalent of 32 PCW in human tissue, there should be a decline in risk for WMI. This will be indicated by the onset of preOL differentiation leading to immature OLs and eventual myelination (2). Given the HI injury, this process of regeneration will occur but less robustly than in Marton et al (2019). Furthermore, in this process, OPCs will be less susceptible to HI than pre-OLs which will be indicated by an increase in OPCs (21). The increase will indicate that new pre-OLs have been created, as preOLs can be detected in the human cortex by 17-27 PCW and newly produced into adulthood (21,22). However, the new pre-OLs will not further develop, leading to myelin loss (21). The temporal and spatial patterning of pre-OLs will correlate with the magnitude and location of WMI in humans and other experimental studies (1). **Potential limitations 1:** Scientists have acknowledged the limitations of brain organoids which, importantly, includes the fact that the *in-vitro* organoid develops in culture with a lack of input from other brain regions. Related to this study, OLs myelinate axons throughout the developing human brain in both “typically”-developing and HIE populations. This model cannot exactly reflect the processes that occur in the human brain. **Aim 2: track hypoxamiR activity throughout OL maturation**. **Hypothesis 2:** *a)* HI will induce dysregulation of

miRNA biogenesis (11). *b)* Furthermore, hypoxamiRs will be differentially expressed following injury (11). Specifically, mature hypoxamiRs will be upregulated in response to HI which may increase the proliferation rate of OPCs and decrease differentiation (11,23). *c)* Three miRNAs will be impacted by HI. MiR-338 will control oligodendrogenesis, miR-138 will increase the time OLs can be maintained during the beginning of differentiation, and miR-219 will regulate differentiation (11). **Rationale 2:** *a)* Chen et al (2020) discuss recent literature that HI regulates miRNA expression at different stages throughout miRNA biogenesis. Epigenetic modifications involved in responding to HI, such as DNA methylation, histone modification, and/or transcription factor binding (HIF, nuclear factor (NF-κB), and p53), can impact transcriptional activities of miRNA genes (24). Furthermore, HI affects expression of specific enzymes, such as Drosha, Dicer, and AGO2, which help to regulate pri-miRNA processing and miRNA maturation (24). In addition, HI modulates configurations of the miRNA-RISC complex (24). Although this experiment will not be investigating all of the mechanisms by which HI can impact the miRNA biogenesis pathway, it is expected that this pathway will be impacted by HI in the hOLS model. *b)* Birch et al (2014) demonstrated that mature miRNAs are involved and required in the white matter loss induced by HI. Specifically, miRNAs which regulate OPC differentiation were significantly upregulated immediately following HI, and this upregulation slowed over time (23). *c)* Cho et al (2019) reviews the role and type of miRNAs implicated in HI and in neuroinflammation. Based on their meta-analysis, Cho et al (2019) highlight miR-338, miR-138, and miR-219 as having critical roles in either oligodendrogenesis or OL differentiation in the context of HI. Furthermore, Birch et al (2014), who conducted an experiment on whether perinatal HI would increase upregulation of miRNAs that regulate OL differentiation in mice, focused on miR-338, miR-138, and miR-219. **Approach 2:** Use the miRNA expression profiling technique proposed by Letzen et al (2010). For mature miRNAs, combine the Letzen et al (2010) technique with the method proposed by Birch et al (2014). Detect miRNAs over eight stages of OL differentiation: hiPSCs, neural embryoid body (EB) cells, NPCs, glial-restricted precursor (GP) cells, oligodendrocyte precursor (OP) cells (OP1, OP2, and OP3), and mature (myelinating) OLs (20). Use a heat map to detect miRNAs which display the highest variance (top 30th percentile) throughout differentiation (20). Validate microarray data for key miRNAs by performing qRT-PCR for samples from the eight stages (20). Perform correlation analysis to compare the miRNA expression profiles using the closest and furthest time points of differentiation (20). Conduct principle component analysis (PCA) to recognize variation between groups (20). For mature OLs, make sure to isolate total RNA, produced cDNA, and amplify miR-338, miR-138, and miR-219 (23). **Predicted results 2:** Using PCA, four primary clusters should be produced: early NPCs, early glial progenitor cells (GP and OL1), developing OL progenitors (OP2, and OP3), and mature OLs (OL) (20). MiR-338 should regulate oligodendrogenesis (11). MiR-338 will be upregulated in mature OLs, and its overexpression should increase OL differentiation (11). MiR-138 expression should be increased in OL precursors (11). MiR-138 will increase the time period OLs are maintained in the beginning stages of OL differentiation which will allow for differentiating OLs to myelinate (11). MiR-219 will be highly expressed in white matter, and this expression should be present in mature OLs (11). MiR-219 should be revealed as an important regulator of OL differentiation (11). Specifically, miR-219 will help to coordinate the transition of OPCs into mature OLs, aiding in myelin formation (11). **Potential limitations 2:** In preterm children, OLs are sensitive to ex utero conditions and can be further damaged by early delivery and exposure to the ex utero environment (21). However, for this model, the external conditions are highly controlled, and so OLs cannot reflect the chemical and mechanical damage which may be incurred in the brain of an infant (21). As miRNAs and OLs are incredibly linked, both in “typical” development and in the context of HI, miRNAs are likely susceptible to changes in the infant’s environment that are not reflected in this model. **Aim 3: miR-219 knockdown in order to study remyelination after HI-induced injury**. **Hypothesis 3:** miR-219 is essential to OL differentiation and, therefore, mature (myelinating) OLs (25). This system is working via a feedback regulatory loop between transcription factors, Sox6 and Hes5, which regulates miR-219 expression (25). Knockdown of miR-219 in hOLS will inhibit OL maturation, and it’s possible that inhibition of miR-219 may block OL differentiation

and, thus, maturation (25). **Rationale 3:** Cortical organoids have been established as valuable for functional gain- and loss-of-function experiments in order to understand miRNAs in human processes (14). Therefore, hOLS presents an excellent model to perform a knockdown experiment of miR-219 to understand how its functioning in relation to Sox6 and Hes5 and inform miRNA-targeted therapeutics. Cho et al (2019) suggest that, as miR-219 is critical for the transition of OPCs to mature OLs, it may have therapeutic potential for promoting myelination following HI-induced brain injury. miR-219 positively regulates OL differentiation in both gain- and loss-of-function experiments *in vivo* and *in vitro* (25). Overexpression of miR-219 promoted OL differentiation in the spinal cord (25). Knockdown of miR-219 inhibited OL maturation in zebrafish and in the spinal cord in mice (25). MiR-219 directly represses negative regulators of OL differentiation (including the transcription factors, Sox6 and Hes5) (25). In order to knockdown miR-219, CRISPR/cas9 will be used, as it can significantly downregulate selected miRNA expression (26). **Approach 3:** Use CRISPR/cas9 to knockdown miR-219 *in vitro*. Similar methods for this experiment used in mice will also be used in hOLS, as animal model methods can often be translated to cell culture methods. Design protospacer sequences of CRISPR/cas9 against miR-219 (26). Amplify and clone target sequence into lenti-CRISPR vectors and verified by DNA sequencing (26). Perform transient transfection of CRISPR/cas9 into cells (26). Isolate RNA (26). Then, the following steps will be performed: SYBR-based qRT-PCR analysis, western blotting, DNA sequencing, and plasmid construction and transfection. (26). **Predicted results 3:** Typically, miR-219 promotes the formation of PDGFRα+ OPC from NPCs (25). Therefore, knockdown of miR-219 should result in a reduction in PDGFRα+ OPC formation (25). MiR-219 loss-of-function should result in an impairment of Olig2+ OPC migration and differentiation, as seen in zebrafish (25). Sox6 will be primarily present in PDGFRα+ OL precursors but not as present in MBP+ mature OLs. This will indicate that Sox6 has a negative regulatory role in OL maturation (25). Hes5 will repress Sox10 and Mash1, two activators of OL differentiation, in order to negatively regulate OL differentiation and myelin gene expression (25). **Potential limitations 3:** Although miR-219 has been found to promote OL differentiation, it is likely that other mechanisms and miRs support miR-219 and this overall process of differentiation. Therefore, although miR-219 is critical to OL differentiation, this model does not completely isolate the function and effects of miR-219 which could impact the efficacy of an miR-219-targeted therapeutic. However, as the goal of Aim 3 is to elucidate how miR-219 is contributing to OL differentiation in hOLS and the relationship between Sox6/Hes5 and miR-219 in this model, the goal is not to design a therapeutic model but rather provide the necessary scientific background in order to do so.

Broader Impacts: There is an uneven distribution of resources in Boston Public Schools (BPS). According to a survey, “More than 40% of students in Boston’s public schools don’t have a library in their building” (27). Furthermore, “Half of all students in K-8 schools and middle schools don’t have a science lab” (27). I propose to work with the Equity and Inclusion Cabinet and the Education Cabinet of the City of Boston to spearhead a four-stage plan. First, we will ensure that all schools in Boston have a dedicated science lab. Second, we will ensure that all students in K-8 schools and middle schools have science as a part of their curriculum throughout each academic year. Third, we will hire science teachers who will develop detailed lesson plans for elementary and middle school students. The content of each year should build on the last. Fourth, scientists from our Department will partner with the under-resourced K-8 schools in Boston in order to teach a weekly lesson on neuroscience. For the younger students, the neuroscience curriculum could include creative art making in which groups of students represent different regions of the brain and its functions and then perform these functions as skits for their class. For the older students, the neuroscience curriculum could be targeted to issues facing middle school students in Boston (e.g., sleep, exercise, and wellbeing. The goal of this outreach program is to encourage students typically underrepresented in STEM to believe in their ability to understand STEM concepts and do work in the fields, if they choose. STEM, and science in particular, is strongest when there are a multitude of perspectives included.

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