The regulation of Oligodendrocyte Precursor-mediated repair in Multiple Sclerosis

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Abstract

English Abstract:

Oligodendrocytes (OL) and their myelin-forming processes are targeted and lost during the disease course of Multiple Sclerosis (MS). With considerable blood-brain barrier (BBB) breakdown, blood resident immune cells and toxins now have access to the central nervous system (CNS). Myelin repair is considered to be dependent on recruitment and differentiation of oligodendrocyte precursor cells (OPCs). OPCs are identified to be present in the adult human brain, where there are pro-inflammatory molecules within the microenvironment. The basis of failure of re-myelination during the disease course of MS remains to be defined. The aim of this study is to determine the impact of inflammatory mediators and blood-derived toxins on the differentiation of OPCs. Selected for this study were interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) and fibrinogen. Each of these molecules have been shown in various models to have a direct effect on OL-lineage cells, although fibrinogen has yet to be studied in the context of human OL-lineage cells. To determine the relevance of fibrinogen to the MS disease state, we report the co-localization of fibrinogen on OL-lineage cells (SOX10+) within various pathological regions. However, fibrinogen did not elicit a specific effect on human adult OLs *in vitro.* As primary human progenitors are difficult to access, we are using human induced pluripotent stem cell (iPSC)-derived as a source to derive OPCs. To generate these cells, we have improved the growth-factor based method previously described by Douvaras et al. 2015. To test the effect of the proinflammatory and blood-derived molecules on OPC differentiation, we treated the cells *in vitro,* following their switch from proliferation to differentiation medium. The effects of the cytokines and fibrinogen were evaluated up to a 6-day time course, in terms of cell viability (propidium iodide), expression of OL-lineage markers (PDGFRα+ and O4+) and diversion into astrocyte

lineage (GFAP+, AQP4+ and CD49f+). Following treatment of human progenitor cultures, there was no significant proportion of dead (PI+) OPCs (O4+). Our analysis indicated that IFNγ, TNFα and fibrinogen decreased the proportion of cells differentiating into the OL-lineage as measured by proportion of O4+ cells; and such findings are consistent with previous reports. Furthermore, with TNFα, we observed a 2-fold increase of AQP4+ cells (a marker of the astrocyte lineage) following treatment for four days. Additionally, in line with previous reports, fibrinogen also increased the proportions of CD49f+ cells in our cultures. A subset of O4+ expressing cells were also found to co-express astrocyte marker CD49f indicating an intermediate phenotype. We document activation of the BMP4 pathway following fibrinogen treatment in the O4+ population which is shown to drive precursor cells toward the astrocyte lineage, which was rescued following addition of the BMP pathway inhibitor DMH1. This signalling effect was recapitulated in the mature human OL, *in vitro*. However, TNFα is not acting via this pathway as addition of the inhibitor (DMH1) does not rescue this effect. In the context of MS, these results imply that OPCs are present but inhibited from differentiating along the OL-lineage, with a subset being diverted towards the astrocyte lineage and therefore, reducing their capacity to contribute to repair. Furthermore, responses to certain pathological insults may vary based on maturation stage along the OL-lineage. These results help define a potential basis for the impaired myelin repair in MS and provide a potential route for regenerative MS treatment. (3657 Characters)

French Abstract:

Les oligodendrocytes (OL) et leurs processus de formation de la myéline sont ciblés et perdus au cours de la sclérose en plaques (SEP). Quand la barrière hémato-encéphalique (BHE) est c dégradée, les cellules immunitaires et les toxines présentes dans le sang ont accès au système nerveux central (SNC). La réparation de la myéline dépend du recrutement et de la différenciation des cellules précurseurs d'oligodendrocytes (OPC), présentes dans le cerveau humain adulte, où le microenvironnement contient des molécules pro-inflammatoires. La base de l'échec de la remyélinisation dans la SEP reste à définir. L'objectif de cette étude est de déterminer l'impact des médiateurs inflammatoires et des toxines dérivées du sang sur la différenciation des OPC. L'interféron gamma (IFNγ), le facteur de nécrose tumorale alpha (TNFα) et le fibrinogène ont été sélectionné. Il a été démontré dans divers modèles que chacune de ces molécules a un effet direct sur les cellules de la lignée OL, bien que le fibrinogène n'ait pas encore été étudié dans le contexte des cellules humaines de la lignée OL. Afin de déterminer l'importance du fibrinogène dans l'état pathologique de la SEP, nous rapportons la colocalisation du fibrinogène sur les cellules de la lignée OL (SOX10+) dans diverses régions pathologiques. Cependant, le fibrinogène n'a pas eu d'effet spécifique sur les OL humains adultes in vitro. Les progéniteurs humains primaires étant difficiles d'accès, nous utilisons des cellules souches pluripotentes induites (iPSC) humaines comme source pour dériver les OPC. Pour générer ces cellules, nous avons amélioré la méthode de Douvaras et al. 2015. Pour tester l'effet des molécules pro-inflammatoires et dérivées du sang sur la différenciation des OPC, nous avons traité les cellules in vitro, après leur passage d'un milieu de prolifération à un milieu de différenciation. Les effets des cytokines et du fibrinogène ont été évalués jusqu'à 6 jours, en termes de viabilité cellulaire (iodure de propidium), d'expression des marqueurs du lignage OL (PDGFRα+ et O4+) et de détournement vers le lignage astrocytaire

(GFAP+, AQP4+ et CD49f+). Après traitement des cultures de progéniteurs humains, il n'y avait pas de proportion significative d'OPC mortes (PI+) (O4+). Notre analyse a indiqué que l'IFNγ, le TNFα et le fibrinogène ont diminué la proportion de cellules se différenciant dans la lignée OL, mesurée par la proportion de cellules O4+ ; et ces résultats sont cohérents avec les rapports précédents. En outre, avec le TNFα, nous avons observé une multiplication par 2 des cellules AQP4+ (un marqueur de la lignée astrocytaire) après un traitement de quatre jours. De plus, conformément aux rapports précédents, le fibrinogène a également augmenté les proportions de cellules CD49f+ dans nos cultures. Un sous-ensemble de cellules exprimant O4+ a également coexprimé le marqueur astrocytaire CD49f, indiquant un phénotype intermédiaire. Nous documentons l'activation de la voie BMP4 à la suite d'un traitement au fibrinogène dans la population O4+, qui entraîne les cellules précurseurs vers la lignée astrocytaire, ce qui a été compensé par l'ajout de l'inhibiteur de la voie BMP, le DMH1. Cet effet de signalisation a été récapitulé dans l'OL humain mature, in vitro. Cependant, le TNFα n'agit pas via cette voie puisque l'ajout de l'inhibiteur (DMH1) ne permet pas de récupérer cet effet. Dans le contexte de la SEP, ces résultats impliquent que les OPC sont présentes mais empêchées de se différencier le long de la lignée des OL, un sous-ensemble étant détourné vers la lignée des astrocytes, ce qui réduit leur capacité à contribuer à la réparation. En outre, les réponses à certaines agressions pathologiques peuvent varier en fonction du stade de maturation le long de la lignée OL. Ces résultats aident à définir une base potentielle pour l'altération de la réparation de la myéline dans la SEP et fournissent une voie potentielle pour le traitement régénératif de la SEP. (3994 Characters)

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Contribution of Authors
Gabriela Blaszczyk – experimental design and execution, generation and maintenance of iPSC derived OL-lineage cells, data analysis and interpretation, composition of thesis
Jack Antel - advice on experiment/project planning, editing of thesis
Valerio Piscopo – training in tissue culture techniques and OL generation, improvement of the OL-generation protocol, assistance with characterization of the SOX10-mOrange reporter cell line, advice on experiment/project planning
Thomas Durcan – allowance for use of facilities (iPSC lines, generation/editing of the SOX10-mOrange reporter iPSC line, tissue culture, reagents, Attune flow cytometer, imageXpress high content screener, single-cell sequencing library preparation)
Taylor Goldsmith – writing sequencing methods, contribution to Parse library preparation, initial genome alignment and normalization of sequencing results
Alexandra Chapleau – general advice, generation of OL-lineage cells from 81280 and AIW iPSC lines
Julien Sirois – Flow cytometry panel design, live FACS on mOrange reporter line, training on Attune flow cytometry acquisition and sample staining
Vanessa Omana - training on Attune flow cytometry acquisition and sample staining, assistance in acquisition of some experiments on the Attune flow cytometer
Qiao-Ling Cui – isolation and maintenance of human primary OL-lineage cells, execution of experiments on human primary OLs, assistance in experimental design
Chao Weng – Assistance in imaging of pSMAD-staining of iPSC-OL cultures, assistance in quantification of nuclear fluorescence intensity (primary and iPSC cultures)
Chloe Plouffe – assistance in quantification of nuclear fluorescence intensity (primary and iPSC cultures), maintenance of iPSC-derived OL-lineage cultures
Wayne Moore – assistance in experimental design of Fibrinogen treatments, classification of multiple sclerosis lesions in tissue sections, conduction of experimental design of IHC staining
Arianna Giurleo – acquisition of images and quantification of SOX10/Fibrinogen staining on human MS tissue sections. Wrote methods sections for objective 3 “human multiple sclerosis brain tissue” and “immunohistochemistry”.

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List of Abbreviations

100 U; 100 international units
A2B5; neuron cell surface antigen
ALS; amyotrophic lateral sclerosis
AQP4; aquaporin-4
BBB; Blood Brain Barrier
BMP4; bone morphogenetic protein-4
BNDF; brain-derived neurotrophic factor
cAMP; cyclic-adenosine monophosphate
CD133; prominin-1
CD49f; integrin, alpha 6 subunit
CHUM; Centre Hospitalier de l'Université de Montréal
CNP; 2',3'-Cyclic nucleotide 3'-phosphodiesterase
CNS; Central Nervous System
CSF; cerebrospinal fluid
CSPG; chondroitin sulphate proteoglycan
D-PBS; Dulbecco's phosphate buffered saline
DMEM-F12; Dulbecco's modified eagle medium nutrient mixture F-12
DMH1; dorsomorphin homolog 1
DMT; disease modifying therapy
EDDU; early drug discovery unit
FACS; fluorescence activated cell sorting
FBS; fetal bovine serum
FMO; fluorescence minus one
GALC; galactosylceramidase
GFAP; glial fibrillary acidic protein
GPR17; uracil nucleotide/cysteinyl leukotriene receptor
HGF; hepatocyte growth factor
IFNγ; interferon gamma
IGF-1; insulin-like growth factor 1

iPSC; induced pluripotent stem cell
JAK/STAT; janus kinase/ signal transducers and activators of transcription
MBP; myelin basic protein
MKI67; antigen Kiel 67
MRI; magnetic resonance imaging
MS; Multiple Sclerosis
NAWM; normal appearing white matter
NG2; neuron-glial antigen 2, chondroitin sulphate proteoglycan 4 (CSPG4)
NKX; NK-homeobox
NPC; neural precursor cell
NT3; neurotrophin-3
O4; sulfated galactocerebroside O4
OL; Oligodendrocyte
OLIG1/2; oligodendrocyte transcription factor 1/2
OPCs; Oligodendrocyte precursor cells
p-SMAD 1/5/9; Phosphorylated-Smad1 (Ser463/465)/ Smad5 (Ser463/465)/ Smad9 (Ser465/467)
PAX6; Paired box 6
PBST; PBS + Tween20
PDGF; platelet-derived growth factor
PDGFRα; platelet derived growth factor receptor, alpha subunit
PFA; paraformaldehyde
PI; propidium iodide
PLP1; proteolipid protein 1
PMS; progressive multiple sclerosis
qPCR; quantitative polymerase chain reaction
RNAseq; RNA sequencing
RRMS; relapse-remitting multiple sclerosis
SB431542; 4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl)-1H-imidazol-2-yl]benzamide
scRNAseq; single-cell RNA sequencing
SHH; sonic hedgehog
SOX10; SRY-box transcription factor 10
STAT3; signal transducer and activator of transcription 3
T3; triiodothyronine
TNFα; tumour necrosis factor alpha

1. Introduction

Multiple Sclerosis (MS) is an autoimmune disease of the CNS characterized clinically by an initial relapse-remitting stages that can evolve over years into a progressive disease course [1]. Initial lesion formation is attributed to targeting and injury of oligodendrocytes (OL) and their myelin membranes by infiltrating immune cells and their effectors [2], such as tumour necrosis factor-alpha (TNFα) and interferon gamma (IFNγ). Progressive disease is linked to ongoing injury and failure of repair mechanisms attributed to decreased of recruitment and differentiation of OL progenitor cells shown to be present in the CNS parenchyma [3]. Also implicated in the injury process are blood-derived factors that may access the CNS due to blood brain barrier breakdown in the continuing injury process and infiltration from the CSF [4], such as fibrinogen. During the MS lesion evolution, there is activation of glial cells, microglia, and astrocytes, that can also be sources of inflammatory molecules and contribute to formation of a metabolically stressed microenvironment [5]. The abundance and availability of disease modifying therapies (DMTs) have increased over the past several decades, where nowadays individuals living with MS have an increased life expectancy. However, there are still no regenerative or curative treatments available for these individuals living with MS. The exact mechanism of regenerative impairment remains to be elucidated. Even with the availability of DMTs, constant medications and doctor's visits still provide a burden for the patient, their family, and the healthcare system. Our ongoing studies have demonstrated the relative susceptibility of mature human OLs to the inflammatory and metabolic insults encountered during the MS process. The focus of this thesis is on the response of OPCs to such insults and how this may contribute to failure of tissue repair during the course of MS.

Hypothesis

Previous *in situ* studies [3, 6] suggest the susceptibility to injury of human OPCs within the MS brain. Increased proportions of progenitors in "normal appearing white matter" (NAWM) were observed in relation to mature OLs and OPC numbers in non-MS controls [6]. However, within an MS lesion, the proportion of OPCs decreased and remyelination fails. Furthermore, specific effects of MS-related molecules have not been studied in a human model of early OL-lineage commitment and differentiation in direct comparison to human primary OLs *in vitro*. *We hypothesize that pro-inflammatory molecules from the MS lesion microenvironment are inhibitive to OPC differentiation, and therefore OPC mediated repair*.

We plan to address the research question with the following aims:

1. Generation of human OPCs from induced pluripotent stem cells
2. Evaluate the effect of pro-inflammatory molecules IFNγ and TNFα on human OPCs
3. Evaluate the effect of fibrinogen on human OPCs

2. Literature Review

2.1 Oligodendrocyte Precursor Cells

During normal developmental processes, OLs are derived from their precursor cells (OPCs). There are multiple sources of OPCs during development, including three waves of differentiation in the spinal cord and sequential waves in the forebrain [7-9]. These waves of differentiation allow for rapid population of the developing CNS, although not all OPCs will survive until adulthood of the organism [10]. From a proliferative early OPC, expressing markers such as PDGFRα, A2B5 and NG2 [11], cells mature and exit the cell cycle. Further along their lineage, each discrete stage can be identified with a combination of markers. Late OPCs and pre-

oligodendrocytes can be identified by the marker O4 [11, 12], and myelinating oligodendrocytes identified by MBP, GALC and PLP1 [11]. Throughout the lineage, the transcription factor SOX10 plays a key role in propelling the OPCs toward maturation and myelination [13]. Proliferative PDGFRα+ OPCs remain resident in the CNS once the brain completes development [10, 12, 14], and act as a reservoir to replace lost OLs in case of injury [14]. However, in the context of MS, individuals with chronic or progressive forms of this disease have this repair mechanism appear to be ineffective. Block of OPC differentiation ability has been observed in situ [3, 15], however the exact cause remains unknown.

2.2 Signalling required in OPC fate specification

It is widely accepted that OPC differentiation during development occurs in three waves; each having different cues, characteristics, and roles they play in the developing CNS [7]. The most studied wave of differentiation occurs in the spinal cord, where differentiation of OPCs is dependent on the patterning factor sonic hedgehog (SHH) [16]. The next wave of OPCs has been shown to be dependent on the presence of fibroblast growth factor (FGF) [17]. Finally, the last wave of OPCs to differentiate in the spinal cord will occur postnatally, and these will remain throughout the life of the organism [10]. Studies have shown that early fetal forebrain OPCs arise from the sub-ventricular zone, which in adulthood remains as a stem-cell niche [18]. The cells are shown to arise and migrate during the late first trimester of human gestation, and only reach terminal differentiation during mid-gestation [18].

During development, additional signalling cues are important for the specification of OPCs. Extracellular signalling molecules which have been implicated in OPC proliferation and recruitment during CNS development include BNDF[19], PDGF[20] and IGF-1[21]. While there are also signalling molecules inhibitory to OPC proliferation and differentiation such as CSPGs

and BMPs [22]. A unique balance between inhibition and promotion is required for optimal population of OPCs within the CNS.

In addition to the extracellular signalling cues, there are intracellular signalling pathways that lead to the control of oligodendrocyte development. Newly generated OPCs will express NK2 Homeobox family of transcription factors (NKX6.1, 6.2 and 2.2) as well as oligodendrocyte transcription factor 1 and 2 (OLIG1 and OLIG2), however the exact roles of these transcription factors will differ depending on the developmental origin of the cells (CNS location as well as developmental wave) [23-25]. These transcription factors in turn, will activate the essential transcription factor for OL differentiation, SRY-Box 10 (SOX10) [13], which will be expressed throughout the OL-lineage. SOX10 and OLIG2 transcription factors participate in a positive feedback loop, where SOX10 acts downstream of OLIG2 and modulates its expression in a dose-dependent manner [26]. SOX10 plays an essential role in the maturation of OPCs to myelinating OLs [13, 26], as it controls the transcription of essential components of the myelin sheath [27].

2.3 OPC multilineage potential

Following the discovery of the OPC, it was noted that certain *in vitro* conditions allow these cells to generate astrocytes (GFAP+) [28] and sometimes neurons [29]. Studies in perinatal rodent cultures performed by Kondo and Raff [29] introduced the notion of fetal bovine serum (FBS) as well as certain bone morphogenetic proteins (BMPs) could induce this developmental switch of OPCs. OPCs isolated from the adult rodent brain were also found to have the capacity of generating astrocytes, albeit at a slower rate [30]. A study in a human model presented fetal late OL-lineage cells expressing GFAP, although this was noted to be a possible result of low turnover of GFAP [31]. It has also been commented on that there is a large overlap of phenotypes between astrocytes and OLs/OPCs [32]. However, detailed analysis of the developing fetal CNS state that

although a combination of astroglial markers are best to identify these cells, the cell surface marker AQP4 is the most reliable in distinguishing cells destined for the astroglial lineage [33].

With the onset of fate-mapping techniques in mouse models, the plasticity of OPCs was further demonstrated although with some conflicting evidence. Zhu and colleagues [34] showed the generation of astrocytes by cells expressing a reporter under the OPC-specific transcription factor OLIG2. Another study by Suzuki and colleagues [35] showed a similar effect with a reporter of SOX10 expression. Conversely, several articles state that under normal adult conditions, OPCs do not retain the capacity to generate astrocytes but rather are confined to replace myelinating OLs. For example, using a PDGFRα reporter line [36, 37], an NG2 reporter line [38], as well as an OLIG2 reporter line [39]. Most interesting and relevant to this thesis, is the idea that OPCs may retain the capacity to generate astrocytes in adulthood as a response to pathological insults. As mentioned previously, certain *in vitro* conditions were found to induce the developmental trajectory of OPCs towards an astrocytic fate [29]. Early studies by Dyer and colleagues [40, 41], introduced the notion of increased presence of "mixed phenotype glia" in a mouse model of phenylketonuria (PKU) and shiverer mice as a mechanism for decreased myelination. It was noted that MBP+ cells co-expressing GFAP failed to produce myelin sheaths, and these cells were found in the PKU mouse in almost double the proportion of a normal mouse brain [41]. Several subsequent studies of spinal cord injury [42], brain ischemia [43], and glioma [44] have shown this is possible in other disease models as well.

2.4 MS Microenvironment

There are two classifications of MS, relapse remitting (RRMS) and progressive (PMS) [1]. RRMS is classified by periodic inflammation and lesion formation, while PMS has a consistent and gradual disease course [1]. Before clinical symptoms manifest, the blood-brain barrier is

disrupted which leads to infiltration of blood derived molecules and inflammatory cells [4]. It has been shown that a majority of MS lesions are located around disrupted blood vessels, hinting at the impact of blood derived cells and their products on MS lesion formation [45, 46]. This series of steps contribute to the unique MS microenvironment, which has the tendency to become progressive and potentially inhibitive for endogenous repair mechanisms.

Once leukocytes have infiltrated the CNS, they release pro-inflammatory molecules such as tumour necrosis factor-alpha (TNFα) and interferon gamma (IFNγ) which have been shown to directly affect myelinating OLs as well as activate other resident glial cells [5, 47]. These cytokines, among others, are found to be increased in peripheral blood of MS patients as well as CSF [47-49]. Furthermore, the lymphocyte gene expression of both TNFα and IFNγ have been found to be correlated with lesion load, as seen by MRI in RRMS patients [49] showing the relevance of these molecules to MS disease pathogenesis.

In addition to BBB breakdown, there is also breakdown of the blood-CSF barrier within the subarachnoid space and meninglial layer [50], which would allow immune cells and toxic blood molecules into the CSF and brain. Under normal conditions, this is a site of molecular transport and immune monitoring [51, 52], where the CSF composition signals the state of the brain to peripheral immune cells. In cases of MS, extensive immune cell infiltration has been shown in this region along with high levels of their soluble mediators found in the CSF of the same individuals [53]. Furthermore, cortical and grey-matter lesions in the MS brain have been correlated to severe clinical disease, hinting at the effects of menengial immune cell infiltration and breakdown of the blood-CSF barrier in the MS disease course [54].

Fibrinogen is a blood clotting factor composed of polypeptide chains which have polymerization sites for clot formation [55], and various domains known to be involved in

stimulation of multiple cell types [56]. Deposition of fibrinogen has been noted in both active and chronic MS lesions [57, 58] as well as in normal appearing white matter (NAWM) which are visually unaffected regions of the MS brain via magnetic resonance imaging [59]. Most emphasis has been placed on its relation to myeloid cells and axons. It promotes neuroinflammation and glial scar formation by direct effects on microglia, astrocytes, and neurons [60-62]. Elsewhere in the body, fibrinogen has been shown to recruit leukocytes to the site of injury to mediate a response [63] and is potentially contributing to this effect in the CNS as well.

2.5 Previous OPC Studies

Most studies on the biology of OPCs and their regenerative capacity has been performed using rodent models given the limitations of access to primary human OPCs. The latter arise only in 2nd trimester of development [18]. Materials from late stages of human fetal development are not available and currently no technique exists to isolate OPCs in significant numbers from pediatric surgical samples. Early multipotential human fetal derived cells (A2B5+) are very susceptible to metabolic and inflammatory insults [6]. Mature human OLs are more resistant. For example, addition of pro-inflammatory molecules causes process retraction but not killing of primary human OLs *in vitro* [64]. As regards rodent based studies, TNFα is reported to kill primary rodent OL-lineage cells *in vitro* and *in vivo* [65-67], although other papers state that *in vivo* OPC contact with TNFα induces a block of differentiation rather than killing [68]. It has been shown that TNFα effect is dependent on the receptor it is acting upon. In a TNF-receptor 1 knockout model, it was shown that by acting on TNF-receptor 2, TNFα promotes proliferation of OPCs and remyelination capacity *in vivo* [69]. With IFNγ, human fetal OPCs treated *in vitro* were observed to have induced quiescence pathways as a possible reason for an inhibition of re-myelination [70]. *In vivo* and *in vitro* studies have shown IFNγ contribution to an inhibition of rodent OPC

differentiation [71-73], although some show the induction of cell death [74]. Furthermore, with blood-derived molecules such as fibrinogen, it has been noted that this treatment induced rodent OPC commitment to the astrocyte lineage rather than differentiating along their own lineage [75], specifically via the BMP pathway.

2.6 Human iPSC studies

Induced pluripotent stem cells (iPSCs) are cells which are derived from any mammalian somatic cell, and genetically reverted back to an embryonic-like state [76]. This means that they have the capacity to generate any cell type in the body, and since their discovery have been used to model cell types which were previously difficult to obtain. There are two main schools of differentiation from iPSC in 2-dimentional cultures; the use of growth and patterning factors simulating embryonic development, or the introduction of lineage-committing genes which would rapidly induce the cells to differentiate. Several protocols describing approaches on differentiating OL-lineage cells from iPSCs have been published.

Direct-differentiation approaches employ over-expression of lineage committing or late-stage genes allowing for late stage (MBP+) cells to be obtained [77, 78]. The protocol published by Ehrlich and colleagues [77] consists of transducing patterned neural precursor cells (NPCs) with key OL-lineage transcription factors SOX10, NKX6.2 and OLIG2. This 28-day protocol can generate 65% of O4+ cells of which 30% express the mature OL marker MBP [77]. Likewise, the protocol suggested by García-León and colleagues (2018) only utilizes the transcription factor SOX10 directly transduced into iPSCs, and has the efficiency of generating an average of 55% O4+ cells. While the directed differentiation methods allow for generation of late-stage cells at a faster time course, they often skip over intermediate stages in the development of the cells. These

methods allow for the study of adult-onset disorders but do not allow for the study of development or reparative mechanisms which consist of differentiation of resident progenitor populations.

Growth factor based protocols [79, 80] allow for the fine tuning of the differentiation of OL-lineage cells by recapitulating molecular steps seen in the developing embryo. Additionally, these protocols allow for the expansion of cells at any given proliferative stage, allowing for high-throughput assays. However, even the most widely cited protocols have low efficacy in generating these late stage (MBP+) OLs, and result in contaminant cell types such as astrocytes due to the generation of their common progenitor. For example, the Wang protocol [79] has an over 130-day differentiation period resulting in a maximum of 11% O4+ cells within a mixed culture of glial progenitors and astrocytes. The Douvaras and Fossati protocol [80] is 95 days in duration with the efficiency of generating an average of 40% O4+ cells with the capacity for expansion and cryopreservation. Wang and colleagues [79] specified that for the purposes of transplantation or OPC-replacement therapy, their protocol is sufficient to do so in the dysmyelinated mouse and possibly for future human regenerative therapies. For the purposes of studying early OL-lineage commitment and the switch between an early proliferative OPC (PDGFRα+) to late OPC (O4+), these growth factor based methods prove to be the most useful.

3. Methodology

3.1 Objective 1

Generation of Oligodendrocyte Lineage Cells from iPSCs: Briefly (See FIG 1A), from control human iPSC lines generated at the Montreal Neurological Institute's Early Drug Discovery Unit (EDDU) and one obtained from Genome Quebec (FIG S1), patterned neural progenitor cells were generated by dual-smad inhibition after 12 days (with addition of SB431542, DMH1 and retinoic acid). Then, transferring to a non-coated dish, the cells generate 3-dimensional structures, while in the presence of ventralization medium consisting of added Retinoic Acid and Puromorphamine, which will select for OLIG2+ neural progenitors. At day 20 of the protocol, the 3D spheres should be visible, and media is switched to an "OPC" medium consisting of added factors such as PDGF-AA, IGF-1, NT3, HGF, Biotin, T3 and cAMP. At day 30 of the protocol, 3D spheres of appropriate size are selected and plated on poly-ornithine/laminin coated culture vessels, where medium will be changed at half volume every second day.

Passaging and Differentiation of Oligodendrocyte Lineage Cells: At day 75 of the protocol, we have a mixed-glial culture consisting of committed glial progenitors, astrocytes and OPCs (FIG 1B). Passaging our mixed-progenitor culture allows for expansion and use for differentiation as needed. To passage, media is removed and TrypLE is added for 30 mins at 37℃. Cells are counted and replated, for up to a maximum of four passages. For differentiation, a commercially available basal medium is supplemented with growth serum for 10 days, followed by IGF-1 and T3 for the following 11 days to reach a majority of OL-lineage cells at a total of 21 days [81]. To confirm presence of OL-lineage cells, RNA was collected, reverse transcribed and gene expression analyzed by qPCR (FIG 1B).

Sorting and phenotyping using the SOX10-mOrange reporter line: An iPSC reporter line under the promoter of SOX10 was generated in-house at the Early Drug Discovery Unit (FIG 1D). This iPSC line was designed to fluoresce once the cells are committed to and maturing along the OL lineage (FIG 1C). With this fluorescence, we can live cell sort to study our cells in isolation (via FACS and culturing, or via transcriptomics) which would help us ascertain OPC-specific responses. Following OPC generation (as described above) and differentiation, in order to FACS sort or phenotype via flow cytometry, the cells are collected from the culture vessel using TrypLE is added for 30 mins at 37℃. The cells are passed through a 40uM mesh to ensure a single cell suspension. Following resuspension in PBS, the cells are counted using the Attune flow cytometer. For live cell sorting (FACS) the cells are passed to a dedicated technician at the EDDU and are sorted based on reporter intensity. For replating, the sorted mOrange+ cells are recovered in progenitor medium for up to four days. For phenotyping using flow cytometry, at least 100K cells are used for the subsequent staining steps. Cells are stained for viability using the LIVE/DEAD cell viability kit according to manufacturer instructions. Then, cells are blocked using an FcX blocker, which will prevent non-specific binding to naturally occurring Fc receptors on cells. The pre-conjugated antibodies (Table 1) are then diluted accordingly in the FACS buffer (D-PBS and 5% FBS), and appropriate FMO controls prepared as well. Cells are stained with pre-conjugated antibodies for 30 minutes, acquired on the Attune flow cytometer, and data analyzed using the FlowJo software. FACS based on endogenous reporter fluorescence was the most logical method for isolating our OL-lineage cells, as we had the prepared line and a dedicated technician readily available. In case of difficulties in recovery of OL-lineage cells post FACS, we aim to directly analyze post sort

using molecular (qPCR, bulk RNAseq) or phenotyping (flow cytometry) techniques as a readout for our subsequent experiments.

Single-Cell sequencing and analysis of generated human oligodendrocyte lineage cells: Cells were collected and fixed using the Parse fixation workflow that allows samples to be stored at -80°C until the barcoding and library steps are carried out. Between 100,000 and four million cells may enter the fixation. On the day of library preparation, cells are combinatorically barcoded, with each cell receiving three unique barcodes over three consecutive splitting and pooling steps. Barcoded cells are then split into sublibraries, lysed, reverse transcribed, and barcoded once more in a sublibrary-specific manner. Sublibraries are sequenced through the McGill Genome Centre Platform using an Illumina NovaSeq 6000. Generated Fastq files are read into the Parse Bioscience analysis pipeline on the high-performance Compute Canada computing cluster Béluga in order to assign samples to barcodes, combine sublibraries, and undergo initial quality control. The outputs from this pipeline are imported into the R package Seurat for further quality control, analysis, and interrogation of the data.

3.2 Objective 2

Treatment: OL-lineage cultures were treated with molecules for up to 6 days. TNFα was applied at 100 U and supplemented with media changes every other day. IFNγ was applied at the same concentration.

Imaging Analysis: Following treatment, cells were analyzed using immunofluorescence microscopy, staining for the late OPC marker O4, and cell death dye propidium iodide (PI) (Table

2). Briefly, O4 and PI are stained live for 1 hour at 37℃, following three washes and the removal of the cell culture medium. Cells are fixed with 4% PFA for 10 minutes, and the secondary antibody for the O4 primary is applied for 1 hour at 37 ℃. Cells are permeabilized in PBS containing 0.2% Triton X-100 for 10 minutes, cells are blocked for 1 hour at room temperature in 5% BSA w/ 0.05% Triton-X and other primary antibodies stained overnight. The next day, cells are washed and secondary antibody is applied for 2 hours at room temperature. Nuclei are stained with Hoescht (1:4000) for 5 minutes. Images were acquired using a high-content screener (ImageXpress, Molecular Devices) or 5 representative images per well at 20x were acquired manually on a widefield microscope (Zeiss). Images were analyzed with at least one software (ImageXpress, ImageJ). Specifically, OPC cell differentiation was assessed by quantifying the proportion of O4+ cells, while OPC cytotoxicity was assessed by counting the amount of O4+ PI+ cells. Due to the heterogeneity of each replicate (ie starting composition of cells), we may present data in terms of fold-change with values normalized to the untreated control of the same replicate, which may decrease the effect of variation on our results.

Flow Cytometry Phenotyping: Following treatment, cells were analyzed with flow cytometry and a panel of OL-lineage (O4, SOX10neg/med/high), astrocyte lineage (GFAP/AQP4/CD49f) as well as early progenitor markers (CD133, PDGFRα) (Table 1). These markers will be able to determine at which stage of the OL-lineage (based on mOrange fluorescence intensity and combination of markers) are the cells most susceptible to the treatment. The cells will be acquired using the Attune flow cytometer, and data will be analyzed using the FlowJo software. Proportions of OL lineage cells (O4, SOX10neg/med/high) in relation to the untreated control will give us a better idea on where the cells are most affected in their lineage. Flow cytometry has been found to be a more

reliable and time efficient readout for lineage analysis, however in the case of low cell amounts for a certain passage, immunofluorescence analysis will be prioritized.

Statistics: All statistics were measured in a paired manner, statistical test used is described in the figure legends. GraphPad Prism or Excel software were used for statistical testing.

3.3 Objective 3

Human Multiple Sclerosis brain tissue: This study was ethically approved by the Institutional Review Board of the McGill University Faculty of Medicine and tissues from the Centre Hospitalier de l'Université de Montréal (CHUM) were obtained by a Material Transfer Agreement between CHUM and McGill University. Patient information and disease classification is unknown. Fresh-frozen human brain tissue sections were obtained from patient cases with clinically diagnosed multiple sclerosis (n = 8) by rapid autopsy from CHUM.

Immunohistochemistry: Human MS brain tissue sections were fixed in 100% acetone for 10 minutes, followed by 70% Ethanol for 5 minutes. Subsequently, sections were washed with PBS and 0.05% PBST (PBS + Tween20) for 3 minutes. Further, sections were blocked with PBS containing 10% donkey serum. Primary antibodies (Table 3) were diluted in PBS containing 3% donkey serum and left to incubate overnight at 4°C. Following incubation, sections were washed with 0.05% PBST 3 times for 5 minutes each. Secondary antibodies were prepared at a 1:200 dilution in PBS, and incubated in the dark for 60 minutes at room temperature. Sections were washed with 0.05% PBST 3 times for 3 minutes and incubated with Hoechst (1:5000) for 10 minutes at room temperature. Once washed with 0.05% PBST 3 times for 3 minutes, sections were

incubated with 0.3% Sudan black diluted in 70% ethanol for 3 minutes. Slides were washed with PBS and mounted with PermaFluor mounting medium, coverslipped and stored in the dark at 4°C.

Primary human OL isolation and culture: Mature OLs were isolated from brain tissue samples gathered from surgical procedures. De-identified pediatric samples were obtained from the Montreal Children's Hospital, and adult samples via the Neuropathology department at the Montreal Neurological Institute and Hospital, with written consent. Brain tissue from the surgical path of pediatric and adult samples were collected into CUSA bags and subjected to trypsin digestion. Following digestion, the samples were separated into myelin depleted and myelin portions by percoll gradient. The myelin depleted layer consists of the OLs used for subsequent studies, which was enriched overnight via shake off technique. Cells are plated onto tissue culture plates with poly-l-lysine and extracellular matrix, cultured in DMEM-F12 medium supplemented with N1.

Treatment: OL-lineage cultures were treated with molecules for up to 6 days. Plasma-depleted fibrinogen was applied at 2.5 mg/mL, with media change every other day.

Imaging Analysis: Following treatment, cells will be analyzed using immunofluorescence microscopy. In a similar manner to Chapter 3.2, images were acquired using a high-content screener or 5 representative images per well at 20x acquired manually. Analysis performed with at least one software, and O4+ cell proportions as well as O4+/PI+ cell proportions were recorded.

Phenotyping: In a similar fashion to Chapter 3.2, the human iPSC mOrange reporter line will be employed to determine treatment effect on phenotypic population proportions at a more efficient manner than with imaging analysis. Following treatment, cells were analyzed with flow cytometry and a panel of OL-lineage (O4, SOX10neg/med/high), astrocyte lineage (GFAP/AQP4/CD49f) as well as early progenitor markers (CD133, PDGFRα) (Table 1). These markers will be able to determine at which stage of the OL-lineage (based on mOrange fluorescence intensity and combination of markers) are the cells most susceptible to the treatment.

Signalling Pathway Analysis: Activation of the BMP pathway will be confirmed in vitro by staining for signalling proteins such as phosphorylated-SMAD 1/5/9 by immunofluorescence, following treatment with fibrinogen and known inhibitors/activators of this pathway (DMH1/BMP4). Cells will be stained similarly as mentioned in Chapter 3.2, however they will be permeabilized with cold 100% methanol as according to manufacturer instructions. BMP pathway activation will be addressed by the measurement of nuclear signal intensity using the ImageJ analysis software.

Statistics: All statistics for cell culture results were measured in a paired manner, IHC statistics were unpaired, statistical test used is described in the figure legends. GraphPad Prism or Excel software were used for statistical testing.

4. Results

4.1 Objective 1

A proportion of the subsequent results are my own contributions to an article that has been published [81], and used in this thesis with the permission of authors (See Appendix). Following generation of OL-lineage cells based off the Douvaras and Fossati protocol [80], RT-qPCR was utilized to determine the composition and differentiation state of the cultures. Using the 3450 healthy control line for differentiation, we observed the decrease in pluripotency genes (*NANOG*) and the increase in OL-lineage related genes (*NKX-family, OLIG2, SOX10, PDGFRA, CNP)* (FIG 1B). Late-OL stage genes (*MBP*) were not observed but can be expected to be upregulated following the 3-week maturation period (FIG 1A). When differentiating OL-lineage cells from the SOX10-reporter line, mOrange positive cells could be observed following 30 days of differentiation and comprise most of the culture by day 75 in vitro (FIG 1C). Following differentiation and FACS, it was observed that the mOrange reporter line created three distinct groups based on fluorescence intensity: SOX10 neg, medium and high (FIG S3). To determine if reporter intensity could suggest lineage maturation stage, RNA was extracted from the FACS-sorted groups and an undifferentiated control. Following qPCR, it was observed that the NPC gene, *PAX6*, decreased in abundance as reporter intensity increased (FIG 1E). *GFAP*, an astrocyte gene, similarly decreased as reporter fluorescence intensity increased (FIG 1E), suggesting that reporter positive cells are within the OL lineage. We confirmed that reporter fluorescence intensity correlates with *SOX10* expression, and late-OL lineage genes (*CNP, MBP)* (FIG 1E), suggesting that fluorescence intensity can be used to determine overall culture commitment and maturation along the OL-lineage. Cells generated from three different genetic backgrounds (FIG S1), were differentiated, and two were sequenced at the single-cell level in progenitor medium and following

differentiation (FIG 2A). It was observed that there is an increase in number of cells contributing to the “pre-OL” cluster following differentiation (FIG 2A). This cluster was found to be upregulating pre-OL markers such as *GPR17*, *SOX10* and *PLP1* (FIG 2B). Two distinct clusters of early OPCs were observed (proliferative and quiescent), where one has a high expression of the cell proliferation gene, *MKI67* (FIG 2B). Other cell types were observed in the culture, such as astrocytes and glial restricted precursors (GRP) (FIG 2B).

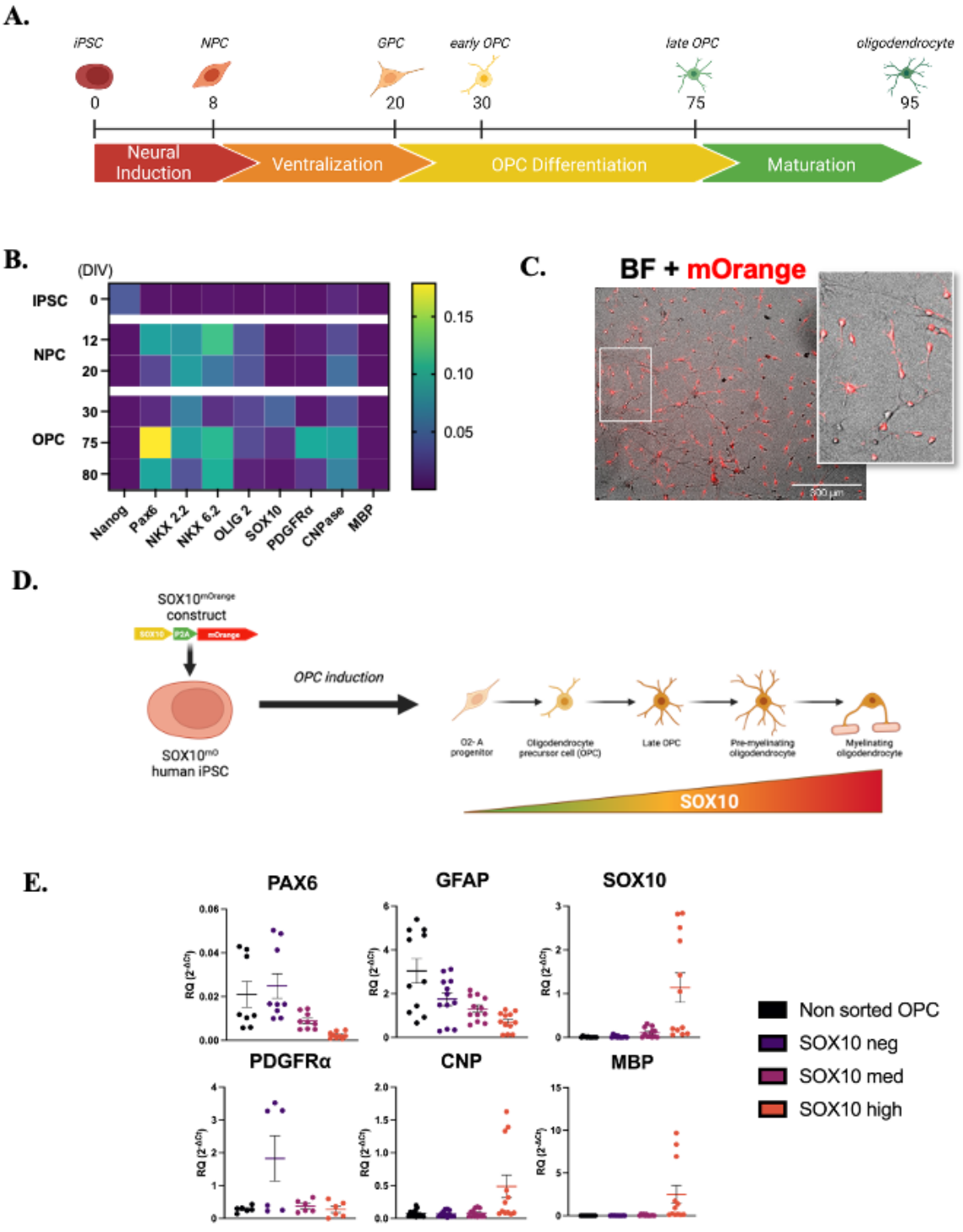


Figure 1. Generation and sorting of iPSC-derived OL-lineage cells. A. Schematic diagram of OL differentiation protocol, adapted from Douvaras and Fossati. **B.** RT-qPCR analysis of genes assessing generation of OPCs following 75-80 days. **C.** Bright field image with mOrange reporter of OPCs at day 75 of the protocol. **D.** Schematic of the generation of the SOX10-mOrange reporter

line. **E.** RT-qPCR of OL-lineage genes following FACS on the mOrange reporter line. Each point represents a single well in a replicated qPCR plate (3 total), bars represent SEM.

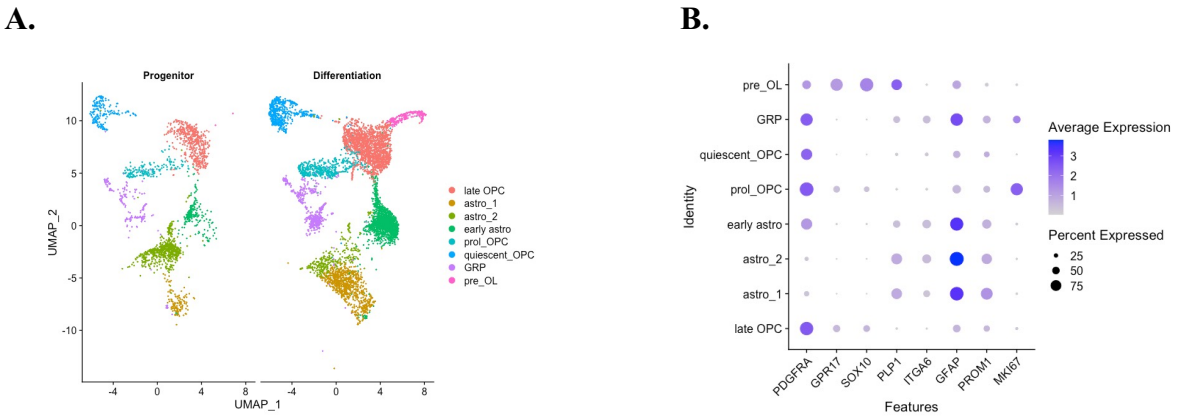


Figure 2. Sequencing and characterization of OL-lineage cells derived from iPSCs. A. Sequenced progenitor and differentiated cells (day 75 vs day 95), two different genetic backgrounds (3450, 81280 cell lines). total number of cells = 8256. **B.** Identification of clusters based on cell-identifying genes.

4.2 Objective 2

Following differentiation and treatment of OPCs with cytokines (FIG 3A), effect on differentiation was evaluated using immunofluorescence microscopy and flow cytometry (FIG 3B). It was observed that treatment with IFNγ for up to 6 days significantly (Dunnett's, p=0.0087) reduced the proportion of late OPCs in our cultures (FIG 3B). Similarly following exposure of OPCs to TNFα for up to 6 days, a 50% reduction was also observed (Dunnett's, p=0.0026). To determine if the decreased proportion of OPCs was due to cytotoxicity, the proportions of O4+ OPCs co-stained with PI were evaluated. Previous studies in our lab suggest that early OL-lineage cells are susceptible to cell death following simulation of metabolic stress conditions, as relevant to chronic MS lesions [82]. Using a glucose deprivation condition (NG) as a positive control (Mann-Whitney test, p=0.0079), we did not observe significant cell death following OPC to exposure to IFNγ or TNFα separately (FIG 3C). To further confirm the decrease in proportion of O4+ cells following cytokine treatment (FIG 3B), cultures were phenotyped using flow cytometry following treatment (FIG 3D).

After differentiation (OPC medium vs Diff medium), we observed the increase of OL-lineage markers PDGFRα and O4, and the decrease of early glial progenitor marker CD133, as expected (FIG 3D). However, following treatment with cytokines, we observed a decrease in OL-lineage markers, confirming our immunofluorescence data (FIG 3B). Interestingly, following treatment with TNFα specifically, a significant (1-way ANOVA <0.0001, post hoc Dunnett; <0.0001) increase in the astrocyte lineage marker AQP4 (FIG 3D, E) was observed. Upon further analysis of the flow cytometry data, a subset of O4+ cells were found to co-express this astrocyte lineage marker AQP4 (1-way ANOVA 0.0014, post hoc Dunnett; 0.0040) following TNFα treatment (FIG 3F, G). These findings suggest that a proportion of OPCs could be contributing to the increase of AQP4+ cells observed (FIG 3D, E).

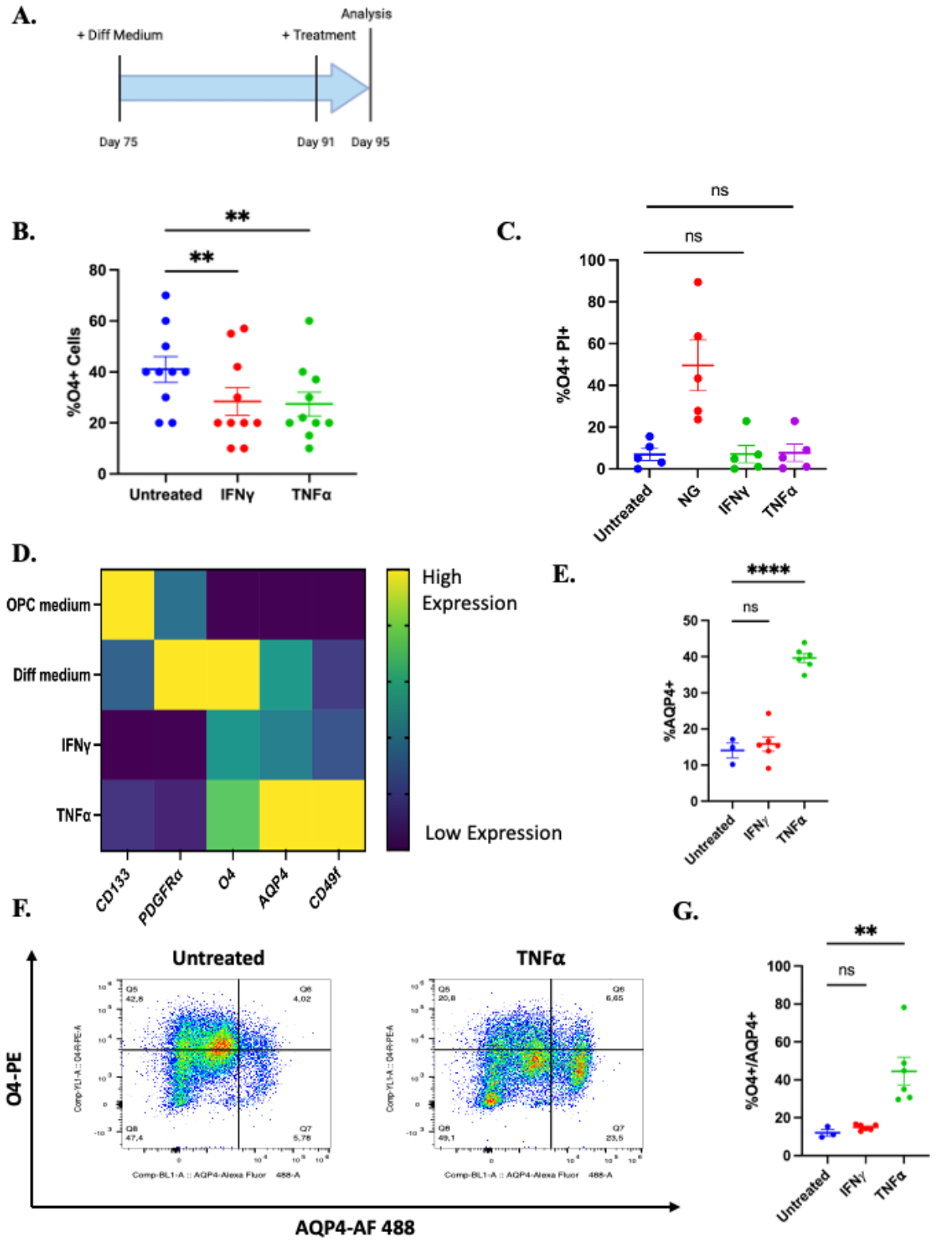


Figure 3. Treatment of OL-lineage cells with pro-inflammatory molecules. A. Schematic of experimental protocol. **B.** %O4+ cells following treatment for 2-6 days, counted by immunofluorescence and flow cytometry. 1-way anova p= 0.0006. post-IFNg: 0.0087, TNFa: 0.0026. n= 10, data collected from 3-4 subsequent passages of 3450, AIW and 81280 cell lines,

bars represent SEM. **C.** %O4+PI+ cells following treatment for 4 days, counted by immunofluorescence. NG = no glucose. n represents subsequent passages of 3450, AIW and 81280 cell lines**. D.** Normalized flow cytometry data of cell surface marker expression, n=4, treatment for up to 6 days. **E.** Quantification of proportions of AQP4+ cells, n=3 passages, treatments up to 6 days, performed with the 3450 cell line, bars represent SEM. One-wayANOVA $p < 0.0001$, post; IFNg: 0.7440 TNFa: <0.0001 **F.** Schematic of gating technique, showing presence of cells of a hybrid phenotype. **G.** Proportion of O4+AQP4+ cells, n=3 passages, three points per condition represent treatments of 4 and 6 days, bars represent SEM. One-wayANOVA $p= 0.0014$, post; IFNg: 0.9078, TNFa: 0.0040.

4.3 Objective 3

To address disease relevance of studying fibrinogen effect on human OL-lineage cells, tissue sections from the MS brain were stained with SOX10 and fibrinogen (FIG 4A). Co-localization of fibrinogen on OL-lineage cells (SOX10+) within MS lesions (FIG 4A) suggest a potential direct effect of this blood-derived molecule. Fluorescence intensity of fibrinogen on OL-lineage cells (SOX10+) in various pathological regions in the MS brain were then assessed (FIG 4B). It was observed that a significant increase in fibrinogen deposition was observed in chronic active and inactive lesions ($p < 0.001$) in comparison to NAWM (FIG 4B). Interestingly, an active lesion which is relatively new, had less fluorescence intensity than the chronic lesions albeit still significantly increased ($p < 0.001$) in comparison to NAWM (FIG 4B). To determine the effect of fibrinogen on mature OLs, the primary human model available in our lab was employed. Cytotoxicity of OLs was evaluated following treatment for 6 days with increasing dilutions of fibrinogen (FIG 5A) and no significant effect was observed. To evaluate sublethal injury to mature OLs, cell area was measured following treatment with fibrinogen at a physiologically relevant concentration (2.5 mg/mL). However, no significant effect was observed (FIG 5B).

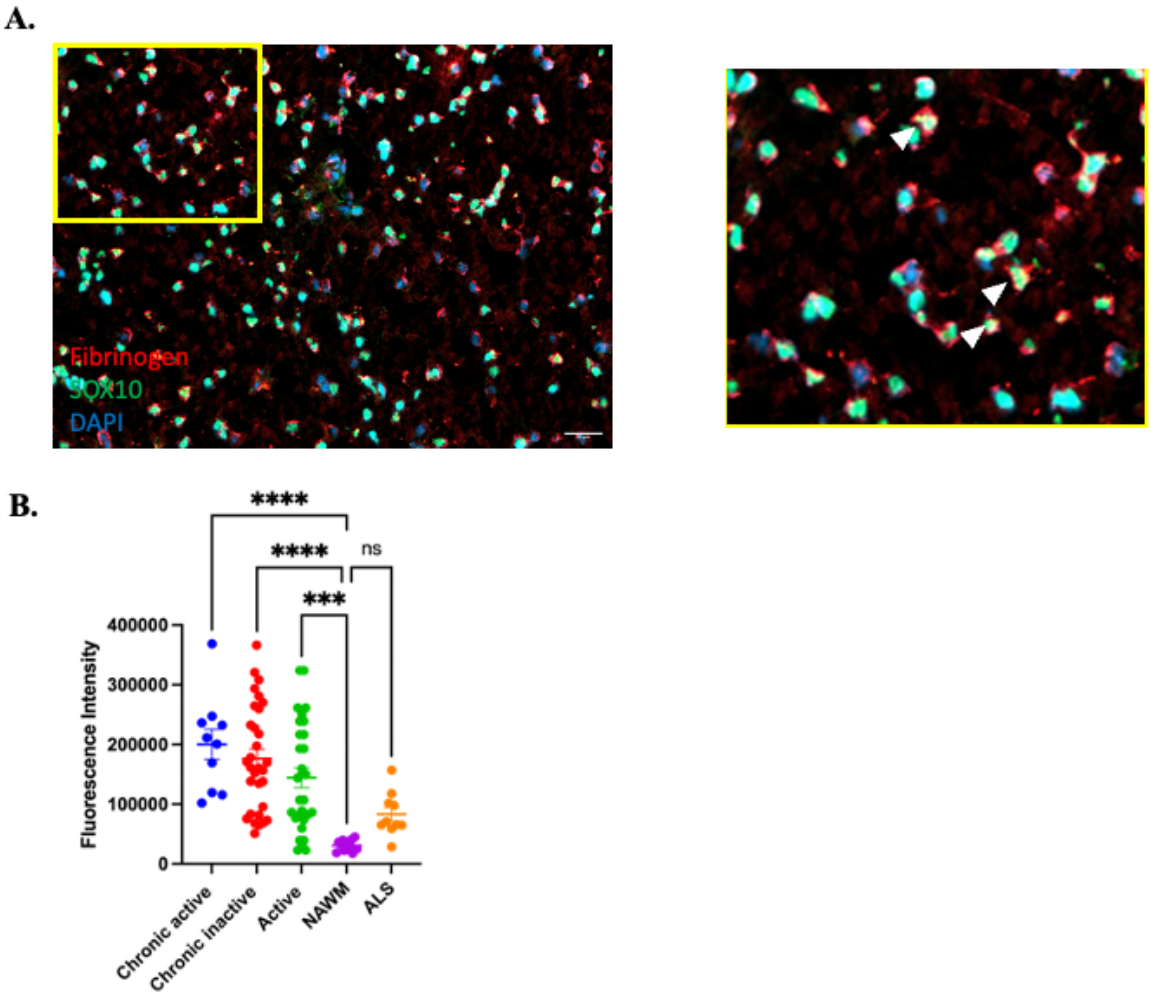


Figure 4. Fibrinogen deposition on OL-lineage cells in the MS brain. A. Edge of a chronic active MS lesion. Fibrinogen stained in Red, SOX10 stained in green, nuclei stained in blue. Arrows point to overlapping of all three markers. **B.** Quantification of fibrinogen fluorescence intensity on SOX10+ cells in relation to pathological regions from MS and non-MS brain tissue. NAWM = normal appearing white matter, ALS = amyotrophic lateral sclerosis. Points represent a single SOX10+ cell within the selected region of donor tissue, bars represent SEM. Kruskal-Wallis test $p < 0.0001$, Dunn's multiple comparisons; **** $p < 0.0001$, *** $p < 0.001$.

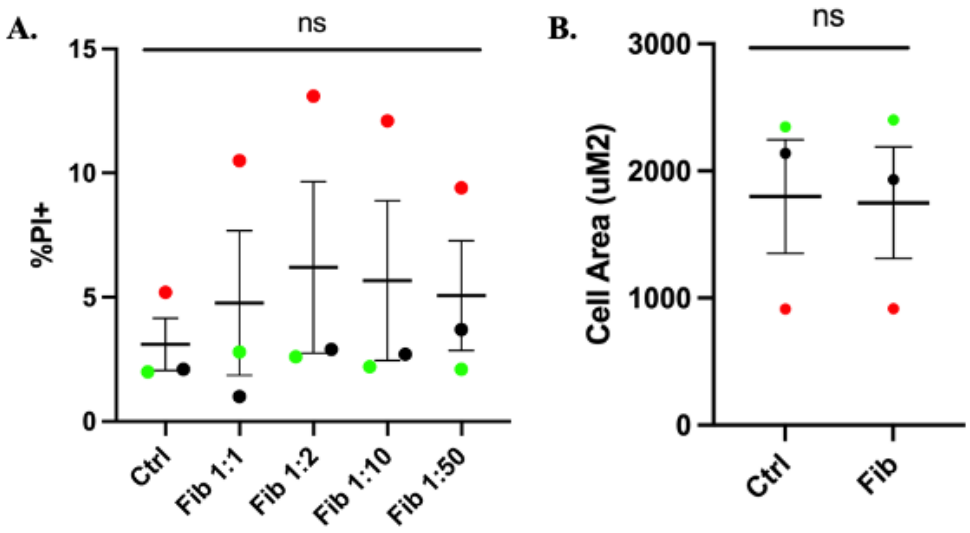


Figure 5. Treatment of mature human OL cells with blood-derived molecules. A. 4 day fibrinogen treatment with increasing dilutions. 1:1= 4 mg/ml 1:2= 2 mg/ml 1:10= 0.4 mg/ml 1:50= 80 ug/ml. **B.** measurement of OL process area following 4 day fibrinogen treatment, 2.5 mg/ml. n represent different adult donors.

Previous literature suggests [75] a direct effect of fibrinogen on rodent OPCs which leads to an inhibition of differentiation and a diversion towards the astrocyte lineage. To test this in a human model, fibrinogen was applied to differentiating iPSC-OPC cultures and differentiation capacity was addressed. A significant (Paired ttest p=0.0360) reduction in the proportion of O4+ cells was observed by immunofluorescence microscopy following treatment (FIG 6A). Although this decrease in proportion of OPCs in culture was not accompanied by increase in OPC cytotoxicity (FIG 6B). To determine where in the lineage the OPCs are stalling, we employed the SOX10-mOrange reporter cell line. During the differentiation period (undifferentiated vs differentiated), we observed an increase in the proportion of mOrange+ cells (FIG 6C), specifically the presence of the mOrange high population. As previously mentioned, (Chapter 4.1), reporter fluorescence intensity correlates to lineage maturity, where mOrange high cells expressed the highest proportion of late lineage genes *MBP* and *SOX10* (FIG 1E). The addition of fibrinogen

decreased the proportion of reporter-high cells (FIG 6C), as did the addition of the known BMP-pathway activator BMP4.

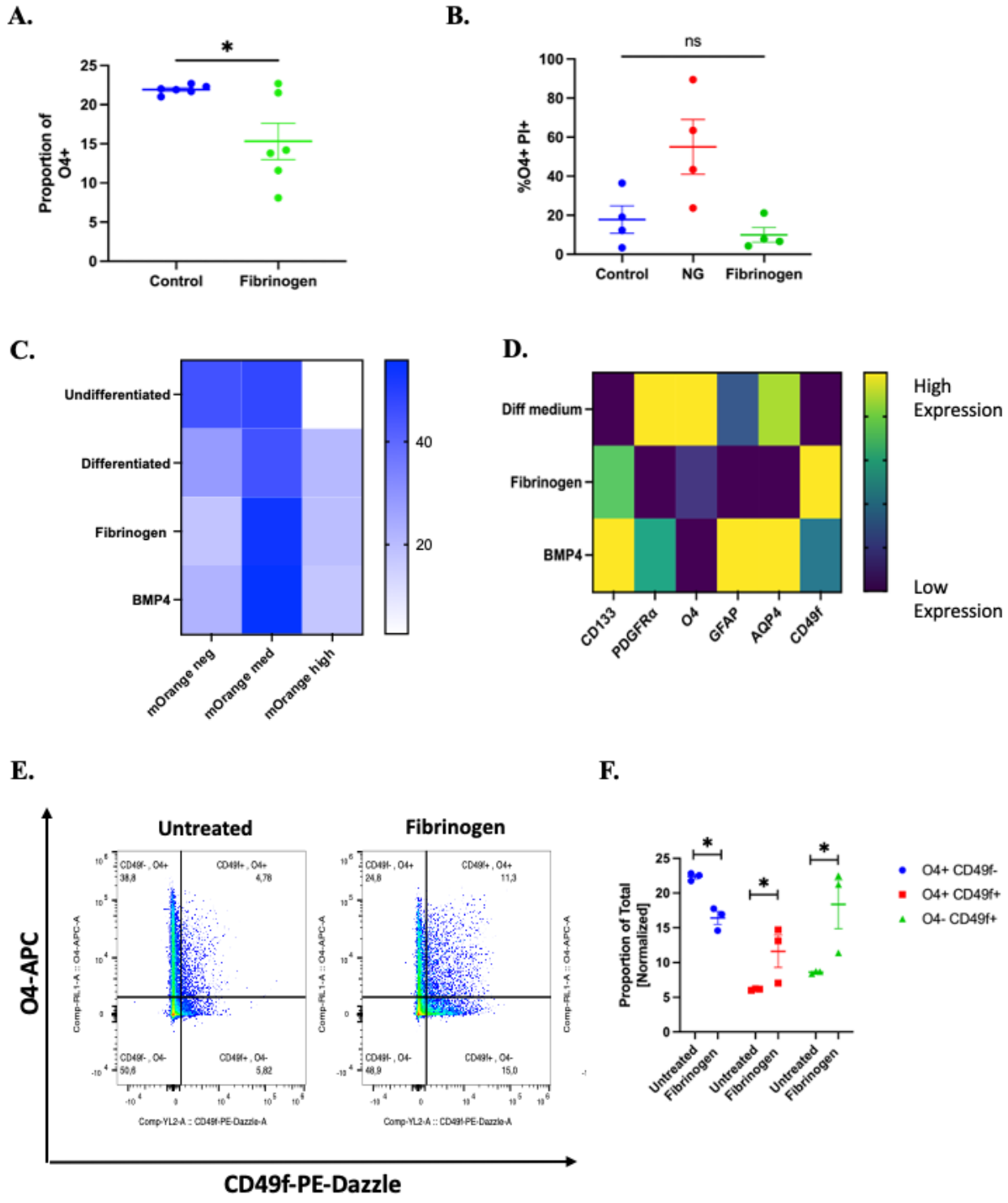


Figure 6. Treatment of OL-lineage cells with blood-derived molecules. A. %O4+ cells following treatment for 4 days, counted by immunofluorescence. Datapoints represent subsequent passages of 3450, AIW and 81280 cell lines, bars represent SEM. Paired ttest p= 0.0360 **B.** %O4+PI+ cells following treatment for 4 days, counted by immunofluorescence. NG = no glucose. Paired ttest, p= 0.1142 **C.** Proportion of reporter positive cells following exposure to fibrinogen, average shown of three subsequent passages. **D.** Phenotyping by flow cytometry. **E.** gating strategy on flow cytometry data for presence of O4+CD49f+ cells. **F.** normalized proportions of O4/CD49f subpopulations as gated in E. n=3 replicates, subsequent passages of mOrange reporter line, bars represent SEM. Paired ttest, O4+/CD49f-: 0.03515, O4+/CD49f+: 0.07818 , O4-/CD49f+: 0.04966.

To determine the effect of fibrinogen on other cell types in our iPSC-derived cultures, we phenotyped our cells with flow cytometry (FIG 6D). Fibrinogen increased the proportions of CD133+ and CD49f+ cells in comparison to the untreated control, while decreasing the proportion of OL-lineage markers (PDGFRα, O4). BMP4 similarly decreased OL-lineage markers (PDGFRα, O4) and increased progenitor (CD133) and astrocyte (GFAP, AQP4, CD49f) markers (FIG 6D). Interestingly, we observed the presence of hybrid OPCs (co-expressing the OPC marker O4 and the astrocyte marker CD49f) following fibrinogen treatment (FIG 6E). Following quantification, it was observed a significant (p =0.03515) decrease in single-positive (O4+ CD49f-) OPCs, an increase in hybrid (O4+/CD49f+) cells (p= 0.07818) and an increase in single-positive astrocytes (O4-/CD49f+, p= 0.04966). To confirm BMP pathway activation across the OL-lineage in response to fibrinogen, both iPSC-derived OPCs and human primary OLs were treated and stained for pSMAD 1/5/9 (FIG 7). Mature human OLs treated with fibrinogen and BMP4 showed a significant increase in nuclear pSMAD fluorescence (Paired ttests; fibrinogen: 0.0300, BMP4: 0.0410) (FIG 7A). Human OPCs (iPSC-derived) showed a similar result (Paired ttests; fibrinogen: 0.0708, BMP4: 0.0501) (FIG 7B).

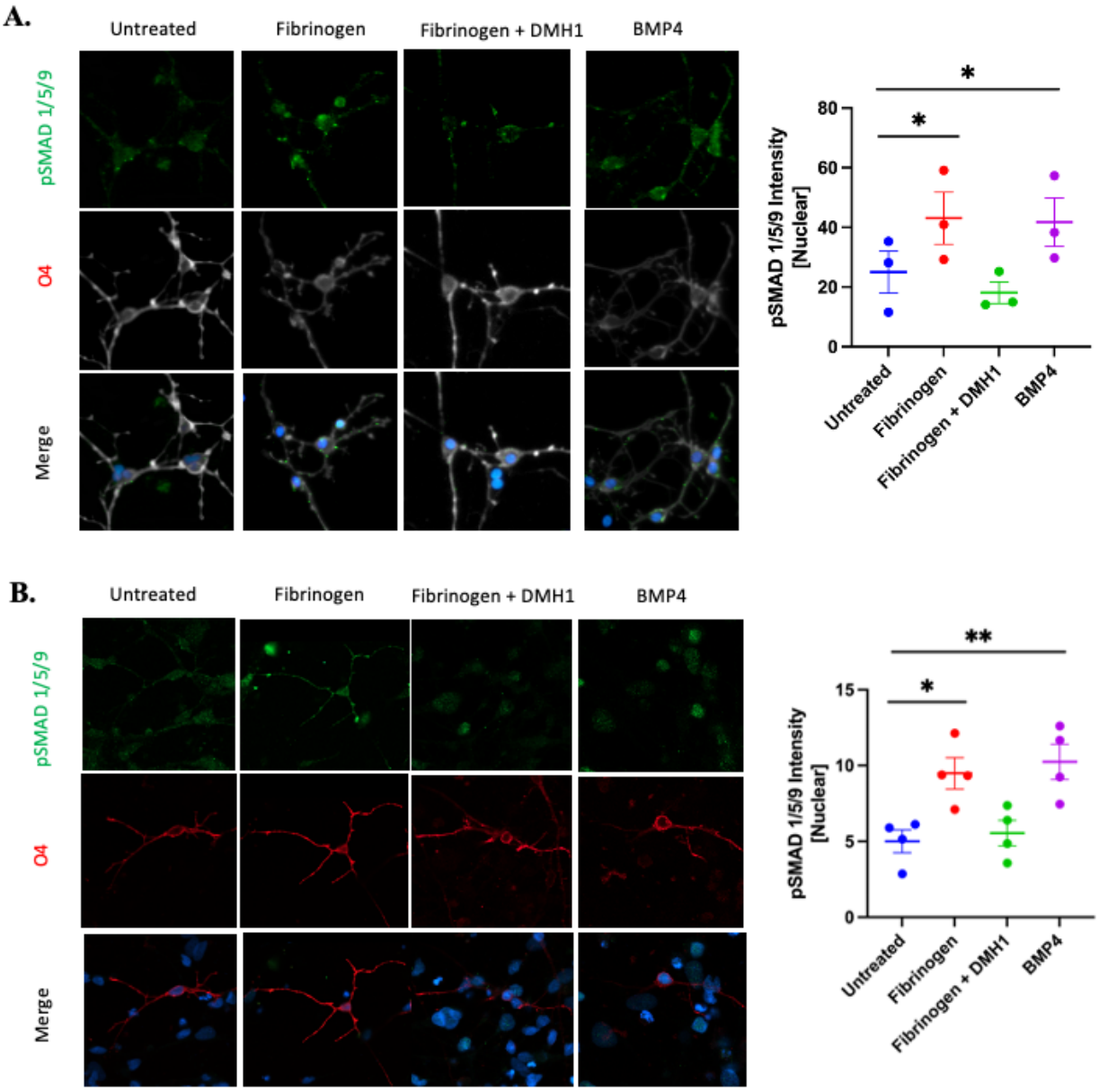


Figure 7. BMP-pathway signalling in human OL-lineage cells. A. pSMAD signalling in O4+ cells from the human adult brain. Nuclear signalling quantified, n=3 adult donors, bars represent SEM. Paired ttests; Fibrinogen: 0.0300, Fibrinogen + DMH1: 0.5791, BMP4: 0.0410. **B.** pSMAD signalling in O4+ cells from iPSC-derived cultures. Nuclear signalling quantified, n=4 passages of 3450 cell line, bars represent SEM. Paired ttests; Fibrinogen: 0.0708, Fibrinogen + DMH1: 0.6855, BMP4: 0.0501.

5. Discussion

The presented work aims to emphasize the importance of human models in the study of disease and development of therapeutic targets. Our emphasis is on MS and how OL lineages cell injury and repair capacity impact on the disease course. MS is characterized by destruction of myelinating OLs with variable repair from OPCs. Our lab has long had access to primary OLs that are either late in the lineage or fully mature. For this thesis, the need to develop a model of OPCs was required as we are unable to get OPCs from primary human sources. Based on previous studies, we then selected potential injury mediators in the form of inflammatory cytokines and fibrinogen to interrogate their effect on early OL-lineage commitment and differentiation.

5.1 Model systems: strengths and weaknesses of rodent studies

The presented work aims to emphasize the importance of human models in the study of disease and development of therapeutic targets. While exploring previous literature on OPC biology and functional responses to disease conditions, it was prominent that findings were highly inconsistent (Chapter 2.5). Rodent studies have allowed us to appreciate the complexity of the OL; however, important studies translating this knowledge to a human context are lacking. Through studies which have been able to assess OPC and OL complexity in the developing human fetus, it has been understood that there are key differences between rodent and humans [83, 84], for example: CNS anatomical differences between humans and rodents [84], vastly different timelines for myelination and development [85], and differing responses to certain cues [86].

Before identifying possible therapeutic avenues targeting OPCs, the effects of the disease context on the targeted cell type need to be thoroughly studied. Following *in vitro* work, effects are studied in a pre-clinical model. Pre-clinical models of MS and demyelinating disease for the study of remyelination include experimental autoimmune encephalomyelitis [87], virus-induced

inflammation [88] and toxin-induced demyelination such as cuprizone [89]. Each model provides a simulation of certain aspects of the MS disease state and demyelination in the human brain. Once a therapeutic is identified, its effect is addressed in a disease-relevant rodent model and finally progress to a clinical trial. However, according to a systematic review [90] on remyelination promoting therapies, of 88 therapeutics only 25 have entered human studies and none received approval by the FDA for the purpose of remyelination. For example, following a promising clinical trial in a cohort of individuals with RRMS [91], Fingolimod, a drug proven to show beneficial effects on remyelination in pre-clinical studies, failed to show effect in a cohort of progressive MS patients [92]. It has since been approved for RRMS as it prevents the formation of new lesions, the increase of preformed lesions as well as prevents physical disability decline [91, 93]. This lack of translatability may be attributed to differences between rodent and human biology. Using current methods on human primary cell isolation employed in our lab, meaningful amounts of OPCs for study have not been isolated successfully. Access to primary human cells for study is increasingly difficult, with the onset of restrictions on use of human fetal tissue. Therefore, the use of human iPSC-models is of increasing importance for the translatability of future therapeutics targeting OPCs and remyelination.

5.2 Generation and characterization of OL-lineage cells

Confirmed by qPCR (FIG 1B) and single-cell RNAseq (FIG 2B), we generated OL-lineage cells from human iPSCs using a protocol adapted from Douvaras and Fossati's 2015 publication [80, 81]. According to scRNAseq, we were able to generate a majority to early- to mid-lineage cells, as suggested by expression of *PDGFRA* and *GPR17, MKI67* (FIG 2B) [11, 94, 95]. One limitation of utilizing the growth-factor based approach (as mentioned in Chapter 2.6) is the inefficiency of generating late-stage OLs. Although we did observe the expression of mature

markers (*SOX10, GPR17, PLP1)* (FIG 2B), we did not observe the decrease/loss of earlier lineage marker *PDGFRA*. Therefore the limitation of this approach is the inability to determine the effect of molecules or conditions across the entirety of the OL-lineage with efficiency. Other approaches such as the transcription-factor based induction methods [77, 78] would be able to circumvent this issue. Although, these protocols tend to skip over the important early progenitor stages, and if the aim of the proposed research is to determine effect on differentiation capacity this is difficult to determine in this type of model. The cells generated by these transcription-factor based induction methods have the constant "push" towards a mature stage, therefore genetically overriding possible external cues (such as disease-relevant conditions). However, the focus of this thesis is to observe the effects of MS-related molecules on early OL lineage commitment and differentiation. Thus, we have shown to efficiently generated early and mid OL-lineage cells with our currently employed protocol [81], which express canonical OL-lineage genes (FIG 1B, 2B). Downstream experiments discussed were focused on early OL-lineage commitment and how disease related molecules affect early differentiation of these cells. In conditions such as fibrinogen treatment where the effect on human mature OLs is previously unknown, we utilized our human primary OLs as a control to study these effects across the entirety of the OL-lineage.

Within our scRNAseq dataset, it was observed that there remains a distinct proportion of GRPs between progenitor and differentiation conditions (FIG 2A). This reservoir of undifferentiated glial precursors could prove to be beneficial for the study of developmental diseases affecting myelination, such as leukodystrophies [96]. As a next step outside the scope of this thesis, this dataset could prove useful in determining key molecular events in the specification of OL-lineage differentiation from the common glial precursor as a tool for the study of these developmental disorders.

Another unique finding is the ability to discern three distinct groups based on SOX10 reporter fluorescence intensity (SOX10 neg/med/high) following the three-week maturation period (Day 95 *in vitro*, see FIG 1A). The SOX10 transcription factor is important in OL-lineage commitment and maturation, as it regulates the expression of key myelination-related genes [25, 97]. It was an unexpected finding that fluorescence intensity of the reporter would correlate to lineage maturation stage, as it was expected to be a positive/negative discernment of OL-lineage commitment. The original CRISPR/Cas9 construct, as designed by EDDU personnel, allows for the self-cleavage of the reporter from the SOX10 transcription factor post translation, thus not interfering with the function of the transcription factor. It was evaluated in our recent publication [81] that the overall expression of the SOX10 transcription factor of this reporter line is similar to that of its parent (un-edited line). As mentioned in Chapter 2.2, transcription factors SOX10 and OLIG2 are involved in a positive feedback loop [25, 97]. This may suggest that SOX10 will be present in increasing levels as cells mature along the OL-lineage. Downstream of SOX10 expression, the protein promotes the transcription of important myelination-relevant genes [25], which require increasing expression for the mature OL to myelinate.

5.3 IFNγ and TNFα on human OPCs

Our findings of IFNγ and TNFα inhibiting the differentiation of human OPCs (FIG 3B) without cytotoxicity (FIG 3C) align with other studies in the field [68, 71, 98]. The discrepancy between results of studies on rodent and human models could rationalize the lack of therapeutic development in the remyelination field. As mentioned in Chapter 2.5, results from previous rodent studies were divided on the exact effect of these cytokines. Previous studies in our lab suggest that these cytokines on human primary OLs result in sublethal injury [64], suggesting that human OL-lineage cells could be more resilient to cytokines than rodent cells. Additional studies on human

OPCs derived from iPSCs [98] utilized different approaches although ultimately publishing the same findings, which strengthens our approach even further for future studies on early OL-lineage commitment in a human model.

Our approach in the generation of early OL-lineage cells with growth factors rather than genetic manipulation, has allowed us to explore the flexibility in the commitment of early OL-lineage cells. Previous studies with rodents suggest that early OL-lineage cells may have the capacity to generate astrocytes [99, 100]. Furthermore, lineage tracing studies suggest that in response to injury, this response may be skewed towards the astrocyte lineage rather than oligodendrocyte [101, 102]. Here we have shown an increase in astroglial markers in our cultures following TNFα exposure (FIG 2D, E) and a surprising emergence of a hybrid OPC subpopulation (FIG 3F-G). This could provide another mechanism in which the endogenous OPC-mediated repair pathway in the CNS is failing in MS-like conditions, where instead of generating myelinating OLs, OPCs generate astrocytes which contribute to the formation of the glial scar. We were unable to observe BMP pathway activation following TNFα treatment (data not shown). It is possible that other pathways contribute to this effect, such as the JAK/STAT pathway [103]. Findings by Sun et al. [103], found STAT3 to be upregulated in OPCs and astrocytes surrounding MS lesion-like sites in a mouse model. In vitro, rodent OPCs in the same study were found to generate GFAP+ astrocytes following JAK/STAT pathway activation [103], showing a possible mechanism of remyelination failure in their mouse model of MS. Further studies with our iPSC-derived OPCs would be required to determine if TNFα is inducing the same pathway in our cultures, as it has been shown to be possible in in human B cells [104] and fibroblasts [105].

5.4 Fibrinogen on human OL-lineage cells

Fibrinogen has been shown to be present in the brain under several neurological disease conditions [106]. It is also known that during the normal ageing process BBB breakdown also occurs [107]. Therefore, these findings could apply to more than just the MS disease state. In MS-disease conditions, it has been shown to localize to the site of an active lesion, and act directly on astrocytes, microglia, and neurons [60-62]. We have provided *in situ* data showing that fibrinogen is also in direct contact with OL-lineage cells within the MS brain (FIG 4). As a control, we applied fibrinogen to mature OLs derived from human parenchymal tissue (FIG 5). We observed that even in increasing concentrations, there was no effect on cell death or morphology. Interestingly, the BMP pathway was activated in these mature OLs (FIG 7A), raising questions for further studies.

Petersen and colleagues [75] determined that fibrinogen-treated postnatal rat OPCs had an impaired differentiation and promoted astrogenesis via the BMP pathway. Additionally, fibrinogen studied *in vivo*, has been shown to promote astrogenesis from the SVZ niche [108] via the same pathway. Our results in the human iPSC model suggest the same phenomenon occurring (FIG 6). Following observation of O4+ cells without accompanied cytotoxicity (FIG 6A, B), we observed that fibrinogen stalls our cells in an early progenitor stage (FIG 6C) while increasing the proportion of astrocyte marker CD49f (FIG 6D). Fibrinogen also increased the presence of cells co-expressing OPC and astrocyte markers (FIG 6E) suggesting that OPCs could be the cause of this increase in astrocyte markers observed. Previous literature has suggested early (PDGFRα+, NG2+) OPCs to still be capable of generating type-2 astrocytes under certain conditions [34, 35, 40, 41]. To my knowledge, this has not been shown in a human model and in response to certain disease-relevant molecules. Interestingly, the BMP pathway has already been identified as a contributor to the inhibition of OPC differentiation [109], and playing an important role in astroglial lineage

development [110, 111]. A limitation of these iPSC studies is the difficulty in growth and recovery of OPCs in isolation. Further work would be required to observe the effect of fibrinogen on OPCs in isolation and compare if we can indeed rescue the observed phenomenon with the addition of DMH1 (the BMP pathway inhibitor), or if there is an additional effect derived by the contaminating cells (astrocytes, glial progenitors).

5.5 General limitations and future directions

The generation and subsequent study of MS-related molecules on human oligodendroglial cells has provided insight into the effect of key molecules in a human context. As mentioned, our current method of generation [81] generates a mixed glial culture consisting of glial progenitors, astrocytes, and OL-lineage cells. This is one of the key challenges proven in the field when growth factor-based iPSC differentiation approaches are employed. Therefore, we cannot be certain that the presented findings of IFNγ, TNFα and fibrinogen on human iPSC-OPC cultures are a result of direct effect on the OPCs or mediated by contaminating glial cells. Further work is required to optimize an isolation technique and growth conditions post-isolation. It has been proposed that astrocytes provide key factors to enhance OPC support and differentiation [112, 113]. In a human context, it is possible that not all cues for optimal OPC differentiation and maintenance have been identified, and therefore mixed glial cultures result in more efficient OPC generation. There may be multiple approaches for the isolation of OL-lineage cells from iPSC-derived cultures. Previous studies employing similar iPSC-OL generation techniques have used FACS-based sorting of reporter lines [114]. Other techniques that can be borrowed from rodent culturing include immunopanning [115], differential adhesion [116], shake off [117] and MACS-beads selection [116]. An optimal isolation technique would not decrease the viability of the cultures, as this could confound subsequent results following treatments.

Pro-inflammatory molecules IFNγ, TNFα and the blood-clotting factor fibrinogen have all been shown to be involved in MS pathogenesis. Previous studies utilizing human iPSC-derived OL lineage cells have shown that IFNγ and TNFα impair differentiation in a transcription-factor mediated differentiation model [98]. Interestingly, in a growth factor based differentiation of OL lineage cells from MS individuals, it has been shown that activated astrocyte supernatant inhibits their differentiation without cytotoxicity [118], where it is known that astrocyte supernatants can contain TNFα [119]. Our presented observations of increased proportions of astrocytes and the presence of OPCs acquiring astrocyte-like phenotypes following TNFα (FIG 3D-G) and fibrinogen (FIG 6D-F) exposure have not been shown as of yet in a human model. In this regard, it is interesting to note that astrocytic precursors have been observed in early MS lesions [120]. The previous studies on human iPSC-derived cells using a transcription-factor mediated differentiation model may not have been optimal to observe these effects. It is important to note that disease-relevant molecules have not been identified to drive this phenomenon in human OL-lineage cells.

Finally, certain experiments would require increased numbers of replicates (either passages or additional iPSC-lines) to achieve increased statistical power. For example, experiments contributing to figure 3G were performed n=3 times where replicates are subsequent passages of the same batch of OPCs. Individual points here were data combined from cells treated for 4 or 6 days with TNFα. Ideally, increasing these values to 5 without the combination of treatment durations in a single iPSC line would improve statistical power. Similarly, flow cytometry experiments treated with fibrinogen were n=3 where each point was a subsequent passage of the mOrange cell line. Here it would be unreasonable to generate another reporter line in a different genetic background, however time-permitting we would have liked to increase these replicates to

5 to increase statistical power. Some experiments (ie contributing to figure 3) were replicated in three lines, without three passages in all lines. With the variability that comes with iPSC-derived cells, all ideal experiments would have been replicated in three different cell lines. For this thesis, we can make concrete conclusions on experiments where replicates are above 3.

6. Conclusion

We have studied well known pro-inflammatory molecules IFNγ and TNFα, as well as a blood derived molecule fibrinogen in a human model of early OL-lineage commitment (FIG 8A), as all molecules have been shown to be relevant to MS disease. Although these molecules have been extensively explored in rodent models, this thesis attempts to highlight the importance of the study of disease related molecules in a human context, which could provide invaluable translational evidence to progress findings to clinical use. Here we have shown data which has contributed to the development of a novel growth-factor based protocol to derive OL-lineage cells from human iPSCs ([81], FIG 1-2). We have generated OL-lineage cells and characterized our cultures on the transcriptomic level (FIG 1B, 1E, FIG 2). We have employed a novel reporter line expressing the fluorophore mOrange under the important OL-lineage transcription factor, SOX10 (FIG 1C-D). We have observed that reporter fluorescence intensity correlates with lineage maturity (FIG 1E), providing a valuable tool for downstream experiments.

To determine the effect of MS-disease related molecules IFNγ and TNFα on early OL-lineage commitment, we applied these factors while the cells were in the presence of maturation cues (FIG 3A). We observed that both molecules, when applied separately, caused a significant reduction in the proportion of late OPCs (O4+) in our cultures without increasing cytotoxicity (FIG 3B-C). Upon further phenotyping, we observed that TNFα increased the proportions of the astrocyte

marker AQP4 (FIG 3D-E). Serendipitously, upon further investigation of our flow cytometry data, we observed the presence and increase of OPCs co-expressing AQP4 (FIG 3F-G), suggesting that this sub-population of OPCs can contribute to this increase in astrocytes following TNFα exposure.

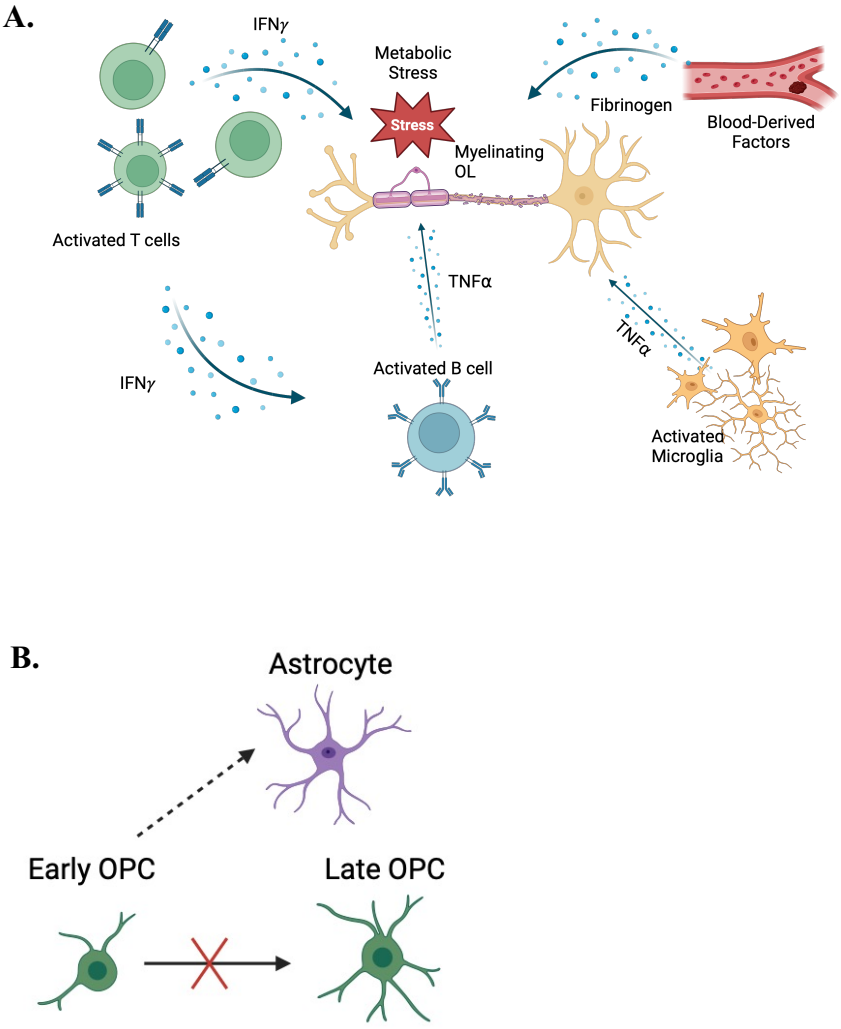


Figure 8. Working model of interrogated molecule effect on human OPCs. A. Schematic of insults present within the MS lesion, all have been shown to directly affect OL-lineage cells in multiple models. **B.** Working theory following findings presented. Figures made with BioRender.

Before exploring the effect of fibrinogen on human OPCs, we determined to evaluate its relevance to OL-lineage cells by investigating MS tissue and treating mature human OLs with this molecule. We observed that fibrinogen is in contact with OL-lineage (SOX10+) cells within the MS brain (FIG 4A), and increased fibrinogen deposition occurs within various types of MS lesions in comparison to NAWM in the MS brain and other pathological disease conditions (ALS) (FIG 4B). Following fibrinogen treatment in increasing concentrations on mature human OLs, we observed no significant effect on cell viability or morphology suggesting that this molecule is not directly toxic to these mature cells. However, when applied to OPCs (FIG 6), we observed a decrease in differentiation capacity (FIG 6A, C) without cytotoxicity (FIG 6B). This more pronounced effect observed in our OPC cultures suggest an increased vulnerability of these cells in comparison to their more mature counterparts, although this molecule acts via the BMP pathway in both instances (FIG 7). In line with previous rodent literature [75] we observed an increase in astrocyte markers following fibrinogen treatment. Furthermore, the presence of hybrid OPCs (O4+ CD49f+) co-expressing OPC and astrocyte markers suggest that this increase in astrocytes is due to a subpopulation of OPCs acquiring these characteristics.

Identifying the key molecules and pathways inhibiting human OPCs from remyelinating the brain in an MS context, is important for the development of translatable therapeutics. We have identified a novel mechanism contributing to this impaired remyelination (FIG 8B) in a human *in vitro* system. We have shown that late OPCs (O4+) retain the capacity to generate astrocytes under stress-like conditions and following exposure to key molecules TNFα and fibrinogen. This mechanism is of importance as we have shown that instead of differentiating into myelinating OLs, or stalling along their lineage, OPCs in the MS brain may be contributing to glial scar formation via the generation of astrocytes. The lineage commitment of OPCs may not be as rigid as we

previously thought. With this novel target identified, in human cells more importantly, these pathways and molecules can be targeted in other models of MS disease and progress towards developing a novel therapeutic strategy.

7. References

1. Lublin, F.D., et al., *Defining the clinical course of multiple sclerosis: the 2013 revisions.* Neurology, 2014. **83**(3): p. 278-86.
2. Rodriguez, M. and B. Scheithauer, *Ultrastructure of multiple sclerosis.* Ultrastruct Pathol, 1994. **18**(1-2): p. 3-13.
3. Kuhlmann, T., et al., *Differentiation block of oligodendroglial progenitor cells as a cause for remyelination failure in chronic multiple sclerosis.* Brain, 2008. **131**(7): p. 1749-1758.
4. Minagar, A. and J.S. Alexander, *Blood-brain barrier disruption in multiple sclerosis.* Multiple Sclerosis Journal, 2003. **9**(6): p. 540-549.
5. Wu, G.F. and E. Alvarez, *The immunopathophysiology of multiple sclerosis.* Neurol Clin, 2011. **29**(2): p. 257-78.
6. Cui Q, K.T., Miron V et al., *Oligodendrocyte Progenitor Cell Susceptibility to Injury in Multiple Sclerosis.* The American Journal of Pathology, 2013. **183**(2).
7. Fogarty, M., W.D. Richardson, and N. Kessaris, *A subset of oligodendrocytes generated from radial glia in the dorsal spinal cord.* Development, 2005. **132**(8): p. 1951-9.
8. Kessaris, N., et al., *Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage.* Nat Neurosci, 2006. **9**(2): p. 173-9.
9. Tripathi, R.B., et al., *Dorsally and ventrally derived oligodendrocytes have similar electrical properties but myelinate preferred tracts.* J Neurosci, 2011. **31**(18): p. 6809-6819.
10. Bergles, D.E. and W.D. Richardson, *Oligodendrocyte Development and Plasticity.* Cold Spring Harb Perspect Biol, 2015. **8**(2): p. a020453.
11. Goldman, S.A. and N.J. Kuypers, *How to make an oligodendrocyte.* Development, 2015. **142**(23): p. 3983-3995.
12. Reynolds, R. and R. Hardy, *Oligodendroglial progenitors labeled with the O4 antibody persist in the adult rat cerebral cortex in vivo.* Journal of Neuroscience Research, 1997. **47**(5): p. 455-470.
13. Küspert, M., et al., *Olig2 regulates Sox10 expression in oligodendrocyte precursors through an evolutionary conserved distal enhancer.* Nucleic Acids Research, 2010. **39**(4): p. 1280-1293.
14. Crawford, A.H., et al., *Oligodendrocyte progenitors: adult stem cells of the central nervous system?* Exp Neurol, 2014. **260**: p. 50-5.
15. Wolswijk, G., *Oligodendrocyte survival, loss and birth in lesions of chronic-stage multiple sclerosis.* Brain, 2000. **123**(1): p. 105-115.
16. Pringle, N.P., et al., *Determination of neuroepithelial cell fate: induction of the oligodendrocyte lineage by ventral midline cells and sonic hedgehog.* Dev Biol, 1996. **177**(1): p. 30-42.
17. Cai, J., et al., *Generation of oligodendrocyte precursor cells from mouse dorsal spinal cord independent of Nkx6 regulation and Shh signaling.* Neuron, 2005. **45**(1): p. 41-53.
18. Jakovcevski, I. and N. Zecevic, *Sequence of oligodendrocyte development in the human fetal telencephalon.* Glia, 2005. **49**(4): p. 480-91.
19. Vondran, M.W., et al., *BDNF+/- mice exhibit deficits in oligodendrocyte lineage cells of the basal forebrain.* Glia, 2010. **58**(7): p. 848-56.
20. Noble, M., et al., *Platelet-derived growth factor promotes division and motility and inhibits premature differentiation of the oligodendrocyte/type-2 astrocyte progenitor cell.* Nature, 1988. **333**(6173): p. 560-2.

21. McMorris, F.A., et al., *Insulin-like growth factor I/somatomedin C: a potent inducer of oligodendrocyte development.* Proc Natl Acad Sci U S A, 1986. **83**(3): p. 822-6.
22. Wheeler, N.A. and B. Fuss, *Extracellular cues influencing oligodendrocyte differentiation and (re)myelination.* Experimental Neurology, 2016. **283**: p. 512-530.
23. Sun, T., et al., *Olig bHLH proteins interact with homeodomain proteins to regulate cell fate acquisition in progenitors of the ventral neural tube.* Current Biology, 2001. **11**(18): p. 1413-1420.
24. Sun, T., et al., *Cross-Repressive Interaction of the Olig2 and Nkx2.2 Transcription Factors in Developing Neural Tube Associated with Formation of a Specific Physical Complex.* The Journal of Neuroscience, 2003. **23**(29): p. 9547-9556.
25. Elbaz, B. and B. Popko, *Molecular Control of Oligodendrocyte Development.* Trends Neurosci, 2019. **42**(4): p. 263-277.
26. Liu, Z., et al., *Induction of oligodendrocyte differentiation by Olig2 and Sox10: evidence for reciprocal interactions and dosage-dependent mechanisms.* Dev Biol, 2007. **302**(2): p. 683-93.
27. Hornig, J., et al., *The transcription factors Sox10 and Myrf define an essential regulatory network module in differentiating oligodendrocytes.* PLoS Genet, 2013. **9**(10): p. e1003907.
28. Raff, M.C., R.H. Miller, and M. Noble, *A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium.* Nature, 1983. **303**(5916): p. 390-396.
29. Kondo, T. and M. Raff, *Oligodendrocyte precursor cells reprogrammed to become multipotential CNS stem cells.* Science, 2000. **289**(5485): p. 1754-7.
30. Wren, D., G. Wolswijk, and M. Noble, *In vitro analysis of the origin and maintenance of O-2Aadult progenitor cells.* J Cell Biol, 1992. **116**(1): p. 167-76.
31. Choi, B.H. and R.C. Kim, *Expression of glial fibrillary acidic protein in immature oligodendroglia.* Science, 1984. **223**(4634): p. 407-9.
32. Alghamdi, B. and R. Fern, *Phenotype overlap in glial cell populations: astroglia, oligodendroglia and NG-2(+) cells.* Front Neuroanat, 2015. **9**: p. 49.
33. Holst, C.B., et al., *Astrogliogenesis in human fetal brain: complex spatiotemporal immunoreactivity patterns of GFAP, S100, AQP4 and YKL-40.* J Anat, 2019. **235**(3): p. 590-615.
34. Zhu, X., et al., *Olig2-dependent developmental fate switch of NG2 cells.* Development, 2012. **139**(13): p. 2299-307.
35. Suzuki, N., et al., *Differentiation of Oligodendrocyte Precursor Cells from Sox10-Venus Mice to Oligodendrocytes and Astrocytes.* Scientific Reports, 2017. **7**(1): p. 14133-14133.
36. Rivers, L.E., et al., *PDGFRA/NG2 glia generate myelinating oligodendrocytes and piriform projection neurons in adult mice.* Nat Neurosci, 2008. **11**(12): p. 1392-401.
37. Kang, S.H., et al., *NG2+ CNS glial progenitors remain committed to the oligodendrocyte lineage in postnatal life and following neurodegeneration.* Neuron, 2010. **68**(4): p. 668-81.
38. Zhu, X., et al., *Age-dependent fate and lineage restriction of single NG2 cells.* Development, 2011. **138**(4): p. 745-53.
39. Dimou, L., et al., *Progeny of Olig2-expressing progenitors in the gray and white matter of the adult mouse cerebral cortex.* J Neurosci, 2008. **28**(41): p. 10434-42.
40. Dyer, C.A., et al., *GFAP-positive and myelin marker-positive glia in normal and pathologic environments.* J Neurosci Res, 2000. **60**(3): p. 412-26.

41. Dyer, C.A., et al., *Evidence for central nervous system glial cell plasticity in phenylketonuria.* J Neuropathol Exp Neurol, 1996. **55**(7): p. 795-814.
42. Hackett, A.R., et al., *Injury type-dependent differentiation of NG2 glia into heterogeneous astrocytes.* Exp Neurol, 2018. **308**: p. 72-79.
43. Kirdajova, D., et al., *Transient astrocyte-like NG2 glia subpopulation emerges solely following permanent brain ischemia.* Glia, 2021. **69**(11): p. 2658-2681.
44. Weng, Q., et al., *Single-Cell Transcriptomics Uncovers Glial Progenitor Diversity and Cell Fate Determinants during Development and Gliomagenesis.* Cell Stem Cell, 2019. **24**(5): p. 707-723.e8.
45. Lassmann, H., *Multiple Sclerosis Pathology.* Cold Spring Harb Perspect Med, 2018. **8**(3).
46. Sati, P., et al., *The central vein sign and its clinical evaluation for the diagnosis of multiple sclerosis: a consensus statement from the North American Imaging in Multiple Sclerosis Cooperative.* Nature Reviews Neurology, 2016. **12**(12): p. 714-722.
47. Filion, L., et al., *Monocyte-derived cytokines in multiple sclerosis.* Clinical & Experimental Immunology, 2003. **131**(2): p. 324-334.
48. Calabresi, P., et al., *ELI-spot of Th-1 cytokine secreting PBMC's in multiple sclerosis: correlation with MRI lesions.* Journal of neuroimmunology, 1998. **85**(2): p. 212-219.
49. Calabresi, P., et al., *Cytokine gene expression in cells derived from CSF of multiple sclerosis patients.* Journal of neuroimmunology, 1998. **89**(1-2): p. 198-205.
50. Zhang, E.T., C.B. Inman, and R.O. Weller, *Interrelationships of the pia mater and the perivascular (Virchow-Robin) spaces in the human cerebrum.* J Anat, 1990. **170**: p. 111-23.
51. Bartholomäus, I., et al., *Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions.* Nature, 2009. **462**(7269): p. 94-8.
52. Weller, R.O., et al., *The meninges as barriers and facilitators for the movement of fluid, cells and pathogens related to the rodent and human CNS.* Acta Neuropathol, 2018. **135**(3): p. 363-385.
53. Magliozzi, R., et al., *Inflammatory intrathecal profiles and cortical damage in multiple sclerosis.* Ann Neurol, 2018. **83**(4): p. 739-755.
54. Calabrese, M., et al., *Cortical lesion load associates with progression of disability in multiple sclerosis.* Brain, 2012. **135**(Pt 10): p. 2952-61.
55. Budzynski, A.Z., *Fibrinogen and fibrin: biochemistry and pathophysiology.* Crit Rev Oncol Hematol, 1986. **6**(2): p. 97-146.
56. Mosesson, M.W., *Fibrinogen and fibrin structure and functions.* J Thromb Haemost, 2005. **3**(8): p. 1894-904.
57. Vos, C.M., et al., *Blood-brain barrier alterations in both focal and diffuse abnormalities on postmortem MRI in multiple sclerosis.* Neurobiol Dis, 2005. **20**(3): p. 953-60.
58. Yates, R.L., et al., *Fibrin(ogen) and neurodegeneration in the progressive multiple sclerosis cortex.* Ann Neurol, 2017. **82**(2): p. 259-270.
59. Kirk, J., et al., *Tight junctional abnormality in multiple sclerosis white matter affects all calibres of vessel and is associated with blood-brain barrier leakage and active demyelination.* J Pathol, 2003. **201**(2): p. 319-27.
60. Adams, R.A., et al., *The fibrin-derived gamma377-395 peptide inhibits microglia activation and suppresses relapsing paralysis in central nervous system autoimmune disease.* J Exp Med, 2007. **204**(3): p. 571-82.

61. Davalos, D., et al., *Fibrinogen-induced perivascular microglial clustering is required for the development of axonal damage in neuroinflammation.* Nat Commun, 2012. **3**: p. 1227.
62. Schachtrup, C., et al., *Fibrinogen triggers astrocyte scar formation by promoting the availability of active TGF-beta after vascular damage.* J Neurosci, 2010. **30**(17): p. 5843-54.
63. Luyendyk, J.P., J.G. Schoenecker, and M.J. Flick, *The multifaceted role of fibrinogen in tissue injury and inflammation.* Blood, 2019. **133**(6): p. 511-520.
64. Pernin, F., et al., *Diverse injury responses of human oligodendrocyte to mediators implicated in multiple sclerosis.* Brain, 2022. **145**(12): p. 4320-4333.
65. Hövelmeyer, N., et al., *Apoptosis of Oligodendrocytes via Fas and TNF-R1 Is a Key Event in the Induction of Experimental Autoimmune Encephalomyelitis1.* The Journal of Immunology, 2005. **175**(9): p. 5875-5884.
66. Akassoglou, K., et al., *Oligodendrocyte Apoptosis and Primary Demyelination Induced by Local TNF/p55TNF Receptor Signaling in the Central Nervous System of Transgenic Mice: Models for Multiple Sclerosis with Primary Oligodendrogliopathy.* The American Journal of Pathology, 1998. **153**(3): p. 801-813.
67. Cammer, W., *Effects of TNFα on immature and mature oligodendrocytes and their progenitors in vitro.* Brain Research, 2000. **864**(2): p. 213-219.
68. Bonora, M., et al., *Tumor necrosis factor-α impairs oligodendroglial differentiation through a mitochondria-dependent process.* Cell Death & Differentiation, 2014. **21**(8): p. 1198-1208.
69. Arnett, H.A., et al., *TNFα promotes proliferation of oligodendrocyte progenitors and remyelination.* Nature Neuroscience, 2001. **4**(11): p. 1116-1122.
70. Wang, J., et al., *Paired Related Homeobox Protein 1 Regulates Quiescence in Human Oligodendrocyte Progenitors.* Cell Rep, 2018. **25**(12): p. 3435-3450.e6.
71. Saraswat, D., et al., *Heparanome-Mediated Rescue of Oligodendrocyte Progenitor Quiescence following Inflammatory Demyelination.* J Neurosci, 2021. **41**(10): p. 2245-2263.
72. Lin, W., et al., *Interferon-γ inhibits central nervous system remyelination through a process modulated by endoplasmic reticulum stress.* Brain, 2006. **129**(5): p. 1306-1318.
73. Chew, L.-J., et al., *Interferon-γ inhibits cell cycle exit in differentiating oligodendrocyte progenitor cells.* Glia, 2005. **52**(2): p. 127-143.
74. Wang, Y., et al., *STAT1/IRF-1 signaling pathway mediates the injurious effect of interferon-gamma on oligodendrocyte progenitor cells.* Glia, 2010. **58**(2): p. 195-208.
75. Petersen, M.A., et al., *Fibrinogen Activates BMP Signaling in Oligodendrocyte Progenitor Cells and Inhibits Remyelination after Vascular Damage.* Neuron, 2017. **96**(5): p. 1003-1012.e7.
76. Takahashi, K., et al., *Induction of pluripotent stem cells from adult human fibroblasts by defined factors.* Cell, 2007. **131**(5): p. 861-72.
77. Ehrlich, M., et al., *Rapid and efficient generation of oligodendrocytes from human induced pluripotent stem cells using transcription factors.* Proc Natl Acad Sci U S A, 2017. **114**(11): p. E2243-e2252.
78. García-León, J.A., et al., *SOX10 Single Transcription Factor-Based Fast and Efficient Generation of Oligodendrocytes from Human Pluripotent Stem Cells.* Stem Cell Reports, 2018. **10**(2): p. 655-672.

79. Wang, S., et al., *Human iPSC-Derived Oligodendrocyte Progenitor Cells Can Myelinate and Rescue a Mouse Model of Congenital Hypomyelination.* Cell Stem Cell, 2013. **12**(2): p. 252-264.
80. Douvaras, P. and V. Fossati, *Generation and isolation of oligodendrocyte progenitor cells from human pluripotent stem cells.* Nat Protoc, 2015. **10**(8): p. 1143-54.
81. Piscopo, V.E.C., et al., *The use of a SOX10 reporter toward ameliorating oligodendrocyte lineage differentiation from human induced pluripotent stem cells.* Glia, 2024. **n/a**(n/a).
82. Fernandes, M.G.F., et al., *Age-related injury responses of human oligodendrocytes to metabolic insults: link to BCL-2 and autophagy pathways.* Communications Biology, 2021. **4**(1): p. 20.
83. Bradl, M. and H. Lassmann, *Oligodendrocytes: biology and pathology.* Acta Neuropathol, 2010. **119**(1): p. 37-53.
84. Jakovcevski, I., et al., *Oligodendrocyte development and the onset of myelination in the human fetal brain.* Front Neuroanat, 2009. **3**: p. 5.
85. Yakovlev, P.I., et al., *Regional development of the brain in early life*. 1967.
86. Filipovic, R., I. Jakovcevski, and N. Zecevic, *GRO-alpha and CXCR2 in the human fetal brain and multiple sclerosis lesions.* Dev Neurosci, 2003. **25**(2-4): p. 279-90.
87. Gold, R., C. Linington, and H. Lassmann, *Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research.* Brain, 2006. **129**(8): p. 1953-1971.
88. Mecha, M., et al., *Viral models of multiple sclerosis: neurodegeneration and demyelination in mice infected with Theiler's virus.* Prog Neurobiol, 2013. **101-102**: p. 46-64.
89. Kipp, M., et al., *The cuprizone animal model: new insights into an old story.* Acta Neuropathol, 2009. **118**(6): p. 723-36.
90. Hooijmans, C.R., et al., *Remyelination promoting therapies in multiple sclerosis animal models: a systematic review and meta-analysis.* Scientific Reports, 2019. **9**(1): p. 822.
91. Kappos, L., et al., *A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis.* N Engl J Med, 2010. **362**(5): p. 387-401.
92. Lublin, F., et al., *Oral fingolimod in primary progressive multiple sclerosis (INFORMS): a phase 3, randomised, double-blind, placebo-controlled trial.* Lancet, 2016. **387**(10023): p. 1075-1084.
93. Sanford, M., *Fingolimod: a review of its use in relapsing-remitting multiple sclerosis.* Drugs, 2014. **74**(12): p. 1411-33.
94. Chen, Y., et al., *The oligodendrocyte-specific G protein-coupled receptor GPR17 is a cell-intrinsic timer of myelination.* Nat Neurosci, 2009. **12**(11): p. 1398-406.
95. He, Y., J. Sandoval, and P. Casaccia-Bonnefil, *Events at the transition between cell cycle exit and oligodendrocyte progenitor differentiation: the role of HDAC and YY1.* Neuron Glia Biology, 2007. **3**(3): p. 221-231.
96. Garcia, L.M., et al., *Glial cells in the driver seat of leukodystrophy pathogenesis.* Neurobiology of Disease, 2020. **146**: p. 105087.
97. Weider, M., et al., *Elevated in vivo levels of a single transcription factor directly convert satellite glia into oligodendrocyte-like cells.* PLoS genetics, 2015. **11**(2): p. e1005008.
98. Starost, L., et al., *Extrinsic immune cell-derived, but not intrinsic oligodendroglial factors contribute to oligodendroglial differentiation block in multiple sclerosis.* Acta Neuropathol, 2020. **140**(5): p. 715-736.

99. Richardson, W.D., et al., *NG2-glia as multipotent neural stem cells: fact or fantasy?* Neuron, 2011. **70**(4): p. 661-73.
100. Zhu, X., D.E. Bergles, and A. Nishiyama, *NG2 cells generate both oligodendrocytes and gray matter astrocytes.* Development, 2008. **135**(1): p. 145-57.
101. Tatsumi, K., et al., *Genetic fate mapping of Olig2 progenitors in the injured adult cerebral cortex reveals preferential differentiation into astrocytes.* J Neurosci Res, 2008. **86**(16): p. 3494-502.
102. Magnus, T., et al., *Evidence that nucleocytoplasmic Olig2 translocation mediates brain-injury-induced differentiation of glial precursors to astrocytes.* J Neurosci Res, 2007. **85**(10): p. 2126-37.
103. Sun, Y., et al., *STAT3 represents a molecular switch possibly inducing astroglial instead of oligodendroglial differentiation of oligodendroglial progenitor cells in Theiler's murine encephalomyelitis.* Neuropathol Appl Neurobiol, 2015. **41**(3): p. 347-70.
104. Miscia, S., et al., *Tumor necrosis factor alpha (TNF-alpha) activates Jak1/Stat3-Stat5B signaling through TNFR-1 in human B cells.* Cell Growth Differ, 2002. **13**(1): p. 13-8.
105. Mori, T., et al., *IL-1β and TNFα-initiated IL-6–STAT3 pathway is critical in mediating inflammatory cytokines and RANKL expression in inflammatory arthritis.* International Immunology, 2011. **23**(11): p. 701-712.
106. Petersen, M.A., J.K. Ryu, and K. Akassoglou, *Fibrinogen in neurological diseases: mechanisms, imaging and therapeutics.* Nat Rev Neurosci, 2018. **19**(5): p. 283-301.
107. Montagne, A., et al., *Blood-brain barrier breakdown in the aging human hippocampus.* Neuron, 2015. **85**(2): p. 296-302.
108. Pous, L., et al., *Fibrinogen induces neural stem cell differentiation into astrocytes in the subventricular zone via BMP signaling.* Nat Commun, 2020. **11**(1): p. 630.
109. Cheng, X., et al., *Bone morphogenetic protein signaling and olig1/2 interact to regulate the differentiation and maturation of adult oligodendrocyte precursor cells.* Stem Cells, 2007. **25**(12): p. 3204-14.
110. D'Alessandro, J.S., J. Yetz-Aldape, and E.A. Wang, *Bone morphogenetic proteins induce differentiation in astrocyte lineage cells.* Growth Factors, 1994. **11**(1): p. 53-69.
111. Agius, E., et al., *Role of BMPs in controlling the spatial and temporal origin of GFAP astrocytes in the embryonic spinal cord.* Dev Biol, 2010. **344**(2): p. 611-20.
112. Kernt, M., et al., *Sorafenib protects human optic nerve head astrocytes from light-induced overexpression of vascular endothelial growth factor, platelet-derived growth factor, and placenta growth factor.* Growth Factors, 2010. **28**(3): p. 211-20.
113. Willis, C.M., et al., *Astrocyte Support for Oligodendrocyte Differentiation can be Conveyed via Extracellular Vesicles but Diminishes with Age.* Scientific Reports, 2020. **10**(1): p. 828.
114. Chamling, X., et al., *Single-cell transcriptomic reveals molecular diversity and developmental heterogeneity of human stem cell-derived oligodendrocyte lineage cells.* Nature Communications, 2021. **12**(1): p. 652.
115. Macintosh, J., et al., *An optimized and validated protocol for the purification of PDGFRα+ oligodendrocyte precursor cells from mouse brain tissue via immunopanning.* MethodsX, 2023. **10**: p. 102051.
116. Janowska, J., J. Gargas, and J. Sypecka, *Pearls and Pitfalls of Isolating Rat OPCs for In Vitro Culture with Different Methods.* Cell Mol Neurobiol, 2023. **43**(7): p. 3705-3722.

117. Medina-Rodríguez, E.M., et al., *Protocol to isolate a large amount of functional oligodendrocyte precursor cells from the cerebral cortex of adult mice and humans.* PLoS One, 2013. **8**(11): p. e81620.
118. Smith, M.D., et al., *Reactive Astrocytes Derived From Human Induced Pluripotent Stem Cells Suppress Oligodendrocyte Precursor Cell Differentiation.* Frontiers in Molecular Neuroscience, 2022. **15**.
119. Choi, S.S., et al., *Human astrocytes: secretome profiles of cytokines and chemokines.* PLoS One, 2014. **9**(4): p. e92325.
120. Prineas, J.W. and S. Lee, *Multiple Sclerosis: Destruction and Regeneration of Astrocytes in Acute Lesions.* J Neuropathol Exp Neurol, 2019. **78**(2): p. 140-156.

8. Appendix

8.1 Agreement between authors for use of data from recent manuscript

Acknowledgement of contribution and agreement of inclusion of data in a thesis

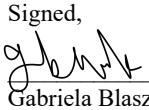
For use of data in the recent publication:

Valerio E.C. Piscopo, Alexandra Chapleau, Gabriela J. Blaszczyk, Julien Sirois, Zhipeng You, Vincent Soubannier, Carol X.-Q. Chen, Geneviève Bernard, Jack P. Antel, Thomas M. Durcan. *The Use of a SOX10 Reporter Towards Ameliorating Oligodendrocyte Lineage Differentiation from Human Induced Pluripotent Stem Cells*. Glia. (2024). DOI:10.1002.

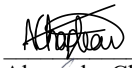
Contributions by students Alexandra Chapleau, Gabriela J. Blaszczyk will be divided for use in thesis as follows:

- Generation, characterization of the SOX10-mOrange reporter line (Figure 1, Figure 5) will be used in Gabriela's thesis
- qPCR data from the 3450 cell line which was generated by Gabriela will be used in her thesis (Figure S5).
- Characterization of the douvaras and fossati protocol, testing of maturation media and related results (Figure 2, 4, 6) was generated by Alexandra and will be used in her thesis

Signed,



Gabriela Blaszczyk



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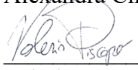

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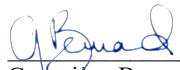
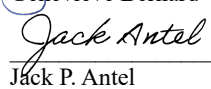


Geneviève Bernard



Jack P. Antel

8.2 Tables

Table 1. Antibodies used for Flow Cytometry

Antibody	Fluorochrome	Dilution used	Manufacturer (CAT #)
PDGFRa	Brilliant Violet 711	1:320	BD optibuild, #752901
PDGFRa	APC	1:320	Biolegend, #323512
O4	PE	1:200	Miltenyi, #130-117-357
O4	APC	1:200	Miltenyi, #130-119-982
CD133	PE-Cy7	1:320	Biolegend, #372810
CD49f	PE-Dazzle	1:1000	Biolegend, #313626
GFAP	AF-488	1:2000	ThermoFisher, #53-9892-82
AQP4	AF488	1:100	Bioss-USA, #bs-0634R-A488

Table 2. Antibodies used for Immunocytochemistry

Name	Host Species	Working Dilution	Manufacturer (CAT #)
O4	Mouse IgM	1:200	R&D, MAB1326
pSMAD-1/5/9	Rabbit	1:50	Cell Signalling, 13820S
PI	n/a	1:200	Invitrogen, P1304MP

Table 3. Antibodies used for Immunohistochemistry

Name	Host Species	Working Dilution	Manufacturer (CAT #)
SOX10	Goat	1:500	R&D systems, AF2864
Fibrinogen	Rabbit	1:400	Agilent, A0080

8.3 Supplementary Figures

Cell Line Name	Sex	Patient Age (years)	Material Source	Type of Reprogramming
AIW002-02	M	37	PBMC	Retrovirus
3450	M	37	PBMC	Episomal
SOX10mOrange (3450)	M	37	PBMC	Episomal
81280 (Genome Quebec)	M	50	Fibroblasts	Sendai

Figure S1. List of used lines for generation of OL-lineage cells.

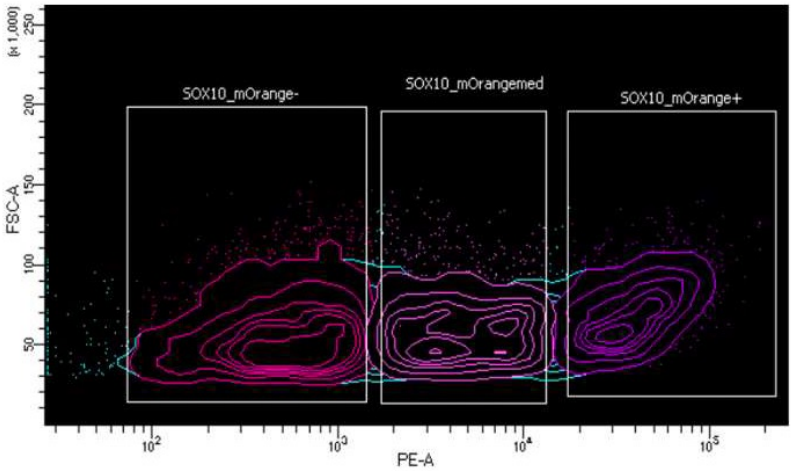


Figure S2. Three distinct groups based on mOrange fluorescence intensity; mOrange negative, medium, and high.